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THE ROLE OF NICOTINIC ACETYLCHOLINE RECEPTORS IN ETHANOL RESPONSIVE BEHAVIORS AND DRINKING

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A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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List of Abbreviations and Symbols

ACh	acetylcholine
AUD	Alcohol Use Disorders
B6	C57BL/6 mice
BAL	Blood Alcohol Level
BAES	Biphasic Effects of Alcohol Scale
BEC	Blood Ethanol Concentration
CA	continuous access group
CPP	conditioned place preference
CRF	corticotropin releasing factor
D2	DBA/2 mice
DA	dopamine
DHβE	dihydro-beta-erythroidine
DID	Drinking-in-the-Dark
DM	Differentiator Model
DSM-IV	Diagnostic and Statistic Manual of Mental Disorders, 4^{th} edition
EDE	Ethanol Deprivation Effect
EPM	Elevated Plus Maze
ES	Embryonic Stem Cells
ETOH	ethanol (alcohol)
FHP+	Family History Positive
FHP-	Family History Negative
FTND	Fagerstrom Test of Nicotine Dependence
HPA	hypothalamic-pituitary-adrenal axis
i.p.	intraperitoneal
IA	intermittent access (group)
IPN	interpeduncular nucleus
KO	knockout
LBD	ligand binding domain
LORR	loss of righting reflex
LLR	Low Level of Response
MDS	mesolimbic dopamine system
MHb	medial habenula
MLA	methyllycaconitine
NAC	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
NIC	nicotine
QTL	Quantitative Trait Loci
SAL	saline
SEM	standard error of the mean
SHAS	Subjective High Assessment Scale
SNP	Single Nucleotide Polymorphism
SR	Subjective Response
s.c.	subcutaneous
VAR	varenicline

VTA	ventral tegmental area
WT	wildtype

Abstract

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By Anton Jerome Dawson, B.S., B.A.

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The high co-morbidity between alcohol (ethanol) and nicotine abuse suggests that nicotinic acetylcholine receptors (nAChRs), which are thought to underlie nicotine dependence, may also be involved in alcohol dependence. A genomic region that encodes the α5* nAChR subtype has recently been shown to be associated with alcohol dependence phenotypes in humans. Therefore, the aim of this study was to determine the role of α5* nAChRs in ethanol-responsive behaviors upon acute administration in mice as well as in their drinking behavior. We conducted tests in mice lacking the α5 coding gene (*Chrna5*) in ethanol-induced hypothermia, hypnosis, anxiolysis, and conditioned place preference. We also assessed drinking behavior in these mice using models of voluntary ethanol consumption, two-bottle choice preference and intermittent access, as well as acute binge drinking behavior in the Drinking-in-the-Dark paradigm.

Our results showed that deletion of the α5* gene enhanced acute behaviors, including ethanol-induced hypothermia, hypnosis recovery time, and the anxiolytic-like response in mice. We also found that α5 gene deletion resulted in decreased ethanol CPP, but had no effect on ethanol consumption in either model of drinking behavior tested under normal conditions. However, we discovered that under conditions of stress from multiple daily injections of saline or nicotine, Drinking-in-the-Dark intake was reduced in α5 null mutant mice. We also examined the role of β2* nAChRs due to the tendency of the α5 subunit to be co-expressed with this subtype, which also plays an important role in nicotine dependence. Our results showed that pharmacological and genetic manipulation of β2* nAChRs modulated some acute alcohol-responsive behaviors, namely, hypnosis, recovery-time and the anxiolytic-like response produced by ethanol, but did not modulate ethanol drinking behavior in mice. These studies provide evidence that α5* subtypes and β2* subtypes, which play a critical role in nicotine dependence, also play a role in acute ethanol-responsive behaviors *in vivo*, thus supporting studies in humans that nicotine and alcohol dependence share common genetic components.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Background and Significance

Alcohol (ethanol) abuse is one of the leading causes of preventable death in society responsible for nearly 3.2% of deaths worldwide (WHO, 2004). It occurs regardless of social or ethnic affiliation. It is estimated that at least one person in every family in Western society has suffered, directly or indirectly, from alcoholism. In the United States alone, 4% of the population is affected with the estimated economic burden exceeding $366 billion a year (Chatterjee *et al.* 2010). Alcohol abuse has vast health consequences contributing to a multitude of medical complications including damage to several important organs such as the liver, pancreas, and brain, as well as immune functions. For example, data show that smoking and alcohol drinking may lead to synergistic increase in risks of various cancers such as those of the head, neck, esophagus, and duodenum (Meyerhoff *et al.* 2006). Alcohol-induced brain damage is a particularly serious problem during pregnancy and adolescence due to the sensitivity of the developing brain to alcohol. Up to 7/1,000 infants are born with fetal alcohol syndrome, one of the most common non-genetic, forms of mental disability (Niccols, 2007). Adolescents, who display as much as a 30% prevalence rate of binge drinking, have higher sensitivities to alcohol-induced brain dysfunction and cognitive impairment of the adult brain, and the onset of adolescent drinking increases the risk for developing alcoholism in the future (Grant & Dawson, 1998; Crews *et al.* 2000, Slawecki *et al.* 2004, Spear *et al.* 2005). Furthermore, alcohol abuse also has a high

comorbidity with psychiatric disorders as well as other addictive behaviors, including nicotine dependence (Kessler *et al.* 1994, Swendsen *et al.* 1998, Funk *et al.* 2006).

There are few FDA approved medications for the treatment of Alcohol Use Disorders (AUDs), which currently are limited to disulfiram (Antabuse®), naltrexone (ReVia®, Depade®, Vivitrol®), and acomprosate (Campral®). Moreover, these drugs have only managed up to a 30% success rate (Spanagel *et al.* 2009, Chatterjee *et al* .2010). Thus, there remains a clear need for further effective pharmacotherapies in dealing with these disorders. While previous treatments have targeted many of the brain neurotransmitter and neural systems classically associated with alcohol's effects including GABA, glutamate, and opioid systems, nicotinic acetylcholine receptors (nAChRs) are emerging as a likely candidate for the development of novel treatments. Indeed, evidence suggests that as many as 80% of alcoholics are smokers and the high incidence of smoking and alcohol co-abuse that nAChRs play an important role in alcohol consumption and relapse-like behavior (Istvan *et al.*1984, Falk *et al.* 2006, Pothoff *et al.* 1983, Blomqvist *et al.* 1996, Lé *et al.* 1996). Furthermore, there is evidence showing that genetic factors are predictors of both long-term alcohol and tobacco consumption (see Schlaepfer *et al.* 2008 for review). Because of the estimation that common genetic factors account for as much as 40% of the covariance between alcohol and nicotine abuse (Swan *et al.* 1997), it is very likely that such genetic factors (i.e. nAChRs) underlying smoking addiction are common to those underlying AUDs as well. This notion is supported by clinical evidence showing that drugs targeting nAChRs such as the nonselective antagonist mecamylamine and the partial agonist and smoking cessation aid varenicline (Chantix™) actually reduce alcohol consumption in human subjects (Chi *et al.* 2003, McKee *et al.* 2009).

The goal of this thesis is to use animal models to explore the role nAChRs play in important pharmacological and behavioral responses to alcohol in the hope of contributing new findings that could lead to the discovery of novel therapeutic targets that may aid in curbing excessive alcohol drinking and, potentially, smoking addiction simultaneously. The following section of the introduction will discuss the structure and function of nAChRs as well as review the relevancy of these receptors to AUDs as discovered by human and animal studies. The next section will provide an overview of the molecular mechanisms by which AUDs are thought to arise. The chapter will then be concluded by reviewing the current evidence suggesting nAChRs as genetic elements in mediating alcohol's behavioral effects.

1.2 Structure and Function of Nicotinic Acetylcholine Receptors

Nicotinic Acetylcholine Receptors are part of the superfamily of ligand-gated ion channels. They have a shared common basic structure that is permeable to mono- and divalent ions under control of extracellular signaling molecules (see Hurst *et al.* 2013 for review). These receptors have been conserved throughout evolution, with human nAChRs sharing a high degree of structural homology with many organisms, from animals all the way down to the simplest forms of life, such as some bacteria (Thany *et al.* 2007). These structures are divided into distinctive subtypes, each retaining specific functions and capable of mediating unique pharmacological effects. To date, 16 genes have been indentified in mammalian genomes that code for distinct nAChR subunits that comprise each receptor subtype found in the body (Dani & Bertrand, 2007; Lukas *et al.* 1999). While it is true these genes code for nAChRs found in both the central and

peripheral nervous systems, this review will focus specifically on those that code for receptors in the brain (neuronal nAChRs). Each neuronal nAChR subtype consists of five subunits comprised of $\alpha(\alpha2-\alpha10)$ and/or $\beta(\beta2-\beta4)$ subunits that form around a central pore (Figure 1A). Each subunit contains a hydrophilic extracelluar amino terminal that faces the synaptic cleft, followed by three hydrophobic transmembrane domains (M1-M3), a large intracellular loop, and a forth hydrophobic (M4) transmembrane domain (Figure 1B). These domains are precisely arranged to facilitate the passage of cations such as Na^+, K^+, and Ca^{2+} through the pore following the binding of an extracellular signaling molecule to the ligand binding domain (LBD). The pinwheel structure forms the five subunit complex consisting of a primary face carrying $\alpha2$, $\alpha3$,$\alpha4$,$\alpha6$, $\alpha7$, $\alpha8$, and/or $\alpha9$ subunits, and a complementary face carrying $\beta2$, $\beta4$, $\alpha7$, $\alpha8$, $\alpha9$ and/or $\alpha10$ subunits (Elgoyhen *et al.* 2001). Each receptor subtype exists as a heteromeric ($\alpha2-\alpha6$,$\alpha10$ and $\beta2-\beta4$, Figure 1C) or homomeric ($\alpha7$, $\alpha8$, and $\alpha9$, Figure 1D) structure containing either two or five binding sites, respectively.

The nicotinic receptor exists in three general states, namely, resting (closed), open, and desensitized at any given moment (Figure 2). When stimulated by agonist binding to the orthosteric site, nAChRs transition from the closed to open state, allowing an influx of cations and thus depolarizing the cell membrane and increasing neuronal excitability. The influx of the cations, particularly Ca^{2+}, leads to changes in a wide array of intracellular signaling cascades. The transition rate of each state is influenced by the binding of endogenous and/or exogenous extracellular ligands, not only at the LBD, but also at allosteric sites on the receptor. Following activation, the receptor will either return to the resting state upon agonist dissociation (deactivation), or transition into a

high affinity, agonist-bound, non-conducting state known as desensitization. The frequency and length of this transition state depends on a variety of parameters, and subunit composition differentially contributes to the rate and probability of desensitization (Giniatullin *et al.* 2005, Quick & Lester 2002). Virtually all nAChRs display low affinity for natural agonist and fast transition from the resting to open state; two critical properties for the fast, high frequency signaling that occurs at synapses.

Nicotinic receptors in the mammalian brain are primarily expressed at presynpatic, peri-synaptic, and extra-synaptic sites where their most common role is to modulate neurotransmitter release (Hurst *et al.*2013). This is a principal reason why nAChRs are theorized to play a critical role in drug dependence since they modulate the release of several other neurotransmitters associated with drug dependence. Receptor distribution is another key factor that determines the neurobiological effects mediated by nAChRs. Various approaches are used to indentify nAChR distribution. These include mRNA expression analysis of different brain regions using specific primers, employing radiolabeled nicotinic ligands with sufficient selectivity and sensitivity for targeted subtypes, using antibodies as specific markers for nAChR subunit proteins, and examining the functional and pharmacological sensitivity of receptors expressed in various brain areas (Hurst *et al.* 2013).

It should be noted that the stoichiometry of a particular nAChR also affects the interaction with its respective ligand. For example, $\alpha4\beta2$* nAChRs, which are highly sensitive to ACh and nicotine, have differing affinities based on the number of α and β subunits. Those with a greater number of β subunits, (i.e.$(\alpha4)_2(\beta2)_3$ nAChRs), have

nearly 100 times greater affinity for its agonist than those with less,(i.e $(\alpha4)_3(\beta2)_2$ nAChRs) (Carbone *et al.* 2009, Zwart *et al.* 1998, Nelson *et al.* 2003).

Many nAChR subtypes are found in brain regions relevant to drugs of abuse such as the ventral mesolimbic pathway, dorsal nigrostriatal pathway, and hanbenulo-interpeduncluar pathways (Koob & Volkow, 2010). The $\alpha7$ and $\alpha4\beta2$* nAChRs are expressed ubiquitously throughout the brain, the latter comprising up to 90% percent of high affinity nAChR expression in the mammalian brain (Gotti *et al.* 2004, Albuquerque *et al.* 2009). $\alpha4\beta2$* nAChRs are also among the principle contributors to nicotine addiction given their critical role in the rewarding and reinforcing properties of nicotine, while $\alpha7$ is known to contribute to nicotine's withdrawal effects (see de Biasi & Salas, 2008 for review). However, there are other noteworthy subtypes relevant to drug addiction with more discrete distribution in the brain, among them $\alpha3$*, $\alpha5$*, and $\alpha6$* nAChRs (Figure 3, also see Gotti *et al.* 2009 for review). The $\alpha3$* nAChR subtype is found to be expressed in high levels in the pineal gland, medial habenula (MHb), hippocampus, interpeduncular nucleus (IPN), and dorsal medulla existing as $\alpha3\beta4$* nAChRs, which acts primarily in conducting fast synaptic transmission (Schlaepfer *et al.* 2008a). Additionally, $\alpha3$* nAChRs are found in the retinal visual pathways expressed mainly as $\alpha3\beta2$*, which can also be found in the MHb (Gotti *et al.* 2007, Grady *et al.* 2009). Next are $\alpha5$* nAChRs, which, much like $\alpha3\beta4$*, are expressed in the pineal gland, MHb, IPN and dorsal medulla, as well as in the ventral tegmental area (VTA) and the prefrontal cortex where high densities of $\alpha4\beta2$* exist (Schleapfer *et al.* 2008a). This is not by chance, as the $\alpha5$ subunit must co-assemble with either of the aforementioned

subtypes, as well as α3β2* in other cases, to form a functioning α5* receptor (Kuryatov *et al.* 2008). The α5 subunit acts as an "accessory" protein where it influences the sensitivity, agonist efficacy, Ca^{2+} permeability, and desensitization properties of its respective receptor (Gerzanich *et al.* 1998, Brown *et al.* 2007, Tapia *et al.* 2007). Furthermore, the α5* subtype was shown to be a critical component of α-Conotoxin MII-resistant dopamine (DA) release in the mesolimbic dopamine system (MDS) because α5 gene deletion resulted in decreased maximal ACh-stimulated dopamine release and α5* nAChRs were found to be expressed in striatal synaptosomes in the mouse (Grady *et al.* 2010, Brown *et al.* 2007). Deletion of the α5 gene also reduced GABA release in mouse striatal synaptosomes, thus demonstrating the important functional implications α5* nAChRs have not only on DA-mediated reward mechanisms, but also on the release of other neurotransmitters relevant to drug dependence (Grady *et al.* 2010). Finally, the α6* nAChR subtype has emerged as another important subtype in determining the contribution of nAChRs to drug dependence due to its high, virtually exclusive expression on DA neurons within the substantia nigra, VTA, and locus coreleus (Yang *et al.* 2009). It is an especially peculiar subtype given its complexity in forming fully functional receptors. For instance, functional subtypes cannot be expressed *in vitro* without co-expression with α4, β4, or β3 subunits, and no naturally expressed receptors have not been observed *in vivo* without the presence of β subunits (Kuryatov *et al.* 2000). Immunopurification data shows two major α6* subtypes, α6α4β2β3* and α6β2β4*, were found in the striatum, retina, and superior colliculus, with the former comprising up to 60% of all α6* nAChRs (Gotti *et al.* 2007). Also, the

presence of the β3, which is also an accessory subunit, seems to be important since deletion of the β3 gene dramatically reduces α6* expression (Gotti *et al.* 2005). Evidence shows that the majority of α6* nAChRs in the striatum are expressed on presynpatic DA neuron terminals and are important for mediating DA release in this brain region, thus underscoring their potentially critical role in drug dependence (Salminen *et al.* 2004). While the majority of the preceding descriptions mainly refer to expression patterns in rodents, data suggests that these do not differ greatly from primates (Gotti *et al.* 2006a). In conclusion, much remains to be discovered about nAChRs, but given their complexity and ubiquitous expression throughout the brain and body, it is no wonder why they remain a focal point in the search for molecular targets for the development of more effective pharmacotherapies for drug dependence.

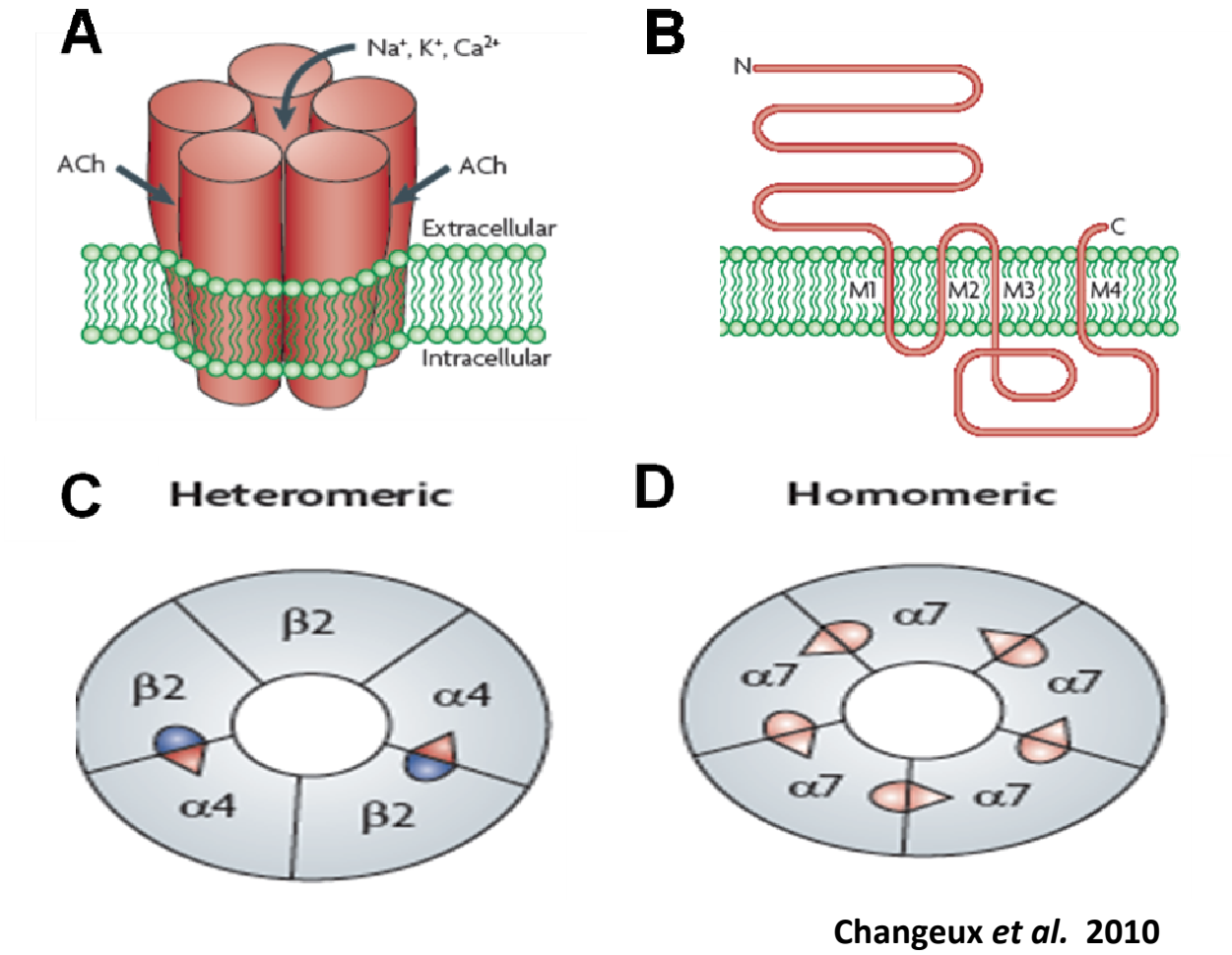
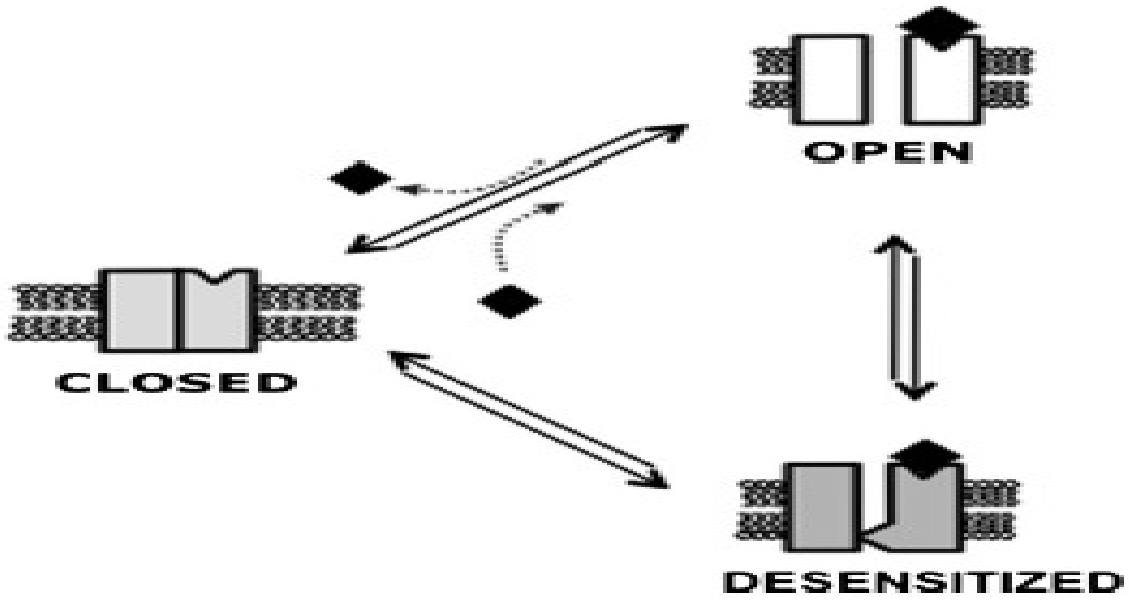


Figure 1. Neuronal nicotinic acetylcholine receptor structure. (A) Each subtype consist of five subunits comprised of α(α2–α10) and/or β(β2–β4) subunits that form around a central pore, which are permeable to Na^+ and $Ca2^+$ cations. (B) Each subunit contains a hydrophilic extracelluar amino terminal facing the synaptic cleft followed by three hydrophobic transmembrane domains (M1-M3), a large intracellular loop, and a forth hydrophobic (M4) transmembrane domain. Receptor subtypes exist as (C) heteromeric pentamers containing α2–α6,α10 and β2–β4 or (D) homomeric pentamers comprised of α7, α8, or α9 subunits. Each structure contains either two or five ACh binding sites, respectively.



Hurst *et al.* 2013

Figure 2. Functional states of neuronal nicotinic acetylcholine receptors. The binding of two agonist molecules (five for homopentamers) causes a transition from the closed state, to the open state, which allows cation influx through the channel pore. Following activation, the receptor will either remain in an agonist-bound, non-conducting state (desensitization) or return to the resting state upon agonist dissociation (deactivation).

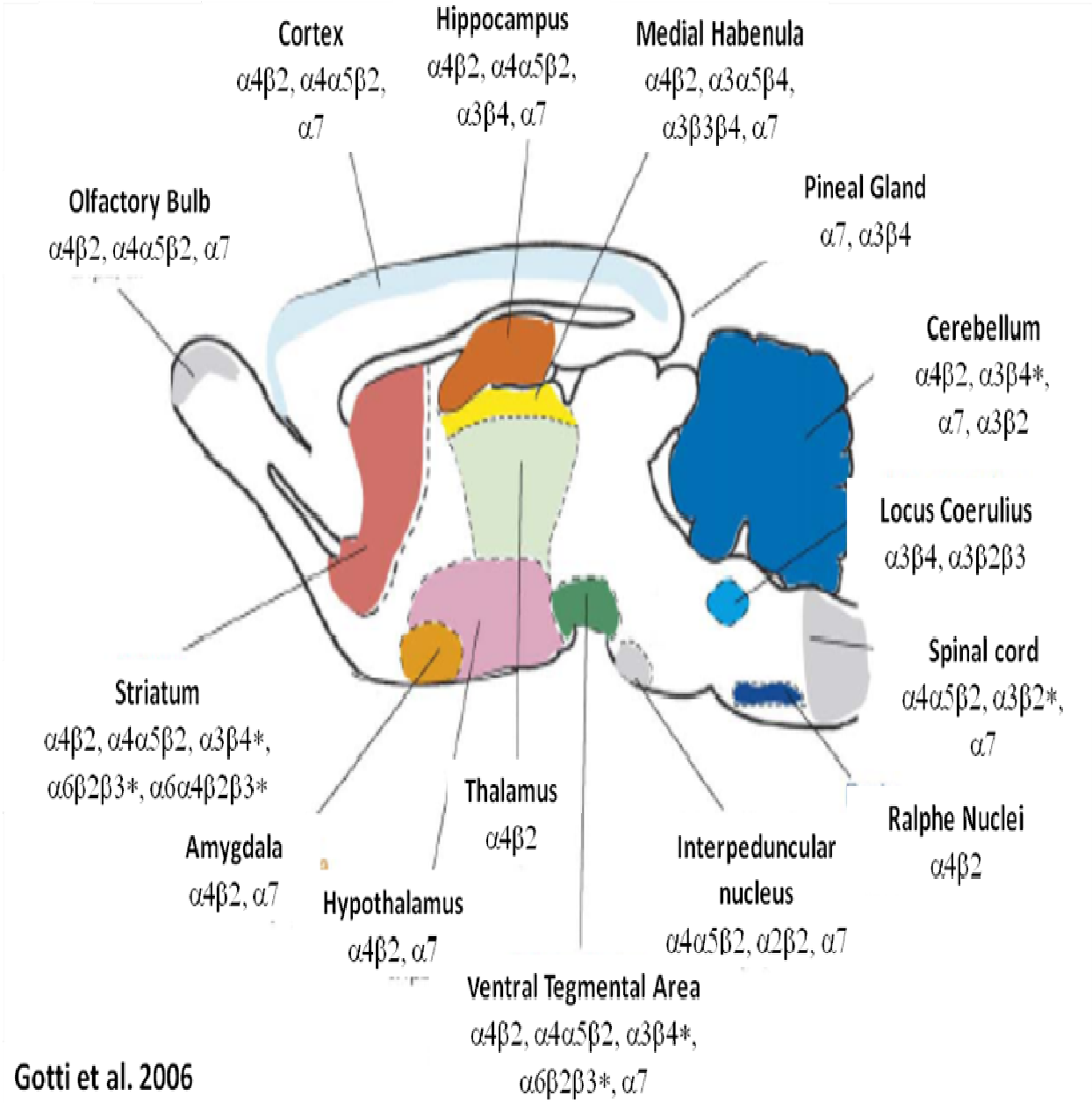


FIgure 3. Distribution of neuronal nicotinic acetylcholine receptors in the rat brain

1.3 Molecular mechanisms involved in Alcohol Dependence

In light of the complexities associated with determining the underlying causes of alcohol addiction, great strides have been made in the last 20 years in advancing our knowledge of alcohol pharmacology (see Spanagel *et al.* 2009 for review). Although, historically, alcohol was thought to be a relatively unspecific pharmacological agent, intense study over the past two decades reveals that this drug has at least a few known primary targets that mediate its more significant effects on brain signaling. While it is true that alcohol can exert a number of significant effects via its metabolic products (i.e. acetylaldehyde), the typical acute behavioral effects associated with acute alcohol exposure are primarily attributed to the first direct hit of ethanol on specific molecular targets followed by numerous indirect effects on a variety of neurotransmitter/peptide systems (Vengeliene *et al.* 2008, Spanagel *et al.* 2009). These effects, in turn, result in alterations in gene expression, leading to lasting neurophysiological changes that can trigger alcohol-seeking behavior with repeated exposure. Eventually, such changes may result in addictive behavior depending on genetic makeup of the individual as well as many environmental factors. While it was a long previously held view that ethanol mediates its action by membrane disruption (lipid theory), evidence has shifted from this view to one that asserts that ethanol has primary targets (protein theory). Among these , ligand-gated ion channels including glutamate (NMDA), $GABA_A$, glycine, $5\text{-}HT_3$ and nACh receptors, though many other notable molecular targets also exist. Studies show that each of these receptor types are differentially affected in a wide range of blood ethanol concentrations (BECs) from those that cause mild behavioral intoxication (~10-20 mM) to loss of consciousness ($\geq$ 300 mM). Generally, the degree of modulation is

directed by the receptor class and subtype. In a concentration range sufficient to produce intoxication, ethanol generally inhibits NMDA receptors and L-Type Ca^{2+} channels, while potentiating $GABA_{A,}$ glycine, 5-HT_3, and nACh receptors (Lovinger *et al.* 1989, 1991; Mihic *et al.* 1997, , Narahasi *et al.* 1999). Additionally, the sensitivity of each receptor type to ethanol depends on both subunit composition and ethanol concentration. For example, most $GABA_A$ receptors are sensitive to ethanol concentrations >60 mM, but those containing a δ subunit can be activated at concentrations as low as 1-3 mM (Wallner *et al.* 2003). Furthermore, $\alpha4/\alpha6\beta3$* subtypes are 10 times more sensitive than $\beta2$* subtypes (Wallner *et al.* 2003). In glycine receptors, $\alpha1$* subtypes appear more sensitive to low concentrations of ethanol than $\alpha2$* (Mihic *et al.* 1997). In nAChRs, $\alpha2\beta4$, $\alpha4\beta4$, $\alpha2\beta2$, and $\alpha4\beta2$ (but not $\alpha3\beta2$ or $\alpha3\beta4$) subtypes are potentiated by ethanol concentrations <100 mM, while the $\alpha7$ subtype is inhibited up to this concentration (Harris *et al.* 1999). The differential distribution of all of these receptors and their respective subtypes will affect some brain regions more than others, which ultimately dictate the net effect ethanol will have on brain signaling and behavior.

The actions of ethanol directly on the aforementioned ion channels, in turn, causes a multitude of indirect effects on a wide array of neurotransmitter/peptide systems crucial for the initiation of drinking behavior (Vengeliene *et al.* 2008). Among these, alteration of signaling involving monoamines, opioiods, and endocannabinoids, seem to have the greatest effect on reward and reinforcement necessary for the acquisition of alcohol drinking. The mesolimbic dopamine system (MDS), in particular, has been characterized as a neurochemical substrate for drug reinforcement, with A10

dopamine neurons identified as a critical component of this event (Schultz, 2007; Wise *et al.* 1989, 2004). A myriad of animals studies strongly support a significant effect of ethanol on the MDS. Though the details of these studies extend past the scope of this thesis, summarily, systemic ethanol mediates its action via neurochemical access points (i.e. $GABA_A$, 5-HT3, glycine and nAChR receptors), which affects dopamine transmission in the nucleus accumbens (NAC), VTA, and its afferents, thus influencing the A10 DA neurons essential to primary alcohol reinforcement processes. In addition to the DA-mediated processes previously mentioned, ethanol triggers the release of numerous endocannabinoids and opioiods, which have been shown to be vital in ethanol's rewarding effects. Furthermore, the mechanisms through which these actions are mediated appear by processes both dependent and independent of the MDS (Koob & Volkow *et al.* 2010)

The complexities of all these interactions are compounded by the fact that ethanol causes both acute and lasting changes in gene expression upon repeated exposure to the drug. There are many changes in signal transduction caused by ethanol exposure, but a primary pathway worth highlighting is the cAMP-PKA pathway that involves the activation of cAMP response binding element (CREB). This transcription factor leads to the expression of numerous ethanol-responsive genes (Diamond *et al.* 1997, Ron *et al.* 2005). These genes control significant physiological functions including neurotransmission, cell structure, signal transduction, metabolism and more (Lonze & Ginty, 2002). Among these CREB-targeted genes, some of the most notable regarding alcohol dependence include corticotropin releasing factor (CRF), prodynorphin, brain-derived neurotrophic factor, and neuropeptide Y, though

there are numerous others (Heilig & Koob 2007, Blednov *et al.* 2006, Martinez *et al.* 2005, Thiele *et al.* 1998, Crabbe *et al.* 2006a). Additionally, there are also CREB-independent genes as well as epigenetic effects induced by ethanol (Aragon *et al.* 1991, Egger *et al.* 2004, Saxonov *et al.* 2006). All these aforementioned changes may occur in a regionally-specific manner or in the brain as a whole. Thus, while the complexities of ethanol's actions on the brain are vast, they also present many opportunities in the way of these molecular targets for discovering more effective treatments for AUDs.

1.4 Nicotinic receptor mechanisms Involved in Alcohol Dependence

1.4.1. Human behavioral and genetic data

Several studies present evidence for nAChRs as potential candidates for mediating phenotypes that characterize alcoholism. The high comorbidity of nicotine and alcohol abuse seem to implicate these receptors in phenotypes related to alcoholism especially due to the knowledge that nAChRs are the principle contributors to the addiction relevant actions of nicotine (Kenny *et al.* 2001, Tuesta *et al.* 2011). It is estimated that up to 45% of alcoholics display nicotine dependence compared to 13% in the general population, and likewise, 13% of nicotine dependent individuals display alcoholism compared to 4% in the general population (Romberger & Grant, 2004; Grucza *et al.* 2006). Furthermore, not only is chronic smoking in alcoholic individuals associated with higher levels of alcohol consumption compared with nonsmoking or former smoking alcoholics, but alcoholic individuals actually seem to display more severe forms of nicotine dependence than that of non-alcoholic smokers (York & Hirsch, 1985; Romberger & Grant, 2004). While it can be argued that co-abuse of these substances is attributable to the easy availability and low social stigma of cigarettes and alcohol, neurobiological evidence suggest a much deeper connection such conditions alone do not fully explain (Feduccia *et al.* 2012, de Fiebre *et al.* 1990, Smith *et al.* 1999, Gould *et al.* 2001, Marubio *et al.* 2003, Tizabi *et al.* 2007). This may be the reason why tobacco is among the most commonly abused substances by actively drinking and recovering alcoholics. However, this clear correlation between alcohol and nicotine abuse presents an opportunity in that it makes nAChRs an attractive target for the

treatment of both AUDs and possibly nicotine dependence simultaneously (Chatterjee *et al.* 2010).

Indeed, preliminary behavioral evidence in the way of this notion has already been demonstrated in humans during clinical trials. For example, mecamylamine, a non-specific nAChR antagonist, appeared to suppress the stimulant effects of alcohol as well as the desire to consume more alcohol in healthy volunteers with no history of substance abuse in several independent studies (Blomqvist *et al.* 2002, Chi *et al.* 2003, Young *et al.* 2005). Similarly, varenicline, a non selective $\alpha4\beta2^*$ nAChR partial agonist approved as a smoking cessation aid by the FDA, recently showed that it reduced the rate of drinking in heavy-drinking smokers, as well as their likelihood of remaining abstinent during the clinical trial (McKee *et al.* 2009). Conversely, studies have also shown that some pharmacotherapies that are prescribed for the treatment of AUDs (e.g. naltrexone) also appear to reduce smoking levels in alcoholic smokers (Rohsenow *et al.*2003). Moreover, nAChRs are known to play a vast role in attention processes, learning and memory, synaptic plasticity, and neuroprotection, all of which are also affected by alcohol consumption; so it is logical to assume they can act as a common site of action for the neurocognitive modulations associated with alcohol abuse. For instance, abstinent alcoholic individuals appear to be more sensitive to nicotine's cognitive enhancing effects than substance abuse users without a history of alcohol dependence (Ceballos *et al.* 2006). Such evidence provides further support for nAChRs as potential targets for the treatment of AUDs.

In addition to the behavioral evidence previously described, genome-wide association (GWA) studies have implicated genes coding for specific nAChR subunits in

alcohol dependence phenotypes. Among these findings, GWA analyses of a chromosome region containing the *CHRNA5/A3/B4* gene cluster, coding for α5, α3, and β4 nAChR subunits, respectively, have produced many intriguing results regarding not only alcohol, but multiple substances of abuse (Lubke *et al.* 2012 for review). While the majority of the data generated pertain to smoking dependence, a few independent studies containing subjects suffering from multiple symptoms of substance abuse also revealed an association with alcohol phenotypes. For instance, a study of young adults testing for association of single nucleotide polymorphisms (SNPs) in the *CHRNA5/A3/B4* genomic region with multiple alcohol and nicotine dependence phenotypes revealed a number of SNPs that were associated with age of initiation of alcohol drinking (Schlaepfer *et al.* 2008a,b). These results were replicated in a separate population-representative adult sample. An independent study corroborated these results, revealing that many alleles within this same chromosome region showed an association with alcohol (and cannabis) dependence (Wang *et al.* 2009). Additionally, two independent samples derived from a panel of subjects diagnosed with nicotine dependence and other psychiatric disorders showed a significant association of two SNPs, rs16969968 and rs1051730, in the *CHRNA5/A3/B4* genomic region with DSM-IV symptom counts for alcohol dependence and the Fagerstrom Test of Nicotine Dependence (FTND), a common assessment of nicotine dependence. Interestingly, the alleles associated with alcoholism risk were the *opposite* of those associated with risk for nicotine dependence as the alleles associated with risk for alcoholism were the G variant of each marker, while the A variant of these same markers were associated with a higher FTND score (Chen *et al.* 2009). Further analysis using both novel and

replicated data sets also supported an association of these same two SNPs with alcohol dependence in subjects diagnosed with multiple substance abuse (Sherva *et al.* 2010). Additional SNPs, in the *CHRNA5/A3/B4* genomic region were also shown to be significantly associated with quantitative level of response, specifically body sway induced by acute alcohol challenge, in normally drinking young adults that were family history positive for alcoholism (Joslyn *et al.* 2008). More recent evidence also shows a SNP in this genomic region, particularly in *CHRNA5*, showed a significant association with SHAS, a measure of subjective feelings of intoxication, in a sibling pair study in which subjects had at least one alcoholic parent (Choquet *et al.* 2013). Thus evidence continues to build that suggests at least a modest contribution of the *CHRNA5/A3/B4* genomic region to influencing the development of AUDs.

GWA studies searching for additional nAChR gene associations with alcohol phenotypes revealed that *CHRNB2, CHRNA6,* and, *CHRNB3,* coding for the β2, α6, and β3 nAChR subunits, respectively, were also associated with various traits such as subjective response to early alcohol exposure and heavy alcohol consumption. More specifically, analysis of the β2 gene revealed an association of a rare SNP with early subjective response to alcohol in a study demonstrating that individuals expressing a variant of the gene recalled a strong negative response to initial episodes of alcohol (and nicotine) use (Ehringer *et al.* 2007). As for *CHRNA6* and *CHRNB3,* three SNPs in the α6 gene and one in the β3 gene showed an association with a handful of alcoholism-related traits including average number of drinks in individuals from a nationally representative sample of households (Hoft *et al.* 2009). Another independent study identified SNPs associated in the α6 (but not β3) gene with heavy alcohol

consumption in a Spanish population of heavy drinkers (Landgren *et al.* 2009). Though more consistent data have been generated with regard to the *CHRNA5/A3/B4* genomic region, it remains evident that at least some nAChRs appear to be common genetic elements for influencing risk for alcoholism, at least to some degree.

1.4.2. In vitro and *In vivo* data

Studies, both *in vitro* and *in vivo,* continue to compile evidence for the involvement of nAChRs in ethanol-responsive behaviors. *In vitro* studies conducted since the mid-1980s recognize that nAChR activity is modulated following ethanol application. This is not surprising considering the structural similarity between these and $GABA_A$ receptors, which are significant targets of ethanol's neuromodulatory action. Studies show that the $\alpha2\beta4$, $\alpha4\beta4$, $\alpha4\beta2$, and $\alpha2\beta2$ nAChRs subtypes are potentiated, while $\alpha7$ nAChRs are inhibited at ethanol concentrations up to 100 mM when expressed in *Xenopus* oocytes, as well as in naturally expressed nAChRs in PC-12 cells, mouse synaptosomes, and cultured cortical neurons, among others (Aistrup *et al.* 1999, Narahashi *et al.* 1999, Harris *et al.* 1999). Data for $\alpha3\beta4$ nAChRs revealed that these receptors are largely insensitive within this concentration range (Harris *et al.* 1999, Aistrup *et al.* 1999, Cardoso *et al.* 1999). However, all nAChRs appear to be potentiated at ethanol concentrations greater than 100 mM.

There are also numerous *in vivo* studies demonstrating nAChR involvement in ethanol's behavioral effects in animals. Studies dating back to the late 1980's demonstrate the ability of nAChR ligands to alter the effects of ethanol after both acute

administration and chronic exposure. For example, nicotine and ethanol produce cross-tolerance to each other's effects in a variety of physiological and behavioral measures including body temperature, heart rate, and hypnosis, as well as in open-field and Y-maze activity (de Fiebre & Collins 1993, Collins *et al.* 1988, 1996). Additionally, many nAChRs ligands are shown to alter ethanol's effects on neurotransmission, suggesting that nAChRs act a common molecular site of action for these drugs. The most notable interactions occur within the MDS (see Soderpalm *et al.* 2009 for review). Studies show that ethanol indirectly activates this system via central nAChRs by elevating ACh levels in the ventral tegmental area (VTA), thereby leading to increased DA levels in the NAC (Ericson *et al.* 2003, Larsson *et al.* 2005). Numerous nicotinic receptor ligands alter changes in DA transmission within the MDS in response to ethanol. For example, ethanol-induced DA elevation in the NAC is blocked when, mecamylamine, a non-specific antagonist is infused in the anterior VTA (Blomqvist *et al.* 2002, Lof *et al.* 2007a, Ericson *et al.* 2008). Similarly, VTA infusion of α-conotoxin MII ($\alpha3\beta2^*$ and $\alpha6^*$ antagonist) and systemic injection of varenicline ($\alpha4\beta2^*$ partial agonist), also block ethanol-induced DA elevation in the NAC in rodents (Ericson *et al.* 2003, 2008, 2009). In contrast, neither Dihydro-β-Erythroidine (DHβE, $\alpha4\beta2^*$ antagonist) nor methyllycaconitine (MLA, $\alpha7$ antagonist) seem to alter ethanol-induced dopamine release (Larsson *et al.* 2002, Ericson *et al.* 2003).

Given such data, it is not surprising to find that manipulation of nAChRs also alters acute ethanol-responsive behaviors as well as self-administration behavior in animals. In line with their observed effects on ethanol-induced DA transmission, mecamylamine

and α-conotoxin MII infused into the VTA and systemic injection of varenicline (but not DHβE or MLA) all demonstrate a reduction of ethanol self-administration behavior in rodents (Ericson *et al.* 2003, 2008, and 2009, Steensland *et al.* 2007, Hendrickson *et al.* 2009, Kamens *et al.* 2010). Furthermore, mecamylamine and α-conotoxin MII also block acute ethanol-induced increase in locomotor activity and conditioned reinforcement of ethanol (Larsson *et al.* 2002, Jerlhag *et al.* 2006, Lof *et al.* 2007b). Nicotine, which targets multiple nAChRs subtypes, also displayed the ability to modulate ethanol drinking behavior in multiple self-administration assays in rodents, although findings have been somewhat conflicting. Some studies report increased ethanol intake (Clark *et al.* 2001, Lê *et al.* 2000, 2003, 2010), while others report a decrease in ethanol intake (Hendrickson *et al.* 2009, Sharpe & Sampson 2002) with discrepancies in the results likely due to the variation in experimental methods including length of exposure to the drugs, route of administration, and drug concentration among others (Burns & Proctor, 2013),

While pharmacological antagonism of α4β2* nAChRs by DHβE and α7 nAChRs by MLA has no effect on ethanol self-administration directly (Lê *et al.* 2000, Hendrickson *et al.* 2009) studies do demonstrate the ability of these nAChRs to alter response to some effects of acute ethanol administration. For instance, both DHβE pretreatment and deletion of the β2 nAChR gene attenuate ethanol-induced loss of righting reflex (LORR), as well as reduction of the acoustic startle response and increased open arm time in the elevated plus maze (Owens *et al.* 2003, Butt *et al.* 2004, Dawson *et al.* 2013). Furthermore, ethanol-induced ataxia was attenuated in mice pretreated with the β2* full

agonists nicotine and metanicotine (RJR-2403), effects that were blocked by DHβE (Al-Rejaie *et al,* 2006 a,b,c; Taslim *et al.* 2008). The α7 nAChR also seems to influence ethanol's acute effects on some level. Deletion of the gene in the mouse resulted in an enhanced response to ethanol's effects in open-field activity, hypothermia, and LORR response, while activation of α7 nAChRs by the agonist PNU-282987 attenuated ethanol-induced ataxia; an effect blocked the α7 antagonist, MLA (Bowers *et al.* 2005, Taslim *et al.* 2010). Additionally, α6* nAChRs were also shown to influence the sedative effects of ethanol as the α6 gene increased sensitivity in the LORR response, while having no effect on two-bottle choice ethanol consumption (Kamens *et al.* 2012).

As for ethanol drinking behavior, however, the data suggests that α3β2* and α3β4* nAChRs appear to be among the likely candidates for influencing ethanol self-administration directly in rodents of the nAChRs assessed so far. As previously mentioned, α-conotoxin MII, which targets α3β2* and α6* nAChRs (Cartier *et al.*1996, Champtiaux *et al.* 2002), was shown to curb ethanol consumption in rodents, but further studies with α6* ruled out the involvement of this subtype due to the observation that α6 knockout mice drank similarly to wild type (Kamens *et al.* 2012). Additionally, studies show that partial agonists targeting α3β4* nAChRs as well as genetic overexpression of this subtype reduce ethanol consumption in rats and mice, respectively (Chatterjee *et al.* 2011, Gallego *et al.* 2012). Moreover, cytisine and its derivative, varenicline, reduced ethanol consumption in rodents in many tests of drinking behavior (Steensland *et al.* 2007, Hendrickson *et al.* 2009, 2010; Kamens *et al.* 2011, Sajja *et al.* 2011, 2012). While both drugs are known primarily as high affinity α4β2* partial agonists, they also

act as full agonists of α3β4* nAChRs and display some activity at α3β2* and α7 nAChRs as well (Papke & Heinemann 1994; Zatónski, 2005, Mihalak *et al.* 2006). Finally, nicotine, which is a full agonist at each of the aforementioned subtypes, has been shown to modulate ethanol drinking behavior in multiple studies (Clark *et al.* 2001, Sharpe & Sampson, 2002; Lê *et al.* 2000, 2003, 2010; Hendrickson *et al.* 2009, Burns & Proctor *et al.* 2013). Interestingly, the genes that code for these receptor subunits implicated by these studies were also identified in human GWA studies.

Another nAChR subtype worthy of mention is that which contains the α4 subunit, although, its involvement in ethanol drinking directly appears more complex. For example, the α4β2* antagonist DHβE had no effect on ethanol drinking behavior in multiple studies (Larsson *et al.* 2004a,b; Lé *et al.* 2000, Hendrickson *et al.* 2009), but these receptors were shown to be sufficient for nicotine and varenicline-induced reduction of ethanol consumption in mice (Lé *et al.* 2000; Hendrickson *et al.* 2009, 2010, 2011). Furthermore, Leu9'Ala mice that express α4* nAChRs hypersensitive to nicotinic agonist (Ross *et al.* 2000, Tapper *et al.* 2004), showed similar consumption between nicotine and varenicline test groups, and their respective controls. Such evidence supports the idea that α4* nAChRs are important in mediating the effects of these drugs on ethanol consumption. This may be explained by the inability of ethanol to further activate DA neurons in the VTA via these nAChRs after agonist (i.e. varenicline, nicotine) binding. This notion is supported by data showing reduced and enhanced DA activation by ethanol in α4 KO and Leu9'Ala mice, respectively, which was corroborated

by respective changes in ethanol CPP behavior, another VTA-dependent mechanism (Hendrickson *et al.* 2010, 2011; Liu *et al.* 2012).

One oversight of increasing significance in the aforementioned *in vivo* studies, however, is the α5* nAChR subtype. While recent data does suggest a potential genetic correlation between α5 mRNA expression levels and ethanol preference (Symons *et al.* 2010), as well as some involvement in ethanol's acute sedative effects in mice (Santos *et al.* 2012), the role of this particular subtype on ethanol-responsive behaviors *in vivo* remains largely unexplored. As described previously, the α5 nAChR gene was identified in numerous GWA studies as having a potential link to several alcohol dependence-related phenotypes in humans (Joslyn *et al.* 2008, Schlaepfer *et al.* 2008a,b; Wang *et al.* 2009, Chen *et al.* 2009, Sherva *et al.* 2010, Choquet *et al.* 2013). Moreover, α5* nAChRs play an inexorable role in nAChR agonist-evoked DA transmission within the MDS (Grady *et al.* 2010), and this subtype was shown to be critically important in mediating *in vivo* self-administration and the rewarding effects of nicotine in rats and mice (Fowler *et al.* 2011, Jackson *et al.* 2010, Tuesta *et al.* 2011). Given that nicotine is a drug whose use displays high co-morbidity with alcoholism, a more detailed *in vivo* characterization of α5* nAChRs may likely reveal some critical insights on their role in alcohol dependence as well. Moreover, the α5 subunit is commonly co-expressed with both α4β2*, α3β4*, and α3β2* nAChRs (Gerzanich *et al.* 1998, Wang *et al.* 1998, Mao *et al.* 2008) and, thus, may be able to explain the deeper mechanisms underlying the effects of these receptors on ethanol consumption.

CHAPTER 2: MODELING ALCOHOL DEPENDENCE

2.1 Modeling Alcohol Dependence in humans

The sheer heterogeneity of AUDs makes alcoholism very difficult to classify and diagnose. Traditionally, alcoholism is diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), which suggests a list of symptomatic criteria the alcoholic individual should exhibit. However, these criteria alone often lead to binary classifications that fail to capture the heterogeneity of AUDs (Hines *et al.* 2005). Generally speaking, alcohol addiction can be thought of as a clinical outcome generated by a complex combination of risk factors, believed to be intergenerationally transmitted via genetic and environmental influences. Nearly 50% of the risk variance for AUDs is determined by genetic influences (Spanagel *et al.* 2009). While the interactions of these genetic components are far too complex for a solitary pathway to explain the genetic link to behavior, scientists have identified endophenotypes that continue to provide important information on susceptibility to AUDs (Knopik *et al.* 2004, Lyons *et al.* 2003, Hines *et al.* 2005). Understanding and modeling specific endophenotypes associated with AUDs could lead to the identification of underlying genes that are critical to diagnosing and treating alcoholism.

While there are countless endophenotypes, ranging from comorbid psychiatric disorders to alcohol metabolic enzymes, five over-arching endophenotypes containing the majority of the genetically-related alcoholism risk factors have been indentified as neuronal/behavioral inhibition, major psychiatric disorders, the opioid system, alcohol

metabolism, and the subjective level of response to alcohol (see Hines *et al.* 2005 for review). Of all the endophenotypes mentioned, subjective response (SR) to alcohol perhaps ranks among the most discussed as of late (see Morean & Corbin, 2010 for review). Since first being identified in the early 1980s, empirical evidence suggest that SR accounts for up to 60% of the variance of AUD risk conferred by genetics (Schuckit *et al.* 1999). SR, which represents the individual differences in sensitivity to the pharmacological effects of alcohol, is typically measured by alcohol challenge paradigms establishing family history or drinker status, then challenging the subject with enough alcohol to produce a targeted breath or blood alcohol level (BAL). These measures are then captured using a self-reporting tool (i.e. Subjective High Assessment Scale, SHAS) to assess the subjective state of the participant. Additionally, physiological assessments such as static ataxia, body sway, heart rate, and many other factors are often used in conjunction with these self-reported measures to more clearly define the level of response in each individual. The two main theoretical views used to explain SR are the Low Level of Response Model and the Differentiator Model. The Low Level of Response (LLR) Model contends that a dampened response to alcohol's effects confer risk for negative alcohol outcomes, suggesting the possibility that LLR reflects a faulty feedback mechanism in which individuals fail to adequately regulate a level of intoxication that normally serves a signal to stop drinking (Schuckit *et al.* 1994b, Schuckit & Smith 2000). This, in turn, may lead to increased tolerance over time, which furthers risk for alcoholism. The most recent and substantial results for LLR have been produced by ongoing studies performed by Schuckit and colleagues since the 1990s, though long previous work on this phenomenon do exist (see Pollack, 1992 for review).

Both non-placebo and placebo-controlled studies have been conducted over the course of more than 25 years, which produced mixed, but nevertheless, intriguing results. For example, studies assessing men family history positive (FH+) for alcoholism report lower SHAS measures than family history negative (FH-) individuals (Schuckit *et al.* 1980). Years later, a cross-generational sample of 25 men from the previously mentioned study and 40 of their offspring showed there was a trending but non-significant positive correlation between the SR of fathers and sons of FH+ groups, which was mirrored by FH- subjects (Schuckit *et al.* 2005). This provided credence to the idea that SR may be heritable trait. Follow up studies were conducted over the course of 25 years, taking assessments at the 10-, 15-. 20-, and 25-year marks, further supporting the predictive power of LLR for future alcohol related problems (Schuckit, 1998; Schuckit & Smith,1996, 1997, 2000, 2001a,b, 2004, 2006; Schuckit *et al.* 2004;Trim *et al.* 2009). The parameters of these studies were regularly updated with additional behavioral domains of functioning (behavioral under-control, coping, drinking environment, etc.) as knowledge in the alcohol research field increased. Some of these behavioral domains independently predicted development of AUDs, but none were related to family history status, as was the case for LLR. None of these domains interacted with LLR in predicting alcoholism indicating that the LLR phenotype is a completely independent risk factor. Moreover, LLR appeared to be unique to AUDs because family history of alcoholism status did not show significant correlation with any other forms of substance abuse or psychopathologies, thus demonstrating that is an alcohol-specific phenomenon (Schuckit 1994a,b). The alternative view of SR, the Differentiator Model (DM), asserts that an individual's risk depends on the degree of

their response to the stimulating and depressing effects of alcohol during the ascending and descending limbs of the BAL time course, respectively (King *et al.* 1993, Newlin & Thompson 1990, Morean & Corbin, 2010). While the DM theory has not been as heavily studied as LLR, evidence does exist that supports this particular view. For instance, a small cohort of FH+ individuals showed significantly increased response to the stimulating effects of alcohol from baseline compared to FH- individuals (Erblich *et al.* 2003). Furthermore, a separate study of non-treatment seeking alcoholics showed that these subjects had an increasing response to the stimulating effects of alcohol over time compared to non-alcoholics whose level of response remained stable during the same period of time (Thomas *et al.* 2004). In placebo-controlled studies, FH+ males reported feeling more intoxicated compared to FH- subjects. Another study also showed that heavy drinkers receiving a high alcohol dose, low alcohol dose, or placebo reported a sharp increase in the stimulant effects from the higher alcohol dose earlier in the ascending limb of the BAL compared to light drinkers, who reported no change between doses (Kaplan *et al.* 1988). While the DM is a relatively less popular theory compared to the LLR model, its importance cannot be overlooked, especially since it does not suffer from some of the caveats found in the LLR model. For instance, the DM uses the BAES as its primary assessment tool, which was specifically designed for the assessment of alcohol's effects, unlike the SHAS measurements used by LLR studies. Another disadvantage in the LLR model is that it mainly focuses on sedative and other "negative" experiences associated with the descending limbs of the BAL curves. This may explain why the strongest results in the LLR studies were only obtained at low doses of alcohol. This issue is addressed by the DM, which incorporates the more

"positive" effects that are commonly associated with motivation for heavy alcohol consumption, thus making this model comparatively more comprehensive. However, both models suffer from caveats, mostly methodical in nature, that hinder their respective interpretations. Such limitations extend from differences in alcohol administration procedures, the number and time of assessments, and measures from SR assessment across studies. Another major (but ethically necessary) limitation is the limited age range of subjects, since they must be at least 21 to participate and often show considerable drinking experience by the time of the study. This creates another variable regarding tolerance. Despite all this, each of these models, their shortcomings notwithstanding, have contributed a great deal to our current understanding of risks for developing AUDs and continue to provide important information in learning how to approach the treatment of these disorders.

2.2. Behavioral Models of Alcohol Dependence in mice

Because addiction is a phenomenon unique to humans, no animal model exists that can encompass all of the characteristics of alcoholism in its entirety. Nevertheless, some of these characteristics can be satisfactorily modeled in laboratory animals enough to provide important information on the neurobiological mechanisms that drive such behaviors (Sanchis-Segura *et al.* 2006). The development of alcohol addiction is viewed as three progressive phases (Vengeliene *et al.* 2008) from the initiation of alcohol drinking (initiation), to the continued maintenance of alcohol consumption (maintenance), and finally, the loss of control of alcohol consumption (compulsive drinking). Again, while addiction cannot be comprehensively replicated in animal

models, a variety of approaches are used to capture aspects of certain behaviors that are also featured in humans during each of these phases. Testing initial sensitivity to the behavioral effects of acute ethanol exposure, for example, can provide insight on genetic contributions to initiation of drinking (see Crabbe 2005, 2010 for review), while ethanol consumption and withdrawal studies can provide information on the maintenance and compulsive phases of drinking (see Sanchis-Segura & Spanagel 2006 for review). The following is an overview of common approaches used in alcohol research for each of these phases, with particular emphasis placed on the models we chose to use for our own studies.

2.2.1. Acute ethanol-responsive behaviors

Models of acute ethanol-responsive behaviors are key to studying the genetics of alcoholism, especially due to the implications that initial sensitivity to ethanol exposure may have on the level of response in humans, a highly predictive phenotype of future drinking behavior (Schuckit & Smith, 2011; Schuckit *et al.* 2011). Ethanol elicits many responses after acute administration in the mouse and different responses to ethanol have different genetic regulation suggesting that alcohol sensitivity cannot be evaluated using a single test (DeFries et al., 1989). The most widely used indices of behavioral sensitivity include ethanol-induced loss of righting reflex (LORR), hypothermia, locomotor activity and behaviors categorically referred to as “ataxia” (i.e. rotorod), (Crabbe *et al.* 2005, 2012). In addition to ethanol-induced hypothermia, we also chose to assess ethanol’s hypnotic effects using the LORR assay for our studies. This was implemented by treating mice with a high dose of ethanol and then determining the

amount of time the subject took to lose its ability to stand upright (LORR onset) and the amount of time it took for the subject to right itself three times within 30 sec, which indicated recovery from these effects (LORR duration). We chose to study both hypothermia and LORR for a few reasons. These behaviors have been used for years as behavioral measures for identifying genetically codetermined responses to ethanol sensitivity, which is an important factor in determining future drinking behavior. Thermoregulation offers an attractive system for studying ethanol sensitivity, tolerance and physical dependence to ethanol (Crabbe *et al.*1989). While exact molecular mechanisms underlying this response is generally unknown, data suggests that ethanol and nicotine are influenced by genetic factors, including nAChRs, which have been shown to play an important role in the maintenance of core body temperature (Crabbe *et al.*1989, Sack *et al.* 2005). Moreover, it was advantageous to use this measure in conjunction with LORR due to the fact that core body temperature has been found to modulate behavioral sensitivity to ethanol-induced LORR, as there appears to be a negative correlation between hypothermia and LORR duration (Crabbe *et al.* 1983, 1994). Another advantage in using these models is that they are relatively straightforward in their implementation and often do not require a great deal of technical knowledge to employ them.

We also sought to explore changes in the reward-related effects and anxiolytic-like behavior induced by ethanol using the conditioned place preference (CPP) and elevated plus maze (EPM) paradigms, respectively. The types of responses these assays model are especially pivotal in the search for understanding motivation for obtaining alcohol. It is known alcohol is often consumed for its rewarding effects, and

alcoholism also appears to be highly co-morbid with anxiety, among several other psychiatric disorders (Loas *et al.* 2002, Segui *et al.* 2001, Hahesy *et al.* 2002, Perugi *et al.* 2002). Furthermore, increased ethanol intake has been positively correlated to anxiety-like behavior in humans and animals (Boyce-Rustay *et al.* 2007; Lopez *et al.* 2011; Ploj *et al.* 2003, Sanna *et al.* 2011, Brown *et al.* 1995, Dawson *et al.* 2005, Kuntsche *et al.* 2009, Linsky *et al.* 1985, Nesic & Duka, 2006, Stevens *et al.* 2008). CPP and EPM are models that have been used extensively in the respective study of the rewarding and anxiolytic properties of drugs of abuse including ethanol (Sanchis-Segura *et al.* 2006, Hart *et al.* 2010). While none of the listed models measure drinking behavior directly, such tests remain important to alcohol research because they provide convergent data on genes and their contribution to the acute effects of intoxicating doses of ethanol. These tests are often used across multiple panels of genetically-inbred rodents in the identification of genomic locations of genes influencing their respective responses, known as Quantitative Trait Loci (QTL). Proving genes within these QTL regions could be important in discovering pathways that can be targeted for pharmacotherapies in humans (Crabbe *et al.* 2005, 2006b). While these approaches provide some important insight in the contribution of particular genes to ethanol-responsive behaviors, the results of such assays must be interpreted with caution due to the sensitivity of these acute assays to procedural variation and the genetic backgrounds of the animals chosen for study (Crabbe *et al.* 2006b, Lim *et al.* 2012).

2.2.2. Mouse models of ethanol intake

Ethanol self-administration models are among the most widely used procedures in research on the maintenance of ethanol consumption, quite simply because they

provide the most direct insight on the conditions that may motivate a subject to drink. Consequently, they can be used as adequate models for discovering common neural mechanisms involved in drinking behavior and can, therefore, help identify therapeutic strategies for treating AUDs (Sanchis-Segura *et al.* 2006). Self-administration models are broadly categorized as either operant or non-operant procedures, the latter commonly reporting the amount of drug consumed while the former focuses on the behavioral response itself. In operant self-administration procedures, subjects are enclosed in an "operant box" consisting of devices that deliver the drug reinforcer (in this case, ethanol) and transmit the operant response. The subject is placed under a schedule of reinforcement where it has to perform a certain task in a given time period to obtain the reinforcer. While operant behavioral models are acknowledged as viable tools for assessing alcohol self-administration, non-operant, oral self-administration procedures are the more frequently featured models in alcohol drinking studies (Spanagel *et al.* 2000). This was the particular model of choice for our experiments. The procedure is traditionally employed using a two-bottle choice paradigm where the subject is presented with one water bottle and one ethanol bottle (normally 10 - 20%), simultaneously, and is monitored for daily intake (in g/kg) and/or preference (expressed as the proportion of ethanol to total fluid intake). Additionally, multiple variables such as temporal accessibility, availability of multiple concentrations of ethanol, and others can be adjusted based on the nature of the data desired. These models do not require sophisticated equipment or major technical expertise making them an attractive method for assessing ethanol consuming behavior. Furthermore, they display high face validity in that they mimic some aspects of real human behavior, as well as predictive validity,

since some drugs identified for reducing ethanol consumption in animals in these paradigms also reduced alcohol consumption when tested in humans during clinical trials (Sanchis-Segura *et al.* 2006; Spanagel *et al.* 2000, 2009). However, a few limitations of this approach are worthy of mention. First, is the inability to make unidirectional interpretations from results since such models provide information only about the consumption of a rewarding drug without being able to distinguish between distinct factors (i.e. drinking patterns) that contribute to the behavior. This is especially true under conditions of unrestricted access, which we used for our studies. Second, human intervention can introduce variability into the experiment, which may obscure results and make reproducibility difficult in some instances. These disadvantages can be addressed by complementing studies with data from operant self-administration procedures, as well as using devices such as lickometers, allowing increased flexibility of experimental design and reducing the need for the experimenter to be present during testing.

Perhaps the most significant shortcoming of the previously mentioned self-administration methods, however, is their inability to compel animal subjects to drink to the point of intoxication, even after prolonged periods of exposure. Thus, while such models are suited for studying maintenance of ethanol drinking behavior, they do not produce key traits that reflect compulsive drinking behavior such as alcohol seeking, and withdrawal. Because of this, work continues to be done to identify methods that are able to model this important phase of the alcohol addiction cycle. In order to achieve the desired behavior, the animal often must first be made physically dependent to ethanol. This has traditionally been achieved through the implementation of ethanol

bolus injections (McQuarrie & Fingl, 1958) or intragastic infusions (Majchrowicz, 1975) , as well as ethanol liquid diet (Tabakoff *et al.* 1977) or vapor chambers (Goldstein & Pal, 1971) to induce more severe dependence. Soon after ethanol administration ceases, observable withdrawal effects, such as handling-induced convulsions appear that can be used to assess the level of dependence. All of these serve as viable options for studying the behavioral effects of ethanol withdrawal directly in rodents because observable withdrawal symptoms similar to humans will appear soon after ethanol administration ceases (Friedman *et al.* 1980). However, the disadvantage to these approaches is that dependence is achieved through passive ethanol administration instead of developing naturally in the subject through active consumption. This not only limits the interpretations made by withdrawal studies, but also, provides no data whatsoever on the drinking patterns required to achieve this state of dependence. Thus, models more conducive to measuring such changes have been developed in the past decade in order to address these concerns. We chose two of these models, Drinking-in-the-Dark and Intermittent Access, in our studies as a way to supplement our results with the traditional two-bottle choice oral self-administration model. Drinking-in-the-Dark (DID) is a limited-access model of acute binge drinking behavior in which animals are briefly exposed to one bottle containing a high concentration of ethanol (~20%). Subjects are exposed to ethanol a few hours into the dark cycle since this is a window of high activity in rodents (Rhodes *et al.* 2005, Goldstein & Kakihana, 1977; Freund, 1970; Millard & Dole, 1983; Kurokawa *et al.* 2000). Studies show that subjects under these conditions, particularly mice selectively bred to prefer ethanol solutions, will reliably self-administer ethanol to achieve blood ethanol concentrations (BEC) in excess

of 1.0 mg/ml, enough to cause measurable behavioral intoxication (Rhodes *et al.* 2005, 2007). While this is a noteworthy improvement on the standard two-bottle choice procedure, there is a trade-off in that it involves the removal of choice, thus diminishing the external validity of the assay much like the other methods of ethanol dependence induction (Hwa *et al.* 2011). This has prompted the development of alternative methods such as the Ethanol Deprivation Effect (EDE) and Intermittent Access (IA) models in an attempt to induce escalated ethanol drinking in a way that more closely reflects human behavior. Both methods are based on consistent observations that renewed access to ethanol after a period of deprivation will cause a pronounced, albeit, temporary escalation of ethanol drinking (Spanagel *et al.* 2000, Rodd *et al.* 2004). The EDE has been observed across many animal species including rats (Khisti *et al.* 2006, McKinzie *et al.* 1998; Rodd-Henricks *et al*. 2000, Sinclair & Senter, 1979), mice (Salimov *et al.*1993), monkeys (Kornet *et al*.1990; Sinclair, 1971), and humans (Burish *et al.* 1981; Mello & Mendelson, 1972) and IA, while comparatively less studies in animals exist, appears to be a common feature in conditions that promote excessive drinking in humans as well (Hwa *et al.* 2011). Each procedure is conducted in a similar manner to standard the two-bottle choice test, but subjects undergo specified deprivation periods where ethanol is removed from their home cages. In EDE studies, the deprivation period implemented can range from days to weeks and often is used as a model of ethanol craving and relapse (Sanchis-Segura *et al.* 2006, Le & Shaham 2002, Khisti *et al.* 2006, Sinclair *et al.* 1968; Spanagel & Holter, 1999)

In the IA procedure, the deprivation period is usually implemented every other day, as was done in our studies. Furthermore, repeated episodes of deprivation over

time may enhance ensuing withdrawal symptoms, which is thought to increase the compulsion to consume more ethanol (Hwa *et al.* 2011). Thus, the method was developed with the intention not solely to induce escalated drinking behavior, but to induce it in such a way that it produces measurable withdrawal symptoms. Indeed, studies show that mice selectively bred for high ethanol preference do achieve intake levels comparable to DID, and can lead to detectable withdrawal symptoms after months of testing (Melendez *et al.* 2006, 2011; Hwa *et al.* 2011).

2.2.3. The mouse as an animal model to study the role of nicotinic receptors in ethanol behavioral effects

Choosing the appropriate animal model for study is another important factor to consider when employing the techniques described above. The utility of the mouse as a model of human disease is substantial due to the nearly 80% similarities between mouse and human genomes (Crabbe *et al.*2005, Doyle *et al.* 2012). Additionally, experimenters can exert a high degree of genetic and environmental control over subjects, and their short intergenerational intervals, robust litter sizes, and defined health histories make them excellent models for examining the genetic underpinnings of mammalian diseases (Doyle *et al.* 2012). There are a wide variety of the genetic backgrounds from which each mouse model originates, with each displaying distinctive phenotypes in their responses to drugs of abuse. Two lines that are particularly highlighted in alcohol studies are those of the C57BL/6 (B6) and DBA/2 (D2) backgrounds. These are two of the most commonly used strains in neuroscience research, and are notable for their pronounced differences in ethanol consumption (Lim

et al. 2012, McClearn *et al.* 1959). Additionally, they show marked differences in their responses to a variety of ethanol's acute behavioral effects including locomotor activity, LORR and ataxia, thus making them excellent models of ethanol sensitivity (Phillips *et al.* 1995, Metten & Crabbe, 1994; Crabbe *et al.* 2003, 2006a, Cunningham *et al.* 1992). For our studies, we chose the B6 model for several reasons. First, highly backcrossed mice from the B6 background are, genetically, nearly homogenous and there is much phenotypic data available for wild-type B6 mice (Lim *et al.* 2012). Second, mice from this background display high preference for ethanol solutions and have long been documented to readily self-administer relatively high quantities of ethanol (McClearn *et al.* 1959). A third important advantage of using B6 mice is the ability to use gene targeting methods, particularly gene deletion to produce knockout (KO) mice that can enhance our understanding of ethanol-induced behavioral changes that occur in the absence of a specified gene. KO mice have been used extensively in alcohol research to identify critical molecular components involved in ethanol-responsive phenotypes. KO mouse studies have been seminal in identifying the contribution of neurotransmitter/neuropeptides systems underlying addiction-related behaviors including GABA, glutamate, monoamines, opioids, and endocannabioids as well as intracellular signaling proteins (see Spanagel *et al.* 2009 for review).

The use of KO mice was especially necessary for our investigation of $\alpha5$* nAChRs since there are currently no available selective antagonists for this particular subtype. These mice are produced in a two-stage process utilizing pluripotent embryonic stem (ES) cells to proliferate induced inheritable genetic changes in many successive generations. They were engineered by replacing the gene of interest with a

neomycin resistance cassette via homologous recombination in ES cells from 129/SvJ mice (Salas *et al.* 2003). The ES cells that have successfully undergone a targeting event are selected and transferred into blastocyst embryos of the B6 surrogate mother from which chimeras containing the KO allele are derived. These chimeras are then mated with B6 mice to transmit the KO allele to later generations. To date, a variety of nAChR KO mice, in addition to α5, have been produced including α2, α3, α4, α6, α7, α10, β2, β3 and β4 (Xu *et al.* 1999a, Marubio *et al.* 1999, Ross *et al.* 2000, Chaptiaux *et al.* 2002, Orr-Urteger *et al.* 1997, Vetter *et al.* 1999, Picciotto *et al.* 1995, Booker *et al.* 1999, Xu *et al.* 1999b).

Despite the enormous utility of these genetic KO mice, however, there are limitations that must be considered when taking this approach. Most notably is the issue of developmental compensation that inevitably occurs with the deletion of a gene. This physiological response can potentially cloud interpretations as the resulting phenotypes could be due either to gene deletion or to developmental changes set in motion by gene deletion that have nothing to do with the drug treatment. A potential example of this is changes in the expression levels of other nAChR subunits when one subunit gene is deleted. Fortunately, however, previous literature show negligible changes in expression level and enzyme function in many nAChR subunit KO animals, including α5 mice, of various backgrounds (Picciotto *et al.* 1995, Marubio *et al.* 1999, Ross *et al.* 2000, Salas *et al.* 1995, Chaptiaux *et al.* 2002). Another minor, but considerable issue that may arise is discrepancies in behavioral results due to the genetic background of the mouse strain used for breeding (Nadeau *et al* 2001). Though most nAChR KO mice are backcrossed with the B6 mouse strain, it is important to note

that the use of other strains for backcrossing the mutations may results in different phenotypes. The effects of these discrepancies can be minimized by ensuring the use of mice with an identical genetic background throughout studies with knowledge of the behavioral phenotypes that characterize the background strain. We made certain to adhere to these conditions in our studies.

2.2.4 Dissertation Hypotheses and Objectives

While it remains to be seen which nAChR subtype(s) is(are) most crucial in mediating ethanol's behavioral effects, it appears evident that cholinergic signaling plays a potentially significant role in ethanol-responsive behaviors. Therefore, the main goal of this thesis is to more fully characterize specific nAChR subtypes involved in mediating ethanol-responsive behaviors in acute administration of ethanol as well as chronic drinking behavior in mice. Particularly, we would like to provide further *in vivo* evidence for the human data suggesting a potential role, if any, for $\alpha5$* nAChRs in influencing the ethanol's acute effects and ethanol consumption in mice as well as identify the specific contribution of these nAChRs to such behaviors. Additionally, we aim to further characterize the role of $\beta2$* nAChRs in mediating ethanol-responsive behaviors, due the close of association of the $\alpha5$ subunit to the $\beta2$ subtype, and their integral role in dependence to nicotine, a drug highly co-morbid with alcohol abuse. We hypothesize that decreased $\alpha5$* nAChR expression by removal of the $\alpha5$ gene will alter the effects of acute ethanol exposure as well as ethanol drinking in high ethanol-preferring C57BL/6J mice. Specifically, we believe that acute ethanol-responsive

behavior will be enhanced in absence of the α5 gene, while drinking behavior will be attenuated by these conditions. We further hypothesize that α5* nAChRs mediate their effects through their association with β2* nAChRs in the acute effects with ethanol, but not necessarily in chronic ethanol drinking.

CHAPTER 3: CHARACTERIZATION OF α5* NICOTINIC ACETYLCHOLINE RECEPTORS IN ETHANOL-RESPONSIVE BEHAVIORS

3.1 Introduction

Alcohol (ethanol) and nicotine are two of the most commonly abused substances in society. These drugs cause over 500,000 deaths annually in the United States, with associated medical costs in excess of $200 billion dollars per year (Harwood *et al.* 1998, Epping-Jordan *et al.* 1998, Li *et al.* 2007). There is 50-80% smoking rate found among alcoholics, and the high co-morbidity of use associated with these two drugs increases the difficulty of achieving long-term abstinence with either (Hurt *et al.* 1994, Pomerleau *et al.* 1997, Romberger & Grant, 2004, Larsson *et al.* 2004a). Evidence in both humans and in animals support that there are at least some common genetic elements underlying these disorders (Swan *et al.* 1997, True *et al.* 1999). In addition, alcohol has been shown to interact with various nicotinic acetylcholine receptors (nAChR) subtypes in expressed systems and in the brain (see Davis & de Fiebre, 2006 for review). In light of such evidence, it is probable that nAChRs play an important role in alcohol-mediated behaviors.

Neuronal nAChRs are ligand-gated ion channels that can form multiple nAChR subtype combinations containing $\alpha\,(\alpha2-\alpha10)$ and/or $\beta\,(\beta2-\beta4)$ subunits. They are expressed as a combination of α2 to α6 and β2 to β4 subunits in the heteromeric form

or as α7 to α10 subunits in the homomeric form. The most widely expressed and best-characterized subtype is α4β2* (* denotes the presence of additional subunits) which has a high affinity for nicotine and is thought to be a principle mediator of its rewarding properties (Picciotto *et al.* 1998; Tapper *et al.* 2004). Mounting evidence suggest that these subtypes modulate several of the acute *in vivo* effects of alcohol in mice (Owens *et al.* 2003, Butt *et al.* 2004; Dawson *et al*. 2013) while playing no significant role in alcohol intake and reward (Hendrickson *et al.* 2009; Kuzmin *et al.* 2009; Kamens *et al.* 2010a,b; Dawson *et al.* 2013). Recent investigations into the CHRNA5-A3-B4 genomic region, a region coding for α5* subunits have yielded some interesting observations with respect to nicotine and alcohol dependence. For example, studies show that functional variation in α5∗ nAChRs and steady state mRNA levels are associated with alcohol dependence risk in humans and ethanol preference in rodents (Wang *et al.*2009, Symons *et al.* 2010). Additionally, variance in this genomic region is also associated with the level of response to ethanol as well as age of initiation of ethanol and tobacco use, which each serve as significant predictors of future drug abuse liability (Joslyn *et al.* 2008, Schlaepfer *et al.* 2008, Schuckit *&* Smith, 2011; Schuckit *et al.* 2011).

Despite their limited distribution, α5* nAChRs can have a substantial functional impact on signaling in the brain (Kuryatov *et al.* 2008, Grady *et al.* 2010). It is noteworthy that α5 subunits are accessory subunits that are known to modify the properties of α4- or α3-containing nAChRs respectively (Grady *et al.* 2010; Kuryatov *et al.* 2011; George *et al.* 2012). The α5 subunits can co-assemble with both α4β2*and α3β4* nAChRs (Gotti *et al.* 2006b, 2007, Collins *et al.* 1996, Picciotto *et al.* 1998) and

have demonstrated significant involvement in nicotine intake, reward, and withdrawal in rodents (Salas *et al.* 2004; Jackson *et al.* 2010, Jackson *et al.* 2008; Fowler *et al.* 2011). Given its functional impact on these particular subtypes, $\alpha 5$ is in a unique position to be an influential component in alcohol dependence-related behaviors. Recent data in support of this notion demonstrates that $\alpha 5$ nAChR gene deletion does, indeed, modulate the sedative effects produced by acute ethanol administration in mice without playing a role in the drug consumption the Drinking-in-the-Dark (DID) paradigm (Santos *et al.* 2012). Despite this initial report, more work is needed to further elucidate the role $\alpha 5$* nAChRs play in mediating phenotypes associated with alcohol dependence.

The aim of these studies was to further characterize the role of $\alpha 5$* nAChRs in ethanol's various behavioral effects in the mouse. In light of genetic and behavioral evidence showing an association of the $\alpha 5$ gene to level of response to acute ethanol exposure, we chose a battery of tests measuring the initial response to acute ethanol administration in the α5 subunit deficient mice. Namely, we chose to test for changes in response to the effects of high ethanol exposure including the hypothermia and loss of righting reflex, as well as test for changes in a more complex behavior, anxiolytic –like behavior in mice. Furthermore, we determined ethanol intake in a variety of ethanol consumption models in mice.

3.2 Methods

Animals

Mice null for the $\alpha 5$ subunit ($\alpha 5^{-/-}$) and their wild-type ($\alpha 5^{+/+}$) littermates were purchased from Jackson laboratories (Bar Harbor, ME) and bred in an animal care facility at Virginia Commonwealth University. Mutant and wild types were obtained from crossing heterozygote ($\alpha 5^{+/-}$) mice with C57Bl/6J mice. For all experiments, mice were backcrossed at least 8 to 10 generations. Mice were housed in a 21℃ humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-approved animal care facility. They were housed in groups of six and had free access to food and water. The rooms were on a 12-h light/dark cycle (lights on at 6:00 a.m.). Mice were 8–10 weeks of age and weighed approximately 20–25 g at the start of all the experiments. All experiments were performed during the light cycle (between 6:00 a.m. and 6:00 p.m.) and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All studies were carried out in accordance with the National Institute of Health guide for the Care and Use of Laboratory animals.

Drugs

(-)-Nicotine hydrogen tartrate salt were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Nicotine was dissolved in 0.9% saline and delivered subcutaneously (s.c.), at a volume of 10 ml/kg body weight. Ethanol was also dissolved in 0.9% saline and prepared as a 20% (v/v) solution which were delivered via intraperitoneal (i.p.) injection for acute

experiments or *per os* (p.o.) for drinking experiments. Ethanol doses (2.0 – 3.5 g/kg) were chosen based on effective doses obtained in dose response curves conducted before each study, which were consistent with those found in literature (Alanka *et al.*1992, Browman *et al.*2000).

Body Temperature Measurement

Hypothermia induced by acute ethanol was measured using a standard rectal thermometer (Fischer Scientific, Pittsburg, PA) with probe (inserted ~24 mm). Five mins after baseline temperatures were recorded, $\alpha5^{-/-}$ and $\alpha5^{+/+}$ mice were administered 3.0 g/kg ethanol or saline (i.p.). Body temperature measurements 15- and 60 min-post ethanol injection were recorded in degrees Celsius (C^{o}). Data was expressed as mean ± SEM of the difference in rectal temperature before and after ethanol treatment. The ambient temperature of the laboratory varied from 21-24℃ from day to day.

Loss of Righting Reflex (LORR)

The sedative-hypnotic effects of ethanol were measured using the loss of righting reflex assay (LORR). $\alpha5^{-/-}$ and $\alpha5^{+/+}$ mice were administered ethanol or saline (i.p.), then were monitored for initial LORR and placed in a supine position in a V-shaped trough. A subject was confirmed to have achieved LORR only after it was on its back for at least 30 seconds. Because this reflects aspects of initial sensitivity and acute functional tolerance

(Ponomarev & Crabbe 2002a), two LORR scores were reported for each subject. The first was the total time from ethanol injection until initial LORR, which was reported as latency to LORR. The second was total time required for the subject to right itself 3 times within 30 seconds from the onset of LORR, which was reported as the time required to right. Mice taking longer than 5 minutes to experience LORR were eliminated from the study due to the possibility of misplaced injection. Data (mean ± SEM) were expressed as latency to LORR and time required to recover in seconds.

Elevated Plus Maze (EPM)

Reduction in anxiety-like behavior induced by acute ethanol was assessed using the elevated plus maze apparatus. This is an elevated platform consisting of two crossbars that create four arms. Two of these arms have walls (closed arms) and the other two arms are exposed (open arms). Because mice commonly display an innate fear of open, elevated places, an increase in the amount of time spent in the open arms is thought to represent a reduction in anxiety-like behavior. Mice were given at least 12 hrs to acclimate to the testing room. On test day, $\alpha5^{-/-}$ and $\alpha5^{+/+}$ mice were administered ethanol or saline (i.p.). Subjects were then returned to their home cage for 15 minutes to allow ethanol to take effect and to avoid any hyperlocomotion from stress caused by the injection. Each subject was then placed briefly in a plastic container and transferred to the center of the maze. The subject was then allowed to freely explore the apparatus for 5 minutes, with time starting immediately after placement in the center of the maze. Data (mean ± SEM)

were expressed as the total time spent in the open arms in seconds. The number of crossovers was also recorded to account for any changes in locomotor activity.

Two-Bottle Choice

Drinking behavior was assessed using a standard two-bottle choice procedure. Mice were housed individually in cages and allowed to acclimate to the test room one week prior to the experiment. Cages contained two 10-ml pipettes filled with water fitted with a double ball-bearing metal sipper tube and rubber stopper on opposite ends. At the end of the acclimation week, one water tube was replaced with 3% (w/v) ethanol providing a choice of ethanol or water with 24-hr access for 4 days. Following exposure to 3% (w/v) ethanol, the ethanol concentration was incrementally increased to 6-, 10-, 15- and 30% (w/v) every 4 days to determine the effects of ethanol concentration on intake and preference. Additionally, ethanol and water tubes were placed on two empty cages, which allowed for measurement of leakage and evaporation from the tubes. The average volume depleted from these "control" tubes was subtracted from the individual drinking volumes each day before data analysis. Intake was reported as g/kg ethanol consumed and preference as the ratio of ethanol consumed divided by total amount of ethanol and water fluid intake (ml), combined. Additional measurements including body weight (g) and total fluid intake (ml) were also recorded. Data (mean ± SEM) were expressed as total intake or preference ratio.

Intermittent Access

Chronic ethanol drinking behavior was also assessed using the intermittent access (IA) procedure as previously described (Hwa *et al.*2011; Dawson *et al.*2013). This procedure is advantageous over the traditional two-bottle choice drinking procedure in that it produces escalation in ethanol drinking by repeated deprivation cycles thus better approximating human drinking behavior (Rodd *et al.* 2004). Briefly, $\alpha5^{-/-}$ and $\alpha5^{+/+}$ mice were housed individually in cages one week prior to testing with *ad libitum* access to food and water. Three days before the end of acclimation week, water bottles were replaced with two drinking tubes, made from 10-ml serological pipettes containing a double bearing sipper tube and a rubber stopper on either end of the tube, filled with water. At the end of the acclimation period, one water tube was replaced with one ethanol filled with 3-, 6-, and 10% w/v ethanol on alternating days (Sunday, Tuesday, and Thursday) while two water tubes were presented on the deprivation days (Monday, Wednesday, Friday, and Saturday; Table 1). After one week, 20% (w/v) ethanol tubes were presented on alternating days (Sunday, Tuesday, and Thursday) for the remainder of the experiment. Control mice were presented with continuous access to ethanol by presenting one 3% (w/v) ethanol tube on Sunday and Monday, 6% Tuesday and Wednesday, and 10% (w/v) ethanol Thursday, Friday, and Saturday. After one week, 20% (w/v) ethanol tubes were presented daily for the remainder of the experiment. Additionally, ethanol and water tubes were placed on two empty cages, which allowed for measurement of leakage and evaporation from the tubes. The average volume depleted from these “control” tubes was subtracted from the individual drinking volumes each day before data analysis. Intake was reported as g/kg ethanol consumed and preference as the ratio between of ethanol consumed divided by total amount of ethanol and water fluid intake (ml), combined. Additional measurements

including body weight (g) and total fluid intake (ml) were also recorded. Data (mean ± SEM) were expressed as total intake or preference ratio.

Drinking-in-the-Dark (DID)

DID is a limited access drinking procedure that was used to assess sub-chronic binge-drinking behavior in mice. As previously described (Hendrickson *et al.*2009), $\alpha5^{-/-}$ and $\alpha5^{+/+}$ mice were housed individually in cages one week prior to testing with *ad libitum* access to food and water. Because mice in general are shown to display maximal expression of consumatory behavior a few hours into the dark cycle (Rhodes *et al.*2005), subjects were housed under a reverse light-dark cycle (7am – 7pm) in order to facilitate for daytime testing. At the end of the acclimation period, the water bottle from each cage was replaced with one drinking tube containing 20% (w/v) ethanol three hours after lights off (10am). Baseline intake was measured at 2- and 4-hrs after ethanol presentation for two days. Additionally, due to evidence that $\alpha5$ gene deletion alters nicotine self-administration behavior, we also wanted to determine the effect of sub-chronic nicotine injections on $\alpha5$ mice in DID drinking behavior. Therefore, after obtaining the previously described baseline intake measurements, each genotype was separated into two groups receiving either saline or 0.5 mg/kg nicotine (s.c.) treatment. Each respective group was treated once a day immediately before presentation of ethanol for a total of 4 days, with measurements reported at 2- and 4-hrs after presentation of ethanol. All mice were treated with injections of saline for two days before testing in an attempt to acclimate them to the stress of the injections. On the last day of testing, one 10 µl blood sample was taken via cheek punch

using 4mm Lancets and stored in BD microtubes to determine blood ethanol concentrations after four hours limited access to ethanol. Data (mean ± SEM) were expressed as total intake (g/kg) and blood ethanol concentration (mg/ml).

Blood Ethanol Concentration (BEC) Analysis

To rule out the possibility of any observed effects being due to deletion of the $\alpha 5$ gene changing BEC levels, we mice tested for their BEC in a two-hour time course after receiving one high dose of ethanol. Drug naïve $\alpha 5^{-/-}$ and $\alpha 5^{+/+}$ mice were given a single challenge injection of 4.0 g/kg ethanol (i.p) and returned to their home cages. Mice were separated into groups in which one 10 µl blood sample was taken via cheek punch using 4mm Lancets (Medipoint, Inc., Minenola, NY) at 15-, 30-, 60- or 120 min time-points after injection. The blood was stored in BD microtainers and analyzed using Gas Chromatography similar to a previously described procedure (Gallaher *et al.*1996). Data (mean ± SEM) were expressed as BEC in mg/ml.

Conditioned place preference (CPP)

An unbiased CPP paradigm was used in this study to assess rewarding characteristics of ethanol. The place-conditioning chambers consisted of two distinct compartments separated by a smaller intermediate compartment with openings allowing access to either side of the chamber. On day 1, animals were confined to the intermediate compartment for a 5-min habituation period, and then they were allowed to move freely between compartments for 15 min. Time spent in each compartment was recorded.

These data were used to separate the animals into groups of approximately equal bias. Days 2 to 4 were the conditioning days during which the saline group received saline in both compartments and drug groups received ethanol (i.p.) in one compartment and saline in the opposite compartment. Drug-paired compartments were randomized among all groups. Activity counts and time spent on each side were recorded via photosensors using interface and software (MED Associates, St. Albans, VT). Data were expressed as difference in time spent on drug-paired side on test day (Day 5) and habiutation day (Day 1). A positive number indicated a preference for the drug-paired side, whereas a negative number indicated an aversion to the drug-paired side. A number at or near zero indicated no preference for either side.

Statistical Analysis

Primary dependent variables for LORR were latency to and duration of LORR and time spent in opent arms for EPM. For CPP, the primary dependent variable was the preference score. Data were analyzed using standard one way analysis of variance (ANOVA) with genotype as the independent variable . For hypothermia, the dependent varable was change in body temperature and analyzed using standard one-way repeated measures (ANOVA) with genotype and time point as the independent variables . Each analysis was followed by Bonferroni post-hoc tests to further analyze significant data with the alpha level set at 0.05. For two-bottle choice, ethanol consumption, water consumption, and total fluid consumption were used as primary dependent variables. Data were analyzed using factorial analysis of variance (ANOVA) with the alpha level set at 0.05 with strain, dose, time, and concentration were possible

variables. Interactions involving multiple factors were examined using successive ANOVAs including fewer factors. Significant two-way interactions were followed up with Bonferroni post-hoc tests to further analyze significant data with the alpha level set at 0.05.

3.3 Results

Body Temperature Measurement

Treatment with 3.0 g/kg ethanol had the intended effect of inducing a significant drop in body temperature (Figure 4). Three-way repeated measures ANOVA (genotype x treatment x time) revealed a significant main effect of genotype [F (1,19) = 5.376; p= 0.0317] and [F (1,19) = 66.028; p< 0.0001], and a marginal, but non-significant effect of time [F (1,19) = 3.300; p= 0.0851]. A genotype x time interaction was the only significant interaction detected [F (1,19) = 5.057; p= 0.031]. Subsequent one-way ANOVA analysis of genotype conducted at each time point revealed that ethanol-induced hypothermia significantly differed in $\alpha5^{-/-}$ mice vs. $\alpha5^{+/+}$ control animals at the 60-min [F (1,10) = 5.976; p= 0.0346], but not 15-min, [F (1,10) = 2.973; p= 0.1154] post-ethanol injection, indicating that $\alpha5^{-/-}$ experienced increased ethanol hypothermia only at the 60 min time point . The data thus shows that deletion of the $\alpha5$ gene confers an enhanced response to the hypothermic effects of acute ethanol that is not evident until at least 15 min after exposure.

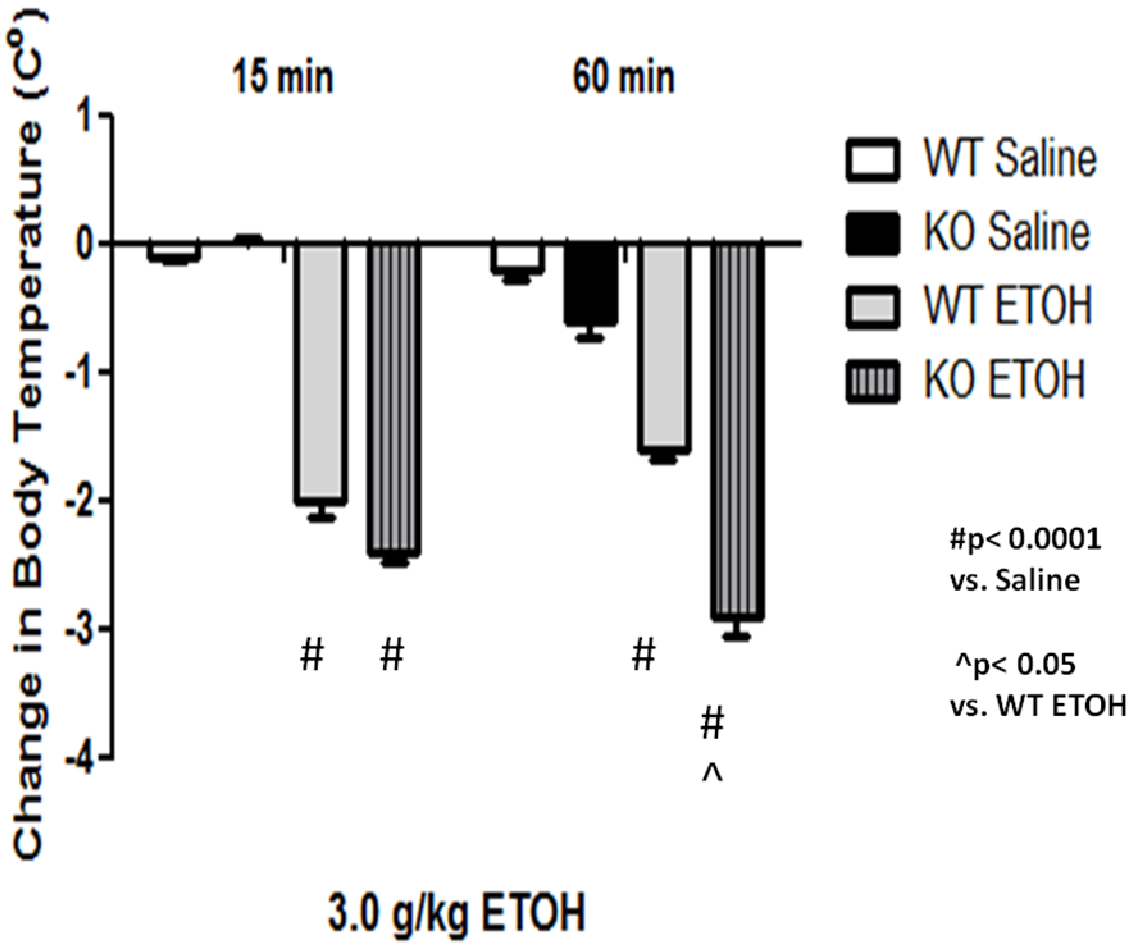


Figure 4. Deletion of the *Chrna5* gene enhances ethanol-induced hypothermia in C57BL/6J mice. Data (mean ± SEM) represent the change in body temperature from baseline in degrees Celsius of α5 KO mice at 15- and 60 min time points after receiving an injection of 3.0 g/kg ethanol. N= 6 per group.

LORR

Delivery of 3.5- and 3.8 g/kg doses of ethanol had the intended effect of inducing LORR (Figure 5). A two-way ANOVA analysis showed no significant main effects of genotype [F(1,29)= 3.144; p= 0.0867], dose [F(1,29)= 0.714; p= 0.4051], nor interaction [F(1,29)= 0.007; p= 0.9326] on LORR onset (Figure 5A). However, two-way analysis of LORR duration revealed a significant main effect of genotype [F(1,29)= 8.298 ; p= 0.0074], post-hoc p< 0.05) and dose [F(1,29)= 11.955; p= 0.017], post-hoc< 0.05), but not interaction [F(1,29)= 0.264; p= 0.6111) in $\alpha5^{-/-}$ and WT mice (Figure 5B). Thus, the data show that while ethanol had a dose-dependent effect on LORR duration in both $\alpha5^{-/-}$ and WT, this response was enhanced in $\alpha5^{-/-}$ mice indicating that deletion of the $\alpha5$ gene may attenuate tolerance to hypnotic doses of ethanol while not necessarily influencing the onset of these effects.

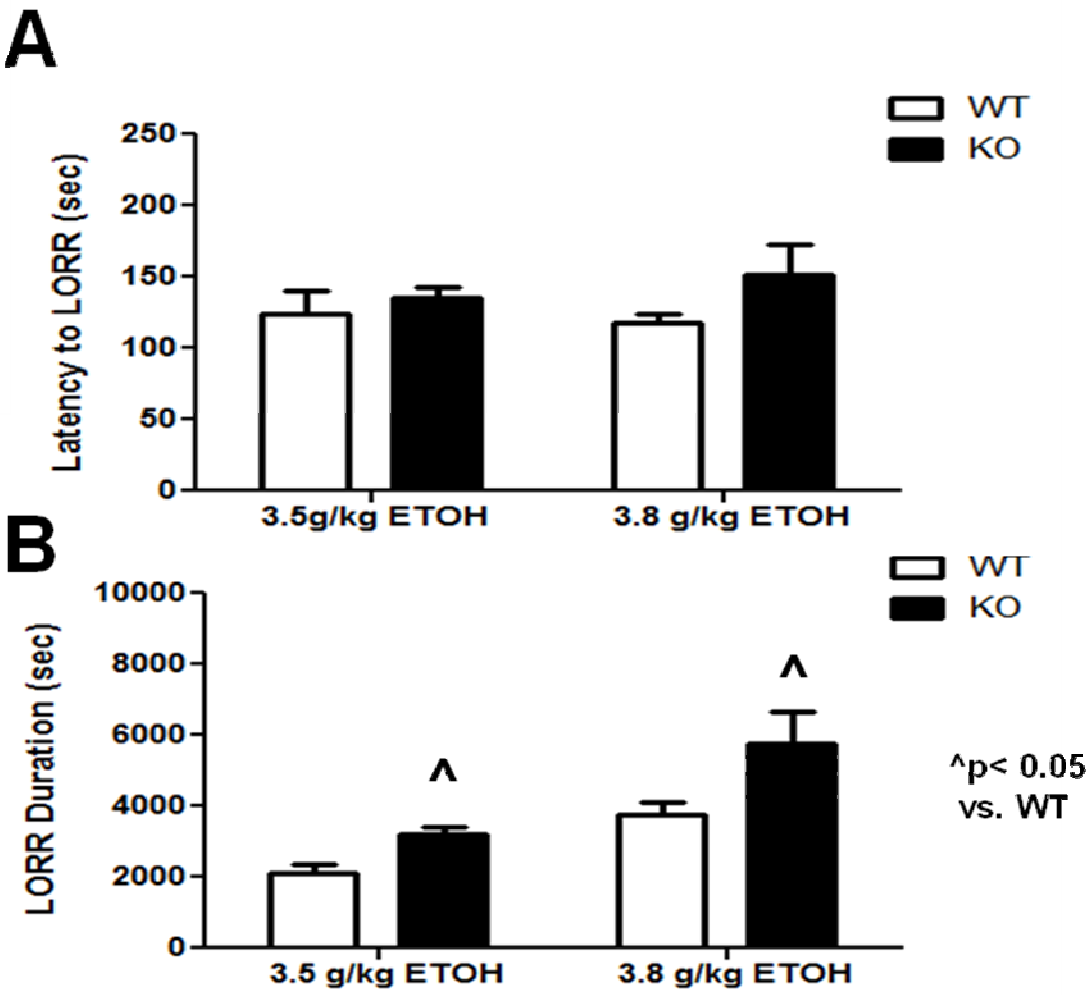


Figure 5. Deletion of the *Chrna5* gene enhances ethanol-induced LORR duration while having no effect on LORR onset in C57BL/6J mice. Data (mean ± SEM) represent (A) latency to LORR onset and (B) total duration of LORR in seconds in $\alpha 5^{-/-}$ mice after receiving an injection of 3.8 g/kg ethanol. N= 6-7 per group.

EPM

As expected, a dose of 2.0 g/kg ethanol caused a significant increase in the amount of time spent in the open arms in the EPM apparatus (Figure 6). Two-way ANOVA analysis of the amount of time spent a significant main effect of treatment [$F(1,34) = 25.236$; $p < 0.0001$], genotype [$F(1,34) = 4.405$; $p = 0.0433$], and interaction ([$F(1,34) = 5.491$; $p = 0.0251$], Figure 6A). Subsequent one-way ANOVA analysis revealed a significant difference in KO mice [$F(1,18) = 5.867$, $p < 0.0262$] compared to ethanol-treated WT mice in the ethanol treated groups. We also analyzed the number of crossovers to determine if these differences were due to changes in locomotor activity (Figure 6B). Two-way ANOVA (genotype x treatment) analysis of the number of crossovers did not show a main effect of either genotype [$F(1,34) = 0.579$; $p = 0.4519$] or treatment [$F(1,34) = 0.1079$; $p = 0.3063$]. There was a marginal interaction, but this was statistically non-significant [$F(1,34) = 3.903$; $p = 0.0564$]. Thus, the data shows that $\alpha 5^{-/-}$ mice spent more time in the open arms of the EPM than their WT counterparts did which indicates that they displayed an enhanced response to those aspects of ethanol-induced anxiolysis reflected in the EPM. This could mean that deletion of $\alpha 5$ gene modulates at least some of the anxiolytic properties produced by acute ethanol exposure.

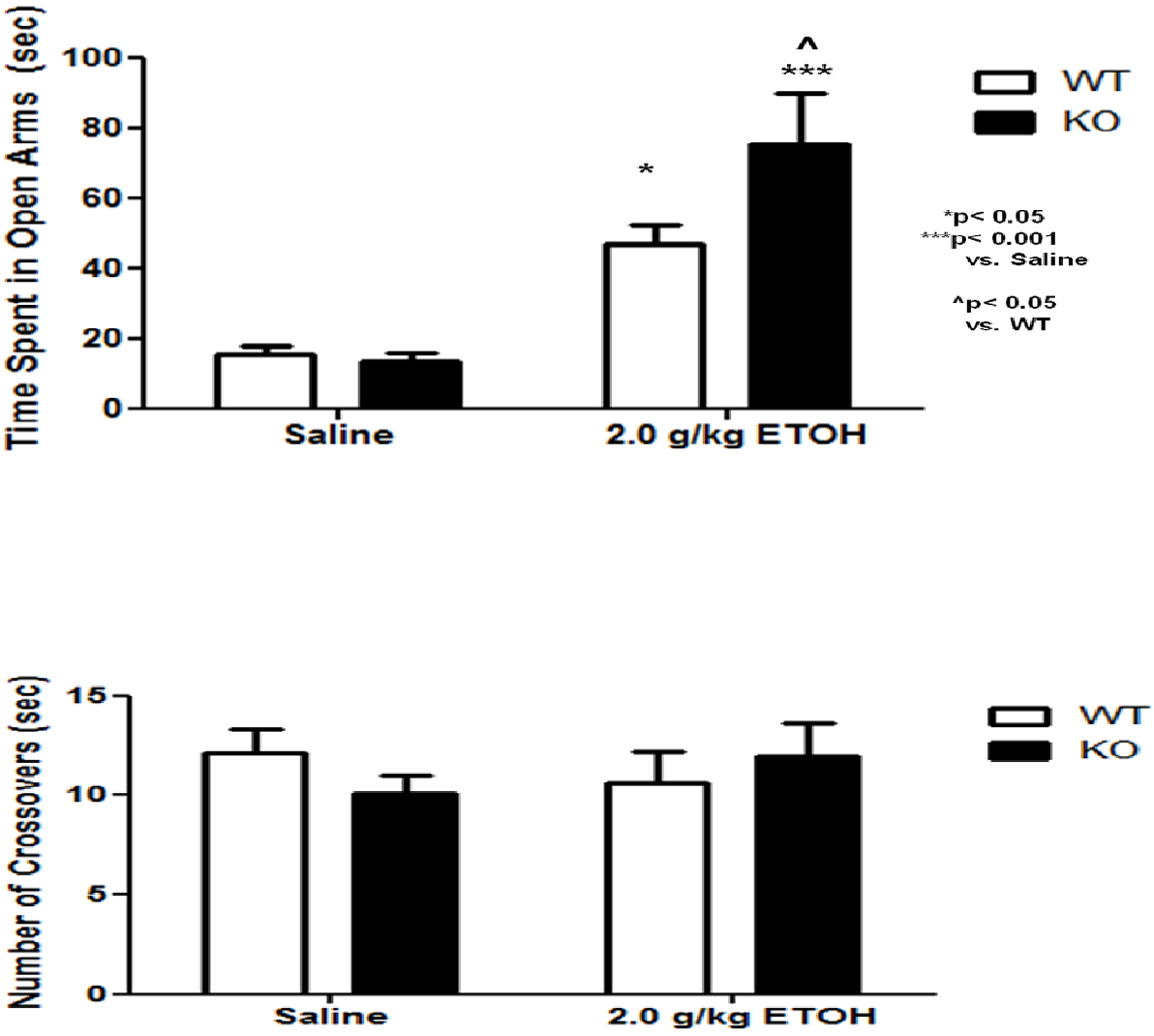


Figure 6. Deletion of the *Chrna5* gene enhances ethanol-induced increase in open arm time without affecting locomotor activity. Data (mean ± SEM) represent **(A)** time spent in open arms in seconds and **(B)** total number of crossovers in $\alpha5^{-/-}$ mice after receiving an injection of 2.0 g/kg ethanol. N= 7-11 per group.

Conditioned Place Preference (CPP)

The administration of 1.0- and 2.0 g/kg ethanol over the course of the conditioning phase produced a preference response on test day, as expected (Figure 7). Two-way ANOVA showed significant main effects of genotype [$F(1,27) = 60.27$, $p < 0.0001$], dose [$F(1,27) = 94.52$, $p < 0.0001$], and interaction [$F(1,27) = 68.50$, $p < 0.0001$]. Further analysis of ethanol –treated groups with one-way ANOVA revealed a significant difference in the preference score between genotypes [$F(1, 6) = 19.975$; $p = 0.0004$] as the preference score displayed by the $\alpha5^{-/-}$ mice was drastically reduced compared to their $\alpha5^{+/+}$ counterparts at each dose. The data show that presence of the $\alpha5$ gene may be required for the full acquisition of ethanol CPP in mice and, thus, some of the reward-like effects produced by ethanol.

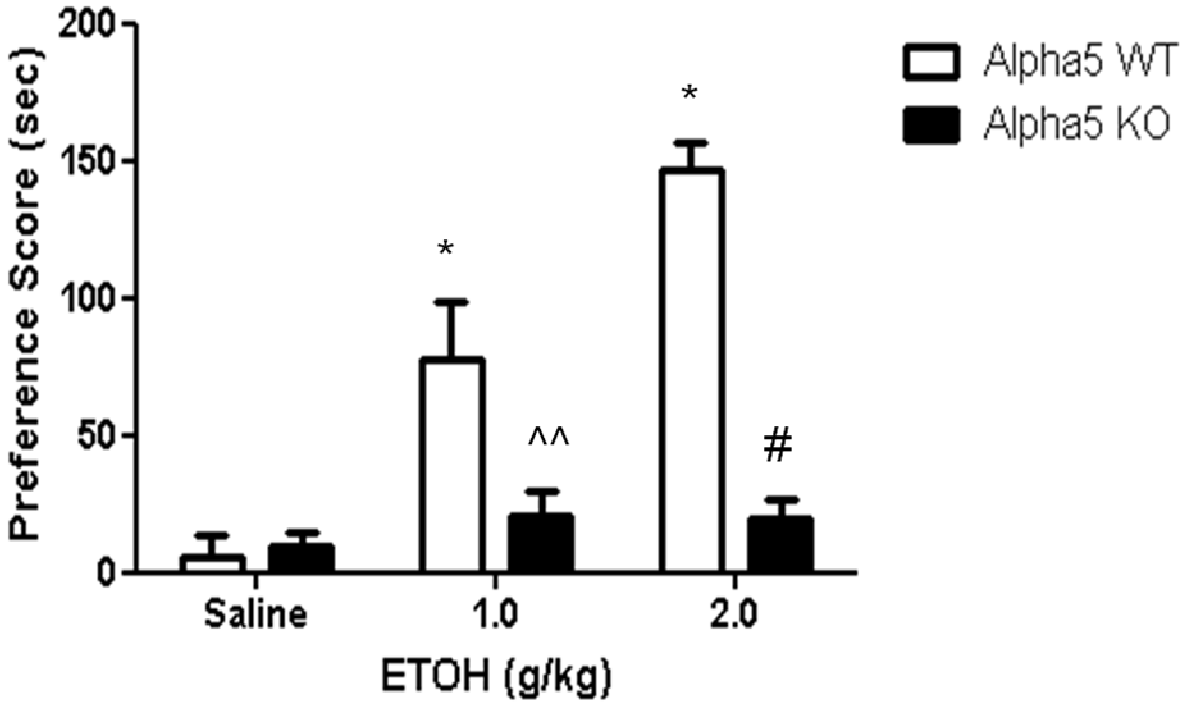


Figure 7. Deletion of the *Chrna5* gene dose dependently reduces ethanol-induced increase in CPP without effecting locomotor activity. Data (mean ± SEM) represent time spent on the ethanol-paired side in $\alpha 5^{-/-}$ mice on test day after conditioning with repeated injections of 1.0- and 2.0 g/kg ethanol. N= 6-10 per group.

BEC Analysis

As expected, a considerable amount of ethanol in blood was detected at each time-point for up to 120 mins after ethanol injection (Figure 5). A one-way ANOVA analysis showed no significant difference between $\alpha 5^{-/-}$ mice and their WT counterparts at 15-min [$F(1,3)= 0.057$, $p= 0.8273$], 30 min [$F(1,3)= 0.2796$, $p< 0.1931$], 60 min [$F(1,3)= 2.733$, $p< 0.1968$], nor 120 min time-points [$F(1,3)= 1.587$, $p< 0.2968$] after injection with 4.0 g/kg ethanol. These data suggest that deletion of the $\alpha 5$ gene does not affect BEC levels in mice, at least at hypnotic doses of ethanol.

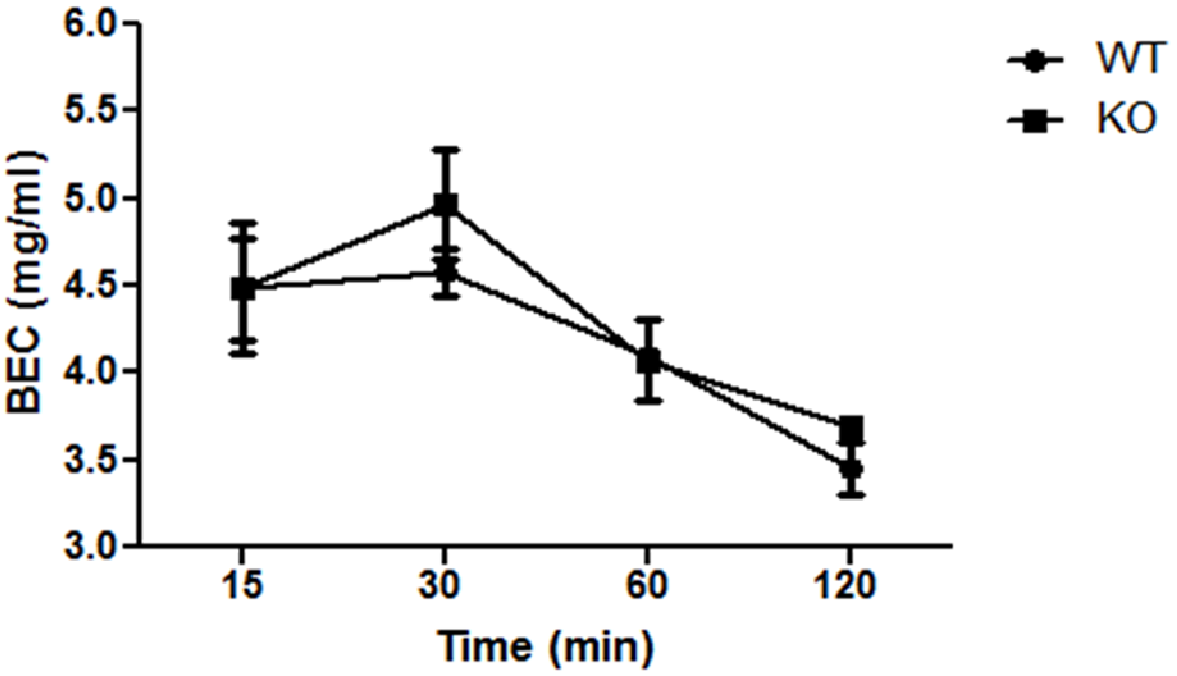


Figure 8. Deletion of the *Chrna5* gene has no effect BEC levels in C57BL/6J mice over two hour time course. Data (mean ± SEM) represent BEC in mg/ml at 15-, 30-, 60- and 120 min time points after receiving an injection of 4.0 g/kg ethanol . N= 3-5 per group.

Two-Bottle Choice

As expected, mice given free access to ethanol and water displayed a concentration-dependent increase in ethanol intake at a range of 3- to 30% (w/v) ethanol (Figure 9). Two-way repeated measures ANOVA (concentration x genotype) revealed main significant effects of concentration [$F(1,19)= 16.321$, $p< 0.0001$], but not genotype [$F (1,17)= 0.474$, $p< 0.5005$] nor interaction ([$F(1,19)= 1.109$, $p< 0.3397$], Figure 9A). Furthermore, the concentration-dependent increase in ethanol intake was not due to differences in total fluid intake between groups as ANOVA analysis showed no difference between groups [$F (2,63)= 0.249$, $p< 0.7807$]. The results for ethanol preference were similar to those of intake as two-way repeated measures ANOVA (concentration x genotype) revealed main significant effects of concentration [$F (1,19)= 13.432$, $p< 0.0001$], but not genotype [$F (1,17)= 0.285$, $p= 0.6984$] nor interaction ([$F(1,19)= 0.875$, $p= 0.4592$], Figure 9B). Taken together, the results show that deletion of the $\alpha 5$ gene has no significant overall effect on ethanol consumption even in a wide range of concentrations.

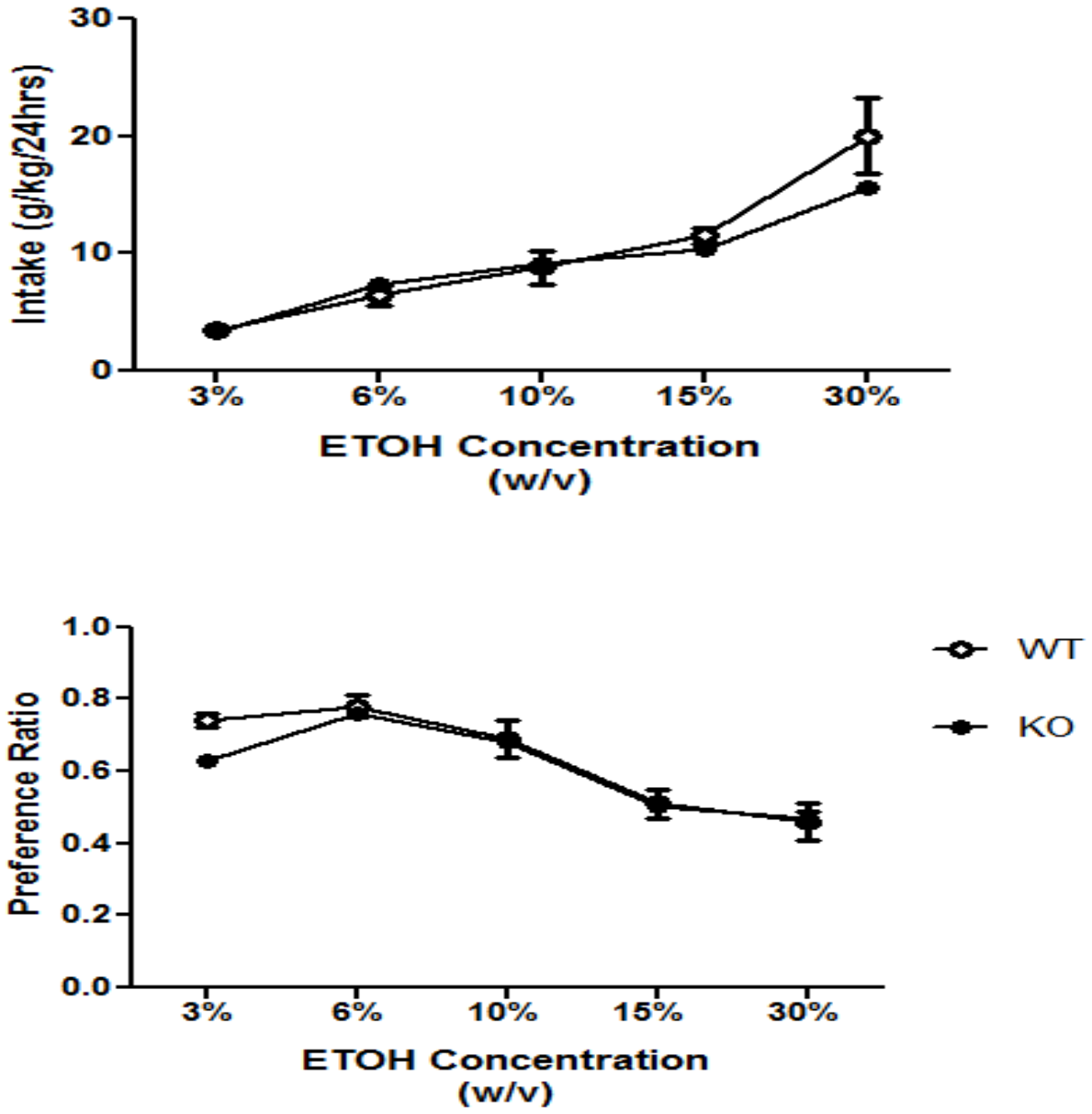


Figure 9. Deletion of the *Chrna5* gene has no effect on two bottle choice drinking behavior in C57BL/6J mice. Data (mean ± SEM) represent **(A)** intake in g/kg and **(B)** preference ratio during 4 weeks of exposure to increasing concentrations ETOH. N= 11 per group.

Intermittent Access

Mice given either intermittent or continuous two-bottle choice access to water and increasing concentrations of ethanol during the acclimation week (Week 1) displayed a concentration-dependent increase in ethanol intake (Figure 10). An initial three-way ANOVA (access group x concentration x genotype) revealed main significant effects of access group [$F(1,72)= 11.313$, $p< 0.0012$, post-hoc $=0.0002$], and concentration [$F(2,72)= 39.361$, $p< 0.0001$, post-hoc $=0.0001$], but not genotype [$F(1,72)= 0.533$, $p= 0.4678$] nor interaction [$F(2,72)= 0.138$, $p= 0.8715$]. Furthermore, the concentration-dependent increase in ethanol intake was not due to differences in total fluid intake between groups as three-way ANOVA analysis showed no difference between groups [$F(2,63)= 0.249$, $p= 0.7807$]. As for the preference results (Figure 11), three-way ANOVA revealed a main significant effect of access group [$F(1,72)= 5.552$, $p= 0.0212$], but not concentration [$F(2,72)= 0.244$, $p= 0.7843$], genotype [$F(1,72)= 2.411$, $p= 0.1249$] nor interaction [$F(2,72)= 0.033$, $p= 0.9864$].

For the maintenance phase of the experiment, weeks 2-4, intermittent access to ethanol had the intended effect of increasing both intake and preference for ethanol in each test group (Table 3). Two-way repeated-measures ANOVA analysis of ethanol intake revealed a significant main effect of access group [$F(1,18)= 49.479$, $p< 0.0001$] but not genotype [$F(1,18)= 1.192$. $p= 0.2886$] nor interaction [$F(1,18)= 0.609$, $p= 0.4448$] over the course of the maintenance phase. Separate one-way repeated measures ANOVA analyses for each week revealed significantly higher intake in intermittent access mice compared to continuous access mice during week 2 [$F(1,26)= 14.605$, $p= 0.007$], week 3 [$F(1,26)= 25.754$, $p< 0.0001$], and week 4 [$F(1,26)= 38.368$, $p< 0.0001$]. The results for preference

were similar with two-way repeated-measures ANOVA analysis revealing a significant main effect of access group [$F(1,18)= 28.156$, $p< 0.0001$] but not genotype [$F(1,18)= 0.959$, $p= 0.3405$] nor interaction [$F(1,18)= 0.350$, $p= 0.5614$] over the course of the maintenance phase (Table 3). Separate one-way repeated measures ANOVA analyses for each week revealed significantly higher preference in intermittent access mice compared to continuous access at mice during week 3 [$F(1,23)= 13.524$, $p= 0.0012$], and week 4 [$F(1,26)= 9.545$, $p= 0.0047$]. Taken together, the results show that intermittent exposure to ethanol induces escalation of drinking behavior of a similar magnitude in both $\alpha5^{-/-}$ and $\alpha5^{+/+}$ mice.

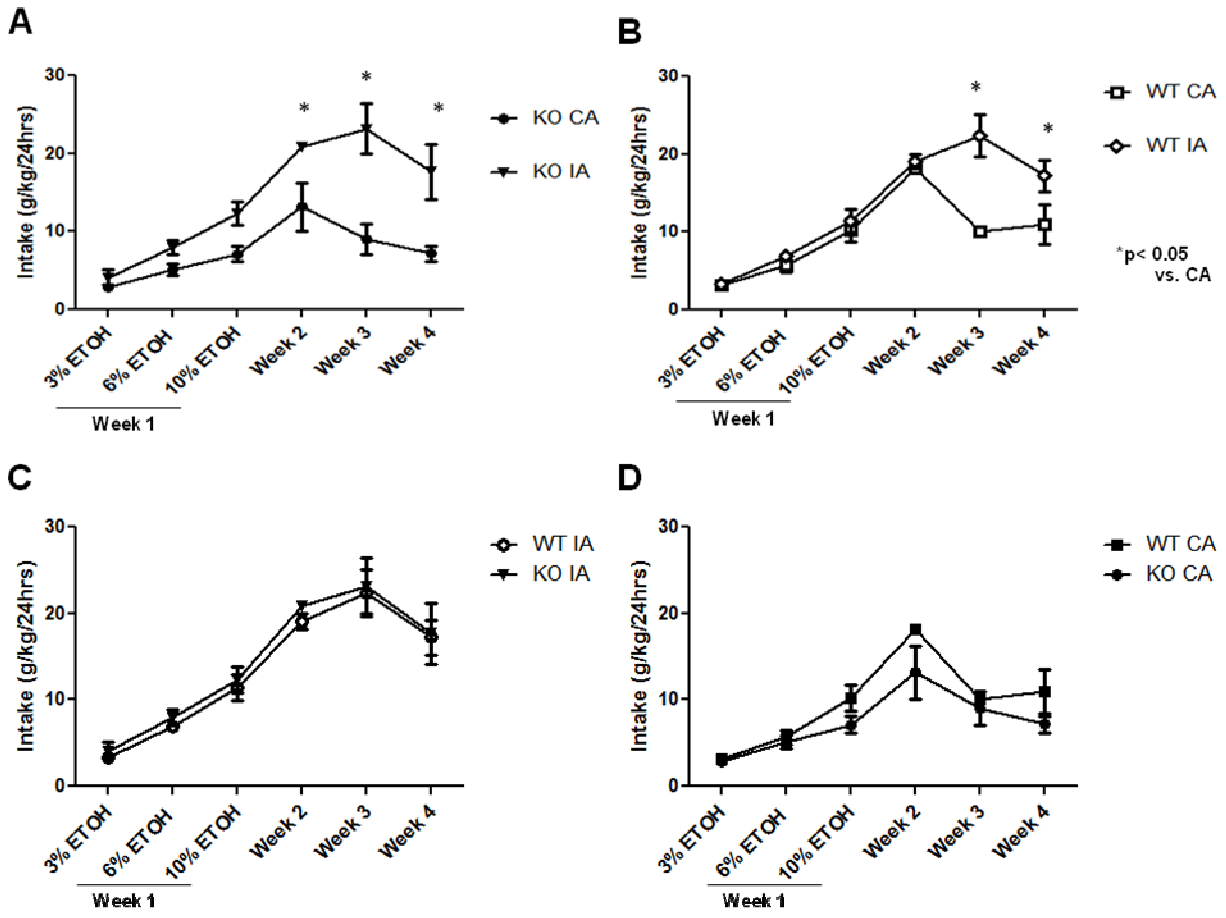


Figure 10. Deletion of the *Chrna5* gene has no effect on intermittent access ethanol intake in C57BL/6J mice. Data (mean ± SEM) in the top graphs represent intake in g/kg in (A) WT and (B) KO intermittent (IA) vs. continuous access (CA) groups at 3-, 6-, 10-, and 20% (w/v) ethanol during weeks 1-5. The bottom graphs display the same data, but rearranged to show comparisons between each genotype during (C) continuous and (D) intermittent access exposure. N= 7 per group.

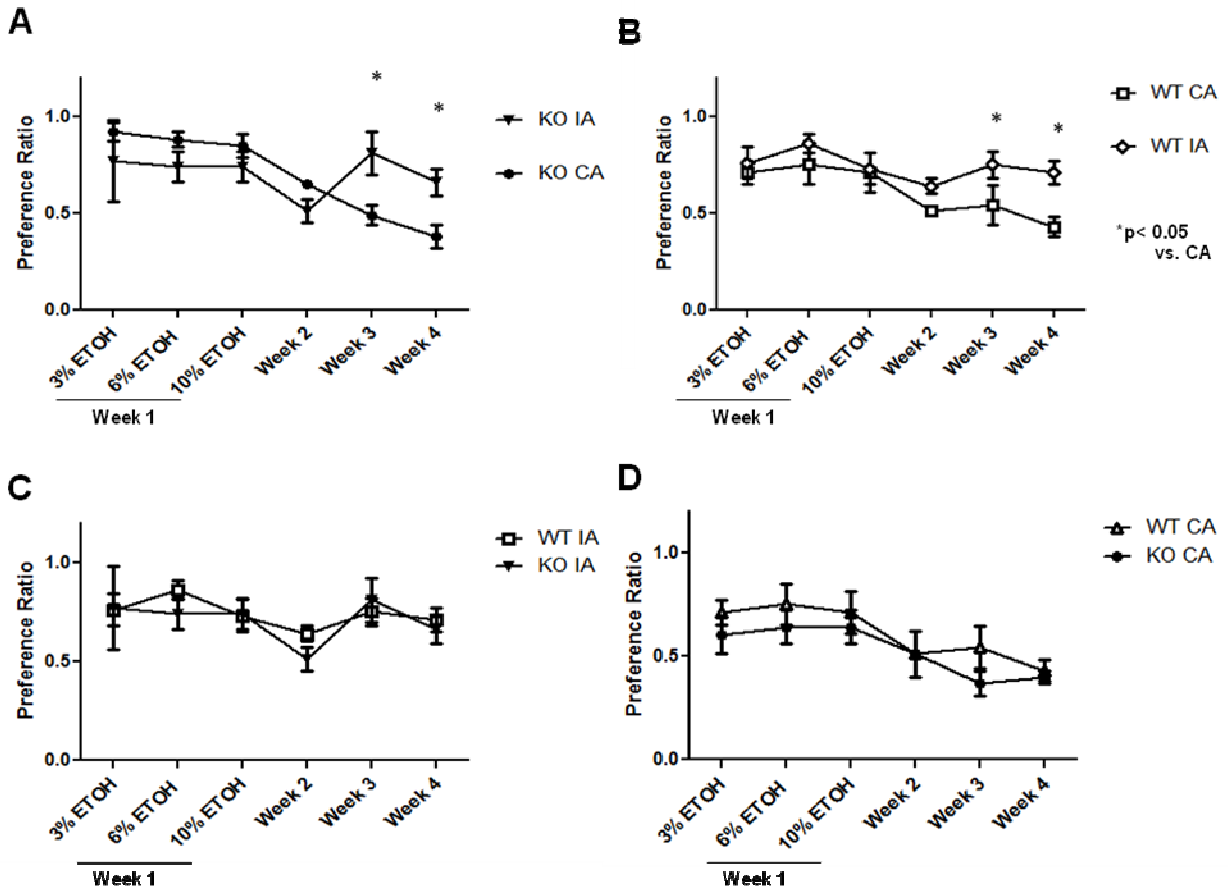


Figure 11. Deletion of the *Chrna5* gene has no effect on intermittent access ethanol preference in C57BL/6J mice. Data (mean ± SEM) in the top graphs represent the preference ratio in (A) WT and (B) KO intermittent vs. continuous access groups at 3-, 6-, 10-, and 20% (w/v) ethanol during weeks 1-5. The bottom graphs display the same data, but rearranged to show comparisons between each genotype during (C) continuous and (D) intermittent access exposure. N= 7 per group.

20% Ethanol Intake and Preference in Alpha 5 WT and KO mice				
Treatment	**CA**		**IA**	
Genotype	**Alpha 5 WT**	**Alpha 5 KO**	**Alpha 5 WT**	**Alpha 5 KO**
Intake Weeks 2 -5	**10.1 ± 1.0**	**9.2 ± 0.7**	**19.4 ± 1.1** [a]	**17.3 ± 1.6**[a]
Preference Weeks 2-5	**0.42 ± 0.04**	**0.42 ± 0.03**	**0.71 ± 0.03**[a]	**0.67 ± 0.06**[a]

[a] Represents significantly higher intake or preference than CA

Table 1. Ethanol Intake and Preference in Alpha 5 WT and KO mice during the maintenance phase of the IA procedure. Data (mean ± SEM) represent intake in g/kg and preference ratio, respectively, for each group.

DID

Finally, we assessed baseline ethanol consumption of the $\alpha5^{-/-}$ and $\alpha5^{+/+}$ mice using the DID paradigm. As expected, $\alpha5^{+/+}$ mice displayed high intake during the 4-hr limited access to ethanol for two days. However, there appeared to be no difference in DID intake between untreated $\alpha5^{-/-}$ and $\alpha5^{+/+}$ mice. Two-way repeated-measures ANOVA (genotype x time) showed a significant effect of time [(F1,1)= 13.208, p= 0.0012], but not genotype [(F1,27)= 0.191, p= 0.6653], nor interaction [(F1,1)= 0.2.868, p= 0.6653]. Thus, we show that deletion of the $\alpha5$ gene has no effect acute binge-drinking behavior in B6 mice during period of forced access to ethanol. However, due to evidence that $\alpha5$ gene deletion alters nicotine self-administration behavior, we also evaluated the effect of nicotine on voluntary ethanol consumption in $\alpha5^{-/-}$ and $\alpha5^{+/+}$ mice using the DID procedure to determine if effects of $\alpha5$ gene deletion on DID would be detected in the presence of an agonist. As intended, $\alpha5^{+/+}$ mice displayed high intake during the 4-hr limited access to ethanol, which was attenuated by repeated nicotine treatments following a three day period of acclimation injections with saline. Three way- repeated measures ANOVA (genotype x treatment x time) showed a significant main effect of genotype [(F1,27)=4.239, p= 0.0346], treatment [(F1,27)= 7.997, p= 0.0104], time [(F1,3)= 3.893, p= 0.0131], but not interaction [(F1,20)= 2.082, p= 0.1645]. Separate one-way ANOVA analyses for each day showed a significant decrease in intake in nicotine-treated $\alpha5^{+/+}$ mice compared to saline control at Day 6 [(F1,10)= 9.265, p= 0.0124], Day 7 [(F1,10)= 8.205, p= 0.0168], Day 8 [(F1,10)= 4.685, p= 0.0557], and Day 9 [(F1,10)= 5.243, p= 0.0450]. One-way ANOVA analyses of nicotine-treated $\alpha5^{-/-}$ mice, however, showed no sigfinicant difference in drinking behavior

compared to saline control for neither Day 6 [(F1,10)= 4.706, p= 0.552], Day 7 [(F1,10)= 0.670, p= 0.4321], Day 8 [(F1,10)= .0019, p= 0.9754], nor Day 9 [(F1,10)= 0.526, p= 0.4851]. Interestingly, this was likely due to the fact that saline-pretreated $\alpha5^{-/-}$ mice *also* appeared to have reduced DID intake as compared to saline-pretreated $\alpha5$ WT mice. Indeed, separate one-way analyses for each day reveal a significant difference on Day 7 [(F1,10)= 5.518., p= 0.261] and Day 9, [(F1,10)= 7.723, p= 0.0195] indicating a reduction in intake in saline-pretreated $\alpha5^{-/-}$ mice compared to $\alpha5^{+/+}$ control for these days. Furthermore, this reduction in drinking behavior did not occur in $\alpha5^{+/+}$ as intake during baseline and the first two test days remained stable. A one-way ANOVA analysis showed no siginificant difference in $\alpha5$ WT mice on Day 6 [(F1,10)= 0.522, p= 0.4761] nor Day 7 [(F1,10)= 0.188, p= 0.6745] compared to baseline intake on Day 1 and Day 2. Thus, the possible effect of nicotine on DID drinking behavior in $\alpha5^{-/-}$ mice, if any, was obscured by similar reduction in saline-pretreated $\alpha5^{-/-}$ mice, which was perhaps induced by injection stress experienced by the $\alpha5^{-/-}$ mice.

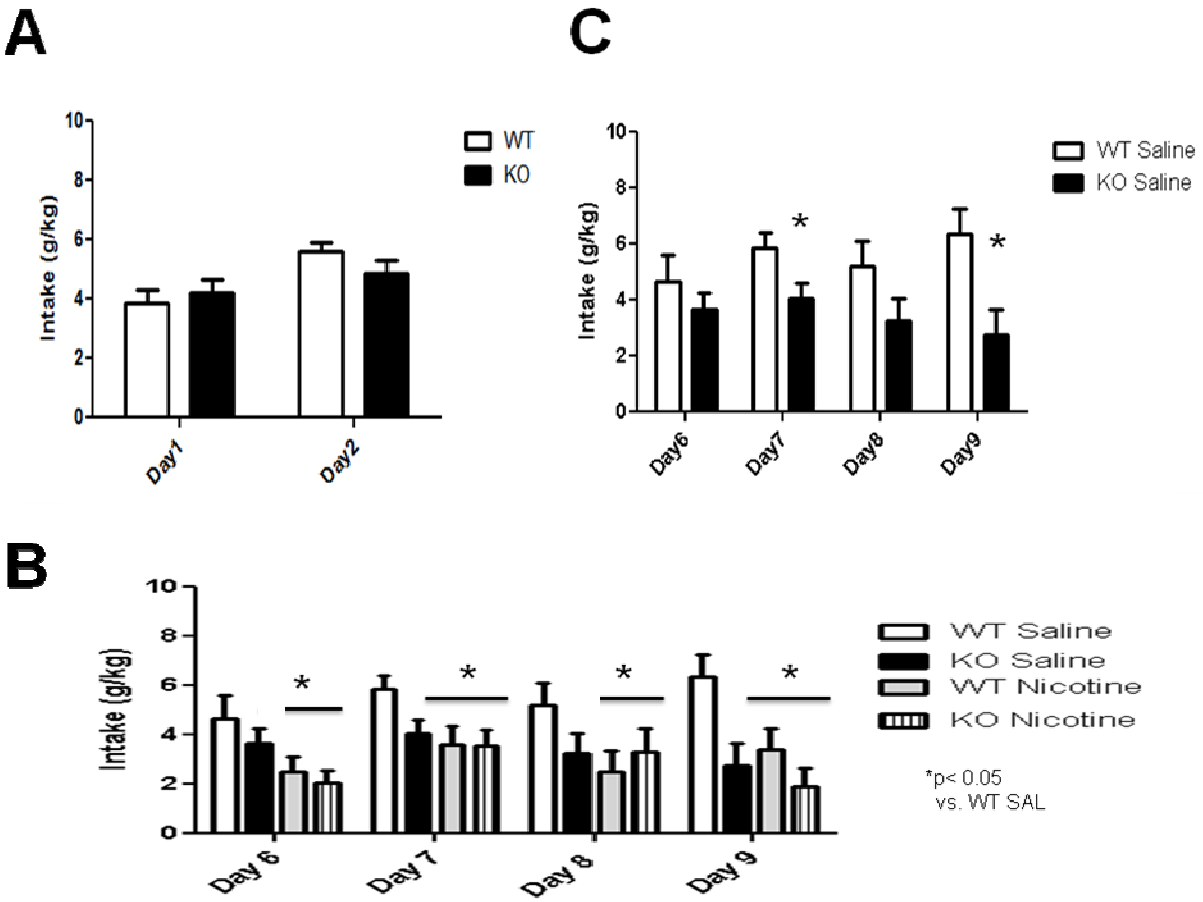


Figure 12. Deletion of the *Chrna5* gene reduces DID drinking behavior after repeated injections of saline or nicotine in C57BL/6J mice. Data (mean ± SEM) represent daily DID ethanol intake in g/kg for 4 hours in $\alpha5^{-/-}$ and $\alpha5^{+/+}$ mice during (A) baseline drinking before injections on days 1 and 2, and (B) drinking after injections of saline or nicotine on Days 6-9 in C57BL/6J mice. Graph (C) shows the same data for days 6-9 but with the nicotine groups removed. N= 6-15 per group.

3.4 Summary

The goal of these studies was to assess the potential influence of the α5 nAChR-coding gene on ethanol-responsive behaviors in an *in vivo* animal model. We examined mice in a battery of tests for acute ethanol-responsive behaviors, including hypothermia, LORR, reduction of anxiety-like behavior in EPM, and reward-like effects in CPP, as well as in chronic drinking behavior in the two-bottle choice model. Because there are no currently available ligands selective for α5* nAChRs, we examined the effects of α5 gene deletion on these behavioral effects in ethanol preferring C57 mice. Our results showed that these mice consistently displayed an enhanced response to hypothermia, LORR, and anxiolysis behavior in the EPM following acute ethanol challenge. We also found, in contrast, that CPP behavior conditioned by repeated ethanol injections was reduced when the mice were observed on test day in a drug free state. Surprisingly, these effects did not translate into changes in ethanol drinking behavior as measured by the two-bottle choice, intermittent access drinking and DID drinking paradigms was altered only *after* repeated injections with either saline or nicotine.

CHAPTER 4: CHARACTERIZATION OF α4β2* NICOTINIC ACETYLCHOLINE RECEPTORS IN ETHANOL-RESPONSIVE BEHAVIORS

4.1 Introduction

Alcohol (ethanol) and nicotine addiction are two of the leading causes of preventable death worldwide. In the United States alone, it is estimated that up to 7% of the entire population is co-dependent upon these substances (Burns & Proctor, 2013; Anthony & Echeagaray-Wagner, 2000; Istvan & Matarazzo, 1984). Studies also show that alcohol dependence is three times more common among smokers than non-smokers and this high co-morbidity of use increases the difficulty of achieving long-term abstinence with either drug (Larsson *et al.* 2004a, Grucza *et al.* 2006). Furthermore, there is a high genetic correlation between alcohol and nicotine dependent individuals suggesting common neurobiological mechanisms mediating co-abuse (True *et al.*1999; Davis & de Fiebre 2006). Nicotinic acetylcholine receptors (nAChRs) may be prime candidates for mediating this vulnerability to alcoholism and nicotine addiction.

Neuronal nAChRs are ligands-gated ion channels consisting of five transmembrane spanning proteins, or subunits. These subunits complex to form many combinations of nAChR subtype consisting of α ($\alpha2-\alpha10$) and/or β ($\beta2-\beta4$) subunits. $\beta2$* nAChRs (* denotes the presence of additional nicotinic subunits) represent the most widely distributed and best-characterized nAChR subtypes to date (Gotti *et al* 2007; Changeux 2009 and

2010). Furthermore, this receptor subtype plays a critical role in nicotine dependence (Picciotto *et al.* 1998). For example, nicotine self-administration is reduced in rats pretreated with dihydro-β-erythroidine (DHβE), an antagonist selective for β2*-containing subtypes (Cohen *et al.*2008). The β2* subtype also plays a major role in nicotine reinforcement (Picciotto *et al.* 1998), nicotine conditioned place preference (Walters et al., 2006) and drug discrimination in rats and mice (Shoaib *et al.* 2002, Smith *et al.* 2007). Therefore, studies on the role of the β2* nAChRs in alcohol phenotypes continue to emerge. For example, β2 genetic Knockout (KO) mice are less sensitive to both ethanol's acute effects in the acoustic startle response and to ethanol withdrawal signs compared with β2 wild-type (WT) mice (Owens *et al.* 2003, Butt *et al.* 2004). Interestingly, the impairment of contextual recall by ethanol was not affected in β2* KO mice (Wehner *et al.* 2004). In addition to the previously mentioned effects, full agonists such as nicotine and RJR-2403 decreased ethanol-induced ataxia while a partial agonist. Varenicline, a non-selective α4β2* nAChRs partial agonist, actually increases the sensitivity to this response (Taslim *et al.* 2007, 2010; Kamens *et al.* 2010). Varenicline also increases sensitivity to the sedative-hypnotic effects of acute ethanol as tested in the rotorod and Loss of Righting Response assays (Kamens *et al.* 2010).

In contrast to the acute administration studies previously mentioned, no changes in chronic ethanol drinking behavior in the two-bottle choice paradigm were found in the β2 KO mice compared to their WT counterparts (Kamens *et al*. 2010). DHβE also had no effect on either ethanol consumption (Hendrickson *et al.* 2009; Kuzmin *et al.* 2009) nor dopamine release in the ventral tegmental area or nucleus accumbens in response to

ethanol-associated cues (Ericson *et al.* 2003, 2008, Larsson *et al.* 2004a,b). Interestingly, a polymorphism in the β2 gene (CHRNB2) has also been associated with the initial subjective response to alcohol in human subjects (Ehringer *et al.* 2007). Collectively these studies suggest that β2* nAChRs may be important for modulating ethanol-responsive behavior, particularly the pharmacological effects after acute exposure to the drug. The initial sensitivity of an individual to the acute effects of early alcohol exposure is becoming an increasingly attractive endophenotype for alcoholism vulnerability. A 25-year study conducted by Schuckit and colleagues (2011) showed that the level of response to acute alcohol exposure early in life was predictive of future drinking behavior and alcohol abuse liability.

The aim of these studies was to more fully characterize the role of β2* nAChRs in ethanol-responsive behaviors in mice after acute exposure to the drug. To do this, we tested mice lacking *Chrnb2* or pretreated with a selective β2* nAChRs antagonist for a range of ethanol-induced behaviors, namely locomotor depression, hypothermia, hypnosis, and anxiolysis. We also tested the effect of activation of β2* nAChRs on these ethanol-induced behaviors through the use of the partial agonists, varenicline and sazetidine-A, as well as the full agonist, nicotine. Finally, , we tested mice lacking *Chrnb2* for voluntary ethanol consumption using an intermittent access two-bottle choice alcohol paradigm since these mice have not previously been tested in this model of ethanol consumption.

4.2 Methods

Animals

Male C57BL/6J mice were purchased from Jackson laboratories (Bar Harbor, ME). $\beta2^{-/-}$ mice; (Institut Pasteur, Paris, France) and their wild-type $\beta2^{+/+}$ littermates were bred in an animal care facility at Virginia Commonwealth University. All the mice used in each experiment were backcrossed at least 10 to 12 generations. Mutant and wild types were obtained from crossing heterozygote ($\beta2^{+/-}$) mice. This breeding scheme controlled for any irregularities that might occur with crossing solely mutant animals. Mice were housed in a 21℃ humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-approved animal care facility. They were housed in groups of six and had free access to food and water under a 12-h light/dark cycle (lights on at 6:00 a.m.) schedule. Mice were 8–10 weeks of age and weighed approximately 20–25 g at the start of all the experiments. All experiments were performed during the normal light cycle (between 6:00 a.m. and 6:00 p.m.) and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All studies were carried out in accordance with the National Institute of Health guide for the Care and Use of Laboratory animals.

Drugs

Dihydro-β-erythroidine (DHβE) was purchased from Sigma- RBI (Natick, MA, USA). (-)-Nicotine hydrogen tartrate salt was purchased from Sigma-Aldrich (St. Louis, MO).

Varenicline [7,8,9,10-tetrahydro- 6,10-methano- 6*H*-pyrazino (2,3-*h*)(3) benzazepine] and sazetidine-A [6-[5-[(2*S*)-2-azetidinylmethoxy]-3-pyridinyl]- 5-hexyn-1-ol] were supplied by the National Institute of Drug Abuse Drug Supply Program (Bethesda, MD). DHβE doses (1.0- and 3.0 mg/kg) were based on published and unpublished studies from our lab that were within an effective range of doses for blocking the behavioral effects of nicotine (Damaj *et al.* 1995, 2003). Varenicline doses (0.10 - 4.0 mg/kg) and Sazetidine A doses (1.0mg/kg) were based on previous studies that were within an effective range of doses for blocking the behavioral effects of ethanol (Kamens *et al.* 2010b,Rezvani *et al.*2010). Nicotine doses (0.1 – 0.5 mg/kg) were chosen based on an effective range of doses behavioral active for CPP preference and self-administration (Walters *et al* 2005). All drugs were dissolved in 0.9% saline and injected intraperitoneally (i.p.), except nicotine, which was delivered subcutaneously (s.c.), at a volume of 10 ml/kg body weight. Ethanol was also dissolved in 0.9% saline and prepared as a 20% (v/v) solution which were delivered via i.p. injection for acute experiments or *per os* (p.o.) for drinking experiments. Ethanol doses (2.0 – 3.5 g/kg) were chosen based on effective doses obtained in dose response curves conducted before each study, which were consistent with those found in literature (Alanka *et al.* 1992, Browman *et al.*2000).

Locomotor Activity Measurement

Locomotor depression induced by acute ethanol was assessed in Omnitech photocell activity cages (28 x 16.5 cm) (Columbus, OH). Each apparatus consisted of two banks of eight cells with locomotor activity recorded as the interruptions of the photocell beams for

the duration of the test. Mice were allowed to acclimate to the room at least 1 hr before the beginning of the procedure. Animals were injected with saline on days 1 and 2, and then injected with 2.5 g/kg ethanol (i.p.) on day 3 after receiving a 10 min pretreatment with DHβE or saline (i.p.). For experiments with $\beta2^{-/-}$ and $\beta2^{+/+}$ mice, subjects were injected with saline on days 1 and 2, and then directly treated with either ethanol (2.5 g/kg) or saline alone on day 3. Locomotor activity scores were defined as the number of interruptions of the photobeam cells measured for 10 minutes. Data were expressed as mean ± SEM of the number of photocell interruptions.

Measurement of Hypothermia, LORR, EPM

We used a similar protocols, respectively, as previously described in Chapter 3

Intermittent Access

Chronic ethanol drinking behavior was assessed using the intermittent access (IA) procedure as described by Hwa and colleagues (Hwa *et al*. 2011). This procedure is advantageous over the traditional two-bottle choice drinking procedure in that it produces escalation in ethanol drinking by repeated deprivation cycles thus better approximating human drinking behavior (Rodd *et al.* 2004). Briefly, $\beta2^{-/-}$ and $\beta2^{+/+}$ mice were housed individually in cages one week prior to testing with *ad libitum* access to food and water. Three days before the end of acclimation week, water bottles were replaced with two drinking tubes, made from 10-ml serological pipettes containing a double bearing sipper tube and a rubber stopper on either end of the tube, filled with water. At the end of the acclimation period, one water tube was replaced with one ethanol filled with 3-, 6-, and 10% w/v ethanol on alternating days (Sunday, Tuesday, and Thursday) while two water

tubes were presented on the deprivation days (Monday, Wednesday, Friday, and Saturday). After one week, 20% (w/v) ethanol tubes were presented on alternating days (Sunday, Tuesday, and Thursday) for the remainder of the experiment. Control mice were presented with continuous access to ethanol by presenting one 3% (w/v) ethanol tube on Sunday and Monday, 6% Tuesday and Wednesday, and 10% (w/v) ethanol Thursday, Friday, and Saturday. After one week, 20% (w/v) ethanol tubes were presented daily for the remainder of the experiment. Additionally, ethanol and water tubes were placed on two empty cages which allowed for measurement of leakage and evaporation from the tubes. The average volume depleted from these "control" tubes was subtracted from the individual drinking volumes each day before data analysis. Intake was reported as g/kg ethanol consumed and preference as the ratio between of ethanol consumed divided by total amount of ethanol and water fluid intake (ml), combined. Additional measurements including body weight (g) and total fluid intake (ml) were also recorded. Data (mean ± SEM) were expressed as total intake or preference ratio.

Statistical Analysis

Data were analyzed using multiple standard analyses of variance (ANOVA) with treatment, dose, and/or genotype as independent variables. All analyses were followed by Bonferroni post-hoc tests where appropriate to further analyze significant data with the null hypothesis rejected at the 0.05 level. For the IA procedure, ethanol intake (g/kg), body weight (g), volume of ethanol intake (ml), water intake (ml), total fluid intake (ml), and ethanol preference ratio during the week of increasing ethanol concentrations (week 1) for each

access group and genotype were analyzed with multiple three-way analyses of variance (ANOVAs), followed by Bonferroni post hoc analysis when significant group effects were found ($p < 0.05$). During the maintenance phase with 20% ethanol (weeks 2-5), ethanol intake (g/kg), body weight (g), volume of ethanol intake (ml), water intake (ml), total fluid intake (ml), and ethanol preference ratio were analyzed between each treatment and genotype with multiple two-way repeated measures ANOVAs followed by Bonferroni post hoc analysis when significant group effects were found ($p < 0.05$).

4.3 Results

Locomotor Activity Measurement

We tested locomotor activity 10 min after 2.5 g/kg ethanol injection (i.p.) since this is sufficient time for blood and brain ethanol concentrations to reach equilibrium (Smolen & Smolen, 1989). Mice treated with ethanol displayed a significant decrease in locomotor activity (Figure 14A). One-way ANOVA analysis showed that ethanol produced a significant decrease in locomotor activity compared with findings for control animals [$(F3,20)= 6.821$, $p=0.0024$]. There was no effect of 3.0 mg/kg DHβE treatment on this ethanol-induced depression [$(F1,10)= 3.47$, $p= 0.0919$]. Similarly, absence of the β2 gene in KO mice did not modulate locomotor depression induced by an acute injection of 2.5 g/kg ethanol (i.p.) (Figure 14B). A two-way ANOVA analysis showed a significant main effect of ethanol treatment [$(F1,19) = 56.85$, $p<0.0001$] but not genotype [$(F1,19)= 0.019$, $p= 0.8913$] nor interaction [$(F1,19)= 3.20$, $p= 0.08$] on locomotor

activity. Thus, the analysis suggests that neither the β2* antagonist, DHβE, nor *Chrnb2* deletion affects acute ethanol-induced locomotor depression in mice.

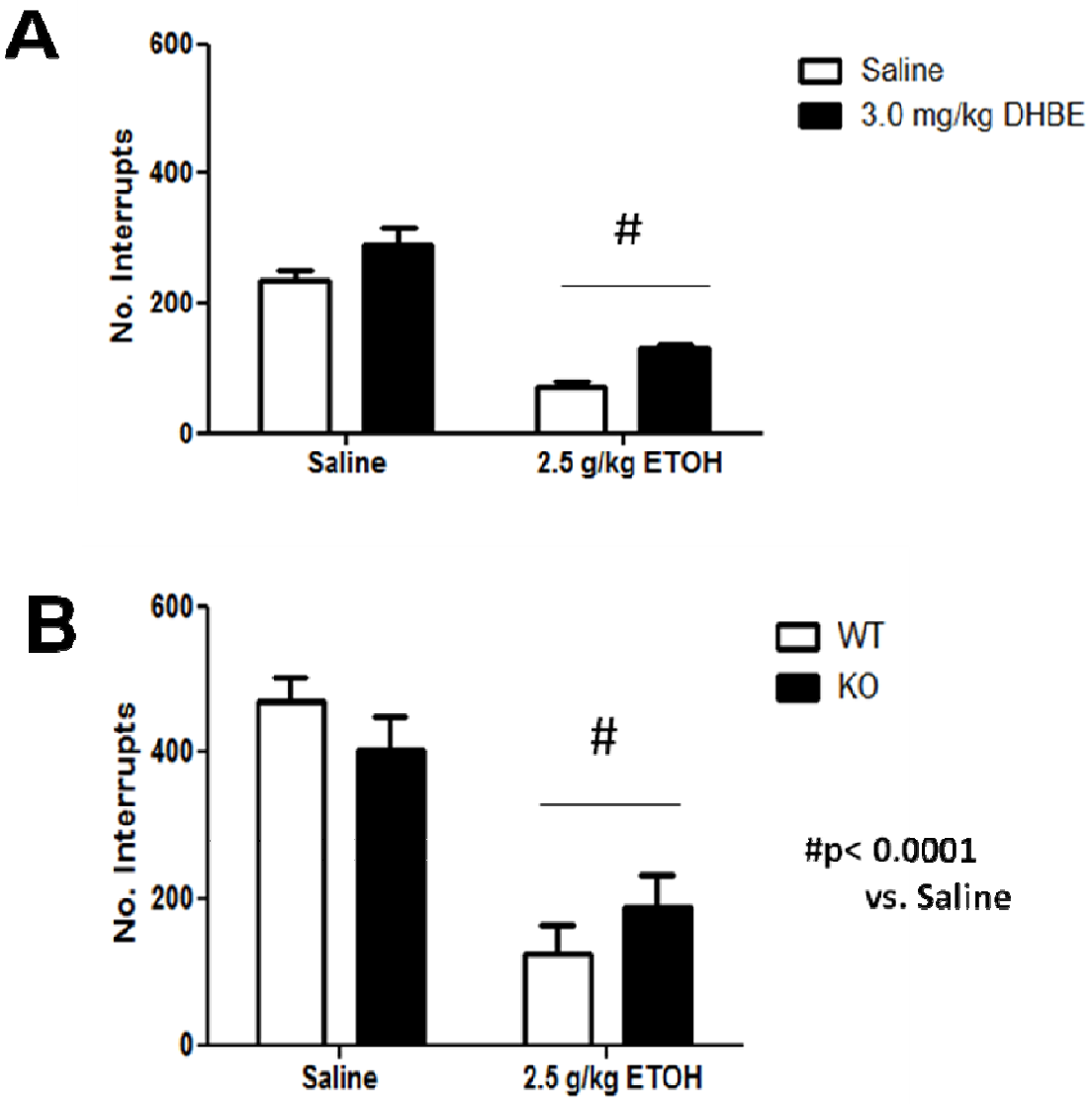


Figure 13. Effects of DHβE pretreatment and deletion of the *Chrnb2* gene on ethanol-induced locomotor depression in mice. Data (mean ± SEM) represent total number of photocell interruptions of **(A)** C57BL/6J mice with 10 min DHβE pretreatment and **(B)** $\beta 2^{-/-}$ mice after receiving an injection of 2.5 g/kg ethanol or saline. N= 6 per group.

Body Temperature Measurement

Treatment with 3.0 g/kg ethanol (i.p.) induced a significant drop in body temperature (Figure 15A). Two-way repeated measures ANOVA (treatment x time) revealed ethanol treatment caused significant hypothermia compared to saline control animals [$F(3,18)= 17.359$; $p< 0.0001$] at 15- and 60-min time points post ethanol injection. A dose of 3.0 mg/kg DHβE treatment did not produce significant changes [$F (1,10)= 0.991$; $p= 0.3430$] in ethanol-induced hypothermia compared to findings with control animals indicating this dose of DHβE has no effect on this measure of acute ethanol-responsive behavior. Deletion of the β2 gene also had no effect on hypothermia induced by an acute injection of 3.0 g/kg ethanol (i.p.) (Figure 15B). Three-way repeated measure ANOVA (genotype x treatment group x time) showed a main significant effect of ethanol treatment [$F(1,20)= 0.126$, $p< 0.0001$), but neither genotype [$F(1,20)= 1.844$, $p< 0.1897$] nor interaction [$F(1,20)= 0.1897$, $p< 0.7263$] on change in body temperature . There were also no genotype differences detected between saline control groups. Taken together, these results suggest that pharmacological and genetic antagonism of β2* nAChRs have no effect on acute ethanol-induced hypothermia.

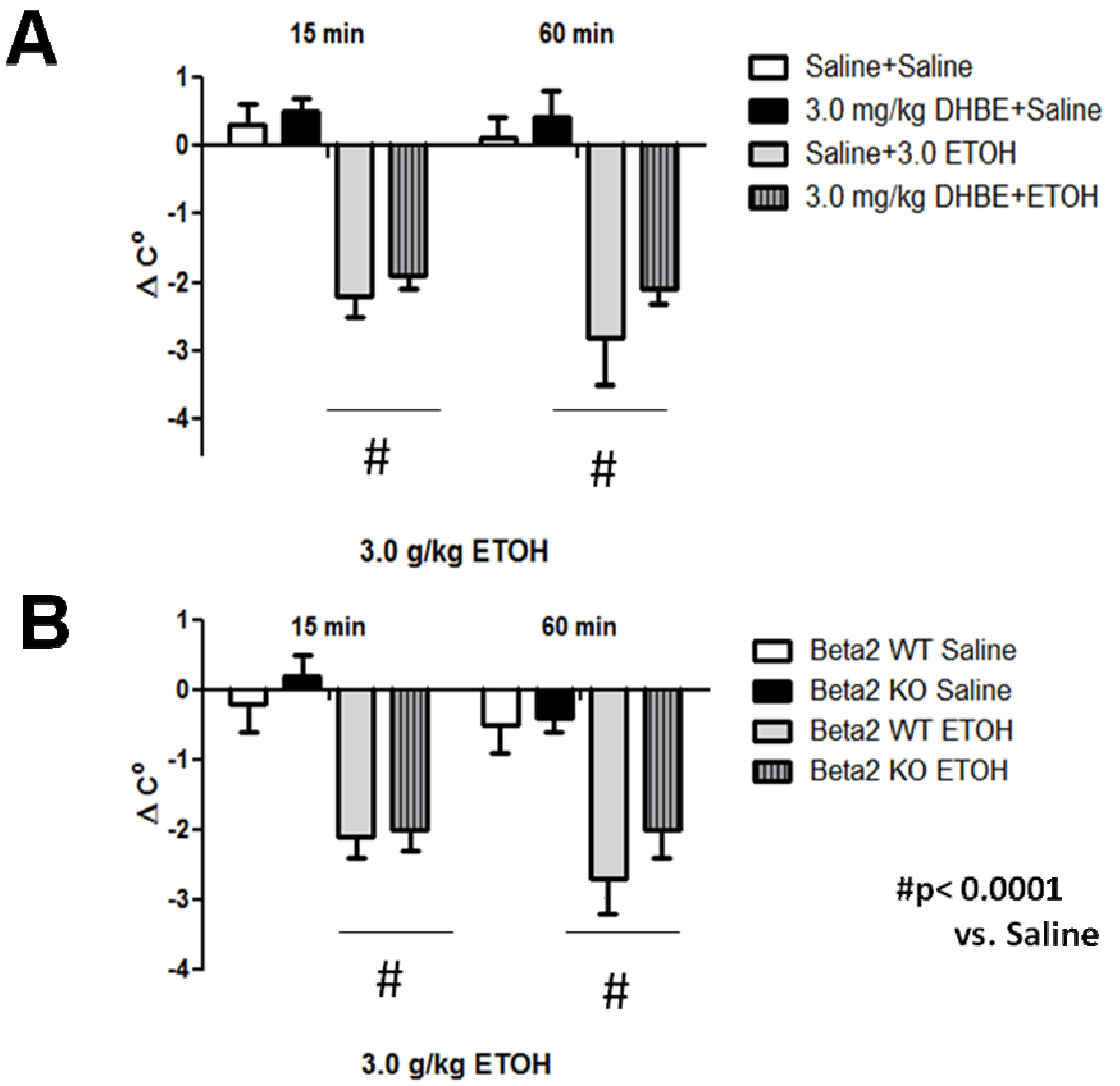


Figure 14. Effects of DHβE pretreatment and deletion of the *Chrnb2* gene have no effect on ethanol-induced hypothermia in C57BL/6J mice. Data (mean ± SEM) represent the change in body temperature from baseline in degrees Celsius of (A) mice with 10 min DHβE pretreatment and (B) $\beta 2^{-/-}$ mice at 15- and 60 min time points after receiving an injection of 3.0 g/kg ethanol. N= 6 per group.

LORR

A dose of 3.5 g/kg ethanol had the intended effect of inducing LORR (Figure 16). A one-way ANOVA analysis revealed that while there was no significant effect of either 1.0- or 3.0 mg/kg DHβE treatment on latency to LORR onset ([F(3,35)= 0.456; p= 0.7144], Figure 16A), the nicotinic antagonist significantly reduced LORR duration at these doses ([F(1,20)= 6.982; p= 0.0156], Figure 16B). $\beta2^{-/-}$ mice showed a similar response (Figure 17) with one-way ANOVA analysis showing genotype did not have an effect on the latency to LORR onset ([F(1,13)= 0.92; p= 0.7669], Figure 17A) but did have a significant effect on LORR duration ([F(1,13)= 5.57; p= 0.346] between KO and WT mice (Figure 17B). $\beta2^{-/-}$ mice took less time to right themselves than $\beta2^{+/+}$ mice, thus displaying decreased response to the hypnotic effects induced by acute ethanol. These results show that pharmacological and genetic antagonism of β2* nAChRs reduces the response to ethanol-induced hypnosis in naïve mice.

Conversely, testing with the partial agonists varenicline and sazetidine-A, and the full agonist, nicotine, produced similar results for LORR onset, but produced opposite results for LORR duration (Figures 18-20). One-way ANOVA analysis revealed no significant effects of sazetidine-A ([F(2,17)= 1.059.; p= 0.3686, Figure 19A), and a marginal but non-significant dose-dependent effect of both varenicline at 1.0 and 4.0 mg/kg ([F(2,18)= 3.747; p= 0.0536], Figure 18A) and nicotine at 0.1- and 0.5 mg/kg ([F(2,10)= 1.724; p= 0.2274, Figure 20A) on latency to LORR onset compared to saline control. However, one-way ANOVA showed a significant dose-dependent enhancement of LORR duration in subjects treated with varenicline at 1.0 and 4.0

mg/kg ([F(2,18)= 7.510; p= 0.0042], Figure 18B), sazetidine-A at 1.0- and 3.0 mg/kg ([F(2,17)= 9.463; p= 0.0017], Figure 19B), and nicotine at 0.1- and 0.5 mg/kg ([F(2,10)= 5.300; p= 0.0270], Figure 20B) compared to saline control. Taken together, it appears evident that activation of nAChRs, by either partial or full agonists consistently enhances the time to recover from ethanol-induced LORR, while differentially affecting LORR onset depending on each drugs affinity for certain nAChR subtypes.

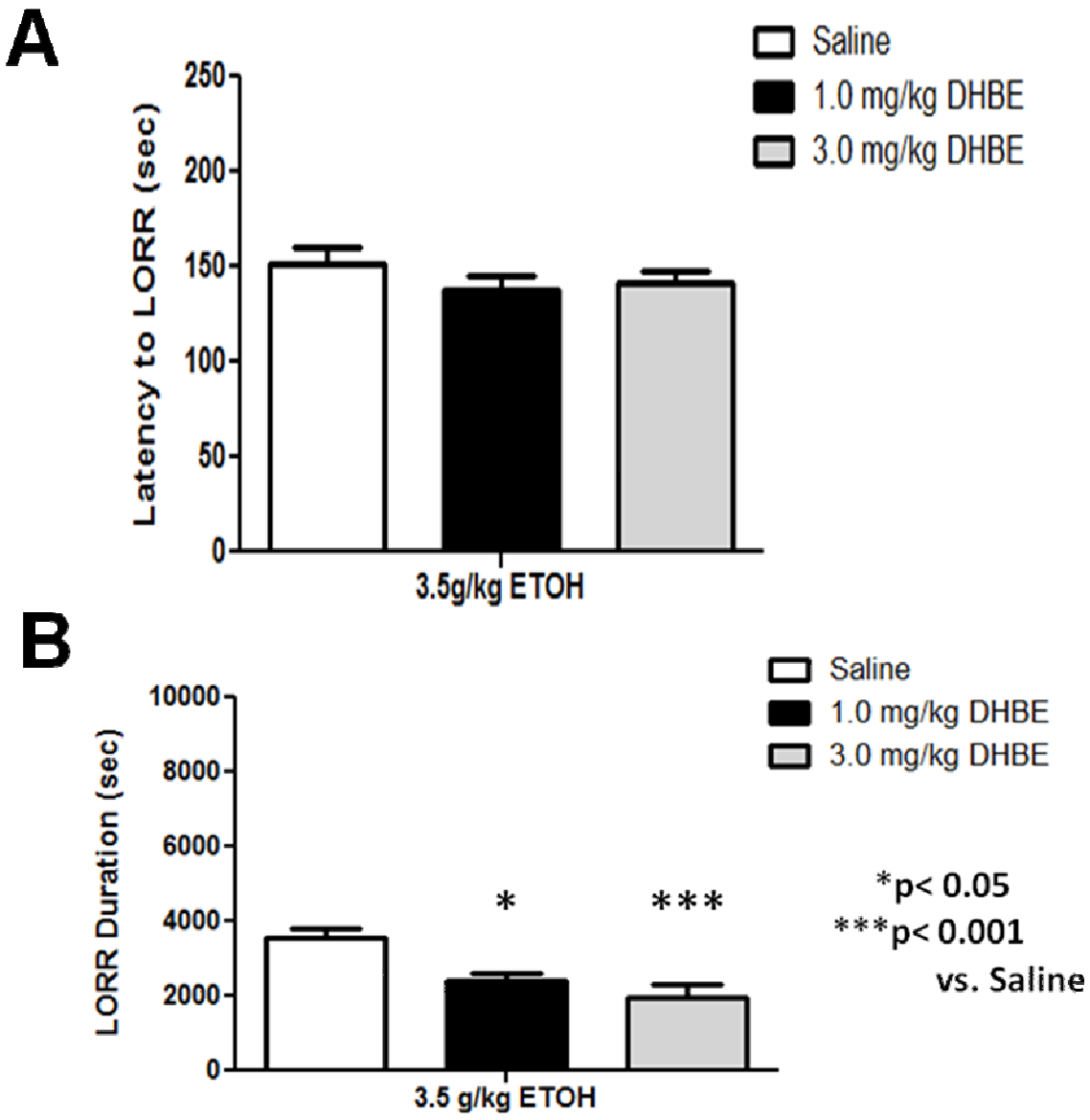


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Figure 15. DHβE pretreatment reduces ethanol-induced LORR duration while having no effect on LORR onset in C57BL/6J mice. Data (mean ± SEM) represent (A) latency to LORR onset and (B) total duration of LORR in seconds in mice with 10 min DHβE pretreatment after receiving an injection of 3.5 g/kg ethanol. N= 7-10 per group.

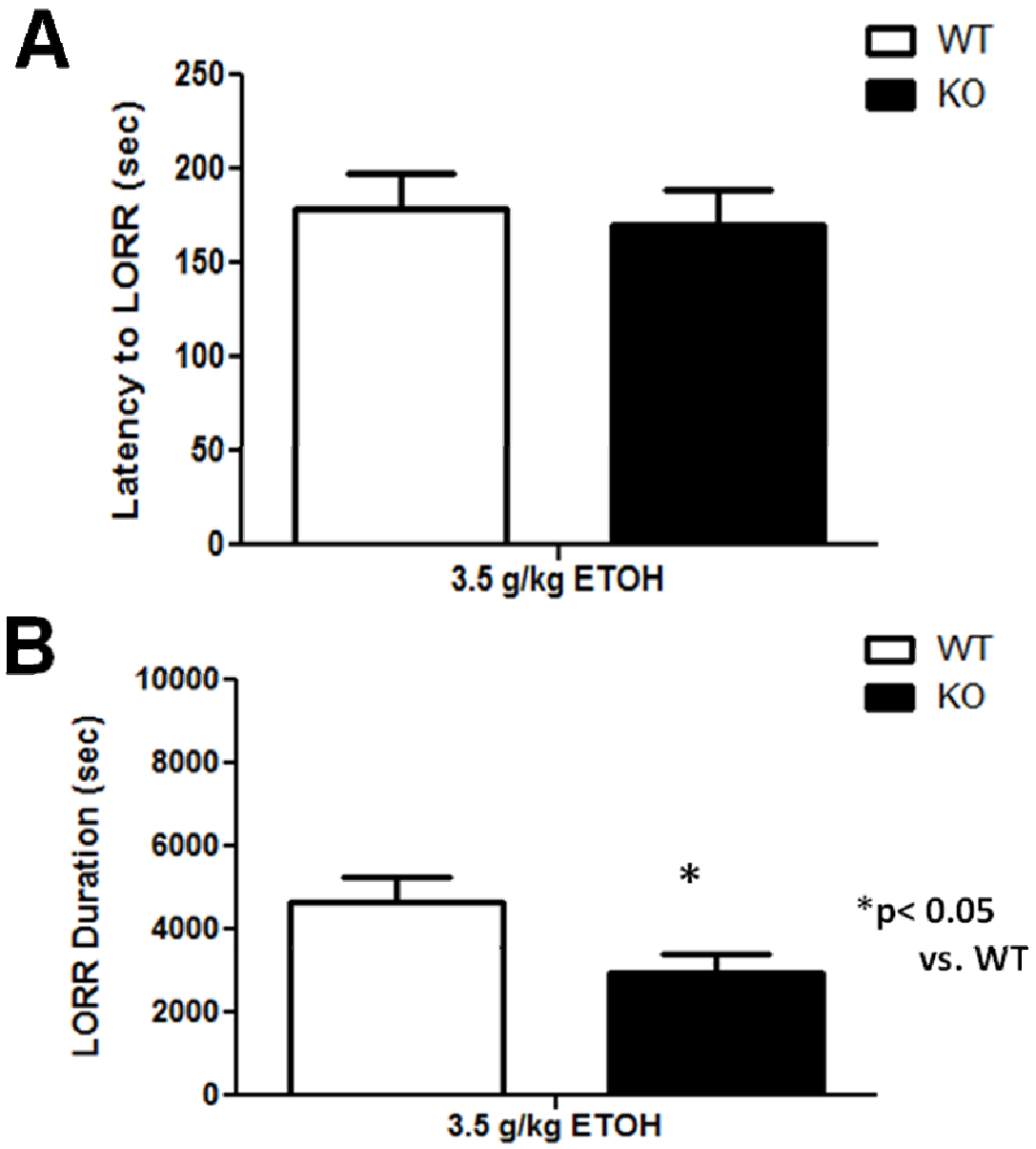

Figure 16. Deletion of the *Chrnb2* gene reduces ethanol-induced LORR duration while having no effect on LORR onset in C57BL/6J mice. Data (mean ± SEM) represent (A) latency to LORR onset and (B) total duration of LORR in seconds in $\beta2^{-/-}$ and $\beta2^{+/+}$ mice after receiving an injection of 3.5 g/kg ethanol. N= 7-10 per group.

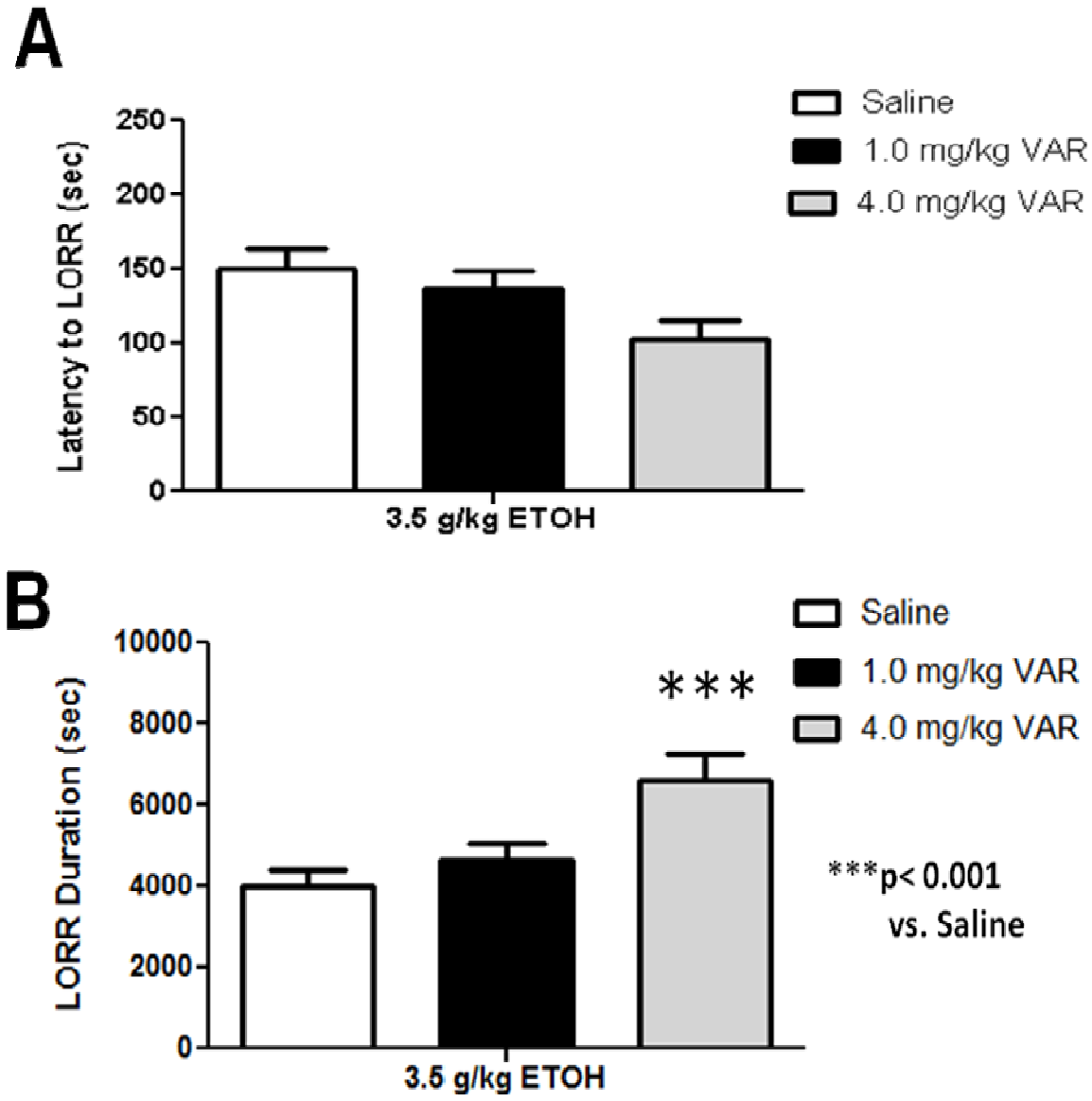

Figure 17. Varenicline pretreatment dose-dependently enhances LORR duration with a tendency to reduce LORR onset time in C57BL/6J mice. Data (mean ± SEM) represent (A) latency to LORR onset and (B) total duration of LORR in varenicline pretreated mice in seconds after receiving an injection of 3.5 g/kg ethanol. N= 7-10 per group.

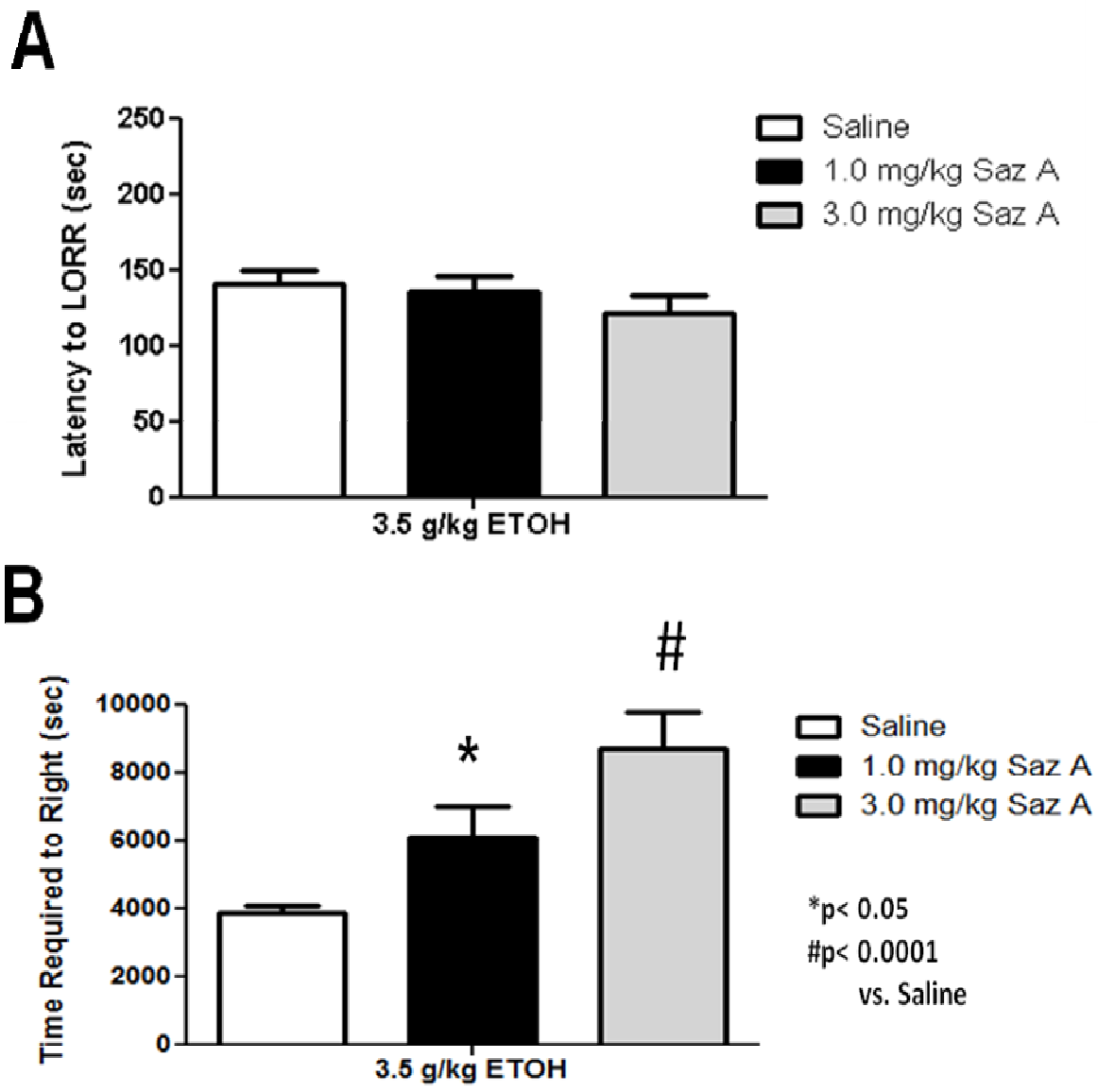

Figure 18. Sazetidine-A pretreatment dose-dependently enhances LORR duration while having no effect on LORR onset in C57BL/6J mice. Data (mean ± SEM) represent (A) latency to LORR onset and (B) total duration of LORR in Sazetidine A pretreated mice in seconds after receiving an injection of 3.5 g/kg ethanol. N= 7-10 per group.

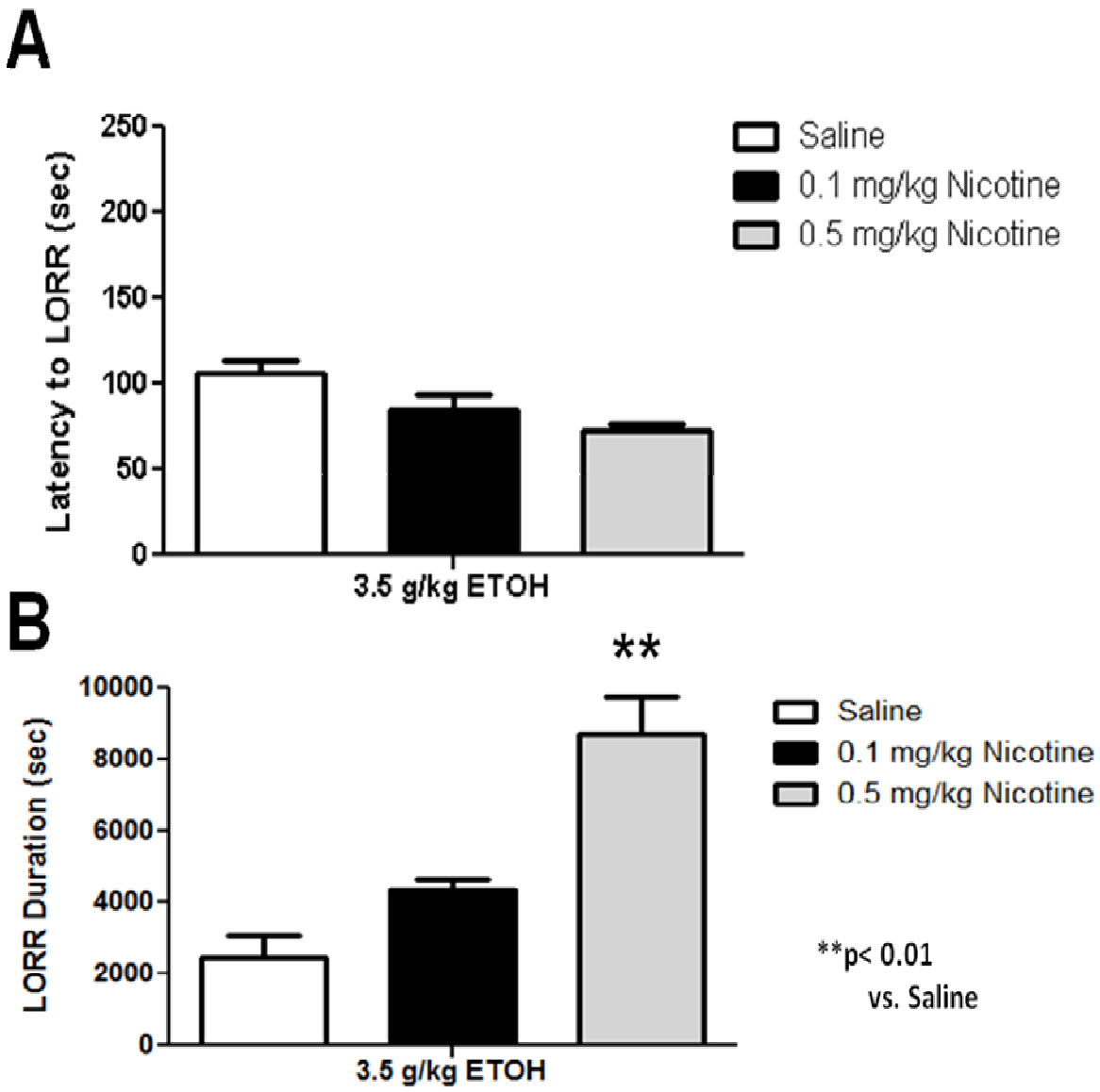

Figure 19. Nicotine pretreatment dose-dependently enhances LORR duration with a tendency to reduce LORR onset time in C57BL/6J mice. Data (mean ± SEM) represent (A) latency to LORR onset and (B) total duration of LORR in nicotine pretreated mice in seconds after receiving an injection of 3.5 g/kg ethanol. N= 7-10 per group.

EPM

As expected, a dose of 2.0 g/kg ethanol caused an anxiolytic-like response in mice compared with saline-treated mice without effecting locomotor activity (Figure 21). Two-way ANOVA analysis showed a main significant of ethanol treatment [$F(1,17)= 24.795$; $p= 0.0001$] as well as interaction between ethanol and DHβE pretreatment [$F(1,17)= 7.669$; $p= 0.0131$, post-hoc $p< 0.05$] but not DHβE pretreatment alone (Figure 21A). One-way ANOVA analysis of the number of crossovers revealed no significant differences between either group [$F(3,18)= 2.16$; $p= 0.1011$] (Figure 21B). The results in $\beta2^{-/-}$ mice showed that ethanol-induced increase in open arm time was also enhanced in these mice compared to WT (Figure 21A). Two-way ANOVA analysis of time spent in the open arms showed a significant main effect of treatment [$F(1,17)= 33.711$; $p<.0001$], genotype [$F(1,17)= 7.38$; $p= 0.0147$], and interaction [$F(1,17)= 7.116$; $p= 0.0162$]. Subsequent one-way ANOVA analysis revealed a significant difference in ethanol-treated KO mice [$F (1,10)= 5.521$, $p< 0.0407$, post-hoc $p< 0.05$] compared to WT mice. No differences were detected in either genotype treated with saline. One-way ANOVA analysis of the number of crossovers revealed no significant differences between either group ([$F (1,17)= 2.729$, $p< 0.1169$], Figure 21B). To confirm if this effect was mediated through β2 nAChRs we also tested the effect of DHβE pretreatment in β2 KO and WT mice. One-way ANOVA analysis revealed a trend for a KO-pretreated mice to spend less time in the open arms than their WT counterparts, though this trend was non-significant [$F(1,10)= 2.770$; $p= 0.1270$]. Furthermore, One-way ANOVA analysis was also used to compare DHβE-pretreated WT mice to WT mice from the previous experiment treated with ethanol alone, revealing that DHβE-pretreated WT mice did indeed spend more time in the open arms than the control

[F(1,11)= 5.084 ; p=0.0455]. Thus, the data reveal that DHβE significantly increased ethanol-induced open arm in $\beta2^{+/+}$ but not $\beta2^{-/-}$ mice. Taken together, these data suggest that functional disruption of β2* nAChRs enhances aspects of the anxiety-reducing effects of acute ethanol administration as measured by the EPM.

Treatment with the partial agonists, by contrast, caused a decrease in ethanol-induced open arm time in drug treated mice. However, it appears that this effect was due changes in locomotor activity (Figures 22 and 23). Two-way ANOVA analysis showed a main significant effect of ethanol treatment [F(1,17)= 6.078; p= 0.0354] as well as interaction between ethanol and varenicline pretreatment [F(1,17)= 5.136; p= 0.0431, post-hoc p< 0.05] but not pretreatment (Figure 22A). Subsequent one-way ANOVA analysis of the ethanol treatment groups revealed a significant difference in varenicline pretreated mice [F (1,8)= 5.641, p< 0.0467, post-hoc p< 0.05] compared to saline pretreated mice. However, this difference seemed to be a caused by significant decline in locomotor activity as one-way ANOVA analysis of the number of crossovers across these groups revealed a significant difference between varenicline pretreated mice [F(1,8)= 2.16; p= 0.1011] and saline control (Figure 22B). Similar behavior was observed in sazetidine-A pretreated mice. Two-way ANOVA analysis revealed a main significant effect of ethanol treatment [F(1,11)= 6.978; p= 0.0354] as well as interaction between ethanol and varenicline pretreatment [F(1,11)= 5.136; p= 0.0431, post-hoc p< 0.05] (Figure 23A), but one-way ANOVA showed a significant decreased in number of crossovers in sazetidine-A and saline pretreated ethanol treatment groups ([F(1,5)= 2.16; p= 0.1011], Figure 23B). Thus, little

conclusion can be drawn from these results due to the locomotor effects of varenicline and sazetidine-A in combination with ethanol in this particular assay.

Interestingly, treatment with full agonist, nicotine, however, produced similar effects on ethanol-induced reduction in open arm time compared to DHβE-treated and β2 KO mice (Figure 24). Two-way ANOVA analysis of time spent in the open arms showed a significant main effect of ethanol treatment [$F(1,17)= 6.988$; $p<0.0312$] and interaction [$F(1,17)= 8.116$; $p= 0.0262$], but not nicotine pretreatment [$F(1,17)= 7.38$; $p= 0.0147$]. Subsequent one-way ANOVA analysis revealed a significant difference in nicotine pretreated [$F(1,8)= 5.821$, $p< 0.0497$, post-hoc $p< 0.05$] and saline-pretreated ethanol groups (Figure 24A). Furthermore. This effect was not due to changes in locomotor activity since one-way ANOVA analysis of the number of crossovers revealed no significant differences between either group ([$F (1,8)= 2.827$, $p< 0.1384$], Figure 24B). Taken together, it appears evident that activation of β2* nAChRs and potentially other subtypes targeted by nicotine, enhances anxiolytic-like activity induced by ethanol in a manner similar to antagonism of β2* nAChRs by pharmacological antagonist or gene deletion.

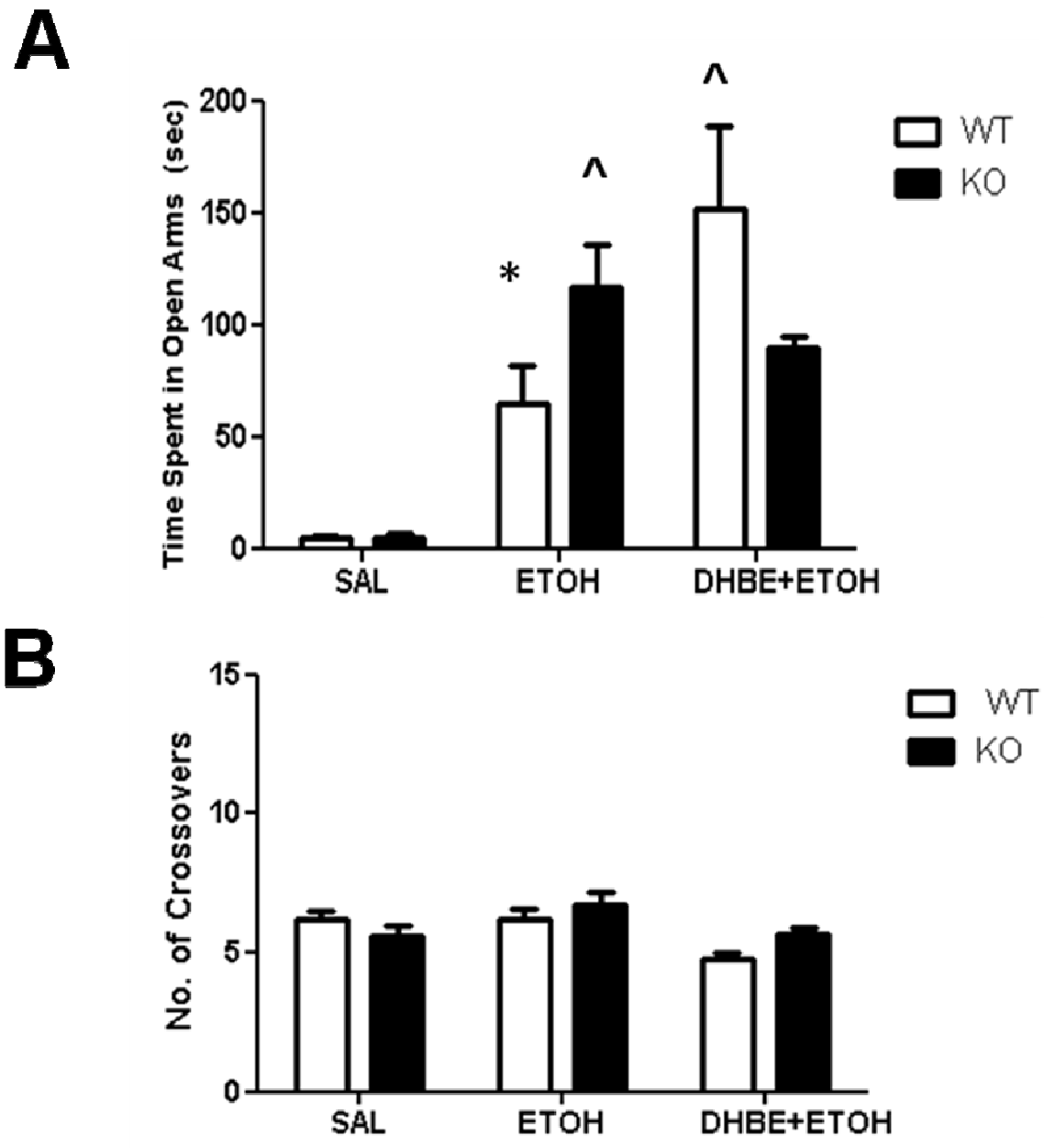

Figure 20. Deletion of the *Chrnb2* gene enhances ethanol-induced increase in open arm time and attenuates DHβE's effect on this behavior without affecting locomotor activity. Data (mean ± SEM) represent (A) time spent in open arms in seconds and (B) total number of crossovers in β2 KO mice after receiving an injection of 2.0 g/kg ethanol. N= 7 per group.

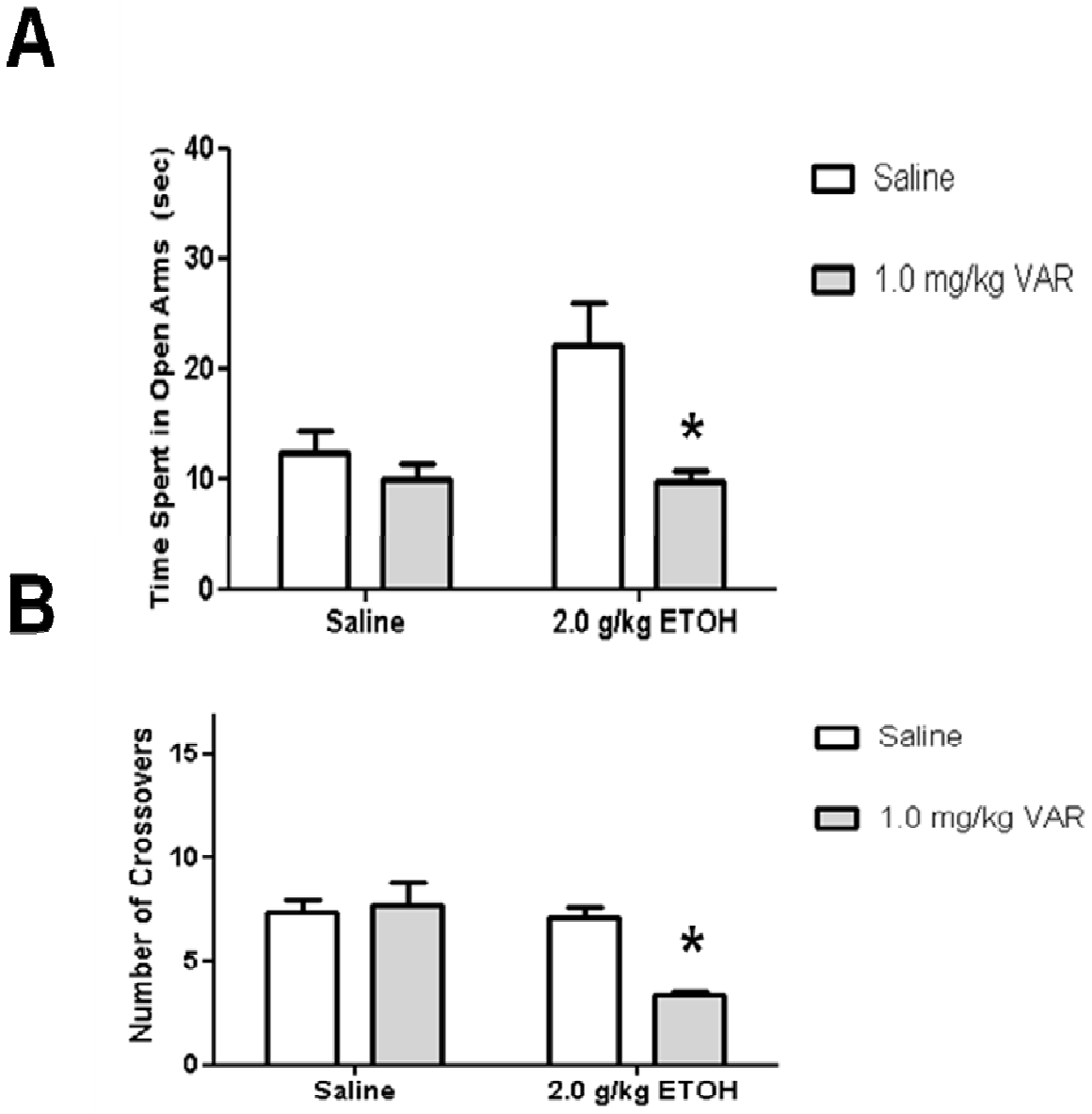

Figure 21. Varenicline pretreatment reduces ethanol-induced increase in open arm time and locomotor activity in C57BL/6J mice. Data (mean ± SEM) represent (A) time spent in open arms in seconds and (B) total number of crossovers in varenicline-pretreated mice after receiving an injection of 2.0 g/kg ethanol. N= 6-7 per group

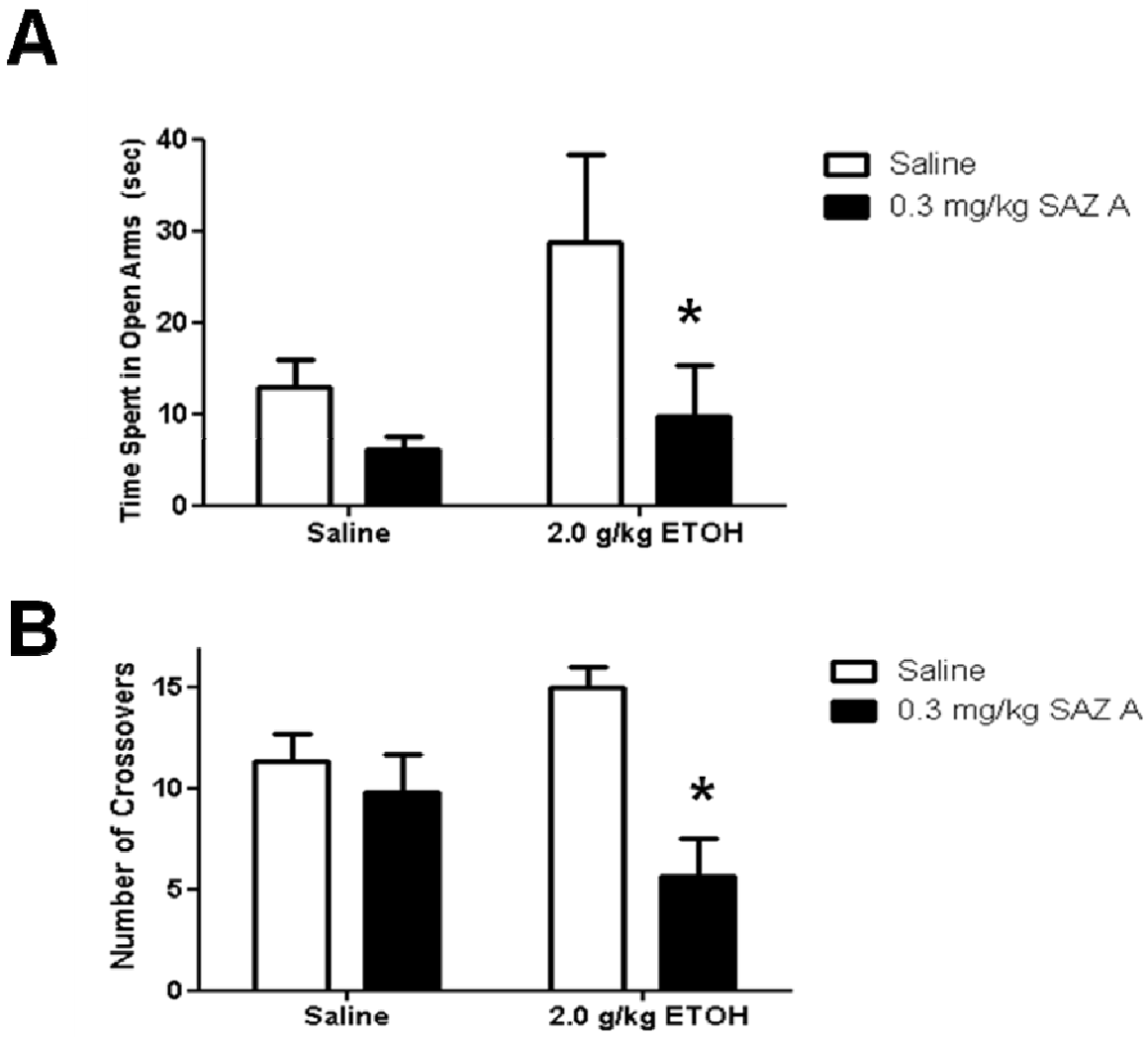

Figure 22. Sazetidine-A pretreatment reduces ethanol-induced increase in open arm time and locomotor activity in C57BL/6J mice. Data (mean ± SEM) represent (A) time spent in open arms in seconds and (B) total number of crossovers in sazetidine-A-pretreated mice after receiving an injection of 2.0 g/kg ethanol. N= 6-7 per group

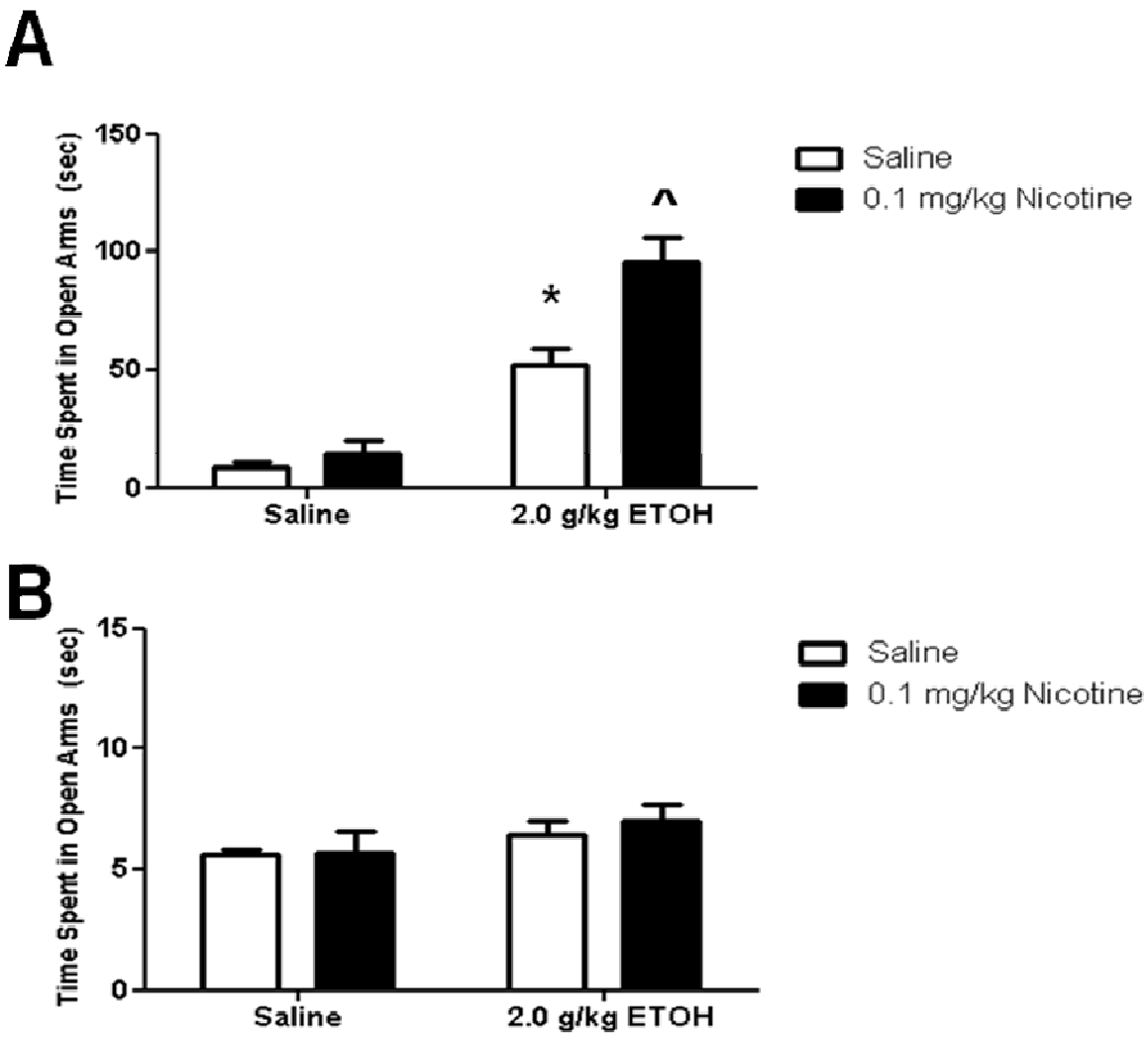

Figure 23. Nicotine enhances ethanol-induced increase in open arm time without affecting locomotor activity in C57BL/J Mice. Data (mean ± SEM) represent **(A)** time spent in open arms in seconds and **(B)** total number of crossovers in nicotine-pretreated mice after receiving an injection of 2.0 g/kg ethanol. N= 7-11 per group.

Intermittent Access

Mice given either intermittent or continuous two-bottle choice access to water and increasing concentrations of ethanol during the acclimation week (Week 1) displayed a concentration-dependent increase in ethanol intake (Figure 25). An initial three-way ANOVA (access group x concentration x genotype) revealed main significant effects of concentration [$F(2,63)=130.457$, $p= 0.0001$, post-hoc $=0.0001$], genotype [$F(1,63)= 5.317$, $p= 0.0244$, post-hoc< 0.05], and interaction [$F(1,63)= 7.892$, $p= 0.0066$]. Separate one-way ANOVA analyses for KO and WT mice at each concentration showed that while WT mice with continuous or intermittent access displayed similar ethanol intake, KO mice with continuous access had significantly higher intake at 6% ethanol [$F(1,10)= 6.011$, $p= 0.034$] than their counterparts with intermittent access. Thus, the data show that while the increase in ethanol intake during the first week was driven largely by concentration, genotype did modestly influence ethanol depending on access conditions. Furthermore, the concentration-dependent increase in ethanol intake was not due to differences in total fluid intake between groups as three-way ANOVA analysis showed no difference between groups [$F(2,63)= 0.249$, $p= 0.7807$]. As for the preference results (Figure 26), three-way ANOVA revealed a main significant effect of access group [$F(1,63)= 4.678$, $p= 0.0344$] and access group x genotype interaction [$F(1,63)= 6.750$, $p= 0.0117$] with further analysis supporting the effect of access group (post hoc, $p= 0.05$) but not genotype (post hoc, $p> 0.05$) on ethanol preference for 3% ethanol ([$F(1,10)= 8.722$, $p= 0.0145$]. Thus, while this effect on preference did not depend on concentration or genotype, it seemed that the conditions of access to ethanol, either intermittent or continuous, did initially affect preference for ethanol in drug naïve mice.

For the maintenance phase of the experiment, weeks 2-5, intermittent access to ethanol had the intended effect of increasing both intake and preference for ethanol in each test group (Table 2). Two-way repeated-measures ANOVA analysis of ethanol intake revealed a significant main effect of access group [$F(1,16)= 18.65$, $p= 0.0005$] but not genotype [$F(1,16)=$, $p= 0.0687$] nor interaction [$F(1,16)= 0.206$, $p= 0.6557$] over the course of the maintenance phase. Separate one-way repeated measures ANOVA analyses for each week revealed significantly higher intake in intermittent access mice compared to continuous access mice during week 3 [$F(1,23)= 5.653.$, $p= 0.261$], week 4 [$F(1,23)= 26.718$, $p< 0.0001$], and week 5 [$F(1,23)= 20.139$, $p= 0.0002$]. The results for preference were similar with two-way repeated-measures ANOVA analysis revealing a significant main effect of access group [$F(1,17)= 20.361$ $p= 0.0003$] but not genotype [$F(1,17)= 0.732$ $p= 0.732$] nor interaction [$F(1,17)= 0.002$, $p= 0.9661$] over the course of the maintenance phase (Table 2). Separate one-way repeated measures ANOVA analyses for each week revealed significantly higher preference in intermittent access mice compared to continuous access at mice during week 4 [$F(1,23)= 15.006$, $p= 0.0007$], and week 5 [$F(1,23)= 20.445$, $p= 0.0002$]. Taken together, the results show that intermittent exposure to ethanol induces escalation of drinking behavior of a similar magnitude in both $\beta2^{-/-}$ and $\beta2^{+/+}$ mice.

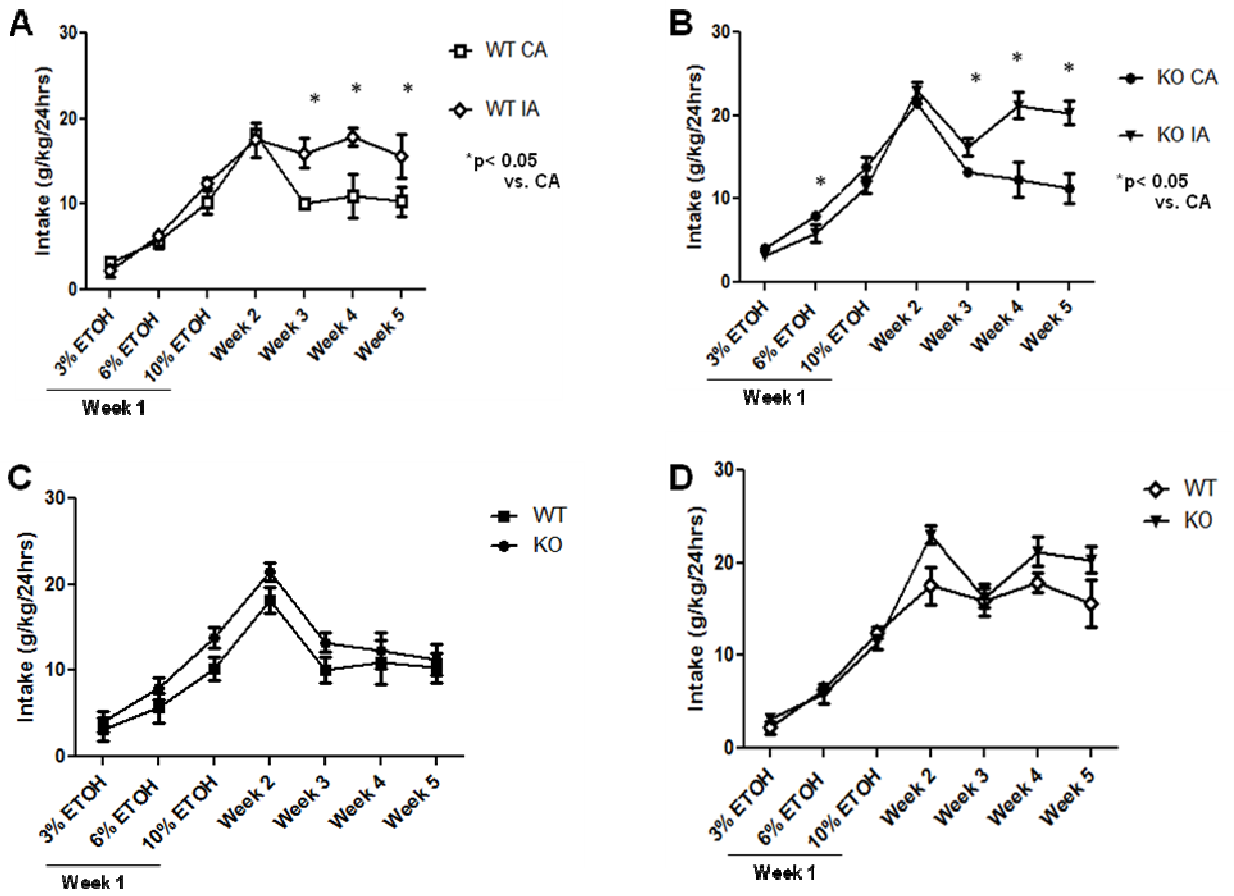

Figure 24. Deletion of the *Chrnb2* gene has no effect on intermittent access ethanol intake in C57BL/6J mice. Data (mean ± SEM) in the top graphs represent intake in g/kg in (A) WT and (B) KO intermittent (IA) vs. continuous access (CA) groups at 3-, 6-, 10-, and 20% (w/v) ethanol during weeks 1-5. The bottom graphs display the same data, but rearranged to show comparisons between each genotype during (C) continuous and (D) intermittent access exposure. N= 7 per group.

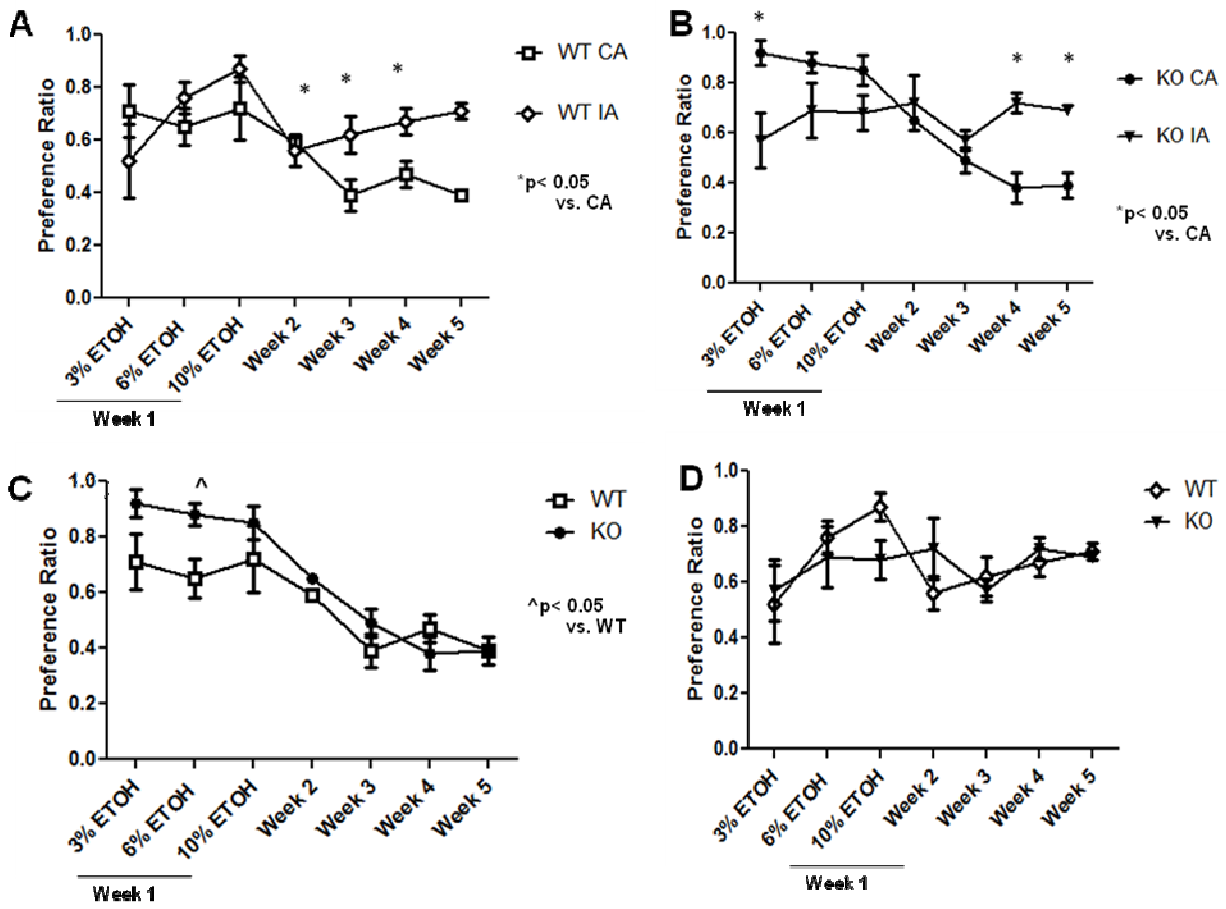

Figure 25. Deletion of the *Chrnb2* gene has no effect on intermittent access ethanol preference in C57BL/6J mice. Data (mean ± SEM) in the top graphs represent the preference ratio in (A) WT and (B) KO intermittent vs. continuous access groups at 3-, 6-, 10-, and 20% (w/v) ethanol during weeks 1-5. The bottom graphs display the same data, but rearranged to show comparisons between each genotype during (C) continuous and (D) intermittent access exposure. N= 7 per group.

20% Ethanol Intake and Preference in Beta 2 WT and KO mice				
Treatment	**CA**		**IA**	
Genotype	**Beta 2 WT**	**Beta 2 KO**	**Beta 2 WT**	**Beta 2 KO**
Intake Weeks 2 -5	**12.35 ± 1.80**	**14.59 ± 1.55**	**16.73 ± 1.48**[a]	**20.20 ± 1.26**[a]
Preference Weeks 2-5	**0.46 ± 0.04**	**0.48 ± 0.04**	**0.64 ± 0.05**[a]	**0.68 ± 0.05**[a]

[a]Represents significantly higher intake or preference than CA

Table 2. Ethanol Intake and Preference in Beta 2 WT and KO mice during the maintenance phase of the IA procedure. Data (mean ± SEM) represent intake in g/kg and preference ratio, respectively, for each group.

4.4 Summary

The goal of our studies was to further characterize the involvement of β2* nAChRs in acute ethanol-responsive behaviors as well as in ethanol drinking behavior. We examined the effect of pharmacological antagonism and genetic deletion of β2* nAChRs on a wide range of acute responses including locomotor depression, hypothermia, LORR, and reduction of anxiety-like behavior in the EPM, as well as in escalated drinking behavior using an intermittent access model. As hypothesized, manipulation of β2* nAChRs modulated some acute behaviors, namely reducing LORR duration and increasing time spent in the open arms in the EPM, while having minimal effect on ethanol drinking behavior.

CHAPTER 5: GENERAL DISCUSSION

We undertook the preceding studies in order to determine the contribution of certain nicotinic acetylcholine receptors (nAChRs) to dependence–related alcohol behaviors in the mouse. Specifically, we set out to more fully characterize the role of $\alpha 5^*$ nAChRs, a subtype whose respective gene has been identified in a number of recent studies as being associated with some phenotypes that characterize alcohol dependence. Furthermore, because $\alpha 5^*$ nAChRs cannot exist without the presence of at least one other α and β subunit, we also examined the $\alpha 4\beta 2^*$ nAChR, a subtype with which the $\alpha 5$ subunit is commonly co-expressed and has also been shown to modulate some ethanol-responsive behaviors. The examination of $\alpha 5^*$ and $\alpha 4\beta 2^*$ subtypes was also beneficial since each are shown to be important mediators of the effects of nicotine, a drug commonly co-abused with alcohol

Overall, we demonstrated that reduced receptor function by $\alpha 5$ gene deletion conferred enhanced sensitivity to the acute behavioral effects induced by acute ethanol. Specifically, $\alpha 5^{-/-}$ mice showed an increased hypothermic response to ethanol, took longer to fully recover from ethanol's hypnotic effects in the Loss of Righting Reflex (LORR) assay, and spent more time in the open arms in the elevated plus maze (EPM) than WT mice indicating increased sensitivity to the anxiolytic-like behavioral effects of

acute ethanol. In contrast, α5 gene deletion blunted the response to ethanol's reward-like effects in the CPP paradigm. Furthermore, none of these effects were due to pharmacokinetic differences between genotypes as $\alpha5^{-/-}$ and WT mice displayed similar blood ethanol concentrations (BEC) over a 2-hr time course after injection with ethanol.

We chose to test the behaviors listed due to their ability to give us an idea of specific brain pathways, and thus possibly the mechanisms involved in mediating sensitivity to ethanol-induced phenotypes. Our results with hypothermia showed that removal of the α5 gene resulted in general increase in body temperature change induced by ethanol, indicating that α5 nAChRs in the hypothalamus may have some role in regulating this response to alcohol. Though the exact molecular mechanisms underlying thermoregulation by the hypothalamus is mammal is unknown, this brain region is part of the hypothalamic-pituitary adrenal (HPA) axis which could have some important implications for stress-related drinking behavior. A particularly noteworthy observation was a significant difference in hypothermia in WT and KO mice was found at the 60 min, but not 15-min time point following ethanol administration. Furthermore, WT mice tended to display a slight decrease in hypothermia, while KO mice tended to display the opposite, thus giving the appearance of enhanced sensitivity. Future studies should include an extended time course, (e.g. up to 240 min) to determine the extent of this effect.

Interestingly, our results seem to be in line with recent human genetic studies regarding the α5 gene. For example, genome wide association studies (GWA) studies identified two single nucleotide polymorphisms (SNPs) in the α5 nAChR-coding gene

that were associated with changes in body sway, a measure of sensitivity to the ataxic effects produced in a subject challenged with acute alcohol (Joslyn *et al.* 2008). These same SNPs were associated with measures of subjective response to acute alcohol challenge, while an additional set of SNPs were identified as being associated with the age of initiation for alcohol use (Schlaepfer *et al.* 2008b). Furthermore, associations were also identified between polymorphisms in the α5 gene with alcohol dependence phenotypes and steady state mRNA levels (Wang *et al.* 2009).

Another striking observation made in these studies was that α5 gene deletion had the opposite effect on acute behavioral response to ethanol when compared to nicotine studies in literature. While our data demonstrate an *enhanced* response to the hypothermic and sedative effects of ethanol, previous literature investigating the effects of α5 gene deletion on nicotine response show that these mice have a *diminished* response to the hypothermic and sedative effects of ethanol (Jackson *et al.* 2010). Moreover, it was also shown that α5 gene deletion led to an increase in nicotine's reward-like effects in CPP and intake (Jackson *et al.* 2010), which we, again, showed is opposite in the case of ethanol. These observations that acute behavioral response to both ethanol and nicotine can be modulated oppositely by deletion of the same gene provides more evidence in support of a link between α5* nAChRs and dependence to these drugs. GWA studies of the genomic region encoding for α5* nAChRs in humans showed the risk variant associated with nicotine dependence bears the opposite alleles of the variant associated with alcohol dependence risk (Chen *et al.* 2009). Interestingly, this variant is known to significantly decrease the maximal response of α5* nAChRs to

agonists by up to 50%, which indicates that change in the functional properties of this subtype could play a role in drug dependence (Bierut *et al.* 2008, Kuryatov *et al.* 2011). Thus, the behavioral data generated by our studies and others corroborate the GWA studies in humans, even if modestly. However, we make such interpretations with caution. As previously explained, there may have been developmental compensation events that occurred with deletion of the $\alpha 5$ gene that could have influenced the behaviors we observed. We believe that our interpretations are strengthened by the fact that expression of many nAChR subunits are not altered by removal of the $\alpha 5$ gene (Mao *et al.* 2008, Kuyatov *et al.* 2008) and that a wide array of basal phenotypes measured in the $\alpha 5^{-/-}$ mouse are similar to WT (Salas *et al.* 2003, Jackson *et al.* 2010). However, we still cannot discount that compensatory mechanisms with other nicotinic subunits or non-nicotinic mechanisms may have potentially affected the results.

We were surprised to see that $\alpha 5$ gene deletion did not confer differences in assays testing multiple aspects of drinking behavior. Our expectations of seeing a change in drinking behavior were bolstered by a previous study showing that polymorphisms in the *Chrna5-Chrna3-Chrnb4* gene cluster co-segregate with ethanol preference in an F_2 population produced from reciprocal crosses of ethanol-preferring B6 and ethanol-avoiding D2 mouse strains (Symons *et al.* 2010). The study further showed that these strains also exhibited differences in $\alpha 5$ mRNA levels, as the ethanol-preferring B6 strains contained significantly higher basal levels of $\alpha 5$ than D2 mice. Given this evidence, we tested the effect of $\alpha 5$ gene deletion on various aspects of drinking behavior in a battery of oral self-administration paradigms in mice. These

included a standard two-bottle choice model, which assessed continuous access drinking behavior within a range of ethanol concentrations, the intermittent access (IA) model to assess escalation of drinking behavior induced by repeated episodes of ethanol deprivation, and the Drinking-in-the-Dark (DID) model to assess acute binge drinking behavior. However, there were no changes in drinking detected in $\alpha5^{-/-}$ mice in either of these paradigms. Our results with DID are in agreement with a previous study showing no change in DID drinking behavior in $\alpha5^{-/-}$ mice (Santos *et al.* 2012). Nevertheless, we knew that ethanol preference is not reflected in the DID paradigm, and therefore, thought it still reasonable to expect that changes in drinking behavior might be still be detected in $\alpha5^{-/-}$ mice when assessed for preference in the two-bottle choice and IA paradigms. Our observations that $\alpha5$ gene deletion did not alter drinking behavior in mice may indicate that this may not necessarily be the case. Given the data previously mentioned about the possible link between the role of $\alpha5$* nAChRs in nicotine and ethanol-responsive behaviors, we repeated our DID experiment to determine if $\alpha5$ gene deletion would alter nicotine-modulated changes in drinking behavior. As expected, nicotine reduced ethanol intake in WT mice compared to saline-treated mice, but unexpectedly, the data also revealed that both nicotine- *and* saline-treated $\alpha5^{-/-}$ mice drank less as well. This led us to speculate the possibility that these effects are too subtle for a standard two-bottle choice procedure, IA, or DID alone to detect under stress-free conditions. Perhaps such effects are not discernible until the presence of a stressor is introduced. Indeed, this was supported by the DID intake data as multiple daily injections of saline in KO mice led to decreased drinking levels similar

to WT mice that were treated with nicotine. In fact, we saw that $\alpha5^{-/-}$ mice both, treated with either saline or nicotine, drank similarly to WT nicotine-treated groups, suggesting that the potential effect of nicotine in the $\alpha5^{-/-}$ mice was masked by the physiological changes induced by the stress of multiple daily injections. Literature suggests that many procedures involved in animal research, including handling and injections, can lead to various stress-related changes mediated by the HPA axis (Drude *et al.* 2011). It may be possible that mild stress caused by these injections leads to HPA axis-mediated changes in drinking behavior that occurs in mice with decreased $\alpha5$* nAChR function. This could suggest a link between $\alpha5$* nAChRs and stress-induced drinking behavior. The HPA axis is a key regulator in the release of hormones in response to stress and is recognized for playing an important role in alcohol dependence (Madiera *et al.* 1999, Heilig & Koob, 2007). Interestingly, compared to WT, $\alpha5^{-/-}$ mice have been shown to express higher basal levels of hypothalamic corticotropin releasing factor (CRF), an HPA axis-mediated hormone that has generated great interest for its potential as a therapeutic target for curbing stress- and anxiety-induced alcohol drinking (Heilig and Koob, 2007, Gangitano *et al.* 2009). Despite this intriguing finding, the data are, so far, only exploratory and there are caveats associated with our findings. In addition to the previously discussed issue of compensation in KO mice, we did not measure changes in stress-related hormones induced by these injections. Furthermore, we only obtained data related to injection-induced stress, which is a mild form of stress (Márquez *et al.* 2002). Much more work must be done to characterize the roles different types of stressors have on the observed effects in these mice because many factors including the type, frequency, and duration of stress among others can influence the end

behaviors examined (Drude *et al.* 2011). For future studies, α5 WT and KO mice should be assessed for drinking behavior following more severe stressors. Tests such as acoustic startle and restraint stress are classified as more severe since they display longer and more profound effects on the HPA-axis (Drude *et al.* 2011, Kiank *et al.* 2008, Depke *et al.* 2008). It will also be necessary to determine differences in basal levels of stress hormones, such as CRF, and changes in the levels of these hormones in response to severe stress in these mice.

Our results with α5 prompted further study of other an additional subtype, α4β2*, with which the α5 subunit is commonly co-expressed. As previously discussed, the majority of our findings on disruption of α5* nAChR signaling on ethanol-responsive behavior via gene deletion showed that this results in the modulation of acute behavioral responses, and not ethanol drinking. This provides the possibility that the modulation of ethanol's acute effects is an α4β2α5* nAChR-mediated phenomenon. Furthermore, there have been multiple studies showing the lack of involvement of α4β2* nAChRs on oral self-administration of ethanol, but that do demonstrate the involvement of α3β4* nAChRs (Lé *et al.* 2000, Hendrickson *et al.*2009, Kuzmin *et al.* 2009, Kamens *et al.*2010a, Chatterjee *et al.* 2011, Gallego *et al.* 2012). Our results, when combined with the literature seem to support this idea. For example, the observation that drinking behavior in the intermittent access paradigm did not differ between β2 WT and KO mice show that β2* nAChRs do not affect chronic drinking behavior, even after repeated cycles of deprivation implemented in the IA assay. As stated earlier, this is the outcome we were expecting and is in line with what the literature suggests using various models of oral self-administration in rodents (Lé *et al.*

2000, Larsson *et al.* 2004a,b; Hendrickson *et al.* 2009).. It is also noteworthy that additional studies using this same drinking model showed a lack of involvement of α6*, a subtype which also often co-assembles with β2* (Yang *et al.* 2009, Kamens *et al.* 2012). Taken together with our results, these data collectively demonstrate that antagonism of β2* nAChRs do not have a discernible effect on ethanol consumption in a wide range of tests of drinking behavior.

In further support of the theory that α4β2* nAChRs are involved in acute ethanol-responsive behaviors we used a panel of high affinity for α4β2* nicotinic ligands including an antagonist (DHβE), a full agonist (nicotine), and two partial agonists (varenicline and sazetidine-A), to show that manipulation of these receptors does modulate sensitivity to acute ethanol administration in drug-naïve mice. Interestingly, these effects did not extend to all the acute responses measured, as no change in ethanol-induced hypothermia, locomotor depression, or latency to LORR were detected. While our results with hypothermia were novel and previously unexplored in literature, our locomotor results were strengthened by the fact that β2* nAChR antagonism also had no effect on latency to LORR, another measure of initial sensitivity to ethanol-induced sedation (Ponomarev & Crabbe, 2002b). Interestingly, past pharmacological studies in mice and rats suggested a role for α3β2/α3β4* nAChR subtypes in ethanol-induced locomotor stimulation (Larsson *et al.* 2002, Kamens *et al.* 2008, 2009). It possible that α5 may be the additional subunit contained in these subtypes that mediate the described effects. Unfortunately, our α5 (and β2) KO mice exist on a C57BL/6 background that shows a minimal stimulant response to

ethanol (Demarest *et al.*1999; Randall *et al,* 1975), and we were thus not able to test this hypothesis.

The results that DHβE-pretreated and β2 KO mice displayed a significant difference in LORR duration and not latency to LORR were very intriguing; even more so by the fact that partial and full activation of α4β2* nAChRs by their respective agonists consistently produced the opposite effect. It is generally accepted that level of response to acute ethanol challenge may represent a combination of initial sensitivity to acute ethanol exposure and acute functional tolerance directly following exposure (Ponomarev & Crabbe 2002b). Because latency to LORR and LORR duration may correlate to these two respective measures, the results may indicate that antagonism of β2* nAChR activity, as shown by DHβE treatment and β2 gene deletion, enhance the rate of the neuronal tolerance that develops minutes to hours after early ethanol exposure without necessarily affecting the initial sensitivity to ethanol's effects. This may explain why a difference in KO mice was only seen in the LORR assay, and not the locomotor depression assay, since the 10 min time window measured in the latter may not have been long enough to observe β2's influence on this behavior. Our results with the partial and full agonists show that the opposite may be true upon activation of α4β2* nAChRs. We are fairly confident that this is, indeed, an α4β2*-mediated effect since, sazetidine-A, a compound with a many fold higher selectivity for the α4β2* subtype than varenicline and nicotine (Xiao *et al.* 2006, Rezvani *et al.* 2010), produced results consistent with the latter drugs. However, the nature of the association of the α5 subunit with these receptors in mediating LORR effects remains a mystery. We were surprised to discover that the effects mediated by α4β2* nAChR

activation in B6 mice matched those observed in KO mice with reduced α5* nAChR function. This was counter to our expectations that α5* nAChRs may mediate its enhancement on the LORR phenotype through association with α4β2* nAChRs. It may be possible, then, that α4β2* nAChRs have opposing effects on α5* nAChRs that *do not* contain α4β2* (i.e. α3β2α5* or α3β4α5* nAChRs) in the case of this ethanol-induced phenotype. Additionally, other nAChRs including α6 and α7 nAChR subunits have also been implicated in the LORR. Deletion of α6* and α7 gene in mice display a significantly higher LORR recovery time compared to WT animals, similar to the results we found with deletion of α5 (Bowers et al., 2005; Kamens et al., 2012). Varenicline and nicotine, in addition to their α4β2* actions, also act as full agonists at α7 and α6 (Mihilak *et al.* 2006, Jackson *et al.* 2009). Thus, one would expect to see effects opposite of those observed in the KO mice (i.e. reduction in LORR recovery time) if these phenomena were principally mediated by α6* or α7 nAChRs. However, our results, taken within the context of the other studies, demonstrates that activation of α4β2* nAChRs by these treatments may mask any effects mediated by α6* and/or α7 nAChRs. Given this data, it is apparent that multiple nAChR subtypes including, α4β2*, α5*, α6*, and α7, differentially influence response to the hypnotic effects of ethanol, with α4β2* acting as the principal mediator of the observed phenotype. Future studies should incorporate the use of $\alpha4^{-/-}$ mice to shed further light on the involvement of α4β2* nAChRs in this behavior. Furthermore, it will be useful to repeat the aforementioned tests over a range of multiple ethanol doses to determine the extent of the effects of nAChR manipulation on ethanol's hypnotic effects. Regardless, the results we have obtained so far underscore the underlying complexities that individual genetic

makeup has on determining the nAChR contribution to initial sensitivity to early ethanol exposure

Our results also showed that both DHβE-pretreated and β2* KO mice demonstrated an enhanced response to ethanol's effect on reducing anxiety-like behavior in these subjects during the EPM test. These data may suggest that functional disruption of β2* nAChRs affects some aspects of the anxiolytic-like effects induced by acute ethanol administration in mice. The experiment showing the attenuated effect of DHβE pretreatment in β2 KO mice seems to support this idea. We also found that α5 gene deletion and nicotine-pretreatment also had similar effects on open arm time in B6 mice as the previously mentioned test groups. Our results with varenicline and sazetidine-A, unfortunately, were inconclusive due their locomotor altering effects. While we were careful to choose pharmacologically-relevant doses of varenicline and sazetidine-A that did not affect locomotor activity by themselves (Kamens *et al.* 2010, Rezvani *et al.* 2010), it seems their combination with ethanol caused a significant reduction in crossovers in the test, which likely affected the time spent in the open arms. However, except for those mentioned groups, none of the behavior observed in any other experiment was due to changes in animal locomotor activity since there was no difference in the number of crossovers across all treatment groups. The fact that $\alpha5^{-/-}$ mice displayed similar behavior in the same paradigm as β2 KO and DHβE-pretreated mice, supports the idea that this may be an α4β2α5*-mediated effect. It is possible that α4β2α5* nAChRs mediate an endogenous pathway(s) that negatively modulate ethanol's effects on this behavioral trait. Interestingly, β2 nAChR subunits are expressed in brain regions including the amygdala,

the hippocampus, and limbic regions, which are known to regulate anxiety behaviors (Silveira *et al.* 1993, Walf *et al.* 2007, Gotti *et al.* 2007).

As for the results with nicotine pre-treatment, while is logical to assume that activation of $\alpha4\beta2\alpha5$* nAChRs would have the opposite effect on that of the KO, which we did not observe, it is possible that nicotine was able to mimic the effects of $\beta2$* and $\alpha5$* nAChR antagonism through *desensitization* of $\alpha4\beta2\alpha5$* receptors. The incorporation of the $\alpha5$ subunit has a significant influence on the desensitization properties of the subtype with which it is co-expressed (Gerzanich *et al.* 1998). For $\alpha4\beta2$* nAChRs, particularly, co-expression with $\alpha5$ can increase receptor desensitization rates, and, furthermore, can affect agonist efficacy and sensitivity of these receptors (Mao *et al* 2008, Kuryatov *et al.* 2008). As mentioned previously, desensitization occurs when agonist binding eventually causes the receptor to transition into an agonist bound, but non-conducting state (Gerzanich *et al.* 1998). The $(\alpha4\beta2)_2\alpha5$ subtype is an important player in neurotransmitter release in the VTA, and is particularly sensitive to activation by nicotine (McClure-Begley *et al.* 2009, Kuryatov *et al.* 2011). Interestingly it theorized that a reduction of $\alpha5$* nAChR function by an amino acid variant in the $\alpha5$ gene results in a deficiency in nicotine-induced $\alpha5$* nAChR signaling that may lead to enhanced nicotine intake to achieve its desired effects (Bierut *et al.* 2009, Kuryatov *et al.* 2011).

Perhaps, the opposite is true with alcohol in which an enhanced response to its anxiolytic properties (as well as sedative effects) reduces the need for further intake. This could provide a possible explanation for the contrasting results discovered in the GWA studies regarding the association of the functional polymorphism with nicotine and alcohol

dependence risk. However, such speculations must be made with caution. When used appropriately, the EPM test can be a very valuable tool in drug testing and our results suggest that one aspect of anxiety and emotional behavior is modulated by alcohol-nicotinic interaction. Nevertheless, we realize it is likely that different tests for anxiety-like behaviors will measure somewhat different forms of emotional behavior and anxiety, which may be mediated by distinct neural circuits and genes. Investigating additional models that test different aspects of anxiety in order to further understand the nature of the role of this nAChR subtype in ethanol's anxiolytic effects are needed. Some suggested models for future studies include light dark box, marble burying, open-field, and startle response, which could each be used to assess the effect of nAChR manipulation on distinct aspects of ethanol-induced anxiolysis. Each of these are reliable models of analyzing anxiety-like behavior in rodents and are distinctly utilized for screening anxiolytic agents like ethanol (Hart *et al.* 2010). The implication of such tests could be important especially when considering that anxiety behavior is thought to serve as a motivating factor for sustained alcohol consumption in humans that may increase risk for excessive drinking in the future (Spanagel *et al.* 1995, Zimmerman *et al.* 2003).

We are confident that the effects we observed in all of our acute studies in the KO mice were not due to changes in ethanol metabolism between the $\alpha 5$ and $\beta 2$ KO genotypes and their WT counterparts. Our data showed no difference in BEC levels over 2-hr time course between $\alpha 5^{-/-}$ and $\alpha 5^{+/+}$ mice treated with a high dose of ethanol. Additionally, while ethanol pharmacokinetics were not directly studied in the $\beta 2$ KO and WT mice, we believe that differences in ethanol metabolism between the two genotypes do not play an

important role in the decrease in the LORR phenotype for a couple reasons. A similar decrease in the LORR response was also seen with the β2* antagonist, DHβE. Also, no difference in hypothermia and hypomotility induced by ethanol was found between β2 KO and WT mice. Several subunits in addition to α5, including α4, α6, and β3 are often co-expressed with β2 subunits generating multiple β2-containing nAChR subtypes (Gotti *et al.* 2007). While our studies did not address the composition of β2* subtypes involved in ethanol's effects, our data likely suggest the involvement of α4β2* subtypes due the similarity of the results displayed between mice pretreated with DHβE, an antagonist with high affinity for α4β2* nAChRs, and $\beta2^{-/-}$ mice. A limitation of our study is the chance that the effects observed in $\beta2^{-/-}$ mice were due to compensatory or developmental changes that occur with the deletion of their respective genes. While the expression of many nAChR subunits, namely α4, α5, β4, and β3, are not altered by removal of the β2 gene (Picciotto *et al.*1995, 1998), compensatory mechanisms with other nicotinic subunits or non-nicotinic mechanisms are still unknown. Importantly, our results with $\beta2^{-/-}$ mice were complemented with those seen with DHβE, a selective β2* nAChR antagonist. We could not make this same confirmation for the results in $\alpha5^{-/-}$ mice due to the lack of an available antagonist selective for this subtype. However, $\alpha5^{-/-}$ mice have been repeatedly assessed for many basal phenotypes and do not appear to differ remarkably from WT mice in these behavioral measures (Salas *et al.* 2003, Jackson *et al.* 2010).

As with our results regarding α5, our assessment of β2* nAChRs in ethanol-responsive behaviors seem to complement the human genetics studies providing support for the role of *Chrnb2* in alcohol behaviors as well. Consistent with what we

observed in animal models, human genetic association studies have implicated *Chrnb2* in alcohol behaviors because an association between polymorphisms in the *Chrnb2* gene and initial subjective response to early alcohol exposure was found (Ehringer *et al.* 2007). Therefore, nicotinic receptors, along with being key components in nicotine dependence, may also present viable candidates in the discovery of molecular underpinnings of behaviors related to alcohol dependence. Our results support the hypothesis that behavioral responses to alcohol and nicotine are likely to be differentially modulated by specific nicotinic subunits. We hope these studies will provide important information for the future development of pharmacotherapies in the ongoing search for a treatment for alcohol dependence, and potentially nicotine dependence simultaneously.

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Vita

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