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Sex, Drugs, and Rodent Reward: An Exploration of the Sex-Specific Roles of Nicotinic Acetylcholine Receptors in Ethanol Reward

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SEX, DRUGS, AND RODENT REWARD: AN EXPLORATION OF THE SEX-SPECIFIC ROLES OF NICOTINIC ACETYLCHOLINE RECEPTORS IN ETHANOL REWARD

A Dissertation Presented

By

Melissa Guildford Derner

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DEDICATION & ACKNOWLEDGEMENTS

Graduate school, much like life itself, is more a journey than a destination. On the road to a PhD, sometimes you are on the autobahn, and sometimes you're anywhere near Boston during rush hour. Whatever the speed, you are not alone on your journey, though sometimes it may feel that way. Even the strongest, most efficient engine in the world could not move an inch without help.

On my journey, I picture myself as the engine. Chugging along, working to get this road traversed, a little worse for wear but still running. I have my inspection sticker TRAC committee, making sure everything is running smoothly each year of my journey, and my lovely lab mates are my radio - making the time fly by, laughing, chatting, commiserating, advising. Paul is the fuzzy dice hanging from the mirror, reminding me not to take myself too seriously, and Andrew is the turbo boost, ready to light a fire under your tailpipe if you are moving too slowly. He's also the GPS, giving (mostly) accurate directions, letting me know when I need to make a U-turn, and reminding me how much longer until I reach my destination.

I've certainly encountered people on my journey who have been fender benders - people that I have followed in error, and not paid attention enough to see that they've stopped. These accidents aren't fun, but they make you realize they are sitting still while you are moving, and make you change course. Fortunately, I've have been so lucky to

have great friends as the fuel in my tank when I was just about running on fumes, or spare tires that, despite not being used often, I knew were always there if I was stuck.

I've had grandparents, some here and some above, as my mirrors, making sure that I am always looking out for those around me, and that I remember where I came from. My in-laws are my windshield wipers, helping me see clearly no matter the weather.

My brother is the random drive to nowhere, the unplanned pit stops, reminding me that the journey isn't just about getting there, it's about the experiences along the way. My father is the open windows, reminding me to listen to the sounds, feel the air, and enjoy the simplicity of nature. My step-father is my jumper cables, my tire iron, and my owner's manual, always ensuring I feel safe, strong, and empowered, no matter the situation. My mom, my headlights, has always been there to illuminate my path and help me navigate in the dark.

My husband Jason is my chassis; he holds all of the pieces together and makes the journey possible. He is the unsung hero, the behind-the-scenes support system. Without a chassis, the engine can run it's heart out and still will not be able to go anywhere. I am where I am because of his unending support.

My son, Henry, is the sunshine on my arm on a warm, breezy, summer day's drive. He's the wind in my hair, the smell of rain, and my favorite song on the radio. He

is the one day a year I actually get down route 9 without hitting all red lights, and the peak of fall foliage. He makes the whole journey worthwhile.

This is dedicated to everyone who has encouraged, supported, guided, and loved me along this crazy, winding road. You are all the reason I was able to make it to my destination. This debt is not one I can pay back, much like my student loans, but I will certainly try. Thank you, from the bottom of my heart.

ABSTRACT

Alcohol, recently named the most dangerous drug in the world, contributes to nearly 40% of violent crimes and fatal traffic accidents, increases risk of roughly 60 different diseases and injuries, and is responsible for 2.5 million deaths each year worldwide. Despite these staggering figures, treatments remain ineffective and riddled with adverse side effects, making successful use of even the most effective treatments unlikely. Moreover, many of the treatments, and the supporting research, have focused only on male subjects, despite sex differences in various alcohol-related behaviors.

Human alcohol use is frequently accompanied by nicotine use, and vice versa, suggesting a common mechanism of the two drugs. In fact, alcohol may act through the same family of receptors as nicotine, the nicotinic acetylcholine receptors (nAChRs), eliciting similar activation of the reward pathway as nicotine and other drugs of abuse. Studies have shown that nAChRs containing the α4 and/or α6 subunits are involved in nicotine-induced activation of the reward pathway, leading to the hypothesis that these same receptor subtypes may be important for alcohol effects in the brain as well.

Using male and female genetic mouse models and various behavioral assays, we have shown not only that these α4 and/or α6-containing nAChRs are involved in alcohol-related behaviors and activation of the reward pathway, but also show sex differences in this involvement. Uncovering the mechanism of alcohol in the brain, in males as well as in females, is an important step in developing targeted treatments for alcohol abuse.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Copyrighted Material

Guildford, M.J., A.V. Sacino, and A.R. Tapper, *Modulation of ethanol reward sensitivity by nicotinic acetylcholine receptors containing the alpha6 subunit.* Alcohol, 2016.

Hendrickson, L.M., M.J. Guildford, and A.R. Tapper, *Neuronal nicotinic acetylcholine receptors: common molecular substrates of nicotine and alcohol dependence.* Front Psychiatry, 2013. **4**: p. 29.

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List of Abbreviations

5-HT3: 5-hydroxytryptamine
α*: α(subunit)-containing
α-Bgtx: α-bungarotoxin
α-CTX: α-conotoxin

ACh: Acetylcholine
AP: Access point/Action potential (?)
AUDs: Alcohol use disorders
aVTA: Anterior ventral tegmental area

BEC: Blood ethanol concentration

CNS: Central nervous system
CPP: Conditioned place preference
CRF: Corticotropin releasing factor

DA: Dopamine
DAergic: Dopaminergic
DHβE: Dihydro-β-erythroidine
DID: Drinking-in-the-Dark

EtOH: Ethanol/alcohol

GABA: Gamma-aminobutyric acid
g/kg: Grams per kilogram

i.p./IP: intraperitoneal
IPN: Interpeduncular nucleus

KO: Knockout

LDT/LDTg: Laterodorsal tegmentum
LH: Lateral habenula

MH: Medial habenula
MLA: Methyllycaconitine

NMDA: N-methyl-D-aspartate
NAc/NAcc: Nucleus accumbens
nAChRs: Nicotinic acetylcholine receptors

PFC: Prefrontal cortex
PPTg: Pedonculopontine tegmental nucleus
pVTA: posterior ventral tegmental area

RDS: Reward deficiency syndrome
s.c.: subcutaneous

SNPs: Single nucleotide polymorphisms

TH: Tyrosine hydroxylase

VTA: Ventral tegmental area

WT: Wild-type

Preface to Chapter I

Figures 1.1, 1.2, and Table 1.1 are from an article previously published in:

Hendrickson, L.M., M.J. Guildford, and A.R. Tapper, *Neuronal nicotinic acetylcholine receptors: common molecular substrates of nicotine and alcohol dependence.* Front Psychiatry, 2013. **4**: p. 1-16. doi: 10.3389/fpsyt.2013.00029.

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Linzy M. Hendrickson, Melissa J. Guildford, and Andrew R. Tapper wrote the paper

CHAPTER I: Introduction

Addiction

The definition of addiction given depends on to whom the question is directed. A quick internet search will declare that addiction is "the fact or condition of being addicted to a particular substance, thing, or activity," with synonyms including habit and problem (google.com). The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) no longer lists drug addiction as a diagnosable condition, instead classifying it as a severe form of an abuse disorder (Hasin, O'Brien et al. 2013). According to the National Institute on Drug Abuse (NIDA 2014), drug addiction is a "chronic, relapsing brain disease that is characterized by compulsive drug seeking and use, despite harmful consequences." With the general population, psychologists, and scientists all having very different definitions of the same condition, it is no wonder that, despite nearly 22.7 million Americans in need of treatment for illicit drug or alcohol use, only about 11% of those ever receive treatment (SAMHSA 2014), and even fewer are able to remain abstinent.

In humans, addiction happens in stages. The initiation phase, also known as binge or intoxication (Becker and Koob 2016), is characterized by use of the drug for its feel-good, or rewarding, properties. This use is considered goal-directed (Barker, Torregrossa

et al. 2010), with the goal being the associated euphoria. Most people will not progress beyond this casual use (NIDA 2014); those that escalate their use, in quantity or frequency or both, may eventually lose control of their drug use (Neasta, Ben Hamida et al. 2010) and qualify as abusers. This hazardous use of the drug of choice can change the brain itself, in structure and/or function (NIDA 2014), and can transition into habitual use (Barker, Torregrossa et al. 2010). What was once euphoria-driven is now habit, driven not by the positive outcome once associated with the drug, but by a preoccupation with and habitual use of the drug (Barker, Torregrossa et al. 2010, Becker and Koob 2016). It is at this point that the drug use is considered compulsive versus impulsive (Hommer, Bjork et al. 2011) - physically reliant on the drug not necessarily for euphoria but for normalcy - a hallmark of dependence.

One of the lingering questions in addiction research is why some end up addicted while others can use casually, or even abuse the drug, without becoming physically dependent. There are many circumstances that can predispose someone to addiction, many of which are governed by an increased likelihood of first use, including education, socioeconomic status, and geographic location, but there are also genetic factors that can influence the transition from use to abuse, including biological sex (SAMHSA 2014).

Biological sex begins with conception, where an egg containing an X ‘sex’ chromosome is fertilized by a sperm cell containing either an X or the male-defining Y chromosome. During development, this chromosomal sex is complimented by gonadal

sex, which relies on the presence or absence of sex hormones, especially testosterone ((Barker, Torregrossa et al. 2010). The chromosomal sex influences the gonadal sex, in that the testes defining *Sry* gene, located on the Y chromosome, initiates formation of the testes and thus production of testosterone (Barker, Torregrossa et al. 2010) although there are instances wherein a developmental issue can cause a disconnect between the two, leading to chromosomal males with female attributes due to a lack of testosterone, for example, which further complicates the role of biological sex in the development of addiction.

Due to the dependent nature of the chromosomal and gonadal sex, with the testes gene present on the male-specific Y chromosome, it can be difficult to determine the function and/or role of each in addiction, for example. However, by physically separating the two in a four core genotype mouse model, Barker et al. (2010) were able to explore each independently of the other. They deleted the *Sry* gene from the Y chromosome and reintroduced it onto an autosome, a chromosome present in both sexes. This allowed the testes-forming gene to sort independently of the sex chromosome, allowing for four genotypes: XX female, XX male, XY female, and XY male, where the sex chromosomes are listed followed by the gonadal sex as determined by the presence or absence of the *Sry* gene (Barker, Torregrossa et al. 2010).

Without the ability to disentangle the various effects of biological sex in humans, observations must be assessed with any number of influencing factors in mind. Human

males are more likely to use drugs, though women proceed from use to abuse and dependency more quickly than their male counterparts (Becker and Hu 2008, Barker, Torregrossa et al. 2010, Lenz, Muller et al. 2012, Agabio, Campesi et al. 2016, Becker and Koob 2016), and enter into treatment more rapidly (Carroll and Lynch 2016). In both males and females, the biggest issue in determining who will become addicted is finding the potential drug abuser before they even use the drug. However, most studies done on drug addiction have been conducted using only male subjects, which, given the differences in the addiction cycle timeline between males and females, is unlikely to translate as well in female addicts. To further complicate the study of sex and addiction, there are different types of behavioral sex differences that lead to the different phenotypes in each sex. For example, quantitative differences, where the same behavioral response occurs with a difference in the magnitude of the response, is fairly easily observed, as is a population difference, wherein more of one sex show a given response than another. However, if the behavior is completely different, a qualitative difference, or identical superficially but due to a completely different mechanism, the interpretation becomes less clear (Becker and Koob 2016).

Sex differences exist not only at the behavioral level, but at the neuronal level. It has been shown, in humans as well as rodents, that sexually dimorphic regions exist and may play a role in the differential effects of drugs in each sex (Retson, Reyes et al. 2015). Two of the main pathways involved in drug addiction are the so-called reward and anti-

reward pathways (Koob and Le Moal 2008), and each has shown sex differences, whether at baseline (Bobzean, DeNobrega et al. 2014) or in response to drugs of abuse (Retson, Reyes et al. 2015), respectively. This dissertation will focus on the reward pathway - the entry point into the addiction cycle.

Reward

There are several hypotheses regarding the risk of addiction, though it is uncertain how accurate they are, or if they can be used across the various stages of addiction. Many of these theories pertain to the very beginning of the addiction cycle during the binge/ intoxication stage and detail the way in which reward is processed in the brain. A reward is something that possesses hedonic value in addition to being a positive reinforcer; it increases the likelihood of an operant response (Becker and Koob 2016). There are various natural rewards, including food, sex, and social interaction, and there are also less natural sources of reward including gambling and drugs of abuse.

Molecularly, reward is the result of activation of the mesocorticolimbic dopamine 'reward' pathway in the brain (Good and Lupica 2009, Tsai, Zhang et al. 2009) and the subsequent increased release of dopamine in the nucleus accumbens (NAc), the quintessential reward area. The three main areas involved in the reward pathway are the prefrontal cortex (PFC), the ventral tegmental area (VTA) and the NAc (**Figure 2)**. The PFC sends glutamatergic projections to the VTA, and receives reciprocal dopaminergic

(DAergic), glutamatergic, and projections containing gamma-aminobutyric acid (GABA) (Fields, Hjelmstad et al. 2007). The VTA itself is comprised of dopaminergic projection neurons as well as GABAergic projection and interneurons. These inhibitory GABAergic interneurons synapse onto DAergic neurons within the VTA, and thus influence activation of NAc-projecting DA neurons as well. In addition to the DA projection, the VTA also supplies glutamate, co-released by DA neurons, as well as GABA, to the NAc, which sends GABAergic projections back to the VTA. Although the pathway in its entirety is vast and complex, with many very specific, discrete connections and many levels of modulatory input, the activity of the DA projection from the VTA to the NAc is ultimately the end result.

Dopaminergic neurons projecting from the VTA provide tonic DAergic input to the NAc under normal conditions, firing at about 1Hz, and switch to burst or phasic firing with a much higher firing rate upon activation (Good and Lupica 2009, Tsai, Zhang et al. 2009, Liu, Zhao-Shea et al. 2012, Liu, Hendrickson et al. 2013); this switch from tonic to phasic is characteristic of reward, representing the increased dopamine being released in the NAc (Pidoplichko, DeBiasi et al. 1997, Rodd, Melendez et al. 2004, Okamoto, Harnett et al. 2006, Good and Lupica 2009, Hendrickson, Guildford et al. 2013).

All known drugs of abuse act through this pathway, and result in a much more robust activation than natural rewards. As such, once drug reward is experienced it tends to be preferred over natural rewards, a critical step in the development of addiction

(Hommer, Bjork et al. 2011). The reward deficiency syndrome (RDS) hypothesis of addiction formation proposes that addicts, or those predisposed to addiction, have a less active reward pathway than their non-addict peers, which is not sufficiently activated by natural rewards such as food, sex, or social interaction. If someone with RDS uses a drug, this drug will be marked as especially salient and prioritized over the ineffective natural rewards (Hommer, Bjork et al. 2011). This and other addiction hypotheses highlight the importance of the binge/intoxication, goal-directed phase of drug use in the development of addiction, but, in order to test these hypotheses, high-risk, drug-naive human subjects that then go on to develop an addiction, would be needed.

Given the inability to decipher deficiencies or dysfunctions that preceded drug use from those resulting from drug use in human addicts, various animal models have been employed in order to uncover the mechanism underlying the development of a drug addiction. A multi-faceted approach is used, investigating the molecular and behavioral effects of the drug, as well as what happens when the drug is subsequently removed. With many of the hypotheses surrounding the development of addiction referencing reward system dysfunction, this is where much of the drug addiction research to date has been focused. Behaviorally, there are many assays used to investigate the various stages of drug addiction. For the intoxication stage, conditioned place preference can be used to assay reward. In this classical conditioning paradigm, the animal is given the drug in one chamber, and vehicle in another. Subsequent preference for the chamber associated with

the drug, in the absence of the drug itself, suggests reward (Gibb, Jeanblanc et al. 2011). Another common assay is operant self-administration, which looks at the tendency to self-administer a drug once the drug is associated with the resultant euphoria or reward. This assay can then be employed in other stages, including maintenance and craving, where the animal is forced to perform a task many times before receiving the drug. The more they are willing to do for each dose, the stronger their motivation is for the drug.

Alcohol

Not all drugs of abuse are the illicit, illegal substances that come to mind when discussing addiction. In fact, alcohol has been labeled the most dangerous drug in the world, being involved in nearly 40% of violent crimes, fatal traffic accidents, and other punishable offenses (Greenfeld and Henneberg 2001). As indicated in the involvement in violent and even deadly encounters, alcohol's effects extend beyond the drinker to those around them and to society as a whole (WHO 2015). Drunk driving, violence, child neglect or abuse, decreased productivity, increased medical costs, all in addition to the increased incidence of cancers, liver cirrhosis, and roughly 60 other diseases and types of injures experienced by the drinker, makes alcohol an especially troublesome drug of abuse (WHO 2015). Alcohol is involved in nearly 2.5 million deaths per year, responsible for roughly 4% of all deaths worldwide, and is the third leading risk factor for preventable injury (McGinnis and Foege 1993, WHO 2015). The greatest risk of alcohol-related issues belongs to those in middle-income, developed countries, especially males,

for whom alcohol is responsible for 6.2% of all deaths, versus 1.1% for females (WHO 2015). Despite all of this, alcohol continues to be not only widely available and easily accessible, but also socially acceptable and legal.

With almost 90% of people in the United States having reported use of alcohol in their lifetime, and over 70% within the past year, alcohol is the most used drug of abuse (SAMHSA 2014). Not all alcohol use is harmful, and moderate use has even been shown to be beneficial, decreasing the risk of ischemic heart disease, stroke, and diabetes (Millen, Abrams et al. 2016). This moderate consumption, defined as up to 1 drink per day for women, 2 for men, is not the type of harmful drinking that typically leads to alcohol dependence (Millen, Abrams et al. 2016). Unlike moderate consumption, harmful drinking is sometimes referred to as heavy episodic or binge drinking, and is defined as drinking at least 60g of pure alcohol on at least once occasion in the past week with the intent of becoming intoxicated (WHO 2015), or four drinks in two hours for a female, five for a male (Coon, Piasecki et al. 2014). Over 11% of drinkers experience these binges weekly, and it is the volume and pattern of consumption which makes the frequency of binge drinking and resulting intoxication the behavior most associated with alcohol dependence (Kendler, Gardner et al. 2014). Males tend to binge drink more frequently than females, and also have higher rates of alcohol abuse, although the gap is closing quickly (Becker and Koob 2016).

Despite higher frequency of binge drinking and abuse in males, females proceed more quickly from alcohol use to abuse than their male counterparts, and are more vulnerable to its negative effects (Becker and Hu 2008, Barker, Torregrossa et al. 2010, Lenz, Muller et al. 2012, Agabio, Campesi et al. 2016, Becker and Koob 2016). Additionally males, when given the same dose of alcohol as females, experience double the dopamine increase in the NAc (Lenz, Muller et al. 2012), while females develop a higher blood ethanol concentration, more motor impairment, and increased sedation (Agabio, Campesi et al. 2016). Some of the differences involve the way in which each sex interacts with their environment, including the different opportunities, and the way in which each sex is viewed in terms of consequences of excessive drinking (Sanchis-Segura and Becker 2016); these differences may also be influenced by the reasons behind why each drinks, with women more likely to drink in response to negative emotions and men drinking to enhance positive (Becker and Koob 2016), but there are certainly genetic components as well.

Animal studies have uncovered sex differences in alcohol consumption and related behaviors, implying that not all differences seen between human men and women can be accounted for via outside influences. Specifically, female mice tend to drink more than males, and unlike humans, male mice will experience greater withdrawal symptoms than will their female counterparts (Barker, Torregrossa et al. 2010, Becker and Koob 2016). Some alcohol-related behaviors have been studied using the four core genotype

mouse model, which allows a dissection of chromosomal and gonadal sex effects, and show differential results depending on the specific behavior. Alcohol intake, for instance, is predicted by gonadal sex independent of chromosomal sex, with males consuming more than females, whereas habitual responding for alcohol depends mainly on the chromosomal sex, with XY switching from goal-directed to habitual responding faster than XX (Barker, Torregrossa et al. 2010). This switch, representing the progression from casual use into the addiction cycle, also reflects a transition from the ventral striatal reward pathway into the dorsolateral striatal areas (Barker, Torregrossa et al. 2010) and suggests sex differences in the formation of addiction may involve the transition out of this reward-driven intoxication/binge stage.

Dopamine

As previously mentioned, all drugs of abuse activate the reward pathway, specifically causing an increase in DA release in the NAc, which is a hallmark of drug reward. Increased NAc DA following alcohol has been shown via micro-dialysis in animals (Gremel and Cunningham 2008, Burkhardt and Adermark 2014). In humans, this presumed increase in DA release correlates with self-reported euphoria following alcohol (Soderpalm and Ericson 2013). Once released in the NAc, the DA binds to various DAergic receptors, causing the downstream drug-related behaviors. If these receptors are unavailable, either antagonized, knocked out, or knocked down, alcohol consumption decreases, alcohol-induced psychomotor stimulation is reduced, and alcohol reward is

attenuated (Bahi and Dreyer 2012, Soderpalm and Ericson 2013). A genetic variation in the D1 subtype dopamine receptor (D1R) can affect alcohol consumption in non-human primates, and levels of NAc DA are decreased in alcohol-preferring mice versus non-preferring, as well as in human alcoholics versus non-alcoholics (Bahi and Dreyer 2012, Soderpalm and Ericson 2013, West, Boss-Williams et al. 2015). In humans, this may predispose abusers to addiction, but it may also be a result of chronic, high-dose alcohol consumption, which has been shown to reduce the number of D1Rs in rodent midbrain and to decrease DA to below the baseline (Ericson, Molander et al. 2003, Bahi and Dreyer 2012, Soderpalm and Ericson 2013).

The increase in DA following alcohol is due to an increase in activation and/or firing rate of DAergic neurons in the VTA that project to the NAc, which has been shown following a dose of alcohol that elicits a reward response in rodents (Doyon, York et al. 2003, Ericson, Molander et al. 2003, Gremel and Cunningham 2008, Hendrickson, Zhao-Shea et al. 2009, Adermark, Clarke et al. 2011, Liu, Hendrickson et al. 2013, Soderpalm and Ericson 2013, Burkhardt and Adermark 2014, Schilaty, Hedges et al. 2014, West, Boss-Williams et al. 2015), but the way in which it activates this pathway remains unknown. Although there is some evidence of direct activation of DAergic neurons via ethanol (Burkhardt and Adermark 2014), most studies agree that the activation is more likely indirect, with ethanol potentiating the response to some other transmitter such as serotonin (Jerlhag, Grotli et al. 2006, Morikawa and Morrisett 2010, Soderpalm and

Ericson 2013) or acetylcholine (Marszalec, Aistrup et al. 1999, Liu, Zhao-Shea et al. 2013, Soderpalm and Ericson 2013, Burkhardt and Adermark 2014). Infusion of ethanol directly into the VTA of rodents leads to an increase in both serotonin and DA, as does inhibition of serotonin reuptake, and serotonergic agonists potentiate the DA increase in response to ethanol (Morikawa and Morrisett 2010). Serotonergic antagonists have the opposite effect, blocking the alcohol-induced increase in NAc DA (Jerlhag, Grotli et al. 2006). With serotonergic receptors present on VTA DA terminals, it is likely that alcohol acts directly on them, making serotonergic antidepressents, specifically specific serotonin reuptake inhibitors (SSRIs), potentially viable for treatment of alcohol use disorders (Jerlhag, Grotli et al. 2006, Morikawa and Morrisett 2010). Acetylcholine (ACh) may also be involved in the alcohol-induced increase in NAc DA, with alcohol unable to increase the firing rate of these DA neurons in the absence of ACh (Marszalec, Aistrup et al. 1999, Liu, Zhao-Shea et al. 2013, Soderpalm and Ericson 2013, Burkhardt and Adermark 2014). Additionally, alcohol has been shown to increase ACh levels in the VTA in parallel with the increased DA in the NAc, suggesting a potential causal relationship (Ericson, Molander et al. 2003, Soderpalm and Ericson 2013). Similarly to serotonin receptors, antagonists of ACh receptors block various alcohol-related molecular and behavioral outcomes, further suggesting a role for ACh.

Acetylcholine and Nicotinic Acetylcholine Receptors

The involvement of ACh in the activation of the reward pathway following alcohol administration is supported by studies showing that mecamylamine, a non-selective antagonist for one class of cholinergic receptors, blocks alcohol-induced activation of the VTA, the resulting increase in NAc DA, and blocks various alcohol-related behaviors in rodents (Hendrickson, Zhao-Shea et al. 2009, Burkhardt and Adermark 2014). The receptors involved, according to the mecamylamine data, are nicotinic acetylcholine receptors (nAChRs), which are located not only in the reward pathway and much of the rest of the brain, but are also on DA and GABA neurons within the VTA (Bowers, McClure-Begley et al. 2005, Morikawa and Morrisett 2010, Hendrickson, Guildford et al. 2013, Schilaty, Hedges et al. 2014, Ngolab, Liu et al. 2015). As the name implies, these receptors are targets of nicotine, the addictive compound in tobacco. These ligand gated cation channels are activated not only be exogenous nicotine, but by endogenous ACh as well. Once ligand is bound, the receptor is activated, and the channel opens, allowing Na+ and Ca2+ to move into the cell along their gradient. The receptor can also exist in two other states: closed, when the receptor is at rest, and desensitized, when the receptor is unresponsive to ligand (Dani and Bertrand 2007). The channel itself is formed by the second of five transmembrane domains in each of five subunits, making the receptor pentameric. The five subunits can be identical,

(homomeric), or can contain a mixture of α and β subunits (heteromeric). Currently, there are twelve known neuronal nAChR subtypes, α2-10 and β2-4. High affinity receptors are heteromeric, and the most ubiquitous of these is the α4β2-containing (α4β2*, where the * indicates the possible presence of other subunits) (Dani and Bertrand 2007). Homomeric receptors, made only of α subunits, are lower affinity, and generally are formed by α7, α9, and α10 (Dani and Bertrand 2007). The subunits included in the nAChR are referred to as the nAChR subtype, and the subtypes each have their own pharmacological and biophysical properties. **(Figure 1.1)**

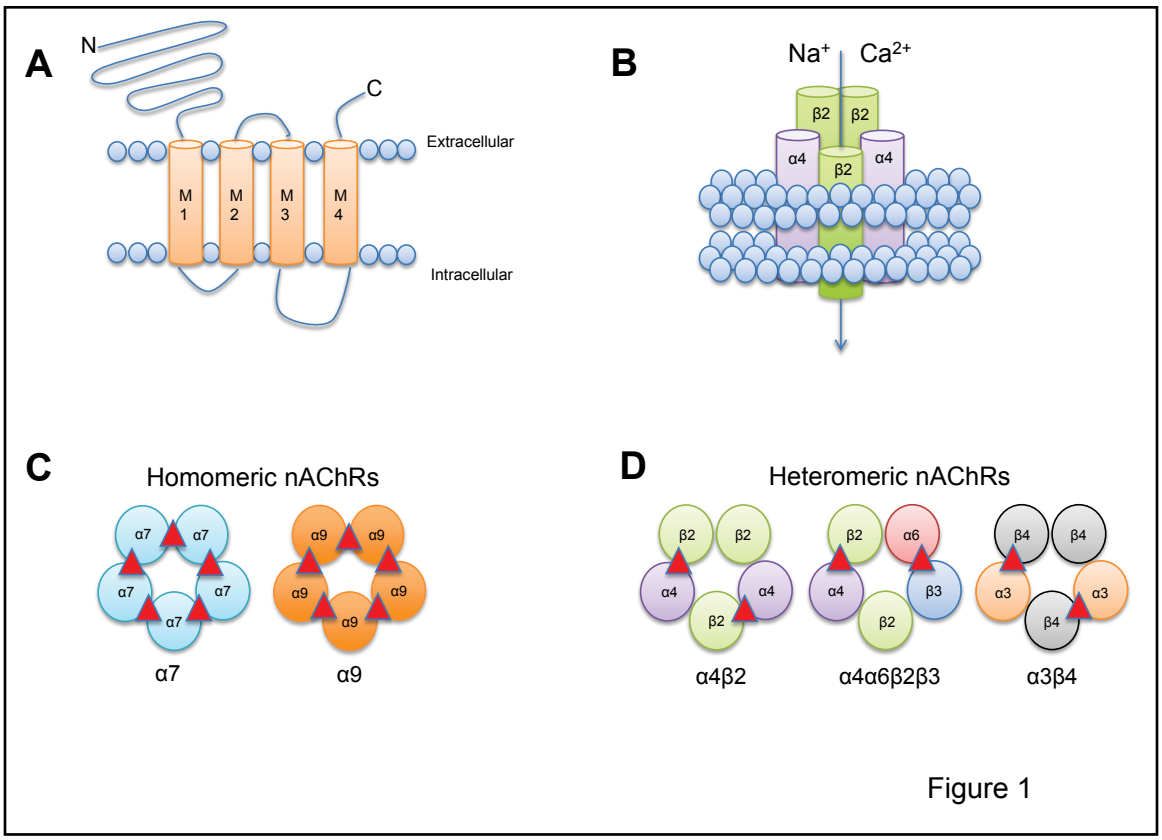

Figure 1

Figure 1.1 Neuronal nAChR Structure. A. Membrane topology of a neuronal nAChR subunit. Each nAChR subunit contains four transmembrane domains (M1-M4), an extracellular amino- and carboxy-terminus, and a prominent M3-M4 intracellular loop of variable length. B. Five subunits coassemble to form a functional subunit. C. Homomeric receptors consist of α subunits only and usually have low affinity for agonist. To date, only mammalian α7, α9, and α10 (not shown) subunits may form functional homomers. D. The majority of high affinity nAChRs are heteromeric and consist of a combination of α and β subunits. Importantly, multiple α subunits have been shown to coassemble with multiple β subunits in the pentameric nAChR complex (illustrated here by α4α6β3β2). ACh binding sites are depicted as red triangles. From (Hendrickson, Guildford et al. 2013)

The potential overlapping of alcohol and nicotine targets is not unexpected, as nicotine and alcohol are the most co-abused drugs, with nearly 90% of alcoholics also being addicted to nicotine, and 60-80% of smokers identifying as alcoholics as well (Bowers, McClure-Begley et al. 2005, Jerlhag, Grotli et al. 2006, Kuzmin, Jerlhag et al. 2009, Lajtha and Sershen 2010, Chatterjee, Steensland et al. 2011, Hendrickson, Guildford et al. 2013). Addiction to each of the two drugs is a risk factor for the other, and although this could be due to the same population being vulnerable to any type of reward or drug of abuse, there could also be an interaction of the drugs or a genetic predisposition to both (Kamens, McKinnon et al. 2009, Lajtha and Sershen 2010, Kamens, Hoft et al. 2012).

Nicotine and Alcohol Interactions

Table 1.1 and the following text excerpt are from and article previously published in:
Hendrickson, L.M., M.J. Guildford, and A.R. Tapper, *Neuronal nicotinic acetylcholine receptors: common molecular substrates of nicotine and alcohol dependence.* Front Psychiatry, 2013. **4**: p. 1-16. doi: 10.3389/fpsyt.2013.00029.

Human studies have shown that individuals dependent on alcohol have higher rates of nicotine dependence (Hughes 1996, Room 2004), and smokers tend to consume more ethanol than non-smoking alcohol users (York and Hirsch 1995). Unlike the majority of clinical studies, nicotine administration can either increase ethanol intake (Potthoff, Ellison et al. 1983, Blomqvist, Ericson et al. 1996, Smith, Horan et al. 1999, Le, Corrigall et al. 2000, Clark, Lindgren et al. 2001, Ericson, Molander et al. 2003), or decrease ethanol intake (Nadal, Chappell et al. 1998, Dyr, Koros et al. 1999, Sharpe and Samson 2002) in rats **(Table 1.1)**. These conflicting results have led to a complex and interesting questions: under what conditions (i.e. time delay between nicotine and ethanol, dose of nicotine, length of ethanol presentation, acute versus chronic nicotine/ ethanol etc.) does nicotine increase ethanol intake, and what conditions cause a decrease in ethanol intake?

Table 1.1 Neuronal nAChR ligands that modulate alcohol behaviors

Table 1 | Neuronal nAChR ligands that modulate alcohol behaviors.

Drug	nAChR subtype target	Route of delivery	Effect on ethanol behavior (in rodents)
Mecamylamine	Non-selective antagonist	i.p.	Decreased ethanol intake in rats (Blomqvist et al., 1996)
		i.p.	Decreased ethanol intake in mice (Hendrickson et al., 2009)
		i.p.	Blocked ethanol-induced DA release in NAc in rats (Blomqvist et al., 1993)
		i.p.	Partially counteracted ethanol-induced enhancements of locomotor activity and brain DA turnover in mice (Blomqvist et al., 1992)
		i.p.	Blocked ethanol-induced activation of DA neurons in mice (Hendrickson et al., 2009)
		i.p.	Reduced operant self-administration and blocked deprivation-induced increase in alcohol consumption in rats (Kuzmin et al., 2009)
		VTA	Reduced ethanol-induced accumbal DA release in rats (Ericson et al., 1998)
		i.p.	Reduced ethanol intake in rats (Le et al., 2000)
Nicotine	Agonist	s.c. (chronic)	Increased ethanol intake in rats (Potthoff et al., 1983; Le et al., 2000)
		s.c. (subchronic/acute)	Increased ethanol intake in rats (Blomqvist et al., 1996; Le et al., 2000)
		s.c. (subchronic)	Increased ethanol preference in rats (Blomqvist et al., 1996)
		s.c. (acute)	Enhanced ethanol-induced locomotor stimulation in mice (Blomqvist et al., 1992)
		s.c. (subchronic)	Enhanced ethanol-induced locomotor stimulation in rats (Blomqvist et al., 1996)
		s.c. (subchronic)	Enhanced DA turnover-increasing effect of ethanol in rats (Johnson et al., 1995)
		s.c. (chronic)	Decreased ethanol intake in rats (Sharpe and Samson, 2002)
		s.c. (chronic)	Decreased ethanol seeking in rats (Sharpe and Samson, 2002)
		i.p. (acute)	Decreased ethanol intake in mice (Hendrickson et al., 2011)
Varenicline	α4β2 Partial agonist high effinity α3β2, α3β4, α6*, α7 low affinity binding	i.p. and VTA	Decreased ethanol intake in mice (Hendrickson et al., 2010; Kamens et al., 2010; Santos et al., 2012)
		i.p.	Decreased ethanol intake in rats (Steensland et al., 2007)
		i.p.	Reduced ethanol seeking and consumption with no rebound increase in ethanol after cessation in rats (Steensland et al., 2007)
		i.p.	Reduced operant ethanol self-administration and blocked deprivation-induced relapse-like consumption in rats (Kuzmin et al., 2009)
		s.c.	Blocks increase in extracellular DA in NAc following acute ethanol injection in rats (Ericson et al., 2009)
α-Conotoxin MII	α6*, α3β2* Antagonist	VTA	Reduced alcohol-induced DA release in mice (Larsson et al., 2004)
		VTA	Reduced locomotor stimulation in mice (Larsson et al., 2004)
		VTA	Decreased self-administration of ethanol in rats (Kuzmin et al., 2009)
		VTA	Blocked deprivation-induced relapse-like ethanol consumption in rats (Kuzmin et al., 2009)
DHβE	α4β2* antagonist	s.c.	No effect on ethanol consumption in rats (Le et al., 2000)
		s.c.	No effect on DA-enhancing effect of ethanol in mice (Larsson et al., 2002)
		i.p.	Inhibited ethanol intake at 4mg/kg in rats (Kuzmin et al., 2009)
		s.c.	No effect on ethanol consumption in rats (Chatterjee et al., 2011)
MLA	α7* antagonist	i.p.	No effect on DA-enhancing effect of ethanol in mice (Larsson et al., 2002)
		i.p.	No effect on self-administration of ethanol or deprivation-induced relapse-like drinking in rats (Kuzmin et al., 2009)
		i.p.	No effect on ethanol consumption in DID in mice (Hendrickson et al., 2009)
α-Conotoxin PIA	α6* antagonist	VTA	No effect on ethanol-induced locomotor stimulation or enhanced DA release in mice (Jerlhag et al., 2006)

CP-601932	α3β4 and α4β2 high affinity partial agonist	s.c.	Decreased ethanol consumption and operant self-administration in rats (Chatterjee et al., 2011)
PF-4575180	α3β4 high affinity partial agonist	s.c.	Decreased ethanol consumption and operant self-administration in rats (Chatterjee et al., 2011)
Lobeline	Non-selective antagonist, particularly at $\beta2^*$ nAChRs	s.c. s.c.	Reduced ethanol consumption in DID and during continuous ethanol access in mice (Farook et al., 2009b; Sajja and Rahman, 2011) Reduced ethanol-induced DA and its metabolite levels in ventral striatum in mice (Sajja et al., 2010)
Cytisine	Low-efficacy partial agonist with high affinity for $\alpha4\beta2^*$ nAChRs. Full agonist at $\beta4^*$ and $\alpha7^*$ nAChRs	s.c. s.c.	Reduced ethanol consumption in DID in mice and during continuous ethanol access in mice (Hendrickson et al., 2009; Sajja and Rahman, 2011) Reduced ethanol-induced DA and its metabolite in mice (Sajja et al., 2010)
Sazetidine-A	Highly selective α4β2 desensitizer	s.c.	Reduces alcohol intake in rats (Rezvani et al., 2010)

Blomqvist et al. demonstrated that daily nicotine during ethanol deprivation and ethanol reinstatement increases ethanol intake and preference in rats shown to have a medium baseline preference (25-65%) for ethanol over water (Blomqvist, Ericson et al. 1996). Similarly, Le et. al. demonstrated that rats increased lever presses for ethanol during the course of daily nicotine injection paired 15 min prior to an operant session (Le, Wang et al. 2003). These data are in agreement with various other experiments in which nicotine was given either constantly or repeatedly (Potthoff, Ellison et al. 1983, Smith, Horan et al. 1999, Ericson, Engel et al. 2000, Olausson, Ericson et al. 2001). In rats, nicotine can also reinstate alcohol seeking after extinction and increase ethanol self-administration when administered during an ethanol deprivation period (Lopez-Moreno, Trigo-Diaz et al. 2004). Interestingly, rats given nicotine only during the relapse period,

once self-administration has resumed after a deprivation period, consume less ethanol, and rats given nicotine during both abstinence and relapse increased ethanol intake compared to control (Alen, Gomez et al. 2009).

In contrast, Sharpe and Samson (2002) demonstrated that ethanol intake and lever pressing during operant ethanol self-administration are both decreased after a high dose of nicotine (0.7 mg/kg, subcutaneous injection (s.c.), expressed as free base nicotine) 30 minutes prior to ethanol self-administration, and with a lower dose of nicotine (0.35 mg/ kg, s.c.). While locomotor depression by nicotine could potentially confound the interpretation of decreased ethanol self-administration, this is unlikely as nicotine injections did not decrease sucrose self-administration. Thus, Sharpe and Samson propose that nicotine could be acting as a reinforcer of ethanol, decreasing the amount of ethanol necessary to achieve satiety (Sharpe and Samson 2002). This is in agreement with other studies in which nicotine is administered either immediately prior to, or within 30 minutes of, ethanol presentation or self-administration (Nadal, Chappell et al. 1998, Damaj 2001).

To reconcile differences in nicotine effects on ethanol consumption and self-administration, Hauser et al. demonstrated that acute nicotine administration affects ethanol seeking and relapse in a time-dependent manner. Nicotine injection immediately prior to an ethanol operant self-administration session in ethanol preferring female rats elicits reduced responding for ethanol compared to a saline injection, whereas nicotine

exposure 4 hrs prior will increase responses (Hauser, Getachew et al. 2012). These data indicate that acute nicotine may initially act as a substitute for ethanol at the immediate time-point causing a reduction in craving for ethanol and, at the later time-point, nicotine may lead to desensitization of nAChRs in the brain, enhancing ethanol seeking.

As in rats, acute nicotine immediately prior to presentation of ethanol in the DID paradigm reduces consumption in mice (Hendrickson, Zhao-Shea et al. 2009), whereas chronic nicotine treatment increases consumption (Sajja and Rahman 2012). The reduction of ethanol consumption is mediated by nAChRs containing the α4 subunit: nicotine fails to reduce consumption in α4 KO mice; whereas acute sub-threshold nicotine doses are sufficient to reduce consumption in Leu9′Ala mice (Hendrickson, Gardner et al. 2011). Acute nicotine activates the posterior VTA as measured by increased c-Fos in mouse VTA DAergic neurons while co-injection of ethanol does not further activate these neurons, consistent with nicotine substituting for ethanol during this treatment schedule (Hendrickson, Zhao-Shea et al. 2009).

The mechanistic basis of chronic nicotine on ethanol consumption is unclear. However, nicotine potentiates the response to ethanol in VTA DAergic neurons in male and female rodents (Clark and Little 2004, Ding, Katner et al. 2012). These data indicate that chronic nicotine treatment may actually increase the reinforcing/rewarding properties of alcohol.

nAChR Subtypes Involved in Alcohol Behaviors

Figure 1.2 and the following text excerpt are from an article previously published in:
Hendrickson, L.M., M.J. Guildford, and A.R. Tapper, *Neuronal nicotinic acetylcholine receptors: common molecular substrates of nicotine and alcohol dependence.* Front Psychiatry, 2013. **4**: p. 1-16. doi: 10.3389/fpsyt.2013.00029.

Studies in VTA responses to *nicotine* indicate that DAergic neurons contain several nAChR subtypes including α4β2*, α4α5β2*, α4α6β2*, α6β2*, α3β2*, and α7 (Picciotto, Zoli et al. 1998, Champtiaux, Han et al. 2002, Marubio, Gardier et al. 2003, Grady, Salminen et al. 2007, Gotti, Guiducci et al. 2010, Zhao-Shea, Liu et al. 2011, Liu, Zhao-Shea et al. 2012) **(Figure 1.2)**. Identifying the precise subunit composition of nAChRs involved in ethanol consumption and activation of VTA DAergic neurons is challenging due to the sheer number of potential subunit combinations that may be expressed in the VTA. However, identifying one or more nAChR subtypes involved in ethanol activation of VTA and/or reward may lead to novel targets to reduce consumption.

Systemic injection or VTA infusion of the competitive α4β2* nAChR antagonist, dihydro-β-erythroidine (DHβE), in rats, fails to reduce ethanol-mediated DA release in the NAc and ethanol intake (Ericson, Molander et al. 2003, Chatterjee, Steensland et al. 2011). In addition, low doses of DHβE also have little effect on operant responding for ethanol in rats, although a higher dose can reduce responding (Kuzmin, Jerlhag et al. 2009). Systemic injection of DHβE does not reduce consumption in mice as measured in

the DID assay nor ethanol-induced NAc DA release (Larsson, Svensson et al. 2002, Hendrickson, Zhao-Shea et al. 2009). Together these data suggest that α4β2* nAChRs may not be critical for ethanol reward and consumption behavior. However, sensitivity of α4β2* nAChR blockade by DHβE is dependent on the stoichiometry of the receptor and the expression of other non-α4β2 subunits that may also be present in an α4β2* nAChR complex (Harvey and Luetje 1996, Harvey, Maddox et al. 1996, Le, Corrigall et al. 2000, Larsson, Svensson et al. 2002, Ericson, Molander et al. 2003, Moroni, Zwart et al. 2006, Lof, Olausson et al. 2007, Kamens and Phillips 2008).

The α7 selective antagonist, methyllycaconitine (MLA), does not affect ethanol-mediated behaviors including consumption, ethanol-induced DA release in NAc and ethanol operant responding in rats, or consumption in mice (Larsson, Svensson et al. 2002, Hendrickson, Zhao-Shea et al. 2009, Kuzmin, Jerlhag et al. 2009). While caution with interpretation of these results is warranted due to data indicating higher concentrations of MLA may also antagonize non-α7 nAChRs (of an unknown nAChR subtype that may include α6 and/or α3 subunits (Mogg, Whiteaker et al. 2002)), homomeric α7 nAChRs may not be involved in ethanol reinforcement (Larsson, Svensson et al. 2002, Hendrickson, Zhao-Shea et al. 2009, Kuzmin, Jerlhag et al. 2009).

On the other hand, the α3β2*, β3*, and α6* subtype-selective antagonist, α-conotoxin MII (Cartier, Yoshikami et al. 1996), when infused into the VTA does inhibit ethanol consumption, operant responding, and DA release in the NAc of rats (Larsson,

Jerlhag et al. 2004, Larsson, Edstrom et al. 2005, Kuzmin, Jerlhag et al. 2009) and reduce ethanol-induced locomotor stimulation and increases in NAc DA release in mice (Larsson, Jerlhag et al. 2004, Jerlhag, Grotli et al. 2006). Importantly, approximately half of α-conotoxin MII-sensitive nAChRs in the striatum also contain the α4 subunit (Grady, Salminen et al. 2007, Salminen, Drapeau et al. 2007) and deletion of β2* nAChRs nearly abolishes α-conotoxin MII binding in the VTA (Marubio, Gardier et al. 2003). However, infusion of α-conotoxin PIA, a version of the toxin which may have more selectivity for α6* nAChRs than α3* nAChRs (Dowell, Olivera et al. 2003), failed to reduce ethanol induced DA release in NAc when infused in the VTA suggesting that α3* nAChRs may be more critical for ethanol reward (Jerlhag, Grotli et al. 2006). Finally, systemic injection of the α3β4* nAChR selective antagonist 18-methoxycoranaridine (18-MC) reduces ethanol consumption in alcohol-preferring rats (Rezvani, Overstreet et al. 1997). However, direct infusion of 18-MC into the VTA fails to reduce alcohol consumption (Carnicella, He et al. 2010) in rats consistent with data indicating low expression of β4* nAChRs in VTA DAergic neurons (Gotti, Guiducci et al. 2010, Zhao-Shea, Liu et al. 2011).

A

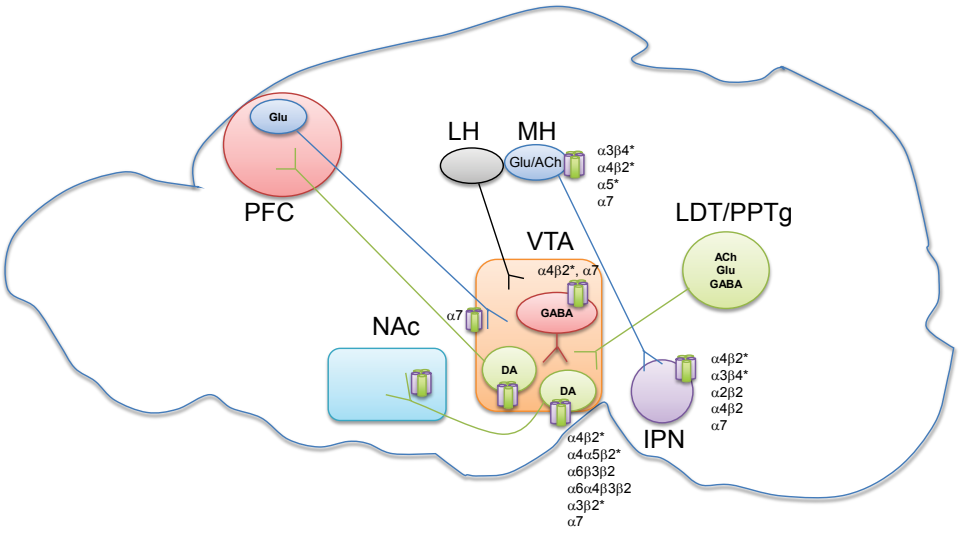

Figure 2A

B

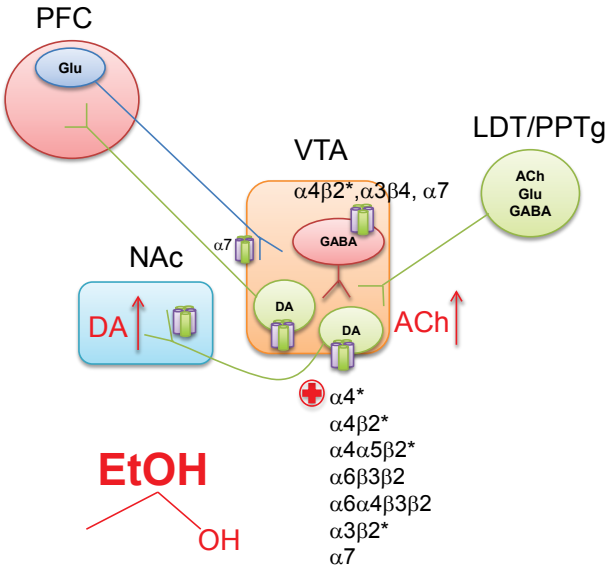

Figure 2B

Figure 1.2 Neuronal nAChR expression in the reward pathway. A. Sagittal rodent section illustrating the simplified mesocorticolimbic and habenulo-peduncular circuitry. Known neuronal nAChR subtypes expressed in different nuclei are indicated (for a review see (Millar and Gotti 2009)). B. In the VTA, alcohol stimulates DAergic neurons at least, in part, via nAChR activation. Ethanol increases ACh release (red arrow, presumably through cholinergic projection from the LDT/PPTg) which in turn activates nAChRs on DAergic neurons driving activity. In addition, ethanol potentiates ACh activation at high affinity α4β2* nAChRs (red plus sign). The effect of alcohol on additional nAChRs in the VTA is unknown. This confluence of events in combination with other effect of alcohol in the VTA ultimately increases DA release in NAc (red arrow). Ventral tegmental area (VTA); Nucleus accumbens (NAc); Prefrontal cortex (PFC); Lateral habenula (LH); Medial habenula (MH); Interpeduncular nucleus (IPN), Lateral dorsal tegmentum (LDT); Pedunculopontine tegmentum (PPTg). (Hendrickson, Guildford et al. 2013)

Thesis Overview

Given the unclear pharmacological data, the following chapters approach the mechanism of ethanol reward, and the role of nAChRs, from a genetic angle in order to elucidate more subunit-specific involvement. Chapters II-IV use mouse models in which a gene encoding a specific nAChR subunit has been knocked out. In Chapter II and IV the CHRNA4 gene, encoding the α4 nAChR subunit, is knocked out as described previously (Ross, Wong et al. 2000), with Chapter II focusing solely on the effect on males. In Chapters III and IV, CHRNA6, encoding the α6 nAChR subunit, is knocked out, as described by Champtiaux et al. (2002).

Preface to Chapter II

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Author Contributions:
L.L., L.M.H., M.J.G., R.Z-S., P.D.G. and A.R.T designed experiments
L.L. performed all electrophysiology experiments
L.M.H. performed BEC experiment
M.J.G. performed CPP and Locomotor experiments
R.Z-S. performed immunofluorescence experiments
M.J.G. and A.R.T wrote the paper

CHAPTER II: Nicotinic Acetylcholine Receptors Containing the α4 Subunit Modulate Alcohol Reward

Abstract

Background: Nicotine and alcohol are the two most co-abused drugs in the world suggesting a common mechanism of action may underlie their rewarding properties. While nicotine elicits reward by activating ventral tegmental area (VTA) dopaminergic (DAergic) neurons via high affinity neuronal nicotinic acetylcholine receptors (nAChRs), the mechanism by which alcohol activates these neurons is unclear.

Methods: Because the majority of high affinity nAChRs expressed in VTA DAergic neurons contain the α4 subunit, we measured ethanol-induced activation of DAergic neurons in midbrain slices from two complementary mouse models, an α4 knock-out (KO) mouse line and a knock-in line (Leu9'Ala) expressing α4 subunit-containing nAChRs hypersensitive to agonist compared to wild-type (WT). Activation of DAergic neurons by ethanol was analyzed using both biophysical and immunohistochemical approaches in midbrain slices. The ability of alcohol to condition a place preference in each mouse model was also measured.

Results: At intoxicating concentrations, ethanol activation of DAergic neurons was significantly reduced in α4 KO mice compared to WT. Conversely, in Leu9'Ala mice, DAergic neurons were activated by low ethanol concentrations that did not increase activity of WT neurons. In addition, alcohol potentiated the response to ACh in DAergic neurons, an effect reduced in α4 KO mice. Paralleling alcohol effects on DAergic neuron activity, rewarding alcohol doses failed to condition a place preference in α4 KO mice, whereas a sub-rewarding alcohol dose was sufficient to condition a place preference in Leu9'Ala mice.

Conclusions: Together, these data indicate that nAChRs containing the α4 subunit modulate alcohol reward.

Introduction

As many as 88-96 % of alcoholics are also smokers and the majority of smokers (~60 %) binge drink or consume significant amounts of alcohol (Batel, Pessione et al. 1995, Hurt, Offord et al. 1996). These statistics suggest that the abusive properties of tobacco and alcohol may, at least partly, share a common mechanism of action. Alternatively, the effects of one drug may modulate the rewarding properties of the other. Indeed, within the mesocorticolimbic reward circuitry of the brain, both drugs stimulate dopaminergic (DAergic) neurons in the ventral tegmental area (VTA), ultimately

increasing dopamine (DA) release in the nucleus accumbens (NAcc), a phenomenon widely associated with drug reinforcement (Pidoplichko, DeBiasi et al. 1997, Rodd, Melendez et al. 2004, Okamoto, Harnett et al. 2006, Tsai, Zhang et al. 2009). However, while nicotine initiates activation of DAergic neurons by binding to and activating neuronal nicotinic acetylcholine receptors (nAChRs) (Pidoplichko, DeBiasi et al. 1997, Maskos, Molles et al. 2005), the mechanism by which alcohol activates DAergic neurons is unclear (McBride, Lovinger et al. 2004, Okamoto, Harnett et al. 2006, Dopico and Lovinger 2009).

Neuronal nAChRs are ligand-gated cation channels that, under normal conditions, are activated by the endogenous neurotransmitter, ACh (Tapper 2006, Albuquerque, Pereira et al. 2009). Twelve vertebrate genes encoding neuronal nAChR subunits have been identified (α2-α10, β2-β4) with five subunits coassembling to form a functional receptor (Laviolette and van der Kooy 2004, Albuquerque, Pereira et al. 2009). The majority of nAChRs with high affinity for agonist are heteromeric consisting of two or three α subunits co-assembled with two or three β subunits while a subset of low affinity receptors are homomeric, mostly consisting of α7 subunits (Albuquerque, Pereira et al. 2009).

While alcohol is not a direct agonist of nAChRs, it has been hypothesized that ethanol induces an increase in ACh release from lateral dorsal tegmentum (LDTg) cholinergic neuron input into the VTA which could potentially drive activation of

DAergic neurons through nAChRs (Larsson, Edstrom et al. 2005). Ethanol also potentiates the response to ACh for high affinity, but not low affinity nAChRs (Cardoso, Brozowski et al. 1999, Zuo, Kuryatov et al. 2002), but whether potentiation occurs in DAergic neurons is unknown. Systemic injection or VTA infusion of the non-selective nAChR antagonist mecamylamine reduces ethanol induced NAcc DA release, alcohol consumption and reinforcement in rodents (Blomqvist, Engel et al. 1993, Blomqvist, Ericson et al. 1997, Ericson, Blomqvist et al. 1998, Hendrickson, Zhao-Shea et al. 2009). Furthermore, mecamylamine has been shown to reduce the voluntary subjective euphoric effects of alcohol (Chi and de Wit 2003). More recently, the FDA approved smoking cessation drug, varenicline, can reduce alcohol consumption and seeking in rodents, partly via an α4* nAChR-dependent mechanism (* denotes that other subunits in addition to α4 are components of the functional receptors), and reduces consumption in heavy smoking alcoholics (Steensland, Simms et al. 2007, McKee, Harrison et al. 2009, Hendrickson, Zhao-Shea et al. 2010, Kamens, Andersen et al. 2010, Hendrickson, Gardner et al. 2011). While these data implicate a role for nAChRs in alcohol consumption and reinforcement, a direct involvement of nAChR function in alcohol-induced activation of VTA DAergic neurons has not been demonstrated.

We sought to test the hypothesis that nAChRs contribute to alcohol-induced activation of VTA DAergic neurons and that alcohol reward could be modulated via α4* nAChR activation.

Materials and Methods

Animals

C57BL/6J male mice (Jackson Laboratory, West Grove, PA, USA) were used in all experiments, in addition to α4 knockout (KO) homozygous mice, Leu9'Ala heterozygous and their respective wild-type (WT) littermates as indicated. All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council (National Research Council 1996), as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Slice preparation

Mice (4-6 weeks old) were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (200 mg/kg) and then decapitated. Brain slices were cut as previously described (Liu, Zhao-Shea et al. 2012).

Electrophysiological recordings

Individual slices were transferred to a recording chamber continually superfused with oxygenated ACSF (30–32 °C) at a flow rate of ~ 2 ml/min. Cells were visualized using infrared differential interference contrast (IR–DIC) imaging on an Olympus BX-50WI

microscope. Electrophysiological recordings were recorded using a Multiclamp 700B patch-clamp amplifier *(*Axon Instruments, Foster City, CA). For a detailed description of recording methodology, see supplemental materials and methods. *Immunohistochemistry*

Adult (8-10 weeks) male α4 KO mice and their WT littermates, as well as heterozygous Leu9′Ala mice and their WT littermates were i.p. injected with saline for three days prior to the start of the experiment to habituate them to handling and to reduce c-Fos activation due to stress. Mice were injected with ethanol and their brains were harvested and processed for immunohistochemistry 90 min post injection (see supplemental material and methods for details).

Conditioned Place Preference

The ethanol conditioned place preference (CPP) assay consisted of a three chamber apparatus (Med Associates). The two conditioning chambers were contextually distinct: One had white walls while the other had black walls and one chamber had mesh metal floors while the other had rod metal floors. The conditioning chambers were separated by a neutral grey chamber. Experiments were conducted over 6 consecutive days as in Gibb et al. (2011).

Ethanol metabolism

Prior to an ethanol injection, blood was obtained from the tail vein (~30 μL each time point) to provide a zero point for each animal. After a 2 g/kg i.p. injection of ethanol,

blood samples were taken at intervals of 30, 60, 90, and 120 min. Blood was collected in heparinized capillary tubes, centrifuged at 1500Xg for 5 minutes and blood analyzed using an alcohol oxidase-based assay. Blood ethanol concentrations were measured on a GM7 Micro-Stat Analyzer (Analox Instruments Ltd.)

Data analysis

AP spikes were detected using a threshold detection protocol contained within pClampfit (pClamp v10.2, Axon Inst., Molecular Devices). Average fold changes in AP frequency are presented as means ± standard errors of means (SEM). A Paired T-test was used to analyze differences between AP frequency at baseline (1 minute prior to drug application) and after a 5-min application of ethanol. Behavioral and immunohistochemistry data were analyzed using One-way or Two-way ANOVAs with genotype and treatment as variables followed by Bonferroni *post hoc* tests as indicated. Results were considered significant at $p < 0.05$. All data are expressed as means ± standard errors of means (SEM).

Results

Cell-attached patch clamp recordings were made from VTA DAergic neurons in C57BL/6J mouse slices. Slices were cut in the sagittal plane allowing for preservation of cholinergic input from LDTg into the VTA. To test the effects of ethanol on DAergic neuron activity, AP frequency was monitored in cell-attached mode at baseline, during

application of an intoxicating concentration of alcohol (100 mM), and after wash-out. Because the focus of our experiments was to uncover the contribution of nAChR activation in response to alcohol, recordings were made in the presence of a cocktail of inhibitors to block muscarinic receptor, AMPA receptor, and $GABA_A$ receptor activities (see methods). Five-minute bath application of 100 mM ethanol produced a significant increase in AP frequency (~33 % increase from baseline, **Fig 2.1A, D**) that was completely reversed upon wash out. To determine if the inhibitor cocktail affected the firing rates of DAergic neurons in response to alcohol, we measured alcohol responses in the absence of antagonists. Bath application of 100 mM ethanol produced a significant increase in AP frequency that was slightly larger than responses in the presence of the inhibitor cocktail but this increase was not statistically significant (**Fig. 2.1D**, bottom panel). Thus, the inhibitor cocktail was included in the remainder of slice physiology experiments. To test the hypothesis that activation of nAChRs is necessary for the observed ethanol-mediated increase in VTA DAergic neuron activity, we bath-applied 10 μM mecamylamine prior to and during application of ethanol. Mecamylamine alone did not affect baseline firing of VTA DAergic neurons (**Fig. 2.1B, D** top panel). However, in the presence of mecamylamine, alcohol failed to significantly increase DAergic neuron activity above baseline (**Fig. 2.1B, D**) indicating that nAChR activation is necessary for alcohol-induced activation of DAergic neurons.

To test the hypothesis that activation of low affinity α7 nAChRs were critical for the observed alcohol-induced increase in DAergic neuron activity, we bath-applied the α7 selective antagonist MLA (10 nM) prior to and during application of ethanol. MLA had little effect on baseline firing of DAergic neurons (**Fig. 2.1C, D** top panel). However, in contrast to mecamylamine, ethanol significantly increased DAergic neuron activity compared to baseline (~33 %) in the presence of MLA suggesting that nAChRs containing the α7 subunit do not contribute to ethanol-mediated activation of VTA DAergic neurons (**Fig. 2.1C, D**).

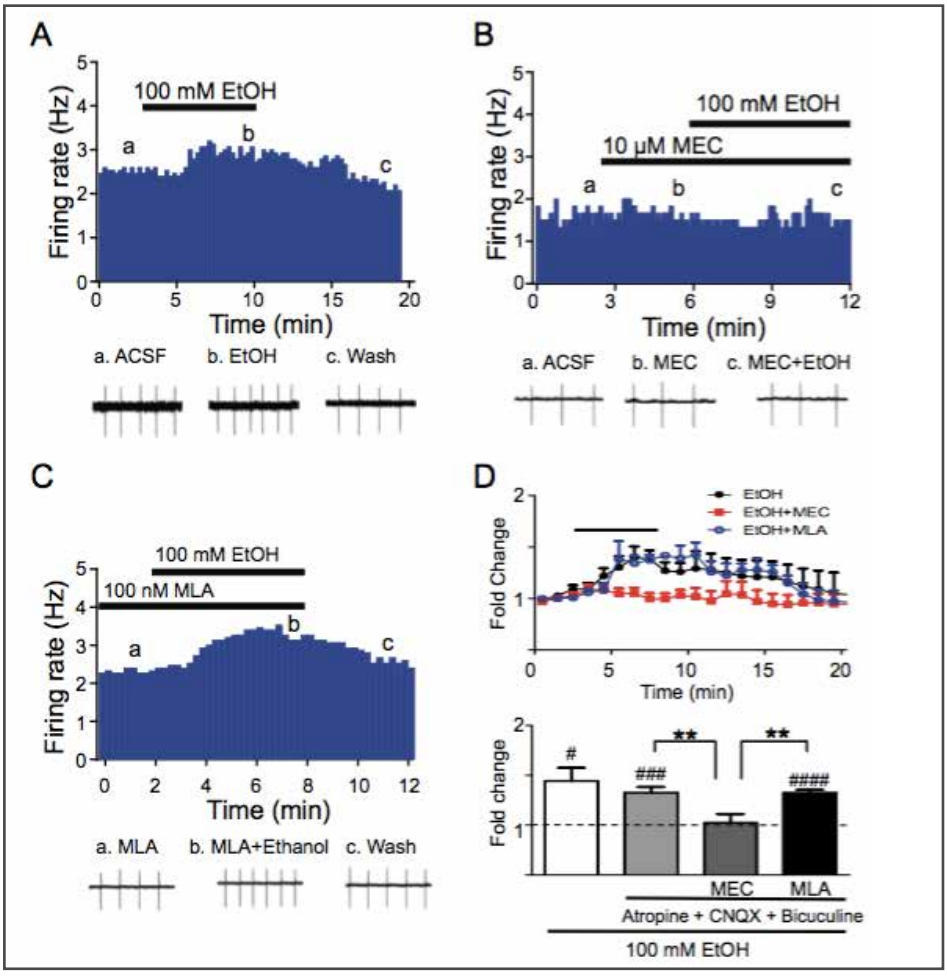

Figure 2.1 Ethanol activation of VTA DAergic neurons. A) Representative action potential firing frequency histogram from a VTA DAergic neuron before, during, and after 5-min bath application of 100 mM ethanol (EtOH) alone (n = 10) or in the presence of B) 10 μM mecamylamine (MEC, n = 7) or C) 100 nM MLA (n = 5). Action potentials were recorded in cell-attached mode. Representative action potential traces (top of each panel, a, b, c) are shown from the corresponding times on the histograms. D. Average time course of ethanol responses in each condition is shown (top panel). Each data point represents the average 1-min firing frequency normalized to baseline for each recording. The bar over the averaged frequency plot represents the duration of ethanol application. Recording times between groups were aligned based on time of ethanol application to facilitate comparison. Antagonists were applied at times indicated in the individual histograms as indicated in B and C. (Bottom panel) Fold-change in average firing frequency at baseline (1 min. prior to alcohol application, dotted line) compared to 5 min of ethanol application for each condition. ### $p < 0.001$, #### $p < 0.0001$, baseline frequency (1 min prior to drug application) compared to 5 min alcohol exposure, paired t-tests. ** $p < 0.01$, Students t-test (effect of ethanol or ethanol + MLA on AP frequency compared to ethanol + MEC). n = 6-10 neurons/condition. (Performed, analyzed by Liwang Liu)

Expression of α4 nAChRs modulates alcohol-induced activation of VTA DAergic neurons.* As previous studies indicate that the majority of high affinity nAChRs in VTA DAergic neurons express the α4 subunit and that nAChRs containing the α4 subunit are both necessary and sufficient for nicotine reward (Tapper, McKinney et al. 2004, Pons, Fattore et al. 2008, Zhao-Shea, Liu et al. 2011), we sought to investigate the contribution of α4* nAChRs to alcohol-induced activation of VTA DAergic neurons in two complementary mouse models, the α4 KO mouse which does not express α4* nAChRs (Ross, Wong et al. 2000) and a knock-in mouse line that expresses a single point mutation

(Leu9'Ala) in the α4 subunit that renders receptors containing the subunit hypersensitive to agonist (Tapper, McKinney et al. 2004, Fonck, Cohen et al. 2005).

No significant difference in baseline firing frequency of DAergic neurons was observed between WT and α4 KO animals (5.0 ± 1.8 and 4.0 ± 1.0 Hz, respectively) as reported previously (Zhao-Shea, Liu et al. 2011, Liu, Zhao-Shea et al. 2012). In WT mice, bath application of 50 or 100 mM alcohol significantly increased DAergic neuron activity (~23 % and ~33 % above baseline, Fig. 2D, top panel). In contrast, 50 mM ethanol did not significantly increase VTA DAergic neuron activity of α4 KO mice compared to baseline; whereas 100 mM ethanol elicited a modest increase (~10 % above baseline, Fig. 2A, C, D top panel) compared to baseline. This increase was significantly lower than the effect of 100 mM alcohol on WT DAergic neurons (**Fig. 2.2D** top panel).

There was no significant difference in baseline DAergic neuron firing rates between Leu9'Ala mice and their WT littermates. However, in contrast to α4 KO mice, Leu9'Ala DAergic neurons were robustly activated by 100 mM alcohol (**Fig. 2.2B, C, D** bottom panel). Whereas WT DAergic neurons responded to 100 mM alcohol by an increased firing rate of ~30 % compared to baseline, Leu9'Ala DAergic neurons increased ~60 %, and was significantly different from WT (**Fig. 2.2B, D** bottom panel). To test the hypothesis that low sub-activating alcohol concentrations are sufficient to increase VTA DAergic neuron firing rates in Leu9'Ala mice, DAergic activity was also recorded in response to 20 mM alcohol. At this concentration, alcohol did not significantly increase

VTA DAergic neurons firing rates compared to baseline in WT slices (**Fig. 2.2D** bottom panel). By contrast, 20 mM alcohol elicited a modest (~15 %), but significant increase in DAergic neuron activity in Leu9'Ala slices. Together, these data indicate that activation of α4* nAChRs can contribute to alcohol-induced activation of VTA DAergic neurons.

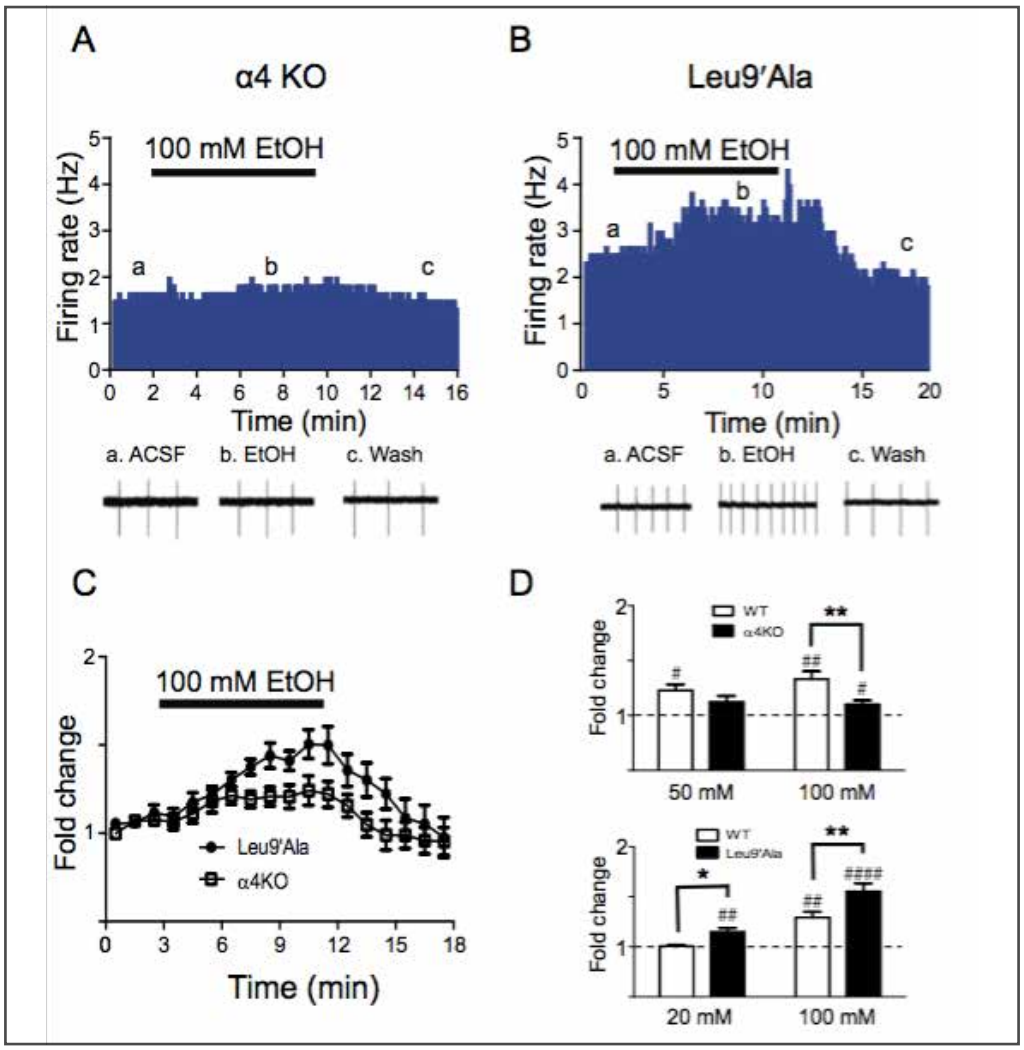

Figure 2.2 Functional α4* nAChR expression modulates DAergic neuron activation by ethanol. Representative action potential firing frequency histogram from a VTA DAergic neuron before, during, and after a 5-min bath application of 100 mM ethanol in sagittal midbrain slices from A) α4 KO and B) Leu9'Ala mice. Representative action potential traces (top of each panel, a, b, c) are shown from the corresponding times on the histograms. C) Time course of the effects of ethanol on average normalized frequency for each genotype are shown (n = 8-10 neurons/genotype). Ethanol (100 mM) was applied at the times indicated by the bar. D. Fold-change in average firing frequency in response to 100 mM ethanol in WT (n = 8) and α4 KO mice (n = 15), 20 mM in WT (n = 5) and Leu9'Ala mice (n = 8), and 100 mM ethanol in WT (n = 10) and Leu9'Ala mice (n = 13). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$, as in 2D. * $p < 0.05$, ** $p < 0.01$ response to alcohol compared between genotypes, One-way ANOVA, Bonferroni post-test. (Performed, analyzed by Liwang Liu)

Alcohol potentiates the response to ACh in DAergic neurons by an α4 nAChR-dependent mechanism.* To test the hypothesis that ethanol may potentiate the response to ACh in VTA DAergic neurons, we bath-applied ACh (300 μM) in the absence and presence of alcohol and measured effects on firing frequency. In WT mice, ACh alone elicited an increase in VTA DAergic neuron firing frequency that was significantly greater than baseline (~25 %, **Fig 2.3A, E, F**). Co-application of either 50 or 100 mM ethanol with ACh elicited a robust increase in firing frequency (~2 fold) which was significantly greater than the response of ACh alone and persisted for several minutes after application before returning to baseline (**Fig. 2.3B, E, F**). Pre-incubation of the slice with DHβE significantly reduced the response to ACh plus 50 mM ethanol (**Fig. 2.3C, E, F**). Finally, the effect of ACh plus 50 mM ethanol on DAergic neuron firing frequency was significantly reduced in slices from α4 KO mice compared to WT slices (**Fig. 2.3D, E,**

F). Together, these data indicate that alcohol potentiates the response to ACh at α4* nAChRs.

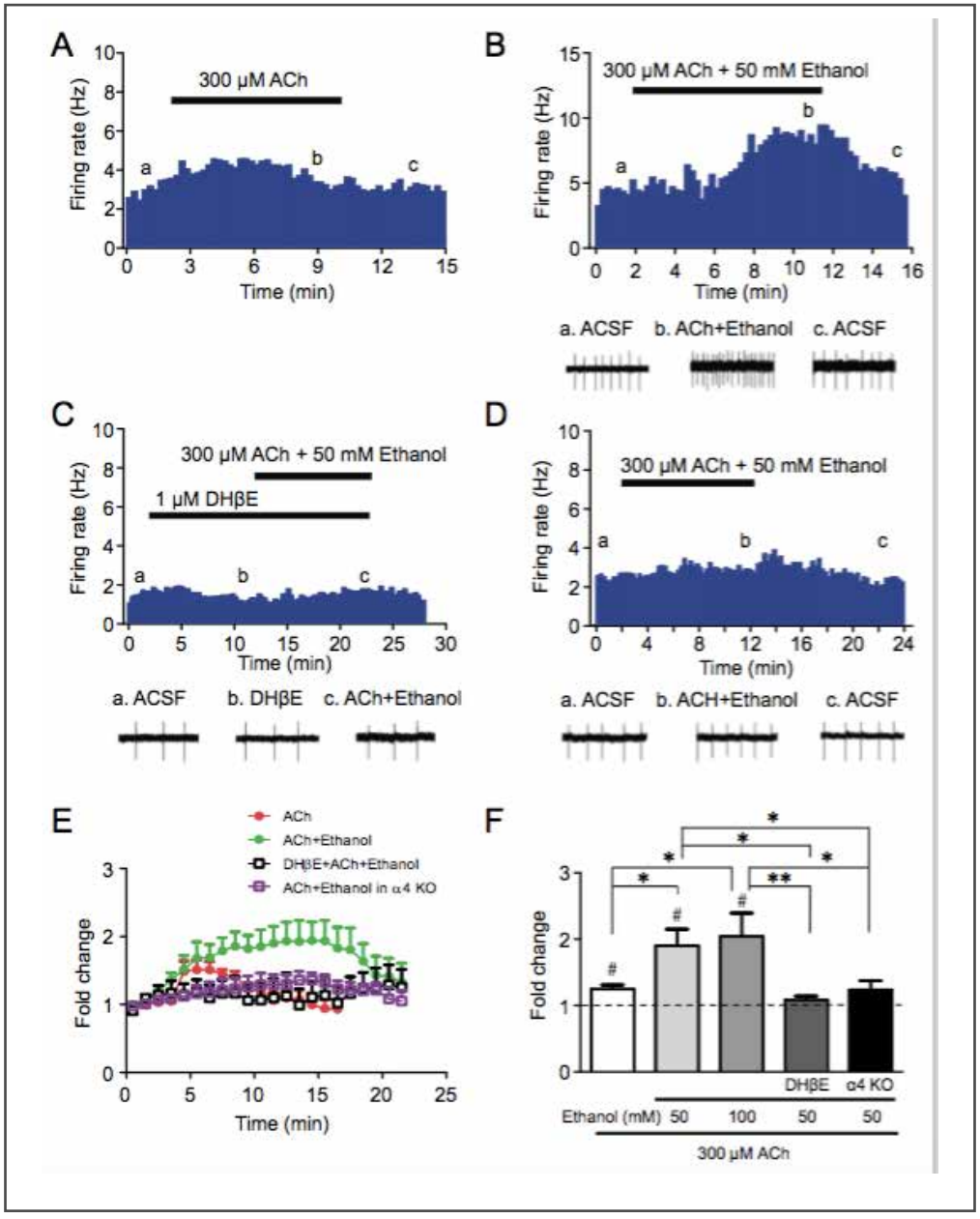

Figure 2.3 Alcohol potentiates the response of DAergic neurons to ACh. A) Representative action potential firing frequency histogram from a VTA DAergic neuron before, during, and after 10-min bath application of 300 μM ACh. Representative action potential traces (top of each panel, a, b, c) are shown from the corresponding times on the histograms. Representative action potential firing frequency histogram from a VTA DAergic neuron before, during, and after 10-min bath co-application of 300 μM ACh and 50 mM ethanol in the absence (B), or presence (C) of 1 μM DHβE. D) Representative action potential firing frequency histogram from an α4 KO VTA DAergic neuron before, during, and after 10 min bath co-application of 300 μM ACh and 50 mM ethanol. E) Time course of the effects of ethanol on average normalized frequency under each condition are shown (n = 6-12 neurons/genotype). ACh ± ethanol was applied at the times indicated by the bar. Recording times between groups were aligned based on time of ethanol application to facilitate comparison. F) Fold-change in average DAergic neuron firing frequency in response to 300 μM ACh alone (n = 6), in the presence of 50 (n = 12) or 100 mM (n = 5) ethanol, in the presence of 50 mM ethanol and DHβE (n = 6), or in the presence of 50 mM ethanol in α4 KO slices (n = 10). $^{\#}p < 0.05$ as in 1D. * $p < 0.05$ response to alcohol compared between treatments/genotypes.

Alcohol induction of c-Fos expression in VTA DAergic neurons requires functional expression of α4 nAChRs.* Previously, using c-Fos as a marker for neuronal activation and TH as a marker for DAergic neurons, we demonstrated that ethanol activates DAergic neurons of the VTA and that this activation can be blocked by a pre-injection of the nAChR antagonist mecamylamine (Hendrickson, Zhao-Shea et al. 2009). To determine if α4* nAChRs are necessary for this activation, we challenged WT and α4 KO mice with saline or 2.0 g/kg ethanol and examined their brains for c-Fos expression within TH(+) neurons 90 min. post-injection (**Fig. 2.4**). Because previous studies indicate alcohol induces c-Fos in DAergic neurons preferentially in the posterior VTA (Rodd, Bell et al. 2004, Hendrickson, Zhao-Shea et al. 2010), we focused on this region

for analysis. Overall there was a significant main effect of genotype ($F_{(1,8)}$ = 8.15, $p<0.05$), treatment ($F_{(1,8)}$ = 12.28, $p<0.01$) and a significant genotype × treatment interaction ($F_{(1,8)}$ = 13.25, $p<0.01$). Bonferroni post-test indicated a significant difference between number of TH(+), c-Fos(+) neurons in WT and α4 KO mice after 2.0 g/kg ethanol, but not saline (**Fig. 2.4A, B, C**). In addition, the % of TH(+) neurons that were also c-Fos(+) significantly differed between WT and α4 KO mice after 2.0 g/kg ethanol, but not saline (**Table 2.1**). WT mice injected with 2.0 g/kg ethanol had significantly higher expression of c-Fos compared to α4 KO mice injected with 2.0 g/kg ethanol (**Fig 2.4C**). One-way ANOVA also indicated that WT mice treated with 2.0 g/kg ethanol had significantly increased c-Fos expression compared to a saline injection (**Fig 2.4C**), whereas the same dose of ethanol had no effect in α4 KO mice compared to a saline injection.

To determine if increasing α4* nAChR agonist sensitivity resulted in activation of VTA DAergic neurons with lower doses of alcohol, we challenged WT and Leu9'Ala mice with two concentrations of ethanol, 2.0 g/kg and a low dose of 0.5 g/kg, and analyzed their brains for c-Fos expression in TH(+) neurons as above. Two-Way ANOVA revealed a significant main effect of treatment ($F_{(2,14)}$ = 22.01, $p < 0.001$), genotype ($F_{(1,14)}$ = 11.65, $p < 0.01$) and a significant treatment × genotype interaction ($F_{(2,14)}$ = 8.97, $p < 0.01$). A Bonferroni post-test indicated that Leu9'Ala mice treated with 0.5 g/kg had significantly increased number of TH(+), c-Fos(+) neurons compared to WT mice (**Fig**

2.4D, E, F). The % of total TH(+) neurons that were also c-Fos(+) was increased in Leu9'Ala mice compared to WT after a 0.5 g/kg ethanol challenge (Data not shown). Additionally, one-way ANOVAs revealed that Leu9'Ala mice treated with 0.5 g/kg and 2.0 g/kg ethanol had significantly increased numbers of TH(+), c-Fos(+) neurons compared to a saline challenge while WT mice treated with 2.0 g/kg had significantly increased numbers of TH(+), c-Fos(+) neurons compared to both saline and 0.5 g/kg alcohol. Together, these data indicate that expression of α4* nAChRs are necessary for alcohol-induced activation of VTA DAergic neurons and that α4* nAChR activation controls DAergic neuron response to alcohol.

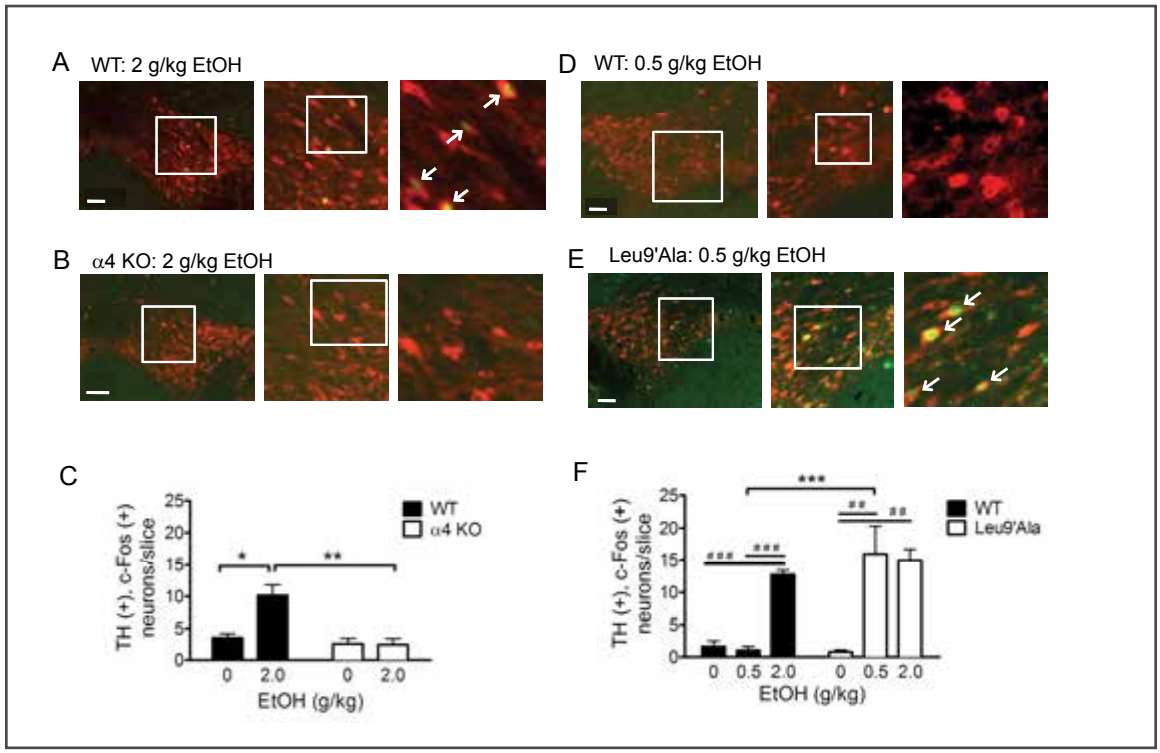

Figure 2.4 Ethanol-induced c-Fos expression in VTA TH(+) neurons is dependent on expression and activation of α4* nAChRs. Representative photomicrographs illustrating midbrain sections of the posterior VTA from A) WT mice and B) α4 KO mice injected with 2 g/kg ethanol. Sections were immunolabeled for TH (red) and c-Fos (green). White boxes delineate slice regions that are magnified in the adjacent photomicrographs. White arrowheads point to neurons that are TH (+), c-Fos (+). Scale bar = 100 μm. Merged images are shown. C) Number of TH (+) c-Fos (+) neurons per slice taken from mice given an i.p. injection of 2 g/kg ethanol. Forty-eight slices/ treatment/mouse were analyzed, n = 3 mice/treatment. D. Representative photomicrographs illustrating midbrain sections of the posterior VTA from WT mice and E) Leu9'Ala mice injected with 0.5 g/kg ethanol. Sections were immunolabeled for TH and c-Fos as in panels A and B. F) Average number of TH (+), c-Fos (+) neurons/slice calculated from mice given an i.p. injection of 0.5 g/kg or 2 g/kg ethanol. Forty-eight slices/treatment/mouse were analyzed, n = 3 mice/treatment. One-way ANOVA and Bonferroni post-test comparing saline to ethanol treatments in WT, α4 KO, or Leu9'Ala mice was used, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$. Two-way ANOVA and Bonferroni post-test comparing treatments in WT and α4 KO mice was also used, ** $p < 0.01$, ***$p<0.001$. (Performed and analyzed by Rubing Zhao-Shea and Linzy M. Hendrickson)

Functional expression of α4 nAChRs modulates alcohol reward.* As activation of VTA DAergic neurons is sufficient for reward (Tsai, Zhang et al. 2009), and both our physiology and immunohistochemical data indicate a role for α4* nAChRs in alcohol-induced activation of VTA DAergic neurons, we evaluated alcohol rewarding properties in α4 KO and Leu9'Ala mouse lines using the CPP assay, a robust behavioral assay of rewarding stimuli (Cunningham, Gremel et al. 2006). In C57BL/6J mice, the background strain for both α4 mouse models, 2.0 g/kg has been established as a rewarding dose of alcohol (Tzschentke 2007). Consistent with this observation, WT mice that received i.p. injections of 2.0 g/kg ethanol in the drug-paired chamber significantly preferred the alcohol-paired chamber over the saline-paired chamber after training compared to

baseline (expressed as the difference score, post-training minus pre-training for each chamber, $F_{(1, 48)} = 58.2$, $p < 0.001$, **Fig. 2.5A**). In addition, the total time spent in the alcohol-paired chamber after training was greater that the time spent in the same chamber before training (Table S2, $F_{(1, 48)} = 22.0$, $p < 0.001$). In α4 KO mice, there was a modest but statistically significant difference in the difference score between ethanol-paired and saline-paired chambers ($p < 0.05$, $F_{(1, 20)} = 5.6$, **Fig. 2.5A**). However, the ethanol difference score in α4 KO mice was significantly lower than the equivalent ethanol difference score in WT mice ($F_{(1, 34)} = 9.49$, $p < 0.01$). In addition, α4 KO mice did not spend significantly greater time in the alcohol-paired chamber during the test day compared to the pre-training, habituation day (**Table 2.1**, NS) indicating that this alcohol dose was weakly rewarding in these animals.

To test the hypothesis that Leu9'Ala mice are more sensitive to alcohol reward, we tested a sub-rewarding dose of alcohol, 0.5 g/kg, in these animals. In WT mice, 0.5 g/kg ethanol failed to condition a place preference (**Fig. 2.5A**, NS). However, Leu9'Ala mice significantly preferred the alcohol-paired chamber compared to the saline-paired chamber (**Fig. 2.5A**, $F_{(1,22)} = 8.57$, $p < 0.01$). Leu9'Ala mice also spent significantly more total time in the alcohol-paired chamber during the post-training test day compared to the pre-training habituation day, indicating a low dose of alcohol was rewarding in these animals (**Table 2.1**, $F_{(1,22)} = 8.33$, $p < 0.01$). In response to 2.0 g/kg alcohol, Leu9'Ala mice displayed a more modest preference for the ethanol-paired compared to the saline-paired

chamber ($F_{(1, 22)}$, $p < 0.05$). However, total time spent in the ethanol paired chamber after training did not reach significance compared to time spent in the alcohol-paired chamber during habituation. Thus, alcohol displayed an inverted "U" shaped dose response relationship in Leu9'Ala mice. As a negative control in Leu9'Ala mice we also measured time spent in the drug paired chamber in response to saline injections in both chambers during training. There was no significant preference for either chamber after training with saline injections nor was there a difference in time spent during the saline-paired chamber during the test day compared to the habituation day, indicating a specific effect of the low alcohol dose in these animals (**Fig. 2,5A, Table 2.1**).

Because genotype differences in locomotor activity could influence CPP results, we measured baseline locomotion in WT, α4 KO, and Leu9'Ala mice. No significant differences in activity were detected over the course of 30 min (**Fig. 2.5B**). Finally, differences in rewarding properties of alcohol between WT, α4 KO and Leu9'Ala mice could be a consequence of altered pharmacokinetics of ethanol between genotypes. To address this possibility, we measured blood ethanol concentrations (BEC) in WT, α4 KO, and Leu9'Ala mice every 30 min after an acute i.p. injection of 2.0 g/kg to monitor alcohol clearance (**Fig. 2.5C**). Two-Way ANOVA revealed an overall significant effect of time ($F_{(4,44)} = 139.0$, $p < 0.0001$) but not genotype on BEC. In addition, there was no significant time × genotype interaction. Together, these data indicate that activation of α4* nAChRs modulates alcohol reward.

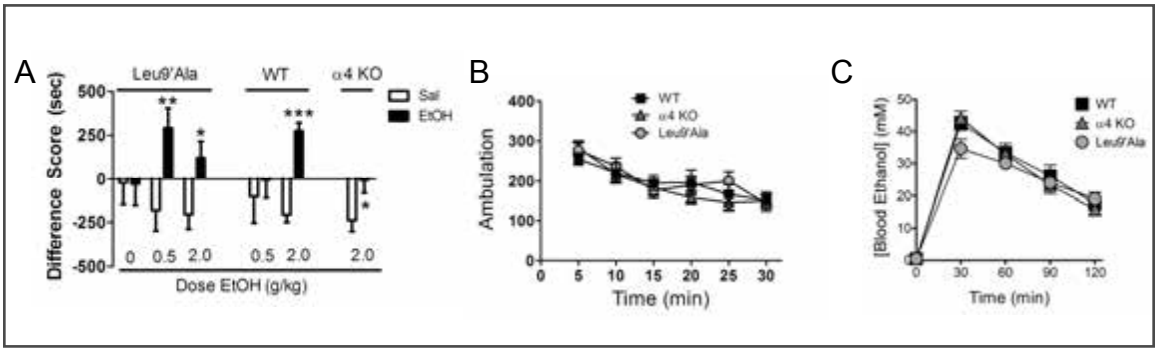

Figure 2.5 α4* nAChR expression modulates alcohol reward. A. Average difference score (test – baseline) in ethanol-paired (black bars) and saline-paired (white bars) chambers in Leu9'Ala, WT, and α4 KO mice in response to the alcohol doses indicated. Because each line has been back-crossed at least ten generations to the C57Bl/6J strain and no differences in alcohol responses between α4 KO and Leu9'Ala WT littermates were detected, WT mice were combined. n = 9 – 22 mice/dose. multiple t-tests * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to saline. B. Locomotor activity (ambulation) in WT, Leu9'Ala, and α4 KO mice. Each data point represents the average total locomotor activity over 5-min (n=5-10 mice/genotype). C. Blood ethanol concentration at 30-min intervals after an acute, 2.0 g/kg i.p. injection of alcohol in WT, α4 KO, and Leu9'ala mice (n = 3-4 mice/genotype).

Genotype (EtOH, g/kg)	SAL(Hab)	SAL(Test)	EtOH(Hab)	EtOH(Test)	Gray(Hab)	Gray(Test)
WT (0.5)	684.1±87.2	539.2±79.3	516.3±44.8	510±80.7	599.6±107.4	750.8±53.9
WT (2.0)	661.2±35.5	458.2±42.6	528.8±21.0	777.8±48.7***	610±31.9	563.9±48.5
α4 KO (2.0)	647.1±32.6	408.8±62.9	520.1±33.6	510.2±89.4	659.7±34.9	582.3±52.4
Leu9'Ala (Sal)	746.3±67.1	724.3±100.6	565.4±64.9	536.2±108.1	488.3±100.3	539.5±131.3
Leu9'Ala (0.5)	775.1±62.3	593.9±91.5	474.3±39.5	766.7±93.3**	550.5±39.8	439.2±33.2
Leu9'Ala (2.0)	569.5±41.1	365.2±80	417.3±40.3	538.2±99.9	760±85.4	834.1±128.5

KO, knock-out; SAL, saline; WT, wild-type.
** $p < 0.01$, *** $p < 0.001$ compared to time spent in EtOH-paired chamber during habituation.

Table 2.1 Total time spent (seconds) in saline-paired, ethanol-paired, and neutral (gray) chamber during habituation (Hab) and after training (Test) in WT, α4 KO, and Leu9'Ala mice.

Discussion

Alcohol and nicotine are often co-abused suggesting that they may share a common mechanism of action in the CNS. Here we show that, in VTA midbrain slices, alcohol significantly increased DAergic neuron activity, an effect that was blocked by mecamylamine indicating a critical role for nAChRs in alcohol-induced activation of these neurons. This observation is also in agreement with our previous data illustrating that mecamylamine prevents alcohol-induced c-Fos expression in VTA DAergic neurons after an alcohol challenge (Hendrickson, Zhao-Shea et al. 2009).

Previously, we demonstrated that VTA DAergic neurons that are activated by alcohol, robustly express α4, α6, and β3 nAChR subunits (Hendrickson, Zhao-Shea et al. 2010). In addition, the smoking cessation aid, varenicline, targets VTA α4β2* nAChRs to reduce alcohol consumption (Hendrickson, Zhao-Shea et al. 2010, Hendrickson, Gardner et al. 2011) although other nAChR subtypes in additional brain regions may also contribute (Kamens, Andersen et al. 2010, Chatterjee, Steensland et al. 2011). Using two complementary genetic mouse models, our data indicate that α4* nAChRs are important for ethanol-induced activation of VTA DAergic neurons. A leftward shift of the agonist sensitivity of these receptors (Lester, Fonck et al. 2003) lowered the concentration of alcohol required to activate these neurons, indicating that α4* nAChR agonist sensitivity

can directly modulate VTA DAergic neuron activation by alcohol. Finally, ethanol potentiated DAergic neuron activation by ACh, a phenomenon that was blocked by DHβE and reduced in α4 KO slices. It is important to note that alcohol responses in α4 KO DAergic neurons were not completely abolished indicating other nAChR subtypes or non-nAChR mechanisms may be involved. However, these data indicate that if alcohol increases ACh concentration in the VTA, then not only will elevated ACh concentrations activate DAergic neuron nAChRs, but alcohol will potentiate this response. One limitation of our physiology data stems from the fact that mesocortical slices may not include critical circuitry influencing VTA activity. In addition, we did not differentiate sub-populations of DAergic neurons that may exist and express distinct nAChR subtypes within the VTA (although the vast majority express α4* nACRs) (Yang, Hu et al. 2009, Zhao-Shea, Liu et al. 2011). Interestingly, expression and function of α4* nAChRs not only contributed to activation of DAergic neurons in slices, but also modulated ethanol induction of c-Fos expression in VTA DAergic neurons. Taken together, these data strongly suggest that expression of functional α4* nAChRs can modulate activation of the mesolimbic reward pathway by alcohol.

Previous studies indicate that the rewarding properties of ethanol, as measured by CPP, is expressed through a VTA-dependent mechanism (Bechtholt and Cunningham 2005). Thus, because alcohol activation of DAergic neurons is reduced in α4 KO mice, alcohol CPP is weak in these animals, although it is possible that alcohol may condition a

place preference in response to higher doses of alcohol. Conversely, a sub-rewarding dose of alcohol is sufficient to condition a place preference in Leu9'Ala mice, presumably because VTA DAergic neurons in these animals are more robustly activated by such low doses of alcohol. Previous data indicate that α4 KO mice acutely consume less alcohol than WT mice in the drinking-in-the-dark paradigm consistent with a role for α4* nAChRs in reward (Hendrickson, Zhao-Shea et al. 2010, Hendrickson, Gardner et al. 2011). However, when given a 2 % or 20 % alcohol bottle in the DID assay, Leu9'Ala mice alcohol consumption did not significantly differ compared to WT mice. This may be due to the fact that consumption was analyzed immediately after saline or drug injections which could influence consumption behavior. Thus, baseline consumption behavior should be measured in Leu9'Ala mice using a variety of alcohol concentrations in the absence of injections. Because blocking alcohol reward might reduce alcohol consumption, our data raise the possibility that α4* nAChRs in the VTA may be useful molecular targets for alcohol cessation therapies. Indeed, in addition to varenicline, recently sazetidine-A, an α4β2-selective nAChR desensitizing agent, has been shown to reduce alcohol consumption in rodents (Rezvani, Slade et al. 2010).

Prior studies using nAChR subtype-selective antagonists have attempted to identify the subunit composition of nAChRs that may influence alcohol-induced DA release in NAcc and alcohol self-administration in rodents. Our lab and others have found that systemic injection of MLA does not reduce alcohol consumption in rodents

(Larsson, Svensson et al. 2002, Larsson, Jerlhag et al. 2004, Hendrickson, Zhao-Shea et al. 2009) indicating that low affinity α7 nAChRs do not play a significant role in alcohol reward. This is also in agreement with our physiology data indicating a lack of effect of MLA on ethanol-induced activation of DAergic neurons. In addition, systemic injection of the α4β2* nAChR competitive antagonist DHβE also fails to reduce ethanol intake in both rats and mice (Larsson, Svensson et al. 2002, Ericson, Molander et al. 2003) at lower doses but has been shown to reduce self-administration in rats at higher doses (Kuzmin, Jerlhag et al. 2009). Thus, it is likely that the ability of DHβE to block α4β2* nAChRs depends on the stoichiometry of the target receptor population (Moroni, Zwart et al. 2006) and subunit composition. For example, we recently identified functional nAChRs in VTA DAergic neurons that are of the α4α6(β2)(β3) subtype (Liu, Zhao-Shea et al. 2012) which we would expect to be more resistant to blockade by DHβE compared to nAChRs consisting of purely α4 and β2 subunits (Grady, Salminen et al. 2007). Although a recent study found that α6 and β3 KO mice did not consume or prefer alcohol differently than WT mice, caution in interpretation is warranted as these KO animals may exhibit compensatory changes in nAChR expression during development that may influence alcohol reinforcement (Kamens, Hoft et al. 2012). Thus, further experiments will need to be done to identify additional nAChR subunits involved in alcohol-mediated activation of VTA DAergic neurons and alcohol reward.

To our knowledge, this is the first study directly implicating α4* nAChRs - molecules that are known to play a primary role in nicotine reward- in the rewarding properties of alcohol. Our data indicate that activation of α4* nAChRs in the VTA modulates alcohol reward, suggesting their potential usefulness as therapeutic targets.

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Preface to Chapter III

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Author Contributions:
M.J.G and A.R.T. designed experiments
M.J.G. performed experiments, analyzed data, and wrote the paper
A.V.S. assisted with experiments

CHAPTER III: Modulation of Ethanol Reward by Nicotinic Acetylcholine Receptors Containing the α6 Subunit

Abstract

The prevalent co-abuse of nicotine and alcohol suggests a common neural mechanism underlying the actions of the two drugs. Nicotine, the addictive component of tobacco, activates nicotinic acetylcholine receptors (nAChRs) containing the α6 subunit (α6* nAChRs) in dopaminergic (DAergic) neurons of the ventral tegmental area (VTA), a region known to be crucial for drug reward. Recent evidence suggests that ethanol may potentiate ACh activation of these receptors as well, although whether α6* nAChR expression is necessary for behavioral effects of acute ethanol exposure is unknown. We compared binge-like ethanol consumption and ethanol reward sensitivity between knock-out mice that do not express chrna6 (the gene encoding the α6 nAChR subunit, the α6 KO line) and wild-type (WT) littermates using the Drinking-in-the-Dark (DID) and Conditioned Place Preference (CPP) assay, respectively. In the DID assay, α6 KO female and male mice consumed ethanol similarly to WT mice at all concentrations tested. In the CPP assay, 2.0 g/kg and 3.0 g/kg, but not 0.5 mg/kg ethanol conditioned a place preference in WT female and male mice; whereas only 2.0 g/kg ethanol conditioned a place preference in α6 KO mice. Acute challenge with ethanol reduced locomotor activity similarly between genotypes in both female and male mice. Together, these data

indicate that expression of α6* nAChRs is not absolutely required for binge-like ethanol consumption and reward, but modulate sensitivity to the rewarding properties of the drug.

Introduction

Alcohol use disorders (AUDs) and the abuse of alcohol lead to 3.3 million deaths annually worldwide, and are responsible for 25% of deaths in people age 20-39 (WHO 2015), making it one of the leading causes of preventable mortality in the world (Mokdad, Marks et al. 2004). Despite the costs of alcohol use and abuse, both in human life and in the economic burden placed on society (CASA 2000), there remain few viable treatment options for those suffering from AUDs (Spanagel 2009) highlighting the need for a better understanding of the mechanism(s) underlying the effects of alcohol on relevant neural circuitry within the brain. The frequent co-abuse of alcohol and nicotine, with an estimated 70-75% of alcoholics also identifying as tobacco-dependent (Miller and Gold 1998), suggest a common mechanism of the two drugs. Nicotine and alcohol activate dopaminergic (DAergic) neurons of the ventral tegmental area (VTA), ultimately leading to an increase in dopamine (DA) in the nucleus accumbens (NAc), which is associated with drug reward and is common for all known drugs of abuse (Hendrickson, Guildford et al. 2013). Nicotine can activate VTA DAergic neurons directly by binding to and activating nicotinic acetylcholine receptors (nAChRs), pentameric ligand-gated cation channels normally activated by the endogenous neurotransmitter, acetylcholine

(ACh), while the mechanism by which alcohol activates these same neurons is complex and likely involves multiple neurotransmitter systems (Pidoplichko, DeBiasi et al. 1997, Xiao, Dong et al. 2008, Morikawa and Morrisett 2010, Theile, Morikawa et al. 2011, Liu, Zhao-Shea et al. 2012, Liu, Hendrickson et al. 2013).

There are twelve known vertebrate genes encoding neuronal nAChR subunits, α2-10 and β2-4. Five subunits co-assemble to form functional receptors, with the majority of high affinity nAChRs heteromeric consisting of 2-3 α subunits coassembled with 2-3 β subunits (Zwart and Vijverberg 1998, Nelson, Kuryatov et al. 2003, Moroni, Zwart et al. 2006).

Although not a direct agonist of nAChRs, ethanol can activate VTA DAergic neurons, in part, through increased release of ACh (Larsson, Edstrom et al. 2005), likely from cholinergic neurons of the lateral dorsal tegmentum (Oakman, Faris et al. 1995), which then leads to increased activation of VTA DAergic neurons via nAChRs (Blomqvist, Ericson et al. 1997, Larsson, Edstrom et al. 2005, Liu, Hendrickson et al. 2013). In addition, ethanol can potentiate ACh-induced nAChR currents (Cardoso, Brozowski et al. 1999, Zuo, Kuryatov et al. 2002, Liu, Hendrickson et al. 2013), although the precise nAChR subtypes critical for ethanol activation of VTA DAergic neurons are unclear.

Of particular interest, nAChRs containing the α6 subunit (α6* nAChR, * denotes the receptor complex contains additional, non-α6 subunits) are uniquely enriched in

catecholaminergic neurons including midbrain DAergic neurons where they are positioned to influence neuronal activity through cholinergic innervation (Champtiaux, Han et al. 2002, Champtiaux, Gotti et al. 2003, Drenan, Grady et al. 2008, Berry, Engle et al. 2015). They also contribute to nicotine dependence-associated behavior including reward (Jackson, McIntosh et al. 2009, Gotti, Guiducci et al. 2010). Given the relationship between nicotine and alcohol, it is not surprising that blocking α6* nAChRs via an intra-VTA infusion of an α6, α3, β3* antagonist, α-conotoxin MII, reduces ethanol-induced DA release in the nucleus accumbens, ethanol consumption, and ethanol reinforcement in rodents (Larsson, Jerlhag et al. 2004, Jerlhag, Grotli et al. 2006, Kuzmin, Jerlhag et al. 2009). Mice lacking *chrna6*, the gene encoding the α6 nAChR subunit (α6 knock-out (α6KO) mice), show a significant increase in the sedative effects of ethanol, taking significantly longer to regain their righting reflex following 4.1g/kg ethanol, but show no differences regarding ataxia with 1.5g/kg ethanol (Kamens, Hoft et al. 2012). These mice have also been studied with respect to ethanol consumption and, although they show no differences in ethanol consumption or preference in a two-bottle free-choice paradigm (Kamens, Hoft et al. 2012), their consumption during a binge-like drinking paradigm, which more closely resembles problematic drinking habits, has yet to be explored. Additionally, Powers et al. (2013) showed a potential role for α6* nAChRs in alcohol consumption and reward behaviors using a hypersensitive, transgenic mouse line, leading to the hypothesis that α6* nAChRs may be involved in these and other

alcohol-related behaviors. To address this hypothesis, we compared binge-like ethanol consumption and the rewarding properties of the drug in α6 KO mice and their wild-type (WT) littermates.

Methods and Materials

Animals

8-20 weeks old α6 KO homozygous mice and their WT littermates were included in all experiments as indicated. This line was backcrossed to C57BL/6J at least 10 generations. The genetic engineering of this line has been described previously (Champtiaux, Han et al. 2002). α6 KO mice were bred homozygous x homozygous or heterozygous x heterozygous. Using Conditioned Place Preference as a representative experiment, mice from each breeding scheme were analyzed separately and then combined in all experiments as there was no significant difference between the two groups (Male CPP 2.0g/kg in Hom from Hom x Hom versus Het x Het [$F(1,10)=0.02110$, $p=0.8874$]). Animals were housed up to five per cage and kept on a 12-hour light/dark cycle prior to the start of each experiment. Mice had access to food and water *ad libitum* except where indicated. All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Drinking-in-the-Dark (DID)

For the binge-drinking assay, α6 KO and WT mice were moved from their colony room into a reverse light-dark room (lights ON at 8 PM and OFF at 8 AM) to habituate for two weeks prior to the start of the experiment. One week prior to the start of DID, mice were single-housed. Three days before DID standard water bottles were replaced with bottles containing sipper tubes with double ball bearing inserts to prevent leakage. In the DID assay, access to ethanol was restricted to 2 h a day, beginning 2 hours into the dark cycle, for 4 days per week (Rhodes, Best et al. 2005). Water bottles were removed and replaced with pre-weighed ethanol bottles (50-mL conical tubes outfitted with a stopper and double ball bearing sipper tube) initially containing 2% ethanol in water (v/v) and increasing to 5%, 10%, and finally 20% over the course of 4 weeks. The ethanol bottles were removed, replaced with water bottles, and weighed after 2 h. Ethanol consumption was measured as grams ethanol per kilogram body weight mouse. Following the 4-week protocol, mice were given 10% sucrose and 10 mM saccharin for 2 hr on 2 consecutive days each as controls.

Conditioned Place Preference (CPP)

The CPP assay used was a 6-day protocol adapted from Gibb et al. (2011). The CPP apparatus (Med Associates, USA) consists of three distinct chambers, a small neutral, grey chamber flanked on each side by a larger conditioning chamber containing a wire

mesh floor and white walls and the other containing a metal rod floor and black walls. Each compartment contains photobeams used to monitor mouse movement in and out of each chamber, and the entire apparatus is contained in a sound-attenuating box with lights and a fan. On day 1, habituation day, α6 KO and WT mice were placed in the middle chamber and allowed free access into all three chambers for thirty minutes during the light phase of their light/dark cycle. Time spent in each chamber was monitored and recorded by Med PC software via photobeam breaks and used to determine any initial bias for either of the conditioning chambers. Mice spending more than 90% of their time in one chamber were excluded from the study. The least preferred chamber was set as the ethanol-paired chamber. On days 2-5, mice were given an i.p. injection of either saline or ethanol (balanced by sex, dose, treatment; dose as indicated; 20 % ethanol solution v/v), and were immediately confined to the corresponding chamber for five minutes. Mice were then returned to their home cage. Four to six h later, each mouse received the other injection (ethanol if they had saline in the morning, saline if they had ethanol) and were confined to the other chamber. Day 6 was the test day, and was the same protocol as day 1. An increase in time spent in the ethanol-paired chamber on test day compared to the habituation day indicated reward.

Locomotor Activity

Mice were given pre-injections of saline in their home cage for two days prior to initiation of the experiment. Locomotor activity was measured in α6 KO and WT mice using a cage rack photobeam system (PAS, San Diego Instruments) and the corresponding PAS software. Mice were placed in a novel cage within the locomotor apparatus, and ambulation (locomotion) was measured as the breaking of two distinct beams 10 cm apart. Locomotor activity was recorded on days 0, 1, 4, and 7, for one h, at which time mice were injected with saline (Day 0) or 2 g/kg ethanol (Days 1, 4, 7) (Martin, Hendrickson et al. 2008). Activity was recorded for an additional hour following the injection, and is presented in 5 minute bins immediately following the injection. Mice received home cage ethanol injections on days 2, 3, 5 and 6.

Data Analysis

Data were analyzed using One- or Two-way ANOVA followed by Bonferroni post-hoc tests or multiple comparison tests as indicated. Results were considered significant at $p < 0.05$. All data are expressed as means ± SEM.

Results

Expression of α6 nAChRs is not necessary for binge-like consumption of alcohol*

We used a restricted-access alcohol consumption assay, drinking-in-the-dark (DID) (Rhodes, Best et al. 2005, Rhodes, Ford et al. 2007) to test whether α6* nAChRs play a role in binge-like alcohol consumption of 2 %, 5 %, 10 %, or 20 % ethanol (see Methods, **Fig. 3.1**) . A two-way ANOVA showed a significant effect of sex on ethanol consumption [$F(1, 39) = 37.33$, $p < 0.0001$], so males and females were separated for independent analysis. In both female and male mice, two-way ANOVA revealed a significant main effect of ethanol concentration [$F_{(3,78)} = 326.3$, $p < 0.001$ and $F_{(3,96)} = 463.9$, $p<0.0001$, respectively], but not genotype [$F_{(1,86)} = 0.1781$, $p = 0.6764$ and $F_{(1,32)} = 0.1770$, $p = 0.6767$, respectively, with no significant interaction **(Fig. 3.1C, D)**. In addition, neither group exhibited a significant difference in sucrose or saccharin consumption **(Fig. 3.1E, F)**. The absence of any effect of genotype on ethanol consumption indicates that expression of α6* nAChRs is not necessary for binge-like ethanol drinking.

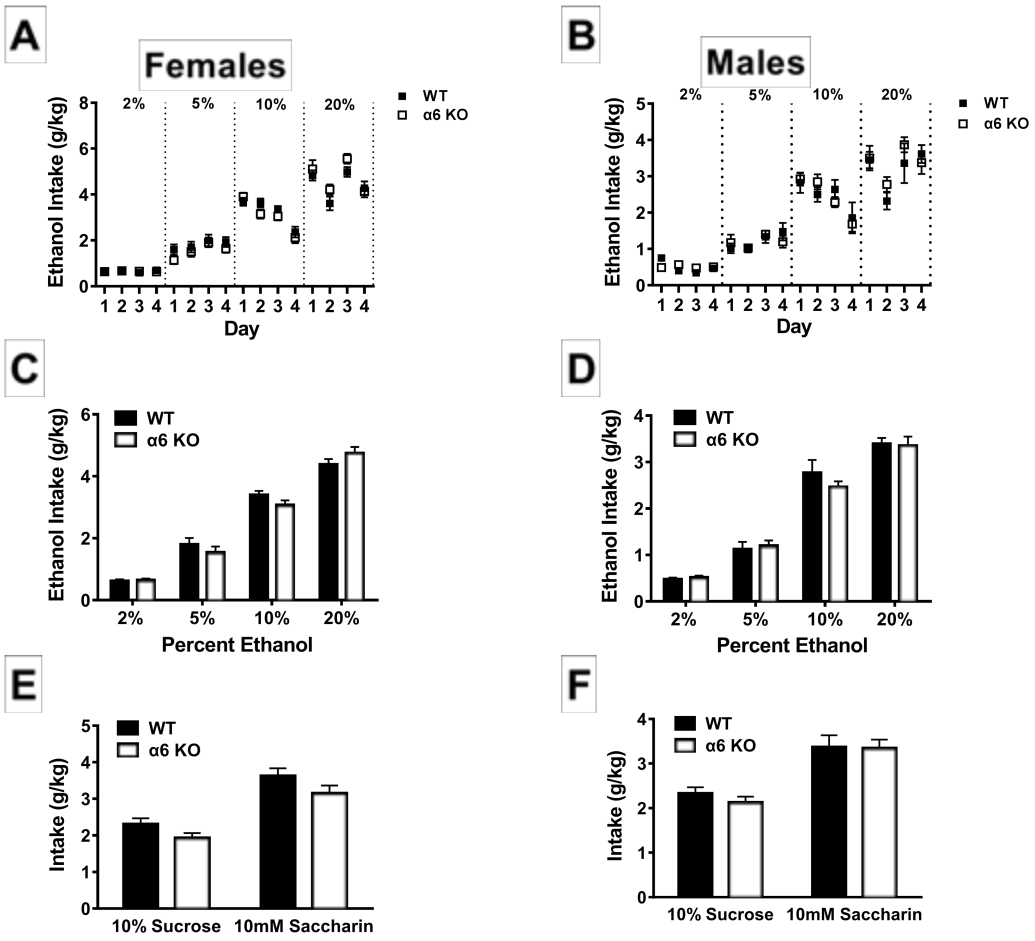

Figure 3.1 Expression of α6* nAChRs is not necessary for binge-like ethanol consumption in the drinking-in-the-dark assay. Daily consumption of 2-20 % ethanol during the DID binge-drinking assay in A) female and B) male WT and α6 KO mice. Average daily ethanol intake for each ethanol concentration for C) female (n=15 KO, n=19 WT) and D) male mice (n=14 KO, n=14 WT) of each genotype. Average sucrose and saccharin consumption in E) female and F) male mice of each genotype.

Sex and Genotype	*n*	Dose EtOH (g/kg)	SAL (Hab) (sec)	SAL (Test) (sec)	EtOH (Hab) (sec)	EtOH (Test) (sec)	Grey (Hab) (sec)	Grey (Test) (sec)
Male WT	**6**	**0**	681.1± 40.6	389.6 ± 41.7	576.7 ± 25.8	452.3 ± 73.7	542.2 ± 33.6	958.0 ± 106.1
	9	**0.5**	650.8 ± 51.8	343.6 ± 34.9	494.5 ± 25.7	483.7 ± 86.3	654.7 ± 66.8	926.0 ± 112.5
	10	**2.0**	696.2 ± 37.5	403.5 ± 72.7	513.1 ± 28.9	*722.4 ± 109.8	590.7 ± 28.9	674.1 ± 103.2
	9	**3.0**	654.1 ± 33.4	397.5 ± 54.9	488.0 ± 24.8	**785.6 ± 86.5	673.3 ± 32.0	616.9 ± 61.8
Male α6KO	**7**	**0**	647.6 ± 63.2	608.4 ± 43.3	414.5 ± 40.4	486.6 ± 56.9	737.9 ± 77.5	704.9 ± 80.6
	6	**0.5**	573.5 ± 53.7	499.0 ± 96.1	504.3 ± 32.8	450.6 ± 87.3	722.2 ± 77.4	850.4 ± 178.2
	9	**2.0**	661.3 ± 29.8	414.3 ± 79.5	439.8 ± 19.3	***820.5 ± 106.2	698.9 ± 28.2	565.1 ± 48.5
	8	**3.0**	676.2 ± 34.3	484.8 ± 68.5	425.9 ± 38.7	580.8 ± 96.3	697.6 ± 62.9	734.4 ± 96.5
Female WT	**9**	**0**	630.6 ± 41.8	472.1 ± 65.8	542.7 ± 32.9	441.0 ± 81.3	626.7 ± 47.9	886.9 ± 77.4
	10	**0.5**	640.8 ± 36.2	402.1 ± 49.5	460.4 ± 34.3	438.3 ± 79.0	698.8 ± 57.9	959.6 ± 106.1
	10	**2.0**	709.1 ± 49.1	361.0 ± 58.5	540.6 ± 22.8	*855.5 ± 115.6	550.2 ± 52.5	583.5 ± 102.6
	7	**3.0**	670.3 ± 35.5	407.6 ± 139.8	495.9 ± 28.9	*907.5 ± 146.1	633.8 ± 44.1	485.0 ± 56.9
Female α6KO	**5**	**0**	571.5 ± 39.7	533.6 ± 46.0	595.0 ± 65.6	391.4 ± 57.4	643.3 ± 79.5	874.9 ± 71.4
	11	**0.5**	626.8 ± 32.1	480.7 ± 37.2	447.9 ± 30.5	599.8 ± 45.0	725.9 ± 41.9	719.4 ± 40.1
	13	**2.0**	683.9 ± 28.3	516.4 ± 49.3	518.7 ± 27.7	**724.4 ± 57.0	597.4 ± 38.4	559.2 ± 51.5
	6	**3.0**	627.0 ± 34.1	761.9 ± 107.9	600.9 ± 42.0	494.6 ± 76.3	572.1 ± 37.8	543.4 ± 74.0

Table 3.1 Time spent in saline and ethanol-paired CPP chambers ± SEM

α6 nAChRs modulate reward response to high-dose ethanol*

To test the hypothesis that expression of α6* nAChRs is necessary for the rewarding effects of ethanol, we tested the ability of the drug to condition a place preference in female and male WT and α6 KO mice using the CPP assay at three distinct doses, 0.5 g/kg, 2.0 g/kg, and 3.0 g/kg ethanol, and with a saline injection **(Fig. 3.2A, Table 3.1)**. Analysis of time spent in the ethanol-paired chamber on test day versus habituation day was done using successive two-way ANOVAs. Analysis of sex x ethanol dose indicated a significant effect of dose [$F(3,121) = 14.40$, $p<0.0001$], but not sex [$F(1,121)=1.205$, $p=0.2722$]. With sexes pooled, analysis of genotype x dose showed an effect of dose [$F(3,127)=12.56$, $p<0.0001$] and an interaction [$F(3,127)=3.142$, $p=0.0276$]. Post-hoc analysis revealed a statistically significant difference in time spent in the ethanol-paired chamber with 3.0g/kg in WT versus α6 KO animals. Together these data indicate that expression of α6* nAChRs is not absolutely required for ethanol reward, but modulates reward sensitivity of the drug.

α6 nAChRs are not necessary for ethanol-induced hypolocomotion*

To test if ethanol-induced locomotor impairment differed between genotypes, we challenged female and male WT and α6 KO mice with an acute injection of saline (Day 0) or 2.0 g/kg ethanol (Days 4 and 7) immediately prior to measuring locomotor activity. Two-way ANOVA of sex x day revealed a main effect of day [$F(3,69)=10.49$, $p<0.0001$] but not sex [$F(1,23)=0.2175$, $p=0.6453$]. Genotype x day also showed only a significant effect of day [$F(3,69)=10.97$, $p<0.0001$], not genotype (**Figure 3.2 B**). These data suggest no significant role of α6* nAChRs in the locomotor response to ethanol, and no difference in the responses of the two sexes.

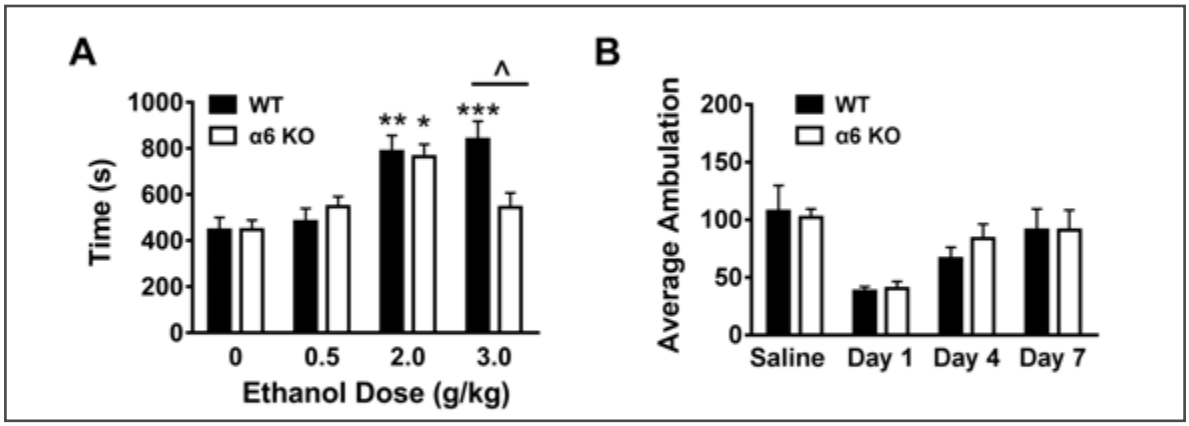

Figure 3.2 Expression of α6* nAChRs modulate sensitivity to ethanol reward, but not ethanol-induced hypolocomotion. A. Time spent in the ethanol-paired chamber on the test day. Mice were conditioned with 0 g/kg (saline), 0.5 g/kg, 2.0 g/kg, or 3.0 g/kg ethanol. *n* = 12-22 mice per genotype/dose (see Table 3.1 for specific n values), two-way ANOVA, Bonferroni post-hoc. B. Average ambulation per 5 minute bin for 30 min following i.p. saline or 2.0g/kg ethanol (Days 1, 4, 7). *n* = 12 WT, *n* = 13 α6 KO. ^compared to WT saline; #compared to α6 KO saline; *WT vs α6 KO; *p<0.5, **p<0.01, ***p<0.001.

Discussion

The present study analyzed the contribution of α6* nAChRs in binge-like ethanol consumption and ethanol reward using a loss of function mouse model, the α6 KO line. Previously, this same mouse line was used to determine if α6* nAChR expression is involved in ethanol preference and consumption (Kamens, Hoft et al. 2012). Using a 24 hr two-bottle choice paradigm, Kamens et al. (Kamens, Hoft et al. 2012) found that α6 KO mice consume and prefer ethanol similarly to their WT littermates. However, binge-like ethanol intake and reward was not analyzed. Using the DID assay, we also did not detect significant differences in ethanol consumption between α6 KO mice and their WT littermates at any ethanol dose tested in male or female mice, though overall females consumed significantly more ethanol than their male counterparts. In addition, ethanol was rewarding in α6 KO mice in that it could condition a place preference in these animals, but only at one dose (2.0g/kg), while a higher dose of ethanol (3.0g/kg) failed to condition a place preference, despite doing so in their WT littermates. Thus, our data indicate that expression of α6* nAChRs is not necessary for ethanol consumption or reward per se, but these receptors do contribute to reward at high concentrations of ethanol.

Interestingly, Powers et al. analyzed ethanol consumption and reward in a "gain-of-function" bacterial artificial chromosome transgenic mouse line expressing α6

subunits harboring a point mutation (a leucine residue at the 9′ position mutated to a serine residue, the α6 L9′S line) that renders α6* nAChRs hypersensitive to ACh (Drenan, Grady et al. 2008, Powers, Broderick et al. 2013). These mice displayed an increase in ethanol consumption in both a two-bottle choice test and the DID assay compared to WT littermates. In addition, mutant mice were more sensitive to the rewarding effects of ethanol (i.e. a sub-rewarding dose of ethanol conditioned a place preference in the mutant mice). Because α6 L9′S mice are exquisitely sensitive to agonist, the increased ethanol consumption and leftward shift in ethanol reward sensitivity of these mice should be interpreted with caution. While the results obtained using the α6 L9′S mice implicate α6* nAChRs in regulating ethanol consumption and reward sensitivity, the impact of endogenous α6* receptors on ethanol behaviors in WT mice may be over-estimated.

Similarly, there are caveats to our interpretations of ethanol behaviors in α6 KO mice. In particular, compensatory expression of non-α6 nAChR subunits may occur in these animals in the absence of *chrna6* expression, thereby minimizing any potential differences in ethanol consumption and reward between genotypes. In addition, α6 nAChR subunits cannot form homomeric receptors; rather, they require at least one additional beta nAChR subunit (in particular β2 or β3) and also can coassemble with the α4 subunit especially in VTA DAergic neurons (Cui, Booker et al. 2003, Drenan, Grady et al. 2008, Zhao-Shea, Liu et al. 2011, Liu, Zhao-Shea et al. 2012, Engle, Shih et al.

2013). Thus, in the absence of *chrna6* expression, VTA DAergic neurons may express a greater number of nAChRs containing α4 subunits compared to WT mice, supported by the fact that the expression of α4, as measured by immunoprecipitation with epidatidine, increases in α6 KO animals (Champtiaux, Gotti et al. 2003). As we have previously identified a role for α4* nAChRs in ethanol behaviors, including reward and acute ethanol consumption, in males, increased α4* nAChRs in α6 KO mice may partially mask the contribution of α6* nAChRs in an α6 KO mouse background in males more than in females (Hendrickson, Zhao-Shea et al. 2010, Hendrickson, Gardner et al. 2011, Liu, Hendrickson et al. 2013). Although Champtiaux, Han et al. (2002) showed no such increase of this and other subunits in the VTA of α6 KO mice at the mRNA level, this was semi-quantitative and done using *in situ* hybridization; compensation may still occur functionally or at the protein level, which could be uncovered via western blot analysis. An alternative approach to circumvent these caveats would be to knockdown α6* nAChRs in adult animals using small interfering RNAs (siRNAs), thereby allowing the animal to develop with the subunit and avoid many compensatory mechanisms that may occur in the knockout animals.

Despite the caveats described above, we did identify a decrease in expression of ethanol reward in α6 KO mice when challenged with a high ethanol dose (3.0g/kg). This is consistent with previous data showing that ethanol-induced activation of VTA DAergic neurons is reduced in ex vivo midbrain slices from α6 KO mice compared to WT slices

(Liu, Zhao-Shea et al. 2013), although this was shown using 100mM ethanol, a concentration that may be different than that reached with a 3.0g/kg dose in a whole animal. Additionally, Kamens et al. (2012) have shown that α6 KO male and female mice show no differences in BEC, compared to WT, and no sex differences, when given 3.0g/kg ethanol, making ethanol metabolism differences unlikely to have caused this result. Future studies using alternative approaches such as viral-mediated siRNA knock-down of individual nAChR subunits in discreet brain regions of adult animals should be done to test contributions of nAChR subtypes in ethanol consumption and reward, as well as to identify if the key nAChRs involved in these behaviors are expressed in sub-populations of VTA neurons, using a Cre-lox expression system and mouse lines with Cre expression driven by GAD or TH, for example. It may be, however, that a different brain region altogether is involved in the effects of α6* nAChRs on ethanol reward. These receptors, expressed on DAergic neurons and not GABAergic neurons in the VTA, are almost exclusively located on the terminals of these DAergic neurons, many of which project to the NAc. It may be that, in simply looking at the VTA, we are missing the main effect of α6* nAChRs in modulating release of DA in the NAc, or in the PFC, which also receives DAergic input from the VTA. To explore the site of DA action, we could use optogenetics to stimulate DAergic neurons within the VTA, using viral-delivery of cre-dependent, channelrhodopsin in a TH-Cre mouse line, and measure DA levels in both the NAc and PFC via microdialysis. This would help pinpoint the important connections

made by VTA DAergic neurons. Once identified, DA release in the area following ethanol administration, including in α6 KO animals, could be explored further. By looking at the terminals in each of these areas, versus the cell bodies in the VTA, we may be more able to decipher the actions of ethanol via α6* nAChRs in the reward pathway.

Given that 2.0g/kg is the generally accepted rewarding dose of ethanol, and yet our results indicate a role for α6* nAChRs at 3.0g/kg, but not 2.0g/kg, it is important to consider the fact that this may be more complicated than simply a lack of reward at this high dose. It has also been previously shown that α6 KO mice are more sensitive to the sedative effects of ethanol, showing a greater latency to regain their righting reflex in the loss of righting reflex (LORR) assay (Kamens, Hoft et al. 2012), but the same paper showed no effects on ataxia with. We saw no significant differences in the response of these mice to 2.0g/kg ethanol on any of the locomotor test days in either sex. This suggests little to no role of α6 in this hypolocomotor response to this dose of ethanol or in baseline locomotion, and consequently no locomotor impact on the CPP data. It may be that, as with reward, α6* nAChRs become important in higher doses of ethanol such as those used for our CPP and the aforementioned LORR (Kamens, Hoft et al. 2012), but this dose, being so close to the dose used for LORR, was not explored in this study.

In conclusion, our data indicate that expression of α6* nAChRs is not necessary for binge-like ethanol consumption, but does modulate ethanol reward sensitivity at high doses.

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Preface to Chapter IV

Nicotinic Acetylcholine Receptors and Sex Differences in Ethanol Behaviors (Unpublished data)

Author Contributions:
M.J.G and A.R.T. designed experiments
M.J.G. performed experiments, analyzed data
A.V.S. assisted with experiments

CHAPTER IV: Nicotinic acetylcholine receptors and sex differences in ethanol behaviors

Introduction

Nearly 38 million Americans experience problematic drinking, but only 1 in 6 will speak to their healthcare provider regarding treatment (CDC 2016). Moreover, of those who do seek treatment, only about one-third will remain symptom free a year later (CDC 2016). In order to successfully treat these patients, we first need an understanding of what alcohol actually does in the brain. We also need to consider that males and females may require different treatments. Human males tend to drink more than females, and are more likely to meet abuse and dependence criteria, but females who do use alcohol tend to move more quickly from use to abuse than do their male counterparts (Nolen-Hoeksema and Hilt 2006). These difference could be due to social perceptions of acceptable drinking, with male alcohol abuse tending to be viewed less harshly than female, or it could be due to genetic or hormonal differences between the two sexes (Nolen-Hoeksema and Hilt 2006). In rodents, females tend to drink more than the males (Barker, Torregrossa et al. 2010, Lenz, Muller et al. 2012, Becker and Koob 2016, Carroll and Lynch 2016), which, although opposite to the human pattern, still suggests the sex differences may be biological rather than societal.

The frequent co-abuse of alcohol and nicotine, with an estimated 70-75% of alcoholics also identifying as tobacco-dependent (Miller and Gold, 1998), suggests a

common mechanism of the two drugs. Nicotine and alcohol both activate dopaminergic (DAergic) neurons of the ventral tegmental area (VTA), ultimately leading to an increase in dopamine (DA) release in the nucleus accumbens (NAc), which is associated with drug reward and is common for all known drugs of abuse (Pidoplichko, DeBiasi et al. 1997, Rodd, Melendez et al. 2004, Tsai, Zhang et al. 2009). Nicotine activates these VTA DAergic neurons by binding to and activating nicotinic acetylcholine receptors (nAChRs) on these neurons (Pidoplichko, DeBiasi et al. 1997, Maskos, Molles et al. 2005), but the mechanism by which alcohol activates these VTA DAergic neurons is as yet unknown.

Neuronal nAChRs are pentameric ligand-gated cation channels that are activated endogenously by acetylcholine (ACh) as well as by exogenous nicotine, the active ingredient in tobacco (Tapper, McKinney et al. 2004, Albuquerque, Pereira et al. 2009). There are twelve known vertebrate genes encoding neuronal nAChR subunits α2-10 and β2-4. Five subunits co-assemble to form functional receptors (Laviolette and van der Kooy 2004, Albuquerque, Pereira et al. 2009), most of which are heteromeric and consist of 2-3 α and 2-3 β subunits (Zwart and Vijverberg 1998, Nelson, Kuryatov et al. 2003, Moroni, Zwart et al. 2006, Dani and Bertrand 2007). Heteromeric receptors display high affinity for agonist, while homomeric receptors, formed only by α subunits, show low affinity (Buisson and Bertrand 2001, Nelson, Kuryatov et al. 2003, Moroni, Zwart et al. 2006, Albuquerque, Pereira et al. 2009). The biophysical and pharmacological properties of the receptor depend on the subunits that make up the receptor, also known as the

receptor subtype (McGehee and Role 1995, Buisson and Bertrand 2001, Nelson, Kuryatov et al. 2003, Moroni, Zwart et al. 2006, Gotti, Moretti et al. 2007, Gotti, Guiducci et al. 2010).

Previously, it has been shown that the activation of VTA DAergic neurons occurs via α4-containing (α*) nAChRs for both nicotine (Tapper, McKinney et al. 2004) and alcohol (Liu, Hendrickson et al. 2013), with a contribution of α6* nAChRs as well (Liu, Zhao-Shea et al. 2012, Liu, Zhao-Shea et al. 2013, Guildford, Sacino et al. 2016). Interestingly, male alcohol reward is also α4* nAChR-dependent, as our lab has shown that α4 knock out (α4KO) male mice show no alcohol reward at the generally accepted rewarding dose of 2.0g/kg while their wild type (WT) littermates do (Liu, Hendrickson et al. 2013). α4 and α6 are both enriched in the VTA (Champtiaux, Han et al. 2002, Champtiaux, Gotti et al. 2003, Pons, Fattore et al. 2008, Liu, Hendrickson et al. 2013, Liu, Zhao-Shea et al. 2013), which is one of only a few regions of the brain that contain α6 (Champtiaux, Han et al. 2002, Gotti, Guiducci et al. 2010), and these subunits frequently co-assemble to make α4α6* nAChRs in this area (Champtiaux, Han et al. 2002, Champtiaux, Gotti et al. 2003, Liu, Hendrickson et al. 2013, Liu, Zhao-Shea et al. 2013). Both α4 and α6 mRNA are higher in VTA DA neurons activated following systemic ethanol than those not activated (Hendrickson, Zhao-Shea et al. 2010), and intra-VTA infusion of an α6*, α3*, β3* antagonist, α-conotoxin MII, reduces ethanol-induced DA release in the nucleus accumbens, ethanol consumption, and ethanol

reinforcement in rodents (Larsson, Jerlhag et al. 2004, Jerlhag, Grotli et al. 2006, Kuzmin, Jerlhag et al. 2009). Powers et al. (2013) showed a potential role for α6* nAChRs in alcohol behaviors using a hypersensitive, transgenic mouse line, leading to the hypothesis that α6* nAChRs may be involved in these and other alcohol-related behaviors, although we found this to be the case only with a high dose of ethanol with no significant sex differences (Guildford, Sacino et al. 2016).

We sought to explore any potential gender-specific nAChR contributions, by examining several alcohol behaviors, including binge drinking, alcohol reward, alcohol-influenced locomotor activity, as well as alcohol-induced activation of the reward pathway in males and females. Various behaviors are associated with alcohol use, but drinking patterns, quantity, and degree of euphoria or reward experienced have been shown in humans to be critical factors in the progression from alcohol abuse to alcohol dependence (Dani and Harris 2005, WHO 2015), making it imperative to decipher the mechanisms behind these behaviors in order to develop more targeted and ultimately more successful therapeutics for AUDs.

Methods and Materials

Animals

8-20 weeks old α4- or α6- knockout (KO) homozygous mice and their wild-type littermates (WT), males and females, were used in all experiments as indicated. The two lines were individually backcrossed to C57BL/6J at least 10 generations. The engineering of the α4 KO line has been described in (Tapper, McKinney et al. 2004), while the α6 KO line was described by Champtiaux et al. (2002). Animals were housed up to five per cage and kept on a 12-hour light/dark cycle prior to the start of each experiment. Mice had access to food and water *ad libitum* except where indicated. All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Drinking-in-the-Dark

For the binge-drinking assay (Rhodes, Best et al. 2005), mice were moved from their colony room into a reverse light-dark room (lights ON at 8PM and OFF at 8PM) to habituate for two weeks prior to the start of the experiment. One week before the experiment, mice were single-housed, and three days before the experiment, mice were given water bottles with the same sipper tube setup (double ball bearing) as the

experimental tubes to habituate. The drinking-in-the-dark (DID) assay was run 2 hours a day, starting 2 hours into the dark cycle, and 4 days a week. Water bottles were removed and replaced with pre-weighed 50-mL conical tubes outfitted with a stopper and double ball bearing sipper tube. This tube contained an ethanol solution, starting at 2% ethanol in water (v/v) and increasing to 5%, 10%, and finally 20% over the course of 4 weeks, with 3 days ethanol-free between each dose. This tube was removed and weighed after 2 hours, and the water bottles were replaced. Ethanol consumption was measured as grams ethanol per kilogram body weight of the mouse. Following the 4-week protocol, mice were given 10% sucrose and 10mM saccharin for 2 days each as controls.

Conditioned Place Preference

The conditioned place preference (CPP) assay used was a 6-day protocol adapted from Gibb et al. (2011) and is described previously (Liu, Hendrickson et al. 2013, Guildford, Sacino et al. 2016). On day 1, habituation day, mice were placed in the middle chamber and allowed free access into all three chambers. There was no injection on the habituation day. Time spent in each chamber was monitored and recorded by the associated software via photobeam breaks and used to determine any initial bias for either of the conditioning chambers. On days 2-5, mice were given an i.p. injection of either saline or ethanol (balanced, dose as indicated, 20% ethanol solution v/v), and were immediately confined to the corresponding chamber for five minutes. Mice were then returned to their home

cage. Four to six hours later, each mouse received the other injection (ethanol if they'd had saline in the morning, saline if they'd had ethanol) and were confined to the other chamber. Day 6 was the test day, and was the same protocol as day 1. An increase in time spent in the ethanol-paired chamber on test day compared to the habituation day indicated reward.

Locomotor Activity

Mice were given pre-injections of saline in their home cage for two days prior to initiation of the experiment. Locomotor activity was measured as previously described (Guildford, Sacino et al. 2016) and was recorded on days 0, 1, 4, and 7, for one hour, at which time mice were injected with saline (Day 0) or 2g/kg ethanol (Days 1, 4, 7). Activity was recorded for an additional hour following the injection, and is presented in 5 minute bins beginning 5 minutes after initiation of the experiment and 5 minutes after the injection.

Immunofluorescence

Mice were i.p. injected with saline for 3 days prior to the experiment to habituate to injections and reduce any stress-induced c-fos. On the day of the experiment, mice were given an i.p. injection of saline or either 2.0g/kg or 3.0g/kg ethanol. Ninety minutes later, the mice were given an i.p. injection of pentobarbital (200mg/kg), were transcardially

perfused, and their brains were harvested as previously described (Liu, Hendrickson et al. 2013). Following a 2-hour post-fix in 4% paraformaldehyde and subsequent equilibration in 30% sucrose, the brains were sliced at 25μm, and the VTA slices were collected. These slices were then stained using antibodies for tyrosine hydroxylase (TH) and c-fos, mounted, and viewed using a fluorescence microscope (Carl Zeiss MicroImaging Inc.). Neurons were considered positive (+) if the signal intensity was at least double that of background. TH+/c-fos+ neurons, as well as TH-/c-fos+ neurons, from 5 VTA slices from each mouse were counted, and the average number per slice was recorded. The experimenter was blind to the drug treatment.

Results

CPP and DID

α4 nAChRs play different role in male and female binge-drinking and reward*

As we have previously shown, the α4 subunit is critical for alcohol reward in male mice (Liu, Hendrickson et al. 2013). Here, we used the same CPP paradigm to study ethanol reward in female mice, including Leu9'Ala heterozygous, α4KO, and WT mice. Each genotype and dose was compared to its own habituation (data not shown), with a significant increase in time spent on test day compared to habituation used as a measure of reward **(Figure 4.1A)**. Female Leu9'Ala heterozygous mice displayed a significant main effect of day (habituation versus test) [$F(1,18) = 6.822$, $p = 0.0177$], but not ethanol

dose [F(3,18) = 2.177, p = 0.1261], nor any significant interaction between the two [F(3,18) = 2.174, p = 0.1264]. Female WT show no significant main effect of day [F(1,29) = 0.2932, p = 0.5922], dose [F(3,29) = 2.762, p = 0.0599], and no interaction between the two [F(3,29) = 2.389, p = 0.0892]. Female α4 KO also show no significant main effect of day [F(1, 33) = 4.079, p = 0.0516] or dose [F(3,33) = 2.135, p = 0.1146], but do show a significant interaction [F(3,33) = 3.26, p = 0.0337] indicative of a conditioned place preference at 3.0g/kg (Sidak's multiple comparisons test *post hoc*). This is in stark contrast to males, where Leu9'Ala males condition a place preference at 0.5g/kg ethanol, WT males condition a place preference at 2.0g/kg ethanol, and α4 KO males show no CPP at any dose observed (Liu, Hendrickson et al. 2013). For this study, 0.5g/kg and 3.0g/kg were studied, and α4 KO showed no significant main effect of day [F(1,15) = 0.01871, p = 0.8930], dose [F(2,15) = 0.2218, p = 0.8037], or interaction between the two [F(2,15) = 0.2724), p = 0.7652] (unpublished MGD) **(Figure 4.1B)**.

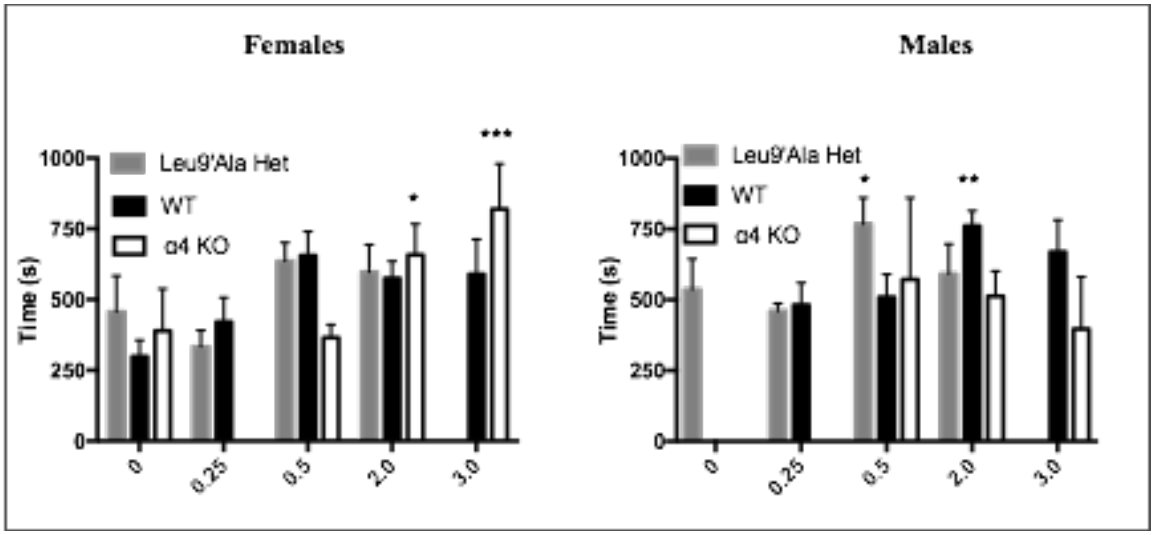


Figure 4.1 α4* nAChRs are critical for ethanol reward only in males. Time spent in ethanol-paired chamber on test day in females **(A,** n=3-14**)** and males **(B,** n=3=18**)** shown with SEM. Unpublished data for females and males (doses 0.25 and 3.0 only, others previously published in Hendrickson et al. 2013). Two-way ANOVA with Sidak's multiple comparisons test. *p< 0.05 compared to own habituation time, not shown.

In addition to the reward data, we have also shown that α4KO males drink less 20% ethanol than WT (Hendrickson, Zhao-Shea et al. 2009). In this experiment, the mice had also received injections of saline prior to intake studies. In the current study, we expanded upon these results. Using the DID assay, we measured ethanol intake using 2, 5, 10, and 20% ethanol, with each concentration being administered for 4 days followed by 3 days with no ethanol. Males showed a significant main effect of ethanol concentration [$F(3,60) = 86.76$, $p < 0.0001$], genotype [$F(1,60) = 36.32$, $p < 0.0001$], and an interaction of concentration x genotype [$F(3,60) = 8.782$, $p < 0.0001$], and α4 KO

mice consumed significantly less 10% and 20% ethanol than WT [Bonferroni's multiple comparisons test *post hoc*], as well as a decrease in saccharin consumption. **(Figure 4.2 D-F)**. Females displayed a significant main effect of concentration [$F(3,36) = 54.73$, $p < 0.0001$], but not genotype [$F(1,36) = 0.2723$, $p = 0.2723$] nor any interaction [$F(3,36) = 0.2105$, $p = 0.8885$], and α4KO mice did not differ from WT females in their ethanol intake at any concentration. **(Figure 4.2 A-C)**. The consumption data suggest that α4* nAChRs may be involved in male, but not female, binge-drinking, and, although the role of α4* nAChRs in ethanol reward in females is unclear, is it different than the role played in male ethanol reward.

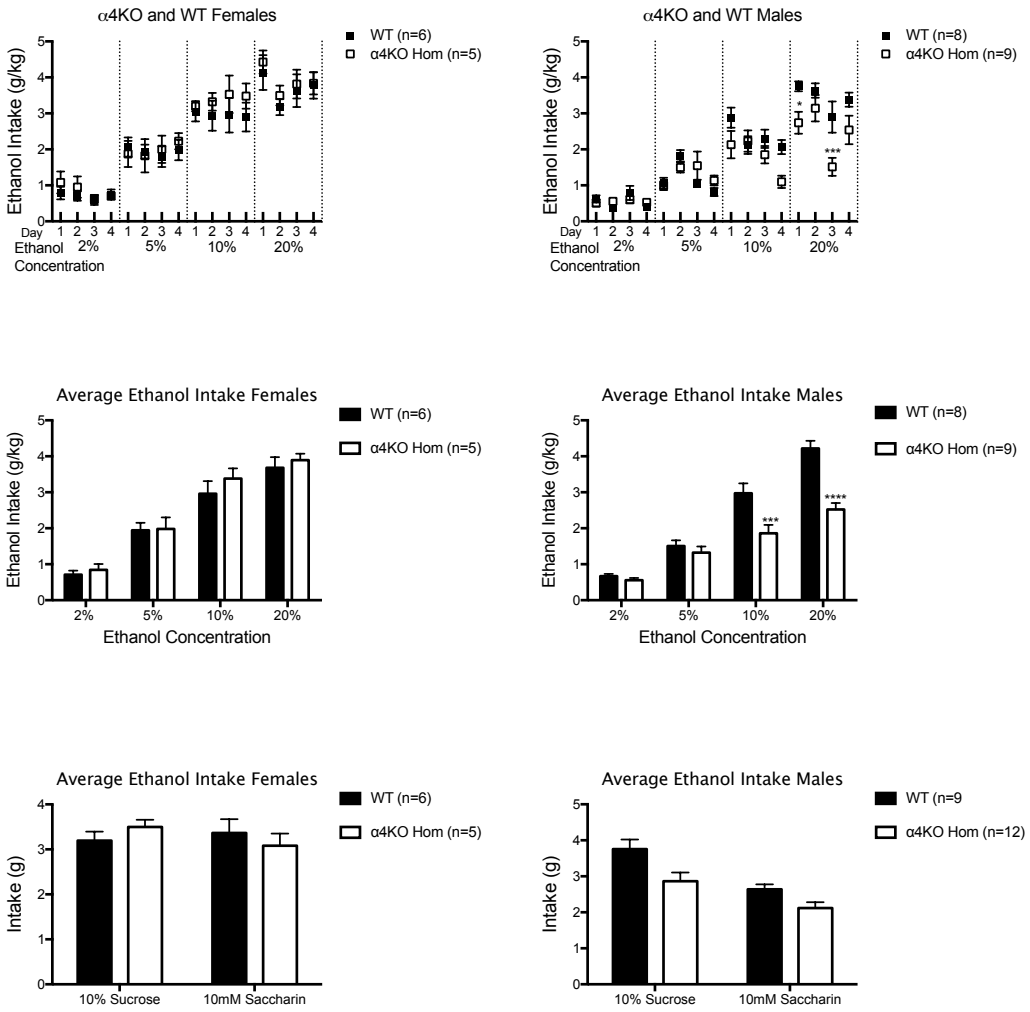


Figure 4.2 α4* nAChRs are involved in ethanol consumption only in males. A. Female WT and α4 KO consume the same amount of ethanol on each day of drinking (top panel) and averaged across the four drinking days (middle panel). B. Male α4 KO consume significantly less 10% and 20% ethanol on individual days (top panel, multiple t-tests) as well as averaged across the drinking days (middle panel, two way ANOVA with Bonferonni's multiple comparisons test). *$p<0.05$

α6 nAChRs may modulate reward response to high-dose alcohol in males and females*

Powers et al. (2013), in addition to their ethanol consumption data, showed a role for α6* nAChRs in alcohol reward using the previously mentioned α6 L9'S mouse line. Results of α6KO and WT CPP were published previously (Guildford, Sacino et al. 2016) and presented in Chapter III with the sexes combined as no significant sex differences were observed. These data are presented separately by sex below to display potential sex differences more clearly.

Female WT mice show a significant main effect of dose [$F(3,32) = 6.303$, $p = 0.0018$], day [$F(1,32) = 7.248$, $p = 0.0112$], and an interaction [$F(3,32) = 4.862$, $p = 0.0067$], and show CPP at 2.0g/kg and 3.0g/kg (Sidak's multiple comparisons test *post hoc*). α6 KO females also show a significant interaction between dose and day [$F(3,31) = 6.891$, $p = 0.0011$], though no main effect of either individually [$F(3,31) = 2.733$, $p = 0.0605$; $F(1,31) = 0.1554$, $p = 0.6962$, respectively]. *Post hoc* analysis showed a significant CPP at 2.0g/kg ethanol only **(Figure 4.3A)**. In males, WT show a significant effect of day [$F(1,30) = 8.071$, $p = 0.0080$] but not dose [$F(3,30) = 1.689$, $p = 0.1903$], and a significant interaction [$F(3,30) = 5.389$, $p = 0.0044$]. Sidak's multiple comparisons test shows a significant CPP at 2.0g/kg and 3.0g/kg ethanol, similar to WT females. α6 KO males also display a significant main effect of day [$F(1,26) = 10.74$, $p = 0.0030$), but not dose [$F(3,26) = 2.198$, $p = 0.1123$], as well as a significant interaction [$F(3,26) =$

4.881, p = 0.0080], and a CPP at 2.0g/kg but not 3.0g/kg (Sidak's multiple comparisons test *post hoc*).

Females **Males**

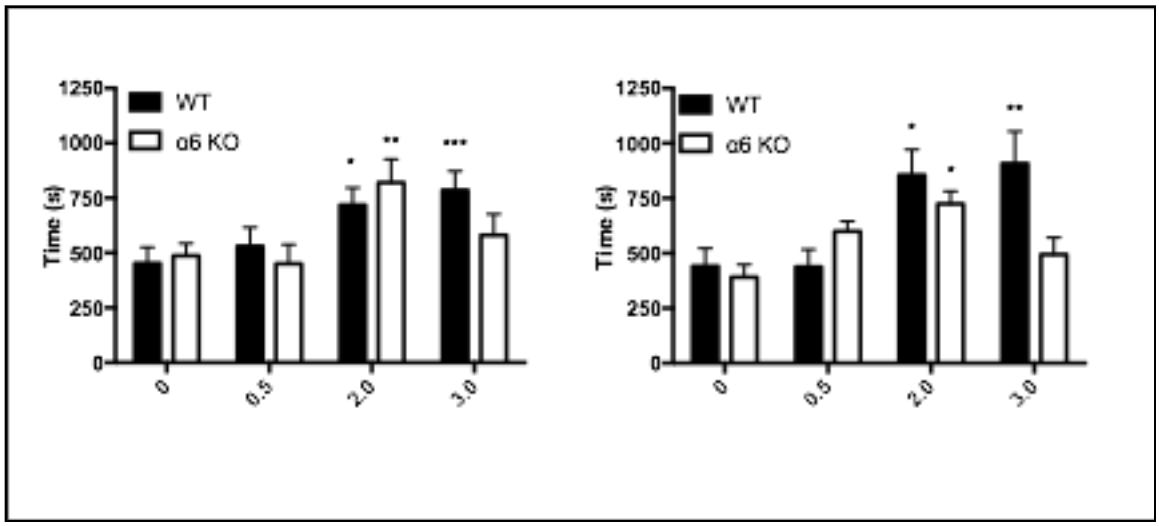


Figure 4.3 α6* nAChRs modulate ethanol reward sensitivity in females and males. WT and α6 KO females (A) and males (B) condition a place preference to 2.0 g/kg ethanol, and WT of both show CPP at 3.0g/kg, which is abolished in KO. (Data previously published in Guildford et al. 2016 with sexes combined.) (Two-way ANOVA with Bonferonngi's multiple comparisons test *p <0.05 compared to own habituation time, which is not shown)

Locomotor Activity

No differences in locomotor activity between α6KO and WT

There were no differences in baseline locomotor activity between genotypes at any point measured (Multiple t-tests, p > 0.7 in all cases) in either sex. **(Figure 4.4)**.

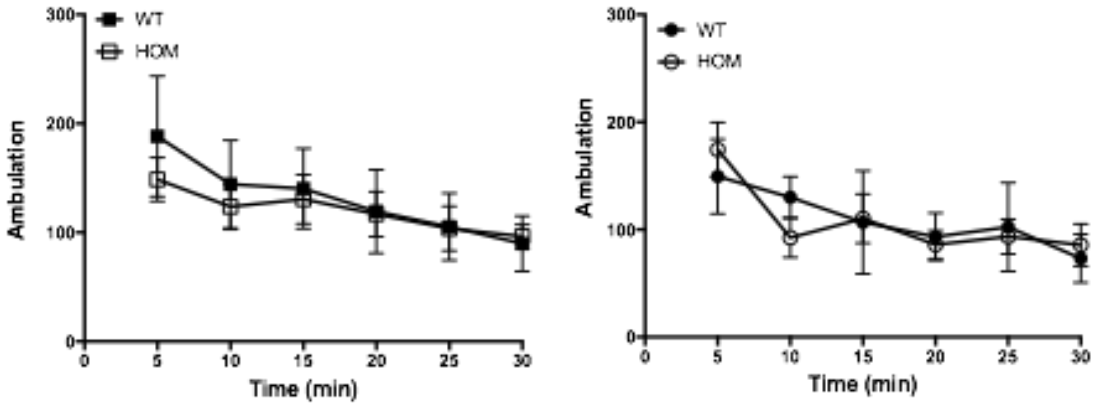


Figure 4.4 No difference in locomotor response to ethanol between WT and α6 KO. WT and α6 KO females (n=5-6) (A) and males (n=7) (B) show no difference in baseline locomotor activity.

Immunofluorescence

Alcohol-induced activation of VTA is blunted in female α4KO

Using c-fos as a marker for neuronal activation and tyrosine hydroxylase as a marker of dopaminergic neurons, we have previously shown that α4* nAChRs are critically involved in alcohol-induced activation of VTA DAergic neurons in male mice (Liu, Hendrickson et al. 2013). In order to assess any sex differences in the role of this receptor subtype, we administered saline or 2.0 g/kg or 3.0g/kg ethanol via i.p. injection to α4KO and WT mice female. We then sectioned the VTA and stained for c-fos and TH, with c-fos/TH double positive cells indicating activated dopaminergic neurons. We

focused on the pVTA, as previous studies have shown that this sub-region is especially important for alcohol-induced activation (Hendrickson, Zhao-Shea et al. 2009, Hendrickson, Zhao-Shea et al. 2010, Liu, Hendrickson et al. 2013, Liu, Zhao-Shea et al. 2013). Whereas male α4KO mice show no increase in c-fos activation in VTA DAergic neurons following 2.0g/kg ethanol (i.p.) while WT show a significant increase (Liu, Hendrickson et al. 2013). Females show a significant main effect of treatment [$F(1,27) = 18.23$, $p = 0.0002$], but not of genotype [$F(1,27) = 2.857$, $p = 0.1025$] and no interaction [$F(1,27) = 1.995$, $p = 0.1693$] **(Figure 4.5 A-C)**. This suggests any role for α4* nAChRs in this activation in females is small and non-critical.

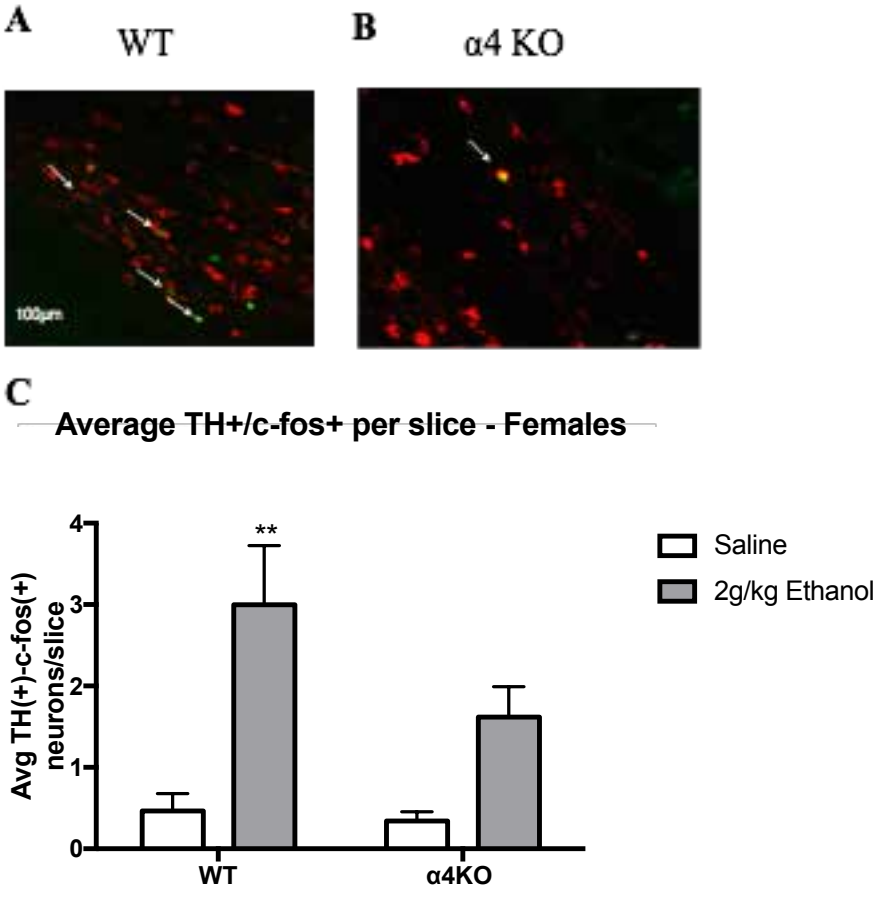


Figure 4.5 α4* nAChRs play a role in ethanol-induced VTA activation, but is not critical in females. A. WT females given i.p. ethanol (2.0g/kg) show increased c-Fos in TH positive neurons compared to saline. B. α4 KO females show a blunted increase in c-Fos. Results quantified in C. ** $p < 0.01$ two-way ANOVA with Tukey's multiple comparisons test.

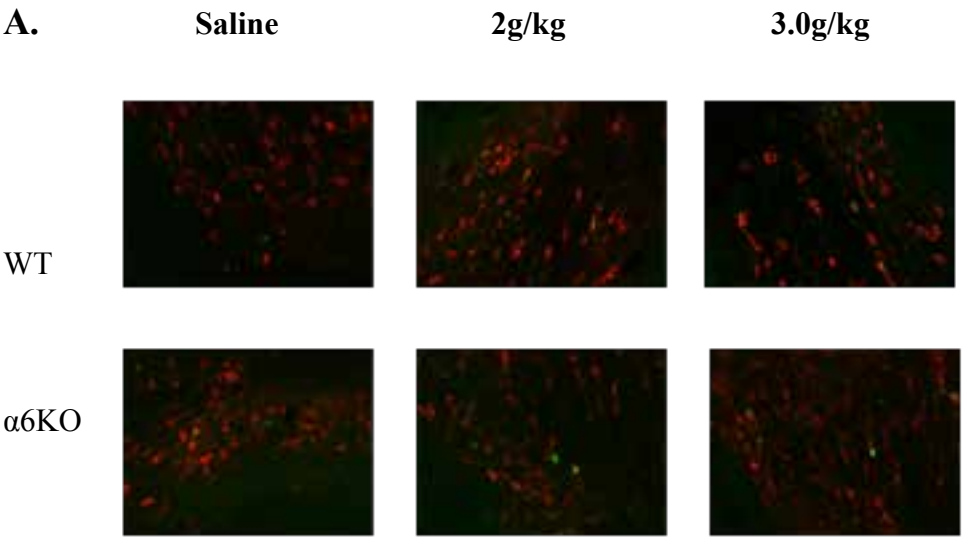
α6 nAChRs modulate activation of VTA*

In addition to the involvement of α4* nAChRs, we have also shown that the neurons that are activated following ethanol administration have higher α4 and α6 mRNA than those not activated following ethanol, suggesting a potential role for α6 as well in this activation (Hendrickson, Zhao-Shea et al. 2010). Therefore, we used the same protocol to assess ethanol-induced c-fos activation in α6 KO mice.

Male mice from the α6 line show a significant main effect of treatment [$F(2,26) = 17.58$, $p < 0.0001$], but not of genotype [$F(1,26) = 2.488$, $p = 0.1268$] nor a significant interaction [$F(2,26) = 1.36$, $p = 0.2744$] **(Figure 4.6 C,D)**. In females, similarly to males, there is a significant main effect of treatment [$F(2,28) = 7.24$, $p = 0.0029$], but not genotype [$F(1,28) = 0.3507$, $p = 0.5584$], and no interaction [$F(2,28) = 1.159$, $p = 0.3285$] **(Figure 4.6 A,B)**. Given these results, the genotypes were pooled within each sex to investigate the sex difference independent of the genotype. With 2.0g/kg ethanol, compared to saline, there is a significant main effect of treatment [$F(1,37) = 44.36$, $p < 0.0001$], but not genotype [$F(1,37) = 4.027$, $p = 0.0521$], and a significant treatment x genotype interaction [$F(1,37) = 6.387$, $p = 0.0159$]. Tukey's multiple comparisons test

show a significant increase in males and in females at 2.0g/kg ethanol, as well as a significant difference between male activation and female activation at this dose (male > female). At 3.0g/kg ethanol, only the significant effect of treatment remains [$F(1,42) = 29.53$, $p < 0.0001$], with no effect of genotype [$F(1,42) = 0.3127$, $p = 0.5790$] and no interaction [$F(1,42) = 0.01303$, $p = 0.9097$].

A.



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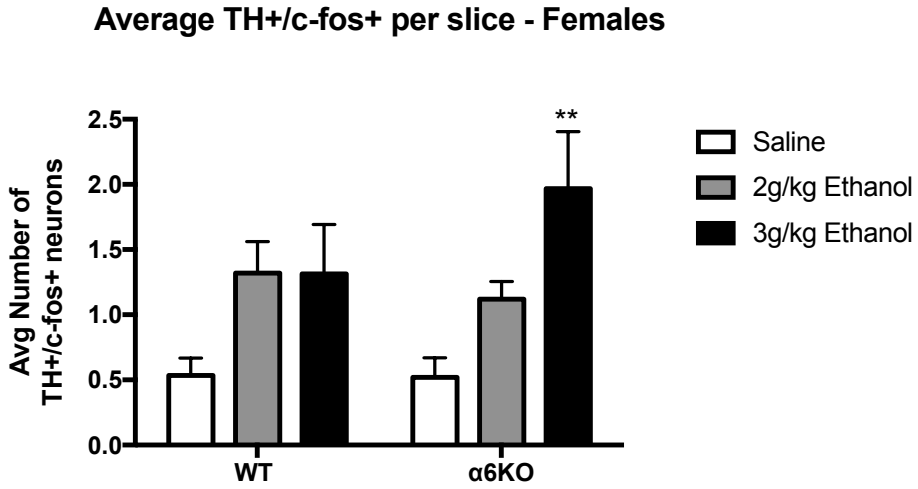

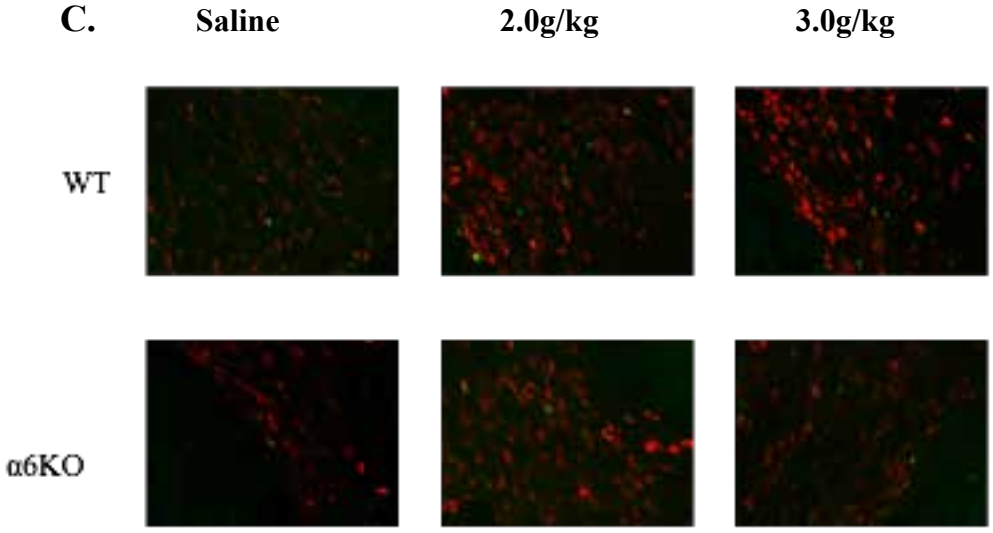

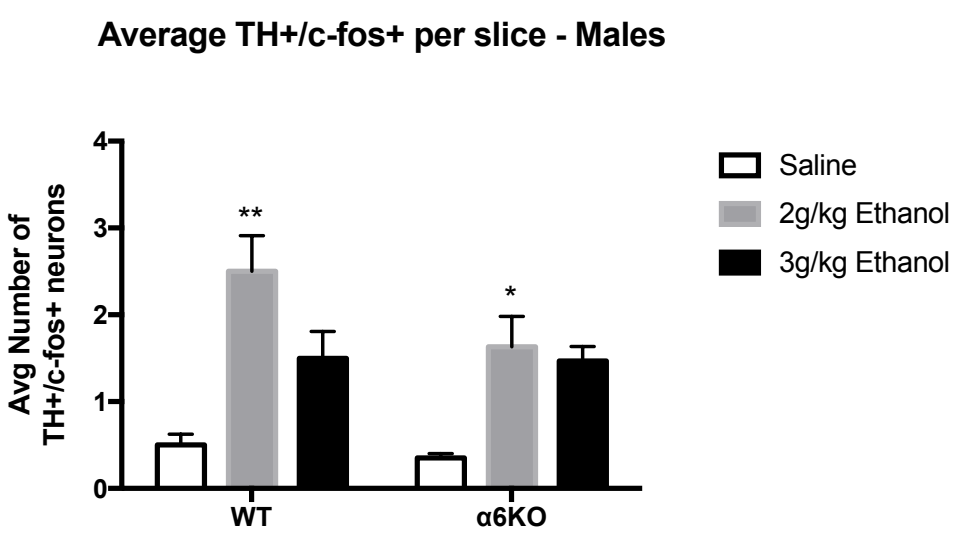

Figure 4.6 α6* nAChRs do not play a role in ethanol-induced activation in females, may play role in males. A. Female WT show increased c-Fos in TH+ neurons of the VTA following 3.0g/kg ethanol, as do α6 KO. Quantified in **B. C**. Male WT show increased c-Fos in TH+ neurons following 2.0g/kg, but α6 KO males show a blunted response at this dose. Quantified in **D.** *$p < 0.05$, ** $p < 0.01$ two-way ANOVA and Tukey's multiple comparison's test.

Discussion

The frequent co-abuse of alcohol and nicotine suggest a common neural mechanism of action of these two drugs. We have previously shown that α4* nAChRs play a critical role in ethanol reward and ethanol-induced activation of the dopamine reward pathway in males (Liu, Hendrickson et al. 2013), and that DAergic neurons activated following ethanol administration have higher α4, α6, and β3 mRNA than those not activated (Hendrickson, Zhao-Shea et al. 2010), suggesting a role for these receptor subunits in the effects of ethanol. In order to develop successful treatments for problem drinking, as well as alcohol dependence and addiction, we first need to understand the way in which ethanol acts both in the brain and in shaping behavior. Drinking patterns, quantity, and degree of euphoria experienced have been shown in humans to be critical factors in the progression from alcohol abuse to alcohol dependence (Dani and Harris 2005, WHO 2015), and uncovering the factors influencing these behaviors is an important step in understanding the progression of harmful alcohol use.

Humans and rodents both show sex differences in alcohol consumption (Lancaster 1995, Middaugh and Kelley 1999, Nolen-Hoeksema and Hilt 2006), which may be due to differences in the mechanisms underlying consumption and other ethanol-related behaviors. In humans, males tend to drink more ethanol and are more likely to meet criteria for alcohol dependence than females, although females tend to move from alcohol use to abuse more quickly than males (Nolen-Hoeksema and Hilt 2006). In rodents, sex

differences manifest differently, with females consuming more alcohol than their male counterparts (Lancaster 1995, Middaugh, Kelley et al. 1999). In our previous studies, we have shown that male mice lacking α4* nAChRs consume less 20% ethanol than their WT littermates following saline injections (Hendrickson, Zhao-Shea et al. 2010), and that the α4* nAChR partial agonist Varenicline (Chantix®) reduced drinking in WT males, but not α4KO males. Using the same DID protocol, we saw reduced consumption of 10% and 20% ethanol in males **(Figure 4.2 D-F)**, and no difference in ethanol consumption between α4KO and WT females at any ethanol concentration **(Figure 4.2 A-C)**. In order to ensure any ethanol consumption differences were specific to ethanol, we used 10% sucrose and 10 mM saccharin as caloric and taste controls, respectively. α4KO males consumed the same amount of sucrose as WT, but less saccharin **(Figure 4.2 F)**. This could be due to an overall reduced amount of drinking, as, although not significant, α4KO males showed a trend toward reduced sucrose intake, but given the similar consumption of 2% and 5% ethanol solutions between the two genotypes, this may not be due to simple hypodipsia. Anhedonia could account for the decrease in ethanol and saccharin, as could a general reward dysfunction. Females showed no differences in consumption of either control solution between α4KO and WT.

Unlike WT, α4KO males do not condition a place preference to the rewarding dose of ethanol, 2.0 g/kg (Liu, Hendrickson et al. 2013). Using the CPP assay, we found that only α4KO females condition a place preference to 3.0 g/kg, with no significant CPP

at any dose in WT females **(Figure 4.1A)**. This could be a result of a rightward shift in the dose response curve in α4KO females compared to WT, although in that case I would expect a CPP at a lower dose in WT females. There could be a difference in metabolism between the two genotypes, or perhaps the estrous cycle causing differences in ethanol reward. These could be explored by assessing blood-ethanol concentrations (BEC) and monitoring the estrous cycles of the females, respectively.

Given the role for α4* nAChRs in ethanol behaviors in males (Hendrickson, Zhao-Shea et al. 2010, Liu, Hendrickson et al. 2013), and the less clear results in females, it is tempting to speculate that perhaps a different subtype of nAChR may be important in females. The α6 subunit is present in only a few brain regions, including the mesolimbic dopaminergic pathway, which highlights the potential importance of this subunit in these regions (Le Novere, Zoli et al. 1996, Champtiaux, Han et al. 2002, Drenan, Grady et al. 2008). Liu et al. (2013) showed that ethanol-induced activation of VTA DAergic neurons *in vitro* may involve α6* nAChRs, a subunit known to co-assemble with α4 (Champtiaux, Han et al. 2002, Champtiaux, Gotti et al. 2003, Liu, Hendrickson et al. 2013, Liu, Zhao-Shea et al. 2013), and Hendrickson et al. (2010) showed elevated mRNA of both α4 and α6 in ethanol-activated VTA DAergic neurons. Additionally, α6KO male and female mice are more sensitive to the sedative effects of ethanol than WT (Kamens, Hoft et al. 2012), although the same study also found no differences in ethanol consumption in these mice. Pharmacological blockade of VTA α6* nAChRs, using the antagonist α-conotoxin MII,

reduces ethanol reward (Larsson, Jerlhag et al. 2004), and it has recently been shown that male and female mice with hypersensitive α6* nAChRs show increased ethanol consumption and sensitivity to ethanol reward. These data make α6* nAChRs good candidates for involvement in ethanol behaviors, especially in females, and perhaps in concert with the α4 subunit.

Using the DID binge-like drinking assay, I explored the role of α6* nAChRs in ethanol consumption. I saw no difference between α6KO mice and their WT littermates at any ethanol dose, in males or females **(Figure 4.4)**. This suggests little to no role of α6* nAChRs in this behavior. Powers et al. (2013), however, found a role for α6* nAChRs using a mouse line containing a hypersensitive α6 subunit. The discrepancy in results could be due to procedural differences or to effects of the genetic changes on the receptors themselves. For example, α6KO mice may display functional compensation by other subunits. Similarly, a hypersensitive α6 subunit may not necessarily portray a physiological phenomenon accurately. These caveats could be ameliorated by using optogenetics (Wang, Szobota et al. 2007) to silence neurons containing α6* nAChRs during behavioral assays, minimizing the effects of developing without the subunit.

We also looked at the role of α6* nAChRs in ethanol reward using conditioned place preference. Given previous results of higher α6 mRNA in ethanol-activated DAergic VTA neurons (Hendrickson, Zhao-Shea et al. 2010) and hypersensitive L9'S mice showing CPP at a sub-rewarding dose of ethanol (Powers, Broderick et al. 2013),

we hypothesized that our α6 KO mice would show reduced CPP compared to WT. We previously presented α6 KO CPP results (Guildford, Sacino et al. 2016), with the sexes combined, but have separated them out here to assess any sex differences. The generally accepted rewarding dose of ethanol is 2.0 g/kg, and this dose elicited a CPP from WT males and females, as well as α6KO males and females **(Figure 4.3)**. A CPP is indicative of reward, and thus it seems, at this dose, α6 may not play an important role. However, at 3.0 g/kg, the male and female WT, but not α6 KO, mice showed a CPP. This suggests a role for α6* nAChRs at higher doses of ethanol or alternatively, a slight leftward shift in the ethanol response, making 3.0 g/kg potentially aversive to the α6 KO animals. Powers et al. (2013) showed L9'S mice condition a place preference at 0.5 g/kg ethanol, a dose which did not show a CPP in WT mice or α6 KO mice, but, were unable to elicit a CPP in WT animals at 2.0 g/kg ethanol. This suggests some role for α6* nAChRs, though it would be expected that a hypersensitive receptor would act in the opposite manner to that of a knocked out receptor. Procedural differences, for example training sessions done on alternating days (saline one, ethanol the next etc.) for a total of 10 days, versus ours with both done each day, 6 hours apart, for 4 days, may account for the different results. It could also be the case that these L9'S α6* nAChRs, or those with the α6 subunit knocked out during development, act differently than physiologically normal receptors.

α4* nAChRs play little to no role in female ethanol consumption and reward, unlike their male counterparts (Liu, Hendrickson et al. 2013), exposing a gender

difference in the way in which mice respond to ethanol. It is important to consider that this gender difference may translate to humans as well, given that current research focuses mainly on males. α6* nAChRs, unlike α4* nAChRs, may play a role in ethanol reward at high ethanol doses (3.0 g/kg) in both males and females, but these receptors are not necessary for alcohol reward at 2.0 g/kg, the generally accepting rewarding dose.

The VTA is a central part of the mesolimbic dopamine reward pathway, and has been shown to be activated following ethanol administration (Blomqvist, Ericson et al. 1997, Larsson, Edstrom et al. 2005, Hendrickson, Zhao-Shea et al. 2010, Liu, Hendrickson et al. 2013, Liu, Zhao-Shea et al. 2013). We have also previously shown a crucial role for α4* nAChRs in activation of the DAergic neurons of this area following ethanol, wherein α4 KO male mice show no increase in c-fos activation following 2.0 g/kg ethanol, while WT littermates show a significant increase (Liu, Hendrickson et al. 2013). Here, we have shown that this role is different in females, as there is no significant interaction between genotype and treatment, although the response in α4 KO females appears blunted compared to WT **(Figure 4.5 A-C)**.

Given the propensity of α4 subunits to co-assemble with α6 in the VTA, and the potential role of α6* nAChRs in *in vitro* activation of VTA DAergic neurons with ethanol (Liu, Zhao-Shea et al. 2013), we explored *in vivo* activation of these neurons using c-fos as a marker of neuronal activation. Following systemic ethanol (2.0 g/kg and 3.0 g/kg), WT males showed a significant increase in c-fos in TH-positive neurons compared to

saline controls, indicating and increase in activation of VTA DAergic neurons with ethanol **(Figure 4.6 C,D)**. α6KO males also showed this activation pattern, indicating no effect of a lack of α6* nAChRs in ethanol-induced activation in these animals. Similarly, there was no difference in alcohol-induced activation in WT and α6 KO females; both showed increased c-fos in VTA DAergic neurons following 2.0g/kg and 3.0g/kg ethanol **(Figure 4.6 A,B)**. Liu et al. (2013) showed a role for α6* nAChRs in ethanol-induced activation of VTA DAergic neurons, but those studies were done *in vitro* via electrophysiology, in which afferents and efferents are not all intact, using higher ethanol concentrations than those occurring physiologically and are not easily translated into a g/kg dose. In addition, the ethanol was directly applied to the VTA, whereas *in vivo*, the ethanol is given i.p. and metabolized. This method of ethanol delivery is more physiologically relevant than ethanol being directly infused onto the VTA, although there are even more relevant delivery mechanisms than those used in this study, for example gavage. Additionally, those results did not differentiate between males and females, which, as we have shown in this study, have differences in activation of the VTA following ethanol administration.

Interestingly, there is a disconnect between activation of the VTA DAergic neurons as measured by c-fos activation, and the expression of ethanol reward, measured by CPP. While both genotypes and sexes show c-fos activation following 2.0 and 3.0 g/kg ethanol, α6 KO males and females show no CPP at 3.0 g/kg while their WT counterparts

do. This suggests that activation of these neurons is not necessarily directly related to the expression of CPP. It may be that at the higher dose of ethanol, versus the rewarding dose of 2.0g/kg, a different neuronal subtype, such as GABAergic neurons, may be involved in the expression of CPP. Although, with α6* nAChRs expressed only on DAergic, and not GABAergic, neurons in the VTA, it is unlikely to be the case here. The Koobian model of addiction suggests there exists not only a reward pathway, but an anti-reward pathway, involved in the adaptive processes to restore normal function in the presence of chronic drug use (Koob and Le Moal 2008). This pathway, which includes the hypothalamic-pituitary-adrenal (HPA) axis as well as other stress-related areas such as the habenula and interpeduncular nucleus (IPN), is mediated in part by corticotropin-releasing factor, CRF, which has been shown to be involved not only in stress responses, but also in anxiety induced by nicotine withdrawal (Zhao-Shea, DeGroot et al. 2015, Pang, Liu et al. 2016). Perhaps 3.0g/kg ethanol activates other areas besides the VTA, specifically areas such as the IPN which not only contains CRF receptors, but also projects to the VTA (Zhao-Shea, DeGroot et al. 2015).

More studies are required to decipher the exact role of α6* nAChRs and how these play into behavioral reactions to ethanol, but this study uncovered an important distinction between ethanol effects on male and female mice, the differential role played by α4* nAChRs, and a potential indirect relationship between the VTA and alcohol reward at higher doses. This idea, explored further, could be used to develop gender-

specific treatments if indeed there are such deep, potentially genetic, differences underlying alcohol's effect on the reward pathway.

CHAPTER V: Discussion

The exact mechanism by which addiction develops remains unclear, but the breadth of the disease is unmistakable. An estimated 22.7 million Americans age 12 and over are problematic drug and/or alcohol users or addicts, 9.9 million persons have driven under the influences of illicit drugs in the past year, 28.7 million have driven under the influence of alcohol in the past year, while only about 2.5 million received treatment as of 2013 (SAMHSA 2014). The effects of addiction reach much further than the addict, impacting their loved ones, increasing violence and child neglect, increasing the risk of traffic accidents while under the influence, leading to absenteeism in the workplace, and place an economic burden on society as a whole (WHO 2015).

Addiction is likely a combination of a genetic and/or physiological disposition mixed with social and environmental influences (Lenz, Muller et al. 2012). Although the human condition of addiction is vastly more complex than anything we can recreate in an animal model, exploring individual aspects of addiction such as drug intake, reward, and drug-induced activation of the reward pathway, allows us to shed light on the biological underpinnings of the devastating human disease. In order to tease out the contributions of genetic makeup and environmental influences, we employed genetic mouse models including two nAChR knockout mouse lines, α4 KO and α6 KO, as well as a hypersensitive nAChR knock-in mouse, Leu9'Ala. While these genetic mouse models are incredibly powerful tools in exploring the contributions of nAChRs in alcohol use and

abuse, they are not without issue. Knockout mice develop without a specific nAChR subunit, allowing for potential compensation issues. For example, α4 KO mice, lacking the α4 subunit, have significantly less α6 expression, measured by immunoprecipitation with epibatidine, while α6 KO mice actually have higher α4 expression (Champtiaux, Gotti et al. 2003). There are also instances of functional compensation, in which some other cells not normally involved in a given action could change their function in order to compensate for the missing subunit, or the compensation could be present but masked entirely by partial redundancies already in place (Picciotto and Wickman 1998). The hypersensitive mouse line, Leu9'Ala, creates an exquisitely sensitive receptor, one which is not physiological in its activation.

There are other technologies available that can circumvent some of these issues, including small interfering RNAs (siRNAs) that could be used to knockdown gene expression in adult animals, minimizing compensation that arises from developmental absence of a receptor subtype, but this would require a more localized target. In our case, a global knockout allowed us to explore the potential role of nAChRs in alcohol reward in general before narrowing down the target areas for exploration. Other tools available include optogenetic activation or deactivation of neurons using light-gated channels, channelrhodopsin and halorhodopsin, respectively, allowing temporal control of neuronal activation as well as spatial and the ability to recreate a behavioral phenomenon simply by activating or inhibiting a specific subset of neurons (Tsai, Zhang et al. 2009).

Not all drug users become addicted, and not all reward leads to addiction, but behavioral assays such as operant self-administration can be used to predict the problematic drug use patterns that may lead to addiction, and allow researchers to study the transition from abuse to addicted (Becker and Koob 2016). Other assays, such as conditioned place preference (CPP) and drinking-in-the-dark (DID), allow for the exploration of drug use from reward and intake angles, allowing the different aspects of drug use and eventual abuse and/or addiction to be separated. All assays have caveats, however, and behavioral assays are especially prone to outside factors influencing the results of the test. For example, a CPP in response to alcohol looks the same as a CPP to the chamber associated with alcohol, as that is what is actually measured. If the CPP is to the light, smells, sounds, etc. of the chamber, and not to the drug itself, it could result in a false-positive result. As such, all CPP experiments are counter-balanced to account for this, and initial chamber bias is taken into account when assigning the drug-paired chamber. With each animal receiving alcohol in the initially non-preferred chamber, it is unlikely that any initial bias would affect results in a positive manner; any CPP resulting in the initially non-favored chamber must first overcome the initial bias against the chamber. Every effort is made to reduce initial bias differences between the two conditioning chambers, the white and black chambers, to ensure a fairly even number of animals having each of the two as their drug-paired chamber, but it is in the nature of the animals to seek out the darker, black chamber, and that chamber is often the initially

preferred and thus vehicle-paired. This could affect the results as the animals may develop a CPP more easily to the white chamber based on sounds or smells, for example, but this was not seen in initial troubleshooting of the assay and would surface in the saline-saline control assays wherein the animal receives saline in both chambers.

Although the predominant areas in the pathway are the VTA, NAc, and PFC, there are other critically involved regions that influence this pathway. Projections from the lateral dorsal tegmentum (LDTg) and the pedonculopontine tegmentum (PPTg) to the VTA provide ACh, Glu, and GABA. The lateral hypothalamus sends GABAergic projections to VTA DA neurons, as does the lateral habenula. It has also been shown that the interpeduncular nucleus (IPN) has GABAergic projections onto VTA DA neurons, which in turn release corticotropin releasing factor (CRF) in the IPN (Zhao-Shea, DeGroot et al. 2015). Amazingly, this is still a simplified version, as many of the projections are neuron- and region-specific. For example, the PFC Glu projections into the VTA synapse exclusively onto DA neurons which then project to the NAc, and not onto those which project back to the PFC, creating a series of direct and indirect circuits within the reward system itself (Fields, Hjelmstad et al. 2007). Each target of the VTA receives projections from a distinct neuronal population within the VTA , adding even more to the complexity of this circuit (Fields, Hjelmstad et al. 2007).

Given the increase in ACh seen concurrently with the alcohol-induced increase in NAc DA (Larsson, Edstrom et al. 2005), it seems likely that the LDTg and/or PPTg may

be influenced by ethanol as well, although this has yet to be shown. It could be that the increased ACh is correlated but not causal, or perhaps the activated DAergic neurons, presumably those causing the increased DA in the NAc, are unable to bind ACh as the receptors are already either in the active or desensitized state, precluding ACh binding; if enough of the ACh receptors are unavailable, the ACh levels may seem elevated. We hypothesize instead that once the ACh is in the VTA, alcohol potentiates the response of DA neurons to ACh via nAChRs. We have previously shown that alcohol does indeed potentiate the response to ACh, though males and females were not investigated separately, and have seen that both α4* and α6* nAChRs play a role in this response (Liu, Hendrickson et al. 2013, Liu, Zhao-Shea et al. 2013). Although I did not see the same reliance on α6* nAChRs in activation of the VTA following alcohol, the electrophysiological approach used in the previous studies explored a temporally controlled response, a weak point of c-fos, although in an incomplete circuit whereas in the c-fos experiment, alcohol was delivered in a whole animal before the brain was sliced.

Interestingly, the nAChR subtypes involved are likely different in males and females. In males, α4* nAChRs are required for alcohol reward and alcohol-induced VTA activation, and play a role in alcohol consumption (Liu, Hendrickson et al. 2013), but α4 KO females show more robust reward than their WT counterparts (MGD unpublished). Both sexes seem to involve α6* nAChRs in alcohol sensitivity (Guildford, Sacino et al.

2016), with α6 KO of both sexes showing no CPP with 3.0g/kg, while their WT counterparts did. Perhaps males rely on an α4α6* nAChR mechanism, while females use a non-α4, α6* nAChR mechanism to modulate activation of the VTA and increase DA in the NAc. These possibilities could be explored either using double knockout animals, lacking both α4 and α6 subunits, or by knocking each subunit down in adult animals to explore the effects of alcohol on NAc DA release.

The c-fos activation data presented here suggest that activation of the VTA is not necessary for alcohol CPP, at least in females, where we saw no significant increase in activation with 2.0g/kg, a dose which induced CPP in (α6) WT. In males, this dose led to both activation and CPP in WT (Guildford, Sacino et al. 2016). Additionally, female α6 KO animals showed significant c-fos activation following 3.0g/kg ethanol, a dose that did not condition a place preference in these animals. This disconnect in females suggests alcohol may cause reward without appreciably activating DAergic neurons in the VTA, perhaps acting via a separate system entirely, or there may be a temporal issue in seeing this effect. As c-fos is not a particularly temporally sensitive measurement of activation, being measured about 90 minutes after an alcohol challenge, it is possible the activation may have occurred outside our experimental timeline. A more precise measure of neuronal activation would be to use calcium or voltage sensors.

The discordant female data could be interpreted a different way, where rather than acting as the accelerator, alcohol could lead to activation of the reward pathway by

instead removing the brakes on the system. Dopaminergic neurons in the VTA are tonically inhibited by GABAergic interneurons (Burkhardt and Adermark 2014); thus, rather than directly activating DA neurons, or even indirectly activating them via potentiation of serotonin or ACh, alcohol may in fact be inhibiting inhibitory GABAergic inputs onto DA neurons, thus disinhibiting these neurons (Gremel and Cunningham 2008, Morikawa and Morrisett 2010, Bahi and Dreyer 2012, Soderpalm and Ericson 2013, Burkhardt and Adermark 2014). It has been shown that alcohol decreases the firing rate of GABAergic neurons at the same dose that is shown to activate DAergic neurons, and stimulating these GABAergic neurons leads to decreased DA release in the NAc (Burkhardt and Adermark 2014). Antagonism of GABA A receptors in the VTA not only leads to increased NAc DA (Soderpalm and Ericson 2013), but rodents will even self-administer these antagonists in the VTA (David, Durkin et al. 1997). Although there is a correlation between decreased VTA GABA and increased NAc DA, the DA increase has been shown in the absence of, or prior to, the GABA decrease (Burkhardt and Adermark 2014). It is likely that the role of GABA changes over the course of the addiction process, perhaps important more in maintenance than initiation/intoxication (Burkhardt and Adermark 2014).

α4* nAChRs are found on GABA neurons in the VTA, but α6* nAChRs are not, indicating that there could also be differential neuronal subtype involvement in each sex where these GABAergic neurons containing α4* nAChRs could be involved more in one

sex than the other, accounting in part for the reliance on the α4 subunit in males but not females. This idea could be investigated using selective activation or inhibition of GABAergic neurons via optogenetics, wherein an animal expressing Cre recombinase only in GABAergic neurons is given an intra-VTA injection of a floxed, light-gated virus, allowing light to be used to either activate or inhibit these neurons, depending on the channel used in engineering the virus (Tsai, Zhang et al. 2009).

Additionally, α6* nAChRs are found predominantly on the terminals of DAergic neurons projecting from the VTA to the NAc, which could affect the c-fos data as we looked in the cell bodies in the VTA and not the NAc terminals. Therefore, an absence of VTA activation at the DAergic cell bodies in α6 KO may not reflect the role of α6* nAChRs in modulating dopamine release in target areas at the terminal level. This could be explored by measuring alcohol-induced DA release in the NAc in α6 KO and WT mice, given that these receptors would be more prominent in this that in activation of the VTA DAergic neurons.

Whatever the role of these nAChRs are in the VTA and/or NAc, it is important to also note that the vast majority of research done prior to this was done solely in males, and there is much work to be done in females. In this study, we started the female exploration at the same point we had reached with our male studies, with the assumption that nAChRs were involved in alcohol consumption and reward. We perhaps could have benefitted from employing pharmacological studies using mecamylamine to first

determine if there were, in fact, any role for nAChRs. Kamens et al. (2010), however, showed a role for α7* nAChRs in alcohol intake in females only, suggesting there may be a role for these nAChR subtypes in females. Given the results in the present study, there is more background research to be done in whether nAChRs do, in fact, play a role in other alcohol-related behaviors before proceeding with additional specific probes.

While sex differences in human alcohol addiction involve societal and other environmental influences, dependence and reward in animals can be studied empirically to uncover purely genetic components. For example, by moving the testosterone-inducing *Sry* gene from the male Y chromosome onto an autosome, Barker et al. (2010) were able to investigate the role of genes and hormones separately by allowing independent assortment of this normally male-only gene. Creating XY gonadal females and XX gonadal males allowed separation of the two components, ultimately showing a role for both in striatal differences between normal genetic males and females (Barker, Torregrossa et al. 2010).

Genetic differences stem not only from the presence or absence of the Y chromosome or the *Sry* gene, but from the presence of one X versus two. With two X chromosomes, females often experience X-silencing, wherein in certain cells, only one X chromosome's genes are expressed. A female will have inherited one X chromosome from her mother and one from her father, and thus either, or both, could be expressed in each cell. In males, there is only one X chromosome and thus no need for X-silencing.

This X is only from the mother, as the father contributes a Y to all sons, and thus all X-related genes will be from the mother alone. This X-silencing, or lack thereof, could influence the expression, and levels, of important genes for alcohol abuse or addiction (Sanchis-Segura and Becker 2016).

Additionally, males and females have different hormones and different levels of shared hormones, including estrogen and testosterone. These hormones have been shown to interact with alcohol (Lenz, Muller et al. 2012), and with various receptors found in the reward pathway (Becker and Hu 2008, Lenz, Muller et al. 2012, Agabio, Campesi et al. 2016, Carroll and Lynch 2016), which could be involved in the sex differences, but with alcohol also able to modulate expression of these sex hormones, the reciprocal relationship makes it difficult to determine a causal relationship.

These sex differences, though unknown in their exact origin, should also be reflected in the treatments offered for alcohol use disorders. Treatments available now are not only ineffective and riddled with adverse side effects in many cases, but they also are based on studies done mainly using male subjects. It has been suggested that females may be more susceptible to the negative reinforcing effects of nicotine than males, with females more likely to relapse in response to withdrawal and negative affect (O'Dell and Torres 2014). Dysregulation of stress response system, including the hypothalamic-pituitary-adrenal (HPA) axis and the CRF system, has been shown with chronic drug use, and stressors that activate this system are also factors in the initiation of, or reversion

back to, drug use (Bobzean, DeNobrega et al. 2014). Interestingly, this stress response system is regulated by ovarian hormones, which is compatible with the idea that females may use drugs to avoid negative outcomes rather than in pursuit of positive outcomes (Bobzean, DeNobrega et al. 2014, O'Dell and Torres 2014). With CRF already implicated in nicotine-withdrawal-induced anxiety, and the reciprocal connection between the CRF-containing IPN and the VTA, it is certainly possible that CRF and the IPN could play a role in the switch from positive- to negative-reinforcement of alcohol, and thus may also be involved in the sex differences seen in the reinforcement behind alcohol use (Zhao-Shea, DeGroot et al. 2015).

With different reasons for drinking, different behavioral and physiological responses, and different hormones to contend with, there should be different treatments available; the more specific and targeted the treatment, the less likely they are to cause so many treatment-ending side effects, and the more likely they are to successfully treat those suffering from addiction.

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