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EXAMINING THE ROLE OF α6β2* NICOTINIC ACETYLCHOLINE RECEPTORS ON ETHANOL-INDUCED BEHAVIORAL RESPONSES IN ADOLESCENT C57BL/6J MICE

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ABSTRACT

Alcohol and nicotine are two of the most commonly abused substances. Prior studies have shown that they share common genes and mechanisms that underlie their reinforcing properties in the dopaminergic (DA) neurons of the ventral tegmental area (VTA). The system of interest that modulates reward behavior is the mesolimbic dopamine system, which includes the VTA, the nucleus accumbens (NAc) and DA neurons. The primary targets for nicotine are neuronal nicotinic acetylcholine receptors (nAChRs) which are found throughout the brain. Studies have suggested that alcohol may be interacting with these receptors directly or indirectly along with nicotine, thus giving a possible mechanism for the co-use of these two substances. In particular, the α6β2* nAChRs are highly expressed within the ventral tegmental area on dopaminergic neurons. Due to the localization of these receptors, researchers have begun to isolate this subset for its possible involvement in modulating alcohol-induced behaviors. In this study, we examined the role of α6β2* receptors in ethanol-induced behaviors by using the antagonist N,N-decane-1,10-diyl-bis-3-picolinium diiodide (bPiDI), a drug that specifically targets α6β2* receptors. In brief, adolescent age C57BL/6J mice were subjected to treatments of saline, 10, 15, 20 or 25 mg/kg doses of bPiDI before being tested in several behavioral experiments: locomotor activity, ethanol consumption, ataxia and sedation. In our locomotor activity studies, bPiDI significantly decreased locomotor activity for specific doses and times during testing in male and female adolescent mice where female mice had higher locomotor activity throughout the experiment in comparison to males. Male and female adolescent mice had initial decreases in ethanol consumption at the highest dose of bPiDI. Initial decreases in tastant consumption for higher doses of bPiDI in male and female mice revealed that the effects on EtOH were not specific to EtOH-mediated effects. The influence of bPiDI on ataxia and sedation were not observed in the experiments reported here for both sexes. The data presented here provide evidence for the involvement of α6β2* nAChRs in modulating locomotor activity and EtOH consumption.

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CHAPTER 1: INTRODUCTION

1.1: Alcohol Use Disorders – AUD

Alcohol is one commonly abused substance consumed by humans (The National Institute on Drug Abuse, (NIDA) 2015). Alcohol use disorder (AUD) is the medical diagnosis given to a patient whose problem drinking has become severe, elevating to points of distress and harm to the patient's well-being (National Institute on Alcohol Abuse and Alcoholism, (NIAAA) 2015). From a census in 2013, about 17 million adults in the United States alone had been diagnosed with an AUD (National Survey on Drug Use and Health, (NSDUH), 2013). Of these 17 million adults 10.8 million were men and 5.8 million were women (NSDUH, 2013). Approximately 87 percent of people age 18 or older among those 17 million adults admitted to binge or heavy drinking at some point during the year on multiple occasions (NSDUH, 2013). Of the 87 percent of people that admitted to binge drinking, 904,000 million men and 444,000 women needed treatment (NSDUH, 2013). Nearly 88,000 people, about 62,000 men and 26,000 women, die from alcohol-related causes annually, making it the third leading preventable cause of death in the United States (NIAAA, 2015). Signs of potential development of alcohol use disorders include failure to fulfill major responsibilities at work, school, or home, drinking in dangerous situations such as driving or operating machinery, being arrested for said behaviors, drinking despite consistent negative impacts in daily life (Alcohol Use Disorder, Mayo Clinic, 2015).

1.2: Alcohol Consumption

Alcohol is a psychoactive drug that acts as a central nervous system depressant which interferes with communication between nerve cells and other cells. It also affects various regions of the brain. Alcohol is rapidly absorbed from the stomach and small intestine into the bloodstream. In the liver, an enzyme called alcohol dehydrogenase mediates the metabolism of alcohol to acetaldehyde. Acetaldehyde is rapidly converted to acetate by aldehyde dehydrogenase and is eventually metabolized to carbon dioxide and water. However, the liver can only metabolize a small amount of alcohol at a time, leaving the excess alcohol to circulate throughout the body (NIAAA, Alcohol Metabolism, 2007). The intensity of the effect of alcohol on the body is directly related to the amount consumed. Depending on the amount consumed, alcohol may alter emotional responses, coordination, balance, speech and may cause nausea and vomiting. When consumption gets

to the point of an overdose, this puts the user at risk for alcohol poisoning, complete loss of coordination and even death (Center for Disease Control and Prevention, (CDC), 2014). Men are more likely than women to drink excessively (CDC, 2014). Men average about 12.5 binge drinking episodes in comparison to women who average about 2.7 (Naimi et al., 2003). Although men naturally consume more alcohol than women, women are a high risk group to develop AUD because they are more vulnerable to the effects of alcohol due to body structure and chemistry (Ashley et al., 1977). Vulnerability to alcohol's effects in women is also due in part to women taking longer to metabolize alcohol (Ashley et al., 1977).

Prior to developing an alcohol use disorder, a person will have already begun to use alcohol. Moderate drinking is set at 1 drink per day for women and 2 per day for men. Heavy drinking is defined as drinking 5 or more drinks on the same occasion on each of 5 or more days in the past 30 days (NSDUH, 2013). One of the most common forms of alcohol consumption is binge drinking which affects all age groups (CDC, 2014). The intensity of this form of drinking is actually at a level between moderate and heavy drinking. A "binge" is a pattern of drinking that brings blood alcohol concentration to 0.08 grams percent or above. Binge drinking is the modern definition of drinking alcoholic beverages with the intent of becoming intoxicated over a short amount of time. For adults, this pattern of drinking is equivalent to 5 or more drinks for males and 4 or more drinks for females within 2 hours (NIAAA, 2004). Along with the major risks posed from AUD, binge drinking adds adverse consequences to the heart, liver, immune system, bone health, and other organ systems (Crabbe 2011).

1.3: Adolescent Alcohol Use

Adolescence is the time of development from puberty to adulthood. The age range is from age 11-21 years (American Academy of Pediatrics, 2015). It can roughly be divided into three stages: early (11-14), middle (15-17) and late adolescence (18-21) (American Academy of Pediatrics, 2015). The major development that occurs during this time deals mostly with self-identity and bridging into adulthood (American Academy of Pediatrics, 2015). Skills developed during this phase are physical, intellectual, emotional and social maturity (American Academy of Pediatrics, 2015). In rodents, this period of development is analogous to human adolescence (Spear, 2000) occurring from postnatal day (PND) 28

until adulthood around PND 60 with puberty beginning around PND 40 (Laviola et al., 2003; Smith, 2003). This age range was established due to this being the timing of the growth spurt and discontinuation of some behaviors associated with childhood (Adriani et al., 2004; Spear, 2000). This phase can be divided into three time periods: early (PND 21-34), middle (PND 34-46) and late (PND 46-59) (Laviola et al., 2003). Although these divisions are common, start dates for each division vary between literatures (Spear and Brake, 1983; Spear, 2000; Laviola et al., 2003; Smith, 2003).

Adolescence is defined as the period characterized by substantial changes in brain structure, connectivity and neurochemical circuitry (Brava and Tapert, 2010; Giedd, 2004; Spear and Brake 1983). Behaviors associated with this stage of growth include high social interaction, play behavior, risk-taking, exploration, impulsivity and sensation seeking (Ernst et al., 2009; Spear 2000). Due to the nature of risk taking behaviors, adolescence has been shown to be the time when people most likely begin to sample and eventually consume alcohol (NIAAA, 1998; Nelson et al., 1995; Spear 2000). About 90% of the alcohol consumed among this age group is in the form of binge drinking (Office of Juvenile Justice and Delinquency Prevention, 2005). Recent data from SAMHSA 2008 showed that drinking rates were similar for males and females (16.0% and 15.9% respectively) (Schulte et al., 2009). Although these rates do not differ during this period, during the young adult stage is when males began to consume more alcohol than females (Schulte et al., 2009). Adolescence in general have low sensitivity to alcoholic responses, which makes it easier, yet more dangerous, for them to consume large quantities with the one effect being a high blood ethanol concentration (Silveri and Spear, 1998). Adolescent drinking has been shown to be linked to cognitive deficits and alterations in brain activity and structures (Tapert and Schweinburg, 2005; De Bellis, Clark and Bears, 2000). Since adolescence is the time alcohol-related behaviors are developing, this leaves the brain vulnerable to neurotoxicity at this crucial time of maturation (Crew et al., 2007; Witt, 2010). Research has shown that there is a strong correlation between early alcohol use and the likelihood of developing alcohol dependence and abuse into adulthood with some cases leading to higher consumption in adulthood (DiFranza and Guerera, 1990; Grant and Dawson, 1997; DeWit et al., 2000; Kraus et al., 2000; Guo et al., 2001; Moore et al., 2010).

1.4: Nicotinic Acetylcholine Receptors – nAChRs

Brain nicotinic acetylcholine receptors (nAChRs) are a family of pentameric, ligand-gated ion channels that respond to endogenous acetylcholine (ACh) or to the ligand nicotine exogenously and are involved in several pharmacological effects depending on subtype composition of the ligand binding site (Albequerque et al., 2009; Dani and Bertrand, 2007; Hurst et al., 2013; Zoli et al., 2014). It was the first neurotransmitter receptor and ion channel to be purified and moleculary elucidated from the Torpedo electric organ (Schmidt and Raftery, 1973; McNamee et al., 1975). They are divided into subunits, α (α2 - α10) and β (β2 – β4). These subunits are arranged in groups of five around a central permeable core (Gotti et al., 2006, 2009; Albuquerque et al., 2009; Chatterjee and Bartlett, 2010). In the mammalian brain, most of the nAChRs contain α4β2* subunits which form heteromeric receptors and the homomeric α7 subunit receptors. Heteromeric receptors usually contain two α and three β subunits whereas the homomeric receptors contain all α subunits (Gotti et al., 2006). The asterisk indicates that the subunits may co-assemble with other subunits (Chatterjee and Bartlett, 2010). The five acetylcholine binding interfaces for the homopentameric receptors lie between the α subunits whereas the two binding interfaces for the heteropentemeric receptors are between the α and β subunits (Gotti et al., 2006).

The binding of acetylcholine to nAChRs brings about their activation. Their primary role is to modulate the release of neurotransmitters, namely dopamine (Chatterjee and Bartlett, 2010; Hurst et al., 2013). The importance of nAChRs in the human brain is determined by the properties and function of the receptor through its diverse subunit composition, location and subunit stoichiometry. For example, receptors composed of α4β2* have a high affinity for nicotine, yet the subunit stoichiometry of the receptor will determine if it will be upregulated (Gotti et al., 2009; Albuquerque et al., 2009). The stoichiometry impacts the pharmacological responses which in turn will determine the effects an agonist or antagonist to the receptor has on particular diseases such as Alzheimer's and epilepsy (Gotti et al., 2009; Nelson et al., 2003; Chatterjee and Bartlett, 2010).

Because nAChRs are the primary targets for nicotine in the brain, this makes it possible to assume that alcohol may also interact with these receptors due to common co-

use of nicotine and alcohol. Studies using *Xenopus* oocytes have shown that alcohol can directly interact on different nAChR subunits. Cardoso and colleagues (1999) used *Xenopus* oocytes to express cloned human nAChRs to study alcohol's interactions and effects at these receptors. The study found that alcohol enhanced the function of α4β2, α4β4, α2β2 and α2β4 nAChRs while having little effect on α3β2 and α3β4 nAChRs. Studies by Cardoso (1999) and others (Yu et al., 1996; de Fiebre and de Fiebre 2005) showed that α7 nAChRs were inhibited by alcohol. Studies done in neuronal cell cultures revealed that naturally expressed α4β2 were enhanced by alcohol and that α7 nAChRs were inhibited by alcohol which was in agreement with the above studies in *Xenopus* oocytes (Narahashi et al, 1999; Narahashi et al, 2001). Within the last decade advances have been made for incorporation of nAChR targeted treatment for alcohol-induced behaviors.

1.5: nAChRs in Alcohol Traits

Several reviews exist showing the role of brain nAChRs in alcohol and drug dependence (Chatterjee and Bartlett, 2010; Feduccia et al., 2012; Rahman, 2013; Hendrickson et al., 2013). Yoshida et al., 1982 discovered the earliest evidence of alcohol interference with nAChRs by investigation of 3H-nicotinic binding in rat brains. In nAChRs, the interaction of ethanol with ligand-gated ion channels was first demonstrated using the Torpedo nAChR resulting in ethanol increasing the affinity of ACh to this receptor (Forman et al., 1989). It has also been demonstrated that ethanol is important for stabilizing the open-close-inactivation kinetic properties of nAChRs (Wu et al., 1994; Forman and Zhou, 1999; Zuo et al., 2004). Several accounts in literature show that brain nAChR subtypes are mediators of the rewarding effects of ethanol and other drugs of abuse (Aistrup et al., 1999; Blomqvist et al., 1993; Cardoso et al., 1999; Ericson et al., 1998; Jerlhag et al., 2006; Steensland et al., 2007; Bell et al., 2009). Although nAChRs are expressed throughout the CNS, those receptors affiliated with the mesocorticolimbic dopamine system are of most interest in studies concerning modulation of ethanol reinforcement (Brodie and Appel, 1998; Brodie et al., 1999). The mesolimbic dopamine system is located in the midbrain where it consists of the ventral tegmental area (VTA), dopaminergic (DAergic) neurons and nucleus accumbal (NAc) projections (Wise and Rompre, 1989). The projections to the NAc have been noted as being important in alcohol reward (Brodie et al., 1999; Gessa et al., 1985). Ethanol's reinforcing effect is

accomplished by activation of DA release into the NAc (Di Chiara and Imperato, 1988; Weiss et al., 1993). Activation of DAergic neurons in the VTA results in increased dopamine (DA) release into the NAc which is a key activity associated with drug reinforcement (Di Chiara and Imperato, 1988; Soderpalm et al., 2009).

Ethanol-induced DA release is critical for the onset and maintenance of dependence. Although ethanol is not a direct agonist of nAChRs, ethanol may induce increased ACh release in cholinergic neuronal inputs in the VTA which result in increased DA neuronal signaling through nAChRs (Ericson et al., 1998, 2003; Nadal et al., 1998; Larsson et al., 2005). Pharmacological blockade studies of nAChRs, using mecamylamine, have been the most consistent findings showing the association between ethanol behaviors and reinforcement. This drug works by blocking ethanol-induced DA release into the NAc and thus reducing ethanol consumption (Blomqvist et al., 1993; Blomqvist et al., 1996). Studies by Hendrickson (2009) and Farook (2009) showed that mecamylamine reduced ethanol consumption in male C57BL/6J mice using a restricted assess drinking-in-the-dark (DID) paradigm and a two-bottle choice consumption assay respectively. Kamens and Phillips (2008) demonstrated that mecamylamine, but not other AChR antagonists, attenuated acute locomotor response to EtOH using male and female FAST and SLOW mice. This data was collapsed due to no significant main effect or interactions with sex. More recently, Lui (2013) demonstrated that ethanol-mediated activation of VTA DAergic neurons in mouse midbrain slices were blocked using mecamylamine as well.

The function of nAChRs has also been implicated previously in ethanol's ataxic and sedative-hypnotic effects (Crabbe et al., 2005; Bowers et al., 2005; Kamens et al., 2010) as well as its effects on locomotor activity (Bowers et al., 2005; Kamens et al., 2010) and voluntary consumption (Finn et al., 2004; Larsson et al., 2004; Jerlhag et al., 2006; Kamens et. al 2010; Kamens et al., 2012). A study done by Crabbe (2005) showed that overall, ataxia is a genetically complex behavioral trait and requires the researcher to incorporate more than two behavioral test, especially when using genetically engineered mice. This study ignored sex differences due to their not being enough mice per sex for each strain of mice used to make those comparison. Result from Bowers (2005) showed that $\alpha 7^{-/-}$ mice were more sensitive to the activating effects of EtOH for the duration of LORR as well as locomotor activity. These data were collapsed across sex due to their being no significant

main effect of sex. Finn (2004) and Kamens (2010) both showed that female mice consumed more ethanol than male mice. Studies using α3β2*, β3*, and α6-specific conotoxins revealed that α-conotoxin-MII attenuated ethanol-induced locomotor stimulation in male mice (Jerhlag et al., 2006 and Larsson et al., 2004). Currently, more studies are needed to pinpoint the specific subunit(s) modulated by ethanol to induce these responses.

In order to isolate the role of specific nAChR subunits, agonists and antagonists had to be made to specifically target them. Pharmacological blockade of the VTA by the nAChR antagonist mecamylamine, blocked alcohol-induced increases in accumbal DA levels and also reduced alcohol drinking in male and female rodents and humans (Blomqvist et al., 1996; Ericson et al., 1998; Hendrickson et al., 2010; Chi and De Wit, 2003). However, this antagonist is not specific for any particular subunit. This raises an issue when trying to deduce which subunits are responsible for alcohol's interaction at nAChRs. Studies for alcohol drinking were also done with the FDA approved smoking cessation drug Varenicline in male and female mice and humans (McKee et al., 2009; Hendrickson et al., 2010; Kamens et al., 2010). This drug is a partial agonist for α4β2* and α6β2* and doesn't yield specific subunit results. A series of N,N'-alkane-diyl-bis-3-picolinium analogs were established as antagonist for nAChRs in (Dwoskin et al., 2008). The C_{10} analog, N,N-decane-1,10-diyl-bis-3-picolinium diiodide (bPiDI), which has been preclinically tested for its antagonistic activity at α6β2* nAChRs using nicotine. Repeated nicotine administration has been shown to initiate changes in receptor state. Along with these changes comes large shifts in ligand affinity (Picciotto et al., 2008). In studies using nicotine, this drug is thought to stabilize or lock the receptor subtypes in a high affinity state after repeated administration (Wooters et al., 2011). In the current study, bPiDI was used to examine its effects on EtOH responses and behavior in adolescent age mice.

1.6: α6* Containing nAChRs

nAChRs containing α6* heteromeric subunits have been given more attention due to possible involvement in the reinforcing properties of alcohol. This happens when ethanol induces an increase in ACh release from cholinergic neuron inputs into the VTA, which drives an increase in DAergic neuron activity via cholinergic signaling through nAChRs (Larsson et al., 2005). There is an abundance of α6*- containing nAChRs in the mesolimbic

dopamine system localized on DAergic neurons in the VTA functioning as regulators of cholinergic control of DA transmission to the NAc (Champtiaux et al., 2003; Larsson et al., 2004; Grady et al., 2007; Drenan et al., 2008) and alcohol's ability to increase DA levels (Söderpalm et al., 2009). The activation of α6*-nAChRs may also play vital roles in central cholinergic circuits, including modulation of locomotor behavior and drug addiction (le Novere, et al., 1999; Larsson et al., 2004). Localization studies of protein expression of α6*-nAChRs became possible with the discovery of the selective antagonist α-conotoxinMII (α-CtxMII) (Cartier et al, 1996). It was later shown that this snail toxin showed high affinity for α6β2* nAChRs. β2* nAChRs represent the most widely distributed and best-characterized nAChR subtypes to date (Gotti et al. 2007; Changeux 2005, 2009, 2010). Although there are limited drugs designed to specifically target α6β2* nAChRs, drugs previously designed to target other subunit combinations, such as mecamylamine and varenicline, have been shown to also interact with α6β2* nAChRs (Exley et al., 2008; Rollema et al., 2007). Immunoprecipitation studies using knockout mice and targeted antibodies revealed that β3 subunits were mainly expressed in α6 complexes due to their mRNA being expressed in the same brain regions (Quik et al., 2000; Cui et al., 2003). The α6 and β3 subunits were found to have limited expression throughout the brain, yet were abundantly expressed in the VTA and the mouse striatum (Champtiaux et al., 2003; Gotti et al., 2006; Grady et al., 2007; Le Novere et al., 1996; Zoli et al., 2002). The three abundant combinations of α6*- containing nAChRs were shown to influence agonist stimulated DA release (Grady et al., 2007). Hendrickson (2010) found that α6 and β3 mRNA expression was increased in this region after an acute injection of EtOH. The α6 and β3 subunits showed higher localization of expression at DA neurons in comparison to α4, β2 and α5 subunits (Pons et al., 2008).

Hoft (2009) and colleagues examined the association of single nucleotide polymorphisms (SNPs) in *CHRNA6* and *CHRNB3* with alcohol consumption using a nationally representative sample of adults. These genes encode the α6 and β3 subunits respectively and are located adjacent to each other on chromosome 8 in humans and mice. Using FBAT-PC software, which allowed for examination of a collections of multiple phenotypes, the results revealed three SNPs in *CHRNA6* and one SNP in *CHRNB3* that were associated with alcohol phenotypes. The four phenotypes used for this study included

average number of drinks, blackouts, total number of DSM-IV abuse and dependence symptoms endorsed and quit attempts. This study along with other human genetic studies established that variations within these genes were associated with alcohol behaviors (Zeiger et al., 2008). Another study by Kamens (2012) using male and female wild-type (WT), heterozygous (HET) and knockout (KO) α6 and β3 mice were tested for 3 behavioral responses: consumption, ataxia and sedation. The results for this study showed that α6 and β3 KO mice consumed EtOH similar to their WT counterparts. No genotypic differences were observed for the mouse *Chrnb3* in any of the behaviors tested. *Chrna6* mice showed no consumption or ataxic influence, yet KO mice took longer to recover from the sedative test called loss-of-righting-reflex in comparison to WT. Female mice consumed more ethanol than male mice in the α6 and β3 null mice. However sex did not interact with genotype. So although females consumed more ethanol, this study showed that *Chrna6* and *Chrnb3* were not involved in EtOH consumption. In contrast, a study done by Powers (2013) revealed that the VTA DA neurons of mice expressing a gain-of-function nAChR mutation (α6L9'S), in male and female mice, showed hypersensitivity to ACh, suggesting that α6 containing nAChRs modulate alcohol consumption and alcohol reward-related behaviors. The α6L9'S female mice drank greater amounts of 3% and 6% EtOH solutions in a two-bottle choice paradigm, whereas both sex of this genotype drank greater amounts of 20% EtOH solution in a Drinking-in-the-Dark paradigm in comparison to their non-transgenic littermates. Powers suggested that the differences in results in comparison to Kamens (2012) may be due to upregulation of α4β2* nAChRs which was seen in α6KO DA terminals by Champtiaux (2003). Another study by Liu (2013) was done with WT and α6 KO mice midbrain slices. Pre-application of the α6 selective nAChR antagonist α-conotoxin MII [E11A] reduced ethanol-induced activity in WT brain slices as it did for α6 KO mice. These studies all support the potential of α6* containing nAChRs in modulating alcohol behaviors with the need of future studies for better characterization.

As previously reviewed, adolescence is a period of growth that is characterized by many behavioral changes and of most importance brain development. Exposure to drugs of abuse at this critical time puts subjects at risk of drug abuse, cognitive, addictive and emotional behavioral effects (Spear and Brake, 1983; Slotkin, 2002; Adriani et al., 2003) and also changes in numbers and expression patterns of nAChRs throughout (Counotte et

la., 2012; Doura et al., 2008). Doura (2008) did a study on adult and periadolescent rats looking at nicotine exposure. In relation to α6, one finding of this study revealed that in comparison to adult rats, α6* nAChRs had a greater response to nicotine with more regions showing greater down-regulation. Another study by Azam (2007) showed that mRNA expression of the α6 subunit peaks at adolescence around PND 21. Around this time, the dopamine systems of the substantia nigra (SN) and the VTA, which contain nAChRs on DA neurons, undergo their last major phase of development. Exposure to abusive drugs during this major SN/VTA development phase is thought to alter DA circuitry which underlies motivated behaviors. This could potentially enhance adolescent vulnerability to addiction (Levin et al., 2003; Chen et al., 2007; Dwyer et al., 2009).

1.7: Mice as subjects to study alcohol-induced behavioral responses

In order to test the involvement of specific nAChR subunits in modulating alcohol-induced behavioral responses, animal models had to be chosen that closely mimic alcohol abuse in adolescent humans. Due to ethical concerns, most research on alcoholism in general use nonhuman animal models such as rodents or primates (Tabakoof & Hoffman 2000). Rodent models using adolescent rats or mice have demonstrated similarities in alcohol-induced behaviors with adolescent humans (Laviola et al., 2003; Spear and Varlinskaya, 2004). Many mouse inbred strains have been well characterized for their responses to alcohol. One earlier study done using C57BL/6J and DBA/2J inbred mouse strains (McClearn and Rodgers 1959) revealed complete opposite behavioral sensitivities. C57BL/6J mice have been shown to readily consume ethanol, while the DBA/2J strain avoids alcohol. Other alcohol-induced behavioral responses have now been characterized for other traits within these same inbred strains and others to include locomotor activity (Shen et al., 1995), motor incoordination (Crabbe et al., 2003) and sedation (Ponomarev and Crabbe, 2002) to name a few. These mouse models were used in this study to deduce the role of α6β2* nAChRs on alcohol-induced behavioral responses.

Locomotor activity measurement is commonly used in mice to study sensitivity to the locomotor activating or depressing effects of a drug (Phillips et al., 1995). Locomotor activation usually corresponds to psychostimulant reinforcement (Wise and Bozarth, 1987). Using this test, it has been shown that ethanol's effects on mouse behavior are dose and strain dependent (Randall, 1975; Lister, 1986). Another animal model we used was the

loss-of-righting-reflex (LORR) method (Crabbe et al., 2006; McClearn and Kitahama, 1981), which measures sedative effects. In brief, LORR is a measure of the time it takes for a mouse to right itself after an injection of a drug of choice. Mice are placed on their backs in V-shaped troughs and monitored until they regain their righting reflex. As it relates to alcohol-induced behavior, studies using these methods have revealed that nAChRs are involved in these effects (Bowers et al., 2005; Kamens et al., 2010, 2012).

Motor incoordination, also known as ataxia, is frequently used as a behavioral index of intoxication by drugs that depress the central nervous system (Crabbe et al., 2003). Alcohol-induced ataxia can be monitored in mice using the dowel test (Crabbe 1983; Crabbe et al., 2005), rotorod (Boehm et al., 2000; Crabbe et al., 2005), the grid test (Dudek & Phillips, 1990) and the parallel rod floor test (Kamens et al., 2005; Kamens & Crabbe, 2007). One other method is the balance beam method. In these experiments, hindpaw slips are scored while a mouse transverses a balance beam after injection of an intoxicating agent. Studies using this method and others to observe alcohol-induced behaviors have shown that the sensitivity to ataxia is genetically different throughout mouse strains (Moore et al., 2007; Linsenbardt et al., 2009 & 2011).

1.8: Project Description

As previously reviewed, alcohol as an abused substance has been studied extensively in relation to its effects on behavior. Studies have shown that males consume and are able to metabolize more alcohol than women (Ashley et al., 1977). Early alcohol abuse during times of critical neuronal development, namely adolescence, can cause prolonged usage into adulthood accompanied by cognitive deficiencies (DiFranza and Guerera, 1990; Guo et al., 2001; Moore et al., 2010). Ninety percent of the alcohol consumed at this age is in binge form which increases risk of other injuries due to altered behavior. Proof of alcohol and nicotine co-abuse (Grant et al., 2004) has been shown as well as their genetic correlation in animal and human studies (Swan et al., 2007; Bergstrom et al., 2003). Nicotinic acetylcholine receptors emerged as targets for nicotine and alcohol interactions, thus studies ensued to determine specific subunits responsible for this interaction. The α6* subunit containing nAChRs have been noted for their involvement in regulating the reinforcing properties of nicotine (Pons et al., 2008), yet their involvement with alcohol-induced behaviors is poorly understood. Though α6*-nAChRs are not as

abundantly expressed throughout the brain as the α4β2* nAChRs, their mRNA is highly expressed in the VTA on dopaminergic neurons within the mesolimbic dopaminergic system (Champtiaux et al., 2003; Grady et al., 2007; Le Novere et al., 1996; Zoli et al., 2002). This system had been noted for its involvement in the rewarding stimulus effects of abused substances which includes alcohol. Given this combination of theory, modulation of α6*-nAChRs may be an important regulator of alcohol's stimulus effects and therefore, ultimately govern alcohol use.

Our goal was to examine the role of α6*-containing nAChRs in modulating alcohol-induced behavioral responses in adolescent male and female C57BL/6J mice. Our objectives were: 1) To determine whether α6*-nAChRs had any effect on locomotor activity; 2) To assess the effects of α6*-nAChRs on alcohol consumption; 3) To determine the effects of α6*-nAChRs on ethanol-induced ataxia; and 4) To observe the effects of α6*-nAChRs on the sedative-hypnotic effects of ethanol. The experimental procedures used to address these aims should help to characterize the role of α6*-nAChRs in modulating alcohol-induced behavioral responses in adolescent mice. We hypothesized that α6*-containing nAChRs may modulate locomotor activity, alcohol consumption and the sedative-hypnotic effects of ethanol and that they have no effect on ataxia as previously reviewed.

CHAPTER 2: METHODS

2.1: Animals

Animal protocols were approved by the Institutional Animal Use and Care Committee of the Pennsylvania State University. Male and female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). This breed of mouse was used for all experiments due to this strain being known to drink large quantities of ethanol (Rhodes 2005; McClearn & Rodgers 1959). Mice were purchased to arrive at PND 25 and allowed to acclimate to the mouse facility for one week before each experiment. All mice were between PND 32-44 at the beginning of each experiment, which is the age range that has been defined as adolescence in mice and is analogous to the human developmental stage of adolescence (Spear, 2000; Spear and Brake, 1983; Laviola et al. 2003). The colony room was maintained at around 21±1°C on a 12-h light dark cycle with lights on at 0700 h. Mice were given water and food (Lab Rodent Diet 5001, PMI Nutrition International, Brentwood, MO) *ad libitum*.

2.2: Drugs and Drinking Solutions

Ethyl alcohol 200 proof – absolute, anhydrous ACS/USP Grade, obtained from VWR-KOPTEC (King of Prussia, PA), was the source of ethanol for all experiments. To make the drinking solutions, the EtOH was diluted in tap water to 20% (v/v) (Rhodes et al., 2005). Similarly, saccharin sodium salt hydrate from Sigma-Aldrich (St. Louis, MO, USA) was diluted in tap water to 0.033% (w/v) (Kamens et al., 2005). N,N'-decane-1,10-diyl-bis-3-picolinium diiodide (bPiDI) obtained from Sigma-Aldrich (St. Louis, MO) was diluted in sterile saline 0.9% Sodium Chloride Irrigation, USP (Baxter, Deerfield, IL) to 2.0 mg/mL. Dosages of bPiDI were adapted from Madsen (2014), Wooters (2011) and previous experiments with varenicline doses by Kamens (2010). The dosing groups used were saline 10, 15, 20 or 25 mg/kg depending on the experiment type. All injections were administered intraperitoneally (i.p.) and were adjusted by body weight for each mouse to 10 mL/kg (10 µL/g) to achieve the desired dose (Kamens et al., 2010).

2.3: Locomotor Activity

Mice were subjected to locomotor activity experiments to determine whether bPiDI had any effect on locomotor activity (Kamens et al., 2010). Forty male and forty female mice PND 32-33 were randomly assigned to four dosing groups based on injection type: saline, 15, 20, or 25 mg/kg bPiDI. On testing days, the mice were weighed and moved to the testing room and allowed to acclimate for 30 to 40 minutes. Standard rat cages were used as locomotor chambers. Ten cages were set up on the floor with cardboard between the cages to keep the mice from seeing each other. Numbers identifying the cages were placed within view of a camera suspended from the ceiling. Groups of 10 mice each were recorded at one time, one mouse per activity chamber. On day one, animals were weighed and each mouse was injected with saline and placed into locomotor chambers with a clear top placed on the chamber to avoid escape. Locomotor activity was recorded for one hour. After recording was done, mice were returned to their home cages. On day two, mice were given assigned bPiDI injections and placed into locomotor chambers and recorded for one hour (Fig 1). After activity was recorded, mice were returned to their home cages and later returned to the colony room. Scoring was done by hand using a grid system where locomotor activity was defined as total body movement, not including the tail, across one line of the grid box. Diagonal movement was included as well. Activity was scored in 5 minute intervals within the hour of recorded activity (Kamens et al., 2010).

Figure 1. Experimental Design - Effects of bPiDI on Locomotor Activity in Adolescent C57BL/6J mice

PND 32				PND 33					
Time	30 – 45 mins	T0	60 mins	30 – 45 mins	T0- bPiDI (mg/kg)				60 mins
Drug	habituation	Saline	Locomotion recorded	acclimation	Saline	15	20	25	Locomotion recorded

2.4: Ethanol Metabolism

To determine if bPiDI altered ethanol metabolism, blood ethanol concentrations were examined using methods from Kamens et al. 2006. Mice from the previous locomotor activity test were weighed, then left to acclimate to the testing room for 30 minutes. For EtOH injections, a 20% (v/v) EtOH solution was made. This solution was used to make 0.1 mL injection solutions. Injection solutions were made by mixing the appropriate EtOH solution volume with sterile saline solution per 10 g of the animal's body weight.

According to the body weight of each animal, individual injections were made at 4.0 g/kg EtOH for each mouse. Mice were divided into four groups, each receiving either 15, 20, or 25 mg/kg bPiDI, or saline as a control. Thirty minutes later, each mouse received an injection of 4 g/kg ethanol. Ten microliters of blood was taken from the tail vein by cutting a small tip off the mouse tail and gently pulling and pressing the tail to allow for blood flow from the tail vein to be captured into one end of the 10 µL heparinized micro-hematocrit capillary tubes. Blood was collected at 30, 60, 120, and 180 minute time points. Using hematocrit tube bulbs, blood was then dispelled into 1.5 mL microfuge tubes that contained 200 µL of 0.55M perchloric acid and stored on ice for later blood ethanol concentration (BEC) readings (Kamens et al., 2010). These samples were used for the following blood alcohol metabolism assay.

2.4a: Blood Alcohol Metabolism Assay

The BEC was measured using a modified enzymatic assay, which couples the conversion of ethanol to acetaldehyde and the conversion of nicotinamide adenine dinucleotide (NAD) to NAD with hydrogen (NADH) by the addition of alcohol dehydrogenase (ADH). EtOH standards were created by diluting a 1000 mg% EtOH stock solution to concentrations ranging from 0 to 700 mg% EtOH. Ten microliters of sample and standards were placed in a 1.5 mL microfuge tubes containing 200 µL of 0.55M perchloric acid. Samples and standards were vortexed and centrifuged at 1500g for 10 minutes. Then, 180 µL of the samples and standards were pipetted into 1.5 mL tubes, and 180 µL of KOH was added to the sample tubes and 200 µL KOH was added to the standard tubes. These tubes were vortexed again at 1500g for 10 minutes. Using a labeled diagram, the assay plate was loaded with 300 µL of water to the water blank wells. 250 µL of Tris/NAD Buffer was added to all the sample blank wells. ADH was added to the remaining Tris/NAD to give 6 units/mL. In wells labeled for the samples, 250 µL of the Tris/NAD/ADH solution was added being sure to avoid contaminating standard wells. Each assay well was filled with 50 µL of standards and samples into the appropriate well starting with the standards to avoid contamination. The plate was incubated at room temperature for 30 minutes, and read for absorbance at 340 nm for NADH production on an ELx808 Absorbance Reader (Biotek) in singlet. Ethanol concentration was calculated

in reference to the standard curve (Ehringer et al., 2009; Smolen et al., 1986). Standard curve ranged from 0.026-2.041 mg/mL.

2.5: Drinking-in-the-Dark Procedure

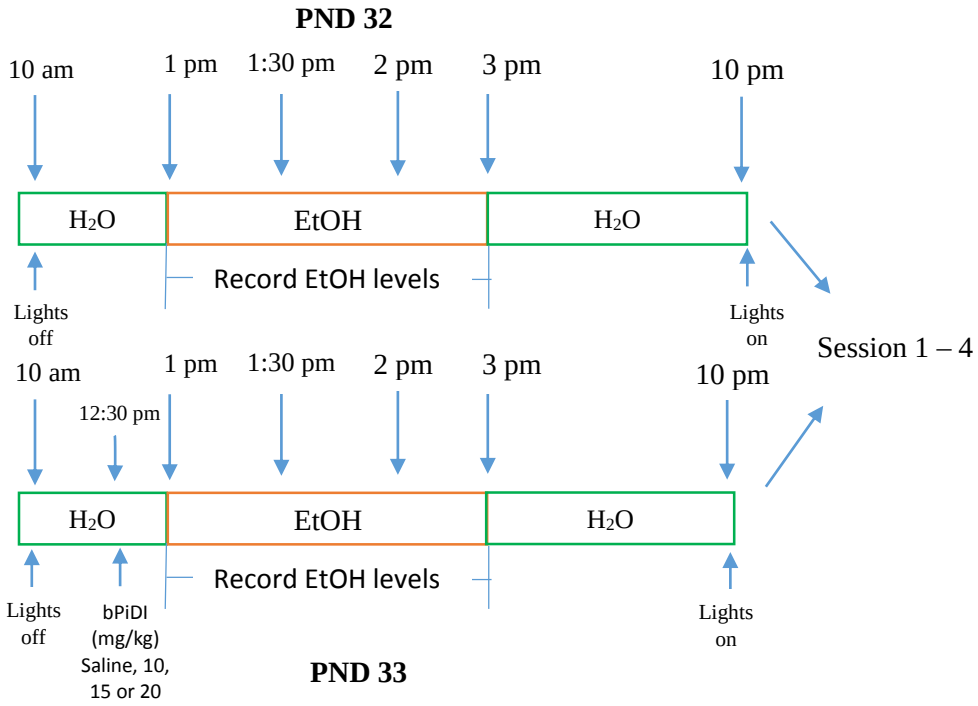
2.5a: Ethanol Consumption

To assess the effect of bPiDI on alcohol consumption, we monitored ethanol consumption of adolescent animals using a 2-day drinking-in-the-dark (DID) paradigm adapted from Kamdar (2007) and Gupta (2008), whose procedures were adapted from Rhodes (2005). This paradigm was set up in four, two-day intervals over a two week period (Fig 2). During the one week acclimation period prior to the experiment, a separate cohort of 24 male and 24 female mice adapted to a 12h reversed light/dark cycle (lights off at 10AM). Mice were housed individually under standard housing conditions to allow for accurate measure of liquid intake. Red incandescent lamps were used to aid in measuring fluid levels during the dark cycle. On Day 1, mice were weighed and their water bottle was removed and replaced with a drinking tube containing 20% EtOH (Rhodes et al., 2005). An EtOH tube was placed on each cage three hours into the dark cycle with an initial reading taken once they were placed on the cage. The EtOH tubes used were made by shortening 10mL serological pipet tubes and attaching a stainless steel double-ball-bearing sipper to one end and a small rubber stopper to the other. EtOH tubes were presented at 3 hours into the dark cycle due to previous studies showing that mice tend to drink more at their peak arousal time as it relates to circadian rhythm of consumatory behavior (Goldstein and Kakihana, 1977). This leads to maximal drinking occurring within the first few hours of the dark cycle (Rhodes et al., 2005). For the EtOH level readings, the observer would record the number on the pipet where the bottom of the EtOH meniscus was nearest. EtOH readings were taken at 30 minute, 60 minute, and 120 minute time points. After the 120 minute time point, the EtOH tube was removed and replaced with a water bottle.

On Day 2, the water bottle on each cage was removed and animals were given intraperitoneal (i.p.) injections of either saline, or one of three doses of bPiDI at 10, 15, or 20 mg/kg 30 minutes before access to EtOH. Each mouse received one of each injection type throughout the two week period using a Latin square design to counterbalance treatments across the animals (Kamens et al., 2010). An EtOH drinking tube was placed on each cage and EtOH level readings were taken at the time points previously mentioned

for Day 1. After the last time point, the EtOH tube was removed and the water bottle was placed back on the cage.

Figure 2. DID Experimental Design - EtOH

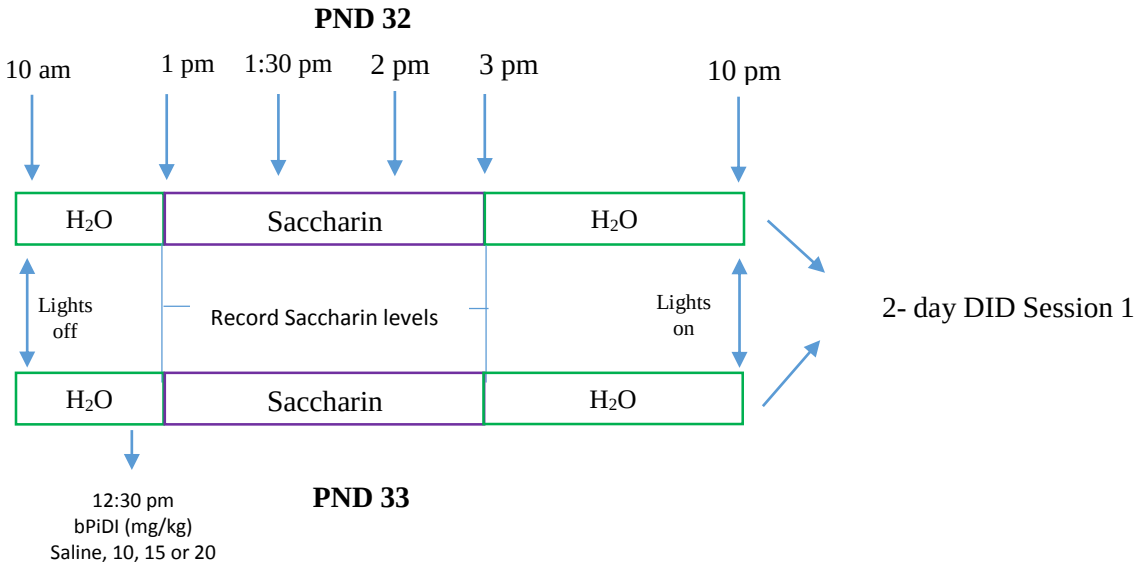


2.5b: Saccharin Consumption

To access the effects of bPiDI on saccharin consumption we used the same 2-day DID paradigm adapted in the previous section (Fig 3). This experiment was done as a control to test whether the effects of bPiDI observed in consumption were specific to EtOH-mediated effects. Bachmanov (1996) showed that C57BL/6J mice have a higher preference for sweet solutions than salt solution meaning that mice would prefer alcohol due to its sweet taste (Belknap et al., 1993; Crabbe et al., 1996). Using this test as a control for taste would show whether the effects of bPiDI are specific to EtOH intake. A separate group of mice were allowed to acclimate to the 12h reverse light dark cycle for one week before the experiment, as previously done for EtOH consumption. On Day 1, mice were weighed, water bottles were removed and drinking tubes containing 0.033% saccharin were placed on the cages three hours into the dark cycle. An initial reading was taken upon placement of the saccharin drinking tubes. Saccharin readings were also taken at 30 minute, 60 minute, and 120 minute time points. After the 120 minute time point, saccharin tubes were removed and replaced with water bottles. On Day 2, animals were given

intraperitoneal (i.p.) injections of either saline, or one of three doses of bPiDI at 10, 15, or 20 mg/kg before access to saccharin. Each mouse received one of each injection type throughout the two week period using a Latin Square design to counterbalance bPiDI doses across the animals. Three hours into their dark cycle, saccharin was placed on cages and an initial reading was taken. Saccharin readings were conducted as mentioned above and after the last reading, water bottles were placed back on the cages.

Figure 3. DID Experimental Design - Saccharin



2.6: Balance Beam

To test for effects on ethanol-induced ataxia we used the balance beam, based on a published procedure (Crabbe et al., 2003; Kamens et al., 2010; Linsenbardt et al., 2009) (Fig 4). The balance beam was constructed from an acrylic sheet by Centre Glass Company, held up by two clamps at a height of 54.6 cm above the floor. The beam's dimensions were chosen at 91.4 cm long by 1.9 cm wide, based off previous literature to determine an appropriate adolescent-mouse-sized balance beam (Linsenbardt et al. 2009). Disposable pads placed under the beam provided cushioning in case the animal fell off the beam (Kamens et al., 2010). During the one week acclimation period, mice were housed 3 to 4 per cage by sex. Before the start of the experiments, mice were moved to individual cages for 30 minutes to allow habituation to the testing area before the experiment. Fifty male and 50 female adolescent age mice (PND 33 and PND 37) were weighed, then trained to transverse the balance beam prior to the actual test. Training was done a day before

testing. To complete the training, each mouse had to traverse the beam twice. If a mouse fell or turned around to move in the wrong direction, the direction of the mouse was corrected by lifting the mouse by its tail and turning it. If the mouse stopped, it was encouraged to complete the test by gently pinching the tail. Previous studies have shown that this training is necessary to acclimate the animal to rapidly transverse the beam with little need for the experimenter to encourage it to keep moving in the appropriate direction (Linsenbardt et al., 2009).

Figure 4. Experimental Design - Effects of bPiDI on Balance Beam and LORR in Adolescent C57BL/6J mice

Balance Beam (PND 32-38)

Time	T0 - Pretreatment					30 mins	T30 - Treatment	T40
	bPiDI (mg/kg)	Saline	10	15	20	Injection	Saline or Ethanol - 1.5 (g/kg)	Test BB

-ONE WEEK-

LORR (PND 39-44)

Time	1 hr	T0 - Pretreatment					30mins	T30 - Treatment	T40
	acclimation	bPiDI (mg/kg)	Saline	10	15	20	Holding cage	Saline or Ethanol 4.0 (g/kg)	Record time to LORR and LORR duration

After successful completion of the training session, mice were returned to their home cages. On testing day, mice were moved to the testing room and weighed. The mice were then acclimated to the environment for one hour before beginning the experiment. Testing was conducted during the light cycle between 2pm and 5pm. Timing for the experiment started with the initial injection of pretreatments of either saline or 10, 15 or 20 mg/kg bPiDI. Mice were placed in individual holding cages for 30 minutes. At the 30 minute time point, mice were given treatment injections of either saline or 1.5mg/kg EtOH (Kamens et al., 2010; Linsenbardt et al., 2009). At the 40 minute time point (10 minutes after the ethanol challenge; Linsenbardt et al., 2009), each mouse was placed on the balance beam, one at a time, and the number of hind paw slips were recorded along with other observed behaviors as the mouse transversed the balance beam for one minute. If the mouse fell it was quickly returned to the balance beam at the fall point to complete the task (Crabbe et al., 2003). After completion of the experiment, all mice were returned to their home cages and retained for a loss of righting reflex experiment.

2.7: Loss of Righting Reflex

The sedative-hypnotic effects of ethanol were measured by observing loss of right reflex (LORR), based on standard published methods (Crabbe et al., 2006; McClearn and Kitahama, 1981) (Fig 4). Mice from the previous balance beam experiment were used for LORR experiments one week later PND 39, 40 and 44. Testing was conducted during the light cycle between 9AM and 5PM. On testing day, the mice were weighed, returned to their home cages and allowed to acclimate to the testing room for one hour before the experiment began. At the initial time point, T=0, mice were injected with a pretreatment of saline or bPiDI at 10, 15 or 20mg/kg. After the pretreatment injection, mice were returned to their home cages for 30 minutes to habituate to the injection. At the 30 minute time point, a 4.0 g/kg EtOH injection (Kamens et al., 2012) was given to each mouse. The mouse was then placed in an empty holding cage until it began to slow down in movement and have trouble keeping balance. When the mouse appeared close to sedation, it was placed in a V-shaped plexiglass trough on its back. LORR was defined as the animal remaining on its back for at least 30 seconds. If a mouse did not succumb to sedation and stay lying down in the V-shaped trough within 3 minutes after the EtOH injection, it was excluded from the study due to possible improper injection (Kamens et al., 2012; Ponomarev and Crabbe, 2002). The initial time of LORR was recorded when the mouse remained sedated with minimal movement in the V-shaped trough. The animal was observed for the duration of LORR and the amount of time it took for the animal to right itself (defined as turning over on all four paws) was recorded. Once the mouse achieved its first righting, it was immediately placed on its back a second time. The mouse has to right itself within 30 seconds to insure that it has regained its righting reflex (Kamens et al., 2010).

2.8: Statistical Analysis

Ethanol consumption (g/kg), saccharin consumption (mg/kg), BEC (mg %), foot slips, duration of LORR and locomotor activity were used as primary dependent variables. Sex, dose, time and concentrations were possible independent variables. Data for ethanol consumption, saccharin consumption and BEC were analyzed using repeated measures analysis of variance (ANOVA) with time as a within-subject variable. Data for balance beam, duration of LORR and locomotor activity were analyzed using factorial ANOVA. All data were normally distributed. All interactions were followed up by ANOVA using fewer factors and Tukey's HSD for post hoc comparisons. All significance tests were two-tailed and the alpha level was set at 0.05. SPSS was used for all analyses.

CHAPTER 3: RESULTS

3.1: The effect of bPiDI on Locomotor Activity

At higher doses, administration of bPiDI reduced locomotor activity in adolescent C57BL/6J mice (Fig 5). Our analysis focused on Day 2 which is when mice were challenged with saline or bPiDI. A three-way repeated measures ANOVA with sex and dose as independent factors and time as the dependent variable revealed a significant main effect of time ($F_{11,319} = 26.46$, $p < 0.001$), a significant time X dose interaction ($F_{33,319} = 5.071$, $p < 0.001$) and a significant time X sex interaction ($F_{11,319} = 2.376$, $p < 0.01$). Due to the significant time X sex interaction, data were separated by sex. In male mice there was a significant time X dose interaction ($F_{33,154} = 2.512$, $p < 0.001$). Post hoc comparisons showed that male mice that received 25 mg/kg doses of bPiDI had decreased activity during the first 30 mins of the experiment in comparison to saline. Male mice that received 15 and 20 mg/kg bPiDI began to gradually increase activity in comparison to the 25 mg/kg doses starting at the 20 min time point. At the 35 min time point, all mice were maintaining activity similar to that of the saline group for the remainder of the experiment. For female mice, there was a significant time X dose interaction as well ($F_{33,165} = 4.125$, $p < 0.001$). Female mice that received 20 and 25 mg/kg doses of bPiDI, post hoc comparisons revealed significantly decreased activity in comparison to saline and the 15 mg/kg dose during the first 15 minutes of the experiment. By the 25 minute time point at the 20 mg/kg dose, activity began to increase to the same level as the 15 mg/kg and saline. By the 35 minute time point, all mice reached similar increased activity levels that were maintained for the remainder of the experiment. The results of this experiment gave us an idea of which time points to use for our consumption experiment and suggests that α6* subunit containing nAChRs are involved in modulating locomotor activity as we hypothesized.

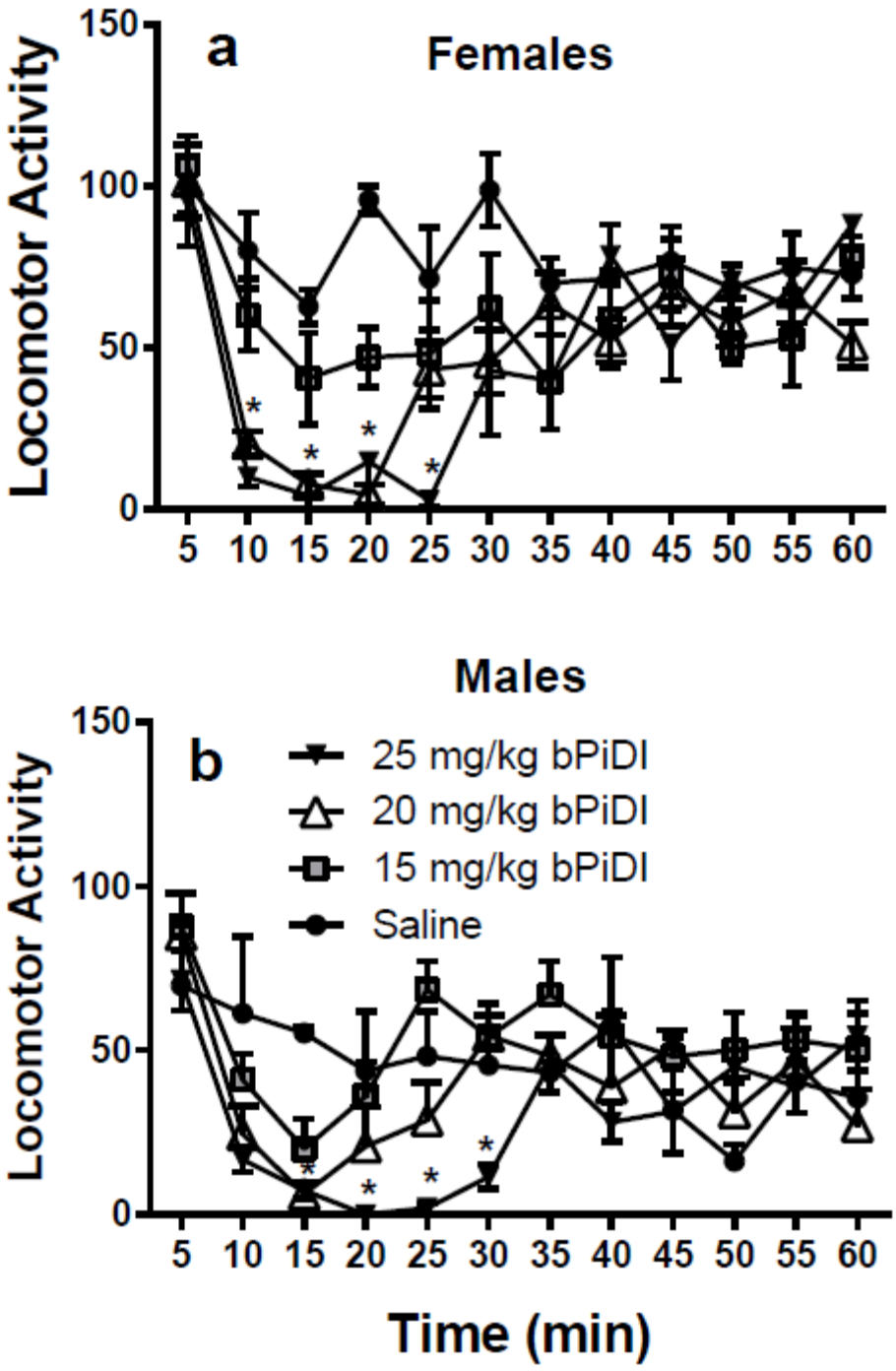


Figure 5. bPiDI decreases locomotor activity. Data (mean ± SEM) represent locomotor activity in **(a)** female and **(b)** male C57BL/6J adolescent mice. *N* = 18-19 per group. **p*<0.05

3.2: The effect of bPiDI on ethanol metabolism

Pretreatments of bPiDI did not alter the clearance of 4 g/kg ethanol (Fig 6). A three way repeated measures ANOVA with sex and dose as the independent factors and time as the dependent variable revealed a significant main effect of time on EtOH metabolism ($F_{3,60} = 208.55$, $p < 0.01$). There was a significant time X sex interaction ($F_{3,60} = 2.81$, $p < 0.05$). Due to the time X sex interaction, male and female mice were analyzed separately. Post hoc comparisons showed that female mice that received saline had significantly higher BEC at the 60 minute time point (417.24 ± 27.9) in comparison to male mice at the same time point for saline injected mice (394.03 ± 17.56). This result suggests that the effects of bPiDI on locomotor activity observed are not due to EtOH metabolism as expected.

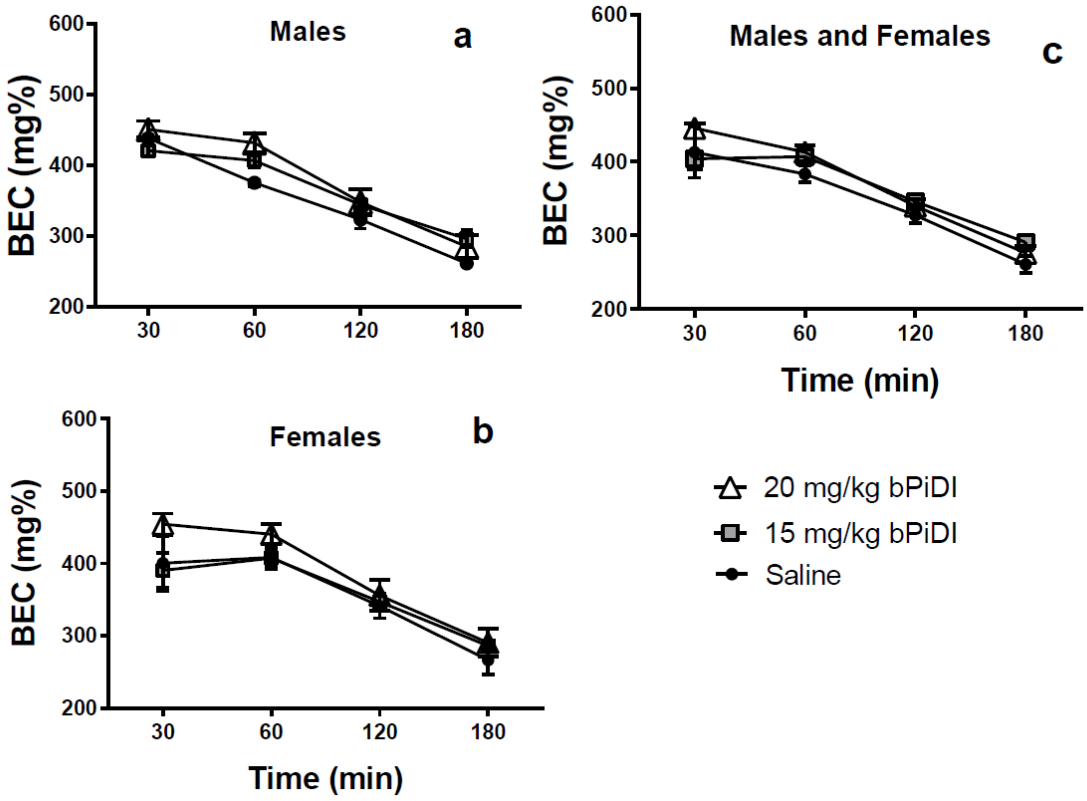


Figure 6. bPiDI does not alter ethanol metabolism in C57BL/6J adolescent mice. Data (mean ± SEM) represent male and female blood ethanol concentration taken after a 4 g/kg injection of EtOH. $N = 14$ per group. *$p < 0.05$

3.3: The effect of bPiDI on ethanol and saccharin consumption

Repeated measures ANOVA showed that there was no significant main effect or interaction of sex on ethanol (EtOH) consumption, therefore data for both sexes was analyzed together. Within subject analysis using a mixed repeated measures ANOVA with dose as the independent factor and time as the dependent variable revealed a significant main effect of dose on EtOH consumption for the 30 minute time point ($F_{3,66} = 4.597$, $p < 0.01$) and at the one hour time point ($F_{3,66} = 4.567$, $p < 0.01$). At the 2 hour time point, there was no main effect of dose on EtOH consumption (Fig 7a). Post hoc comparisons revealed that mice drank significantly less EtOH when injected with the 20 mg/kg dose of bPiDI in comparison to any other dose for the 30 minute (all p -values < 0.05) and 1 hour time points (all p -values < 0.01).

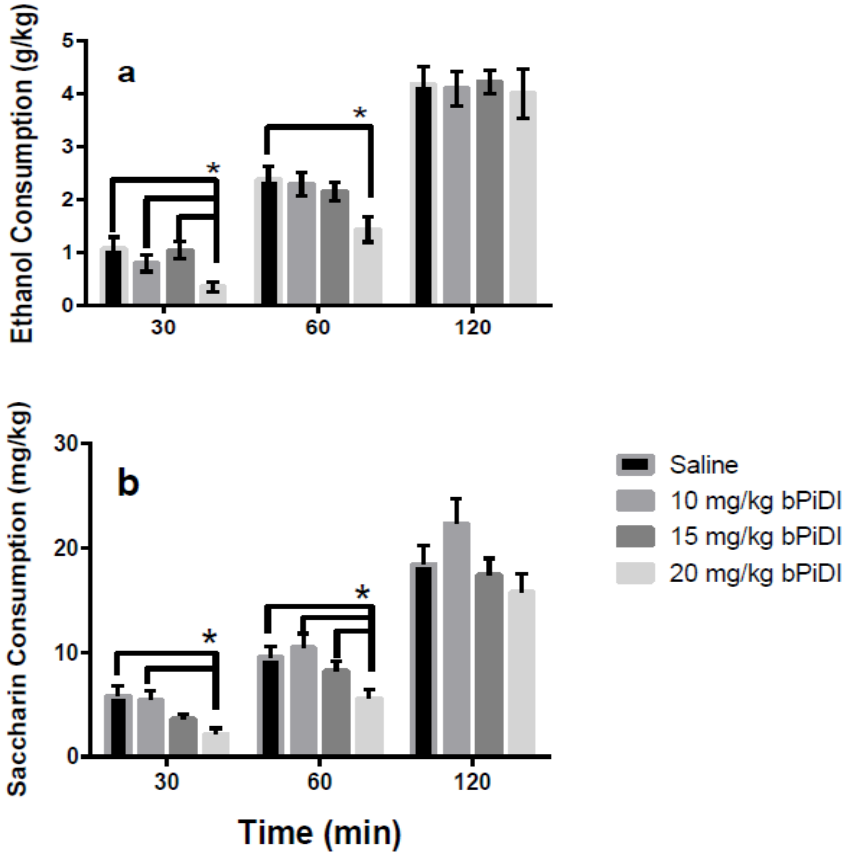


Figure 7. bPiDI (20 mg/kg) significantly reduces EtOH consumption at the 30 minute and 1 hour time point in adolescent C57BL/6J mice. Data (mean ± SEM) represent **(a)** ethanol consumption and **(b)** saccharin consumption. $N = 24$ per group. *$p < 0.05$

To determine if differences in EtOH consumption were being affected by taste preference, we subjected a naïve group of adolescent mice to a similar 2-day DID paradigm with the sweet tastant saccharin (Fig 7b). Repeated measures ANOVA showed that there was no significant main effect of sex on saccharin consumption, therefore data was analyzed together. There was a significant main effect of dose on saccharin consumption for all three time points: 30 minute ($F_{3,66} = 5.72$, $p < 0.01$), 1 hour ($F_{3,66} = 5.54$, $p < 0.01$) and 2 hour ($F_{3,66} = 3.20$, $p < 0.05$). Post hoc comparisons between the doses of bPiDI given at the 30 minute time point showed significant differences in saccharin consumption between saline and 10 mg/kg bPiDI, saline and 20 mg/kg bPiDI, 10 mg/kg bPiDI and 20 mg/kg bPiDI dose against all doses (all p -values < 0.05). Post hoc comparisons between the bPiDI doses given at the 1 hour time point revealed significant differences between the 20 mg/kg bPiDI dose and all other doses (all p-values < 0.05). For the 2 hour time point, significant dose differences were found between 10 mg/kg and 15 mg/kg ($p < 0.05$) and the 10 mg/kg and 20 mg/kg bPiDI doses (all p - values < 0.05). These results suggest that α6* subunit containing nAChRs may modulate EtOH as hypothesized.

3.4: The effect of bPiDI on Balance Beam

bPiDI does not influence ethanol-induced ataxia as measured by the balance beam method (Fig 8). There were no significant main effects or interactions of bPiDI pretreatment on the number of footslips observed following saline challenge ($p = 0.1$). We then corrected for EtOH footslips by subtracting the saline footslips from their respective EtOH footslips (Kamens et al., 2010; Crabbe et al., 2003; Crabbe et al., 1994). Corrected footslips were calculated by subtracting each EtOH-treated animal's footslip score from the baseline footslips each animal made (Kamens et al., 2010). This data was subjected to a 2-way ANOVA with sex and dose as the independent variables. There were no significant main effects or interactions between sex and bPiDI dose. This data suggests that α6* subunit containing nAChRs may not be involved in modulating ethanol-induced ataxia.

3.5: The effect of bPiDI on LORR

Pretreatments of bPiDI did not influence the sedative-hypnotic effects of ethanol as measured by LORR (Fig 9). Data was subjected to a 2-way ANOVA with sex and dose as independent variables. There were no significant main effects or interactions of sex or dose on LORR duration. To assess the effect of bPiDI on time to LORR, data was subjected to a 2-way ANOVA with sex and dose as independent variables. There were no significant main effects or interactions of sex or dose on the time to LORR (data not shown). This result suggest that α6* subunit containing nAChRs may not involved in the modulation of the sedative-hypnotic effects of ethanol which was opposite to what we hypothesized.

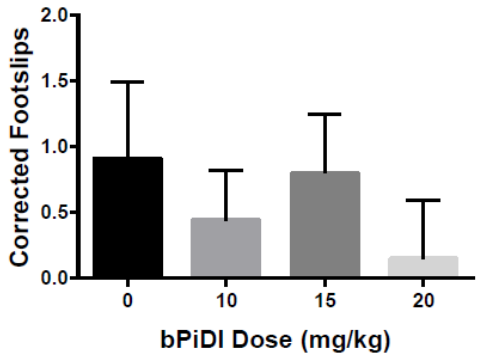


Figure 8. bPiDI does not influence ethanol-induced ataxia. Data (mean ± SEM) represent ethanol-induced ataxia when corrected for baseline ataxia in C57BL/6J adolescent mice. *N* = 11-14 per group.

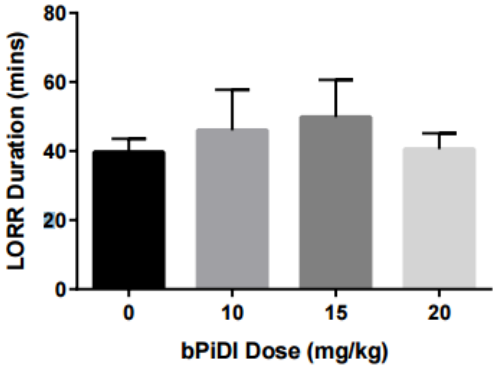


Figure 9. bPiDI does not influence the sedative-hypnotic effects of ethanol as measured by LORR. Data (mean ± SEM) represent ethanol-induced sedation in C57BL/6J adolescent mice. *N* = 18-22 per group.

CHAPTER 4: DISCUSSION

In this thesis, we aimed to characterize the role of α6*subunit containing nAChRs involved in ethanol-induced behavioral responses in adolescent C57BL/6J mice. Using the novel drug bPiDI, designed to specifically target α6β2* nAChRs, we modeled several alcohol-induced behavioral responses which included voluntary consumption using a modified drinking-in-the-dark paradigm, ataxia using the balance beam test, and sedation using the loss-of-righting-reflex test. Using the α6β2* selective drug bPiDI, the data presented here provides evidence that α6* subunit containing nAChRs may modulate ethanol consumption and locomotor activity in adolescent mice.

The influence of bPiDI caused initial decreased locomotor activity at higher doses on its own (Fig 5). We observed significant decreases in male mice locomotor activity dependent on the time and dose of bPiDI administered. At the 10 min time point, there was a significant decrease of activity in females at the 20 mg/kg and 25 mg/kg doses of bPiDI in comparison to male groups that had decreased activity at all doses of bPiDI at the same time point. From the 35 minute time point to the end of the experiment, activity for both sexes reached levels similar to control and remained constant until the last time point. This outcome does not agree with other locomotor studies performed in mice using bPiDI. Only a few studies have been done using this drug yet they were done in relation to its effects on nicotine (Struthers et al., 2009; Madsen et al., 2014; Wooters et al., 2011; Dwoskin et al., 2008). Struthers (2009) findings showed that at a dose of 3.38 mg/kg, bPiDI significantly decreased the conditional stimulus effects of a 0.4 mg/kg dose of nicotine causing a motor impairment effect. In comparison to our results, this was similar to the effect observed in EtOH consumption with our highest dose of 20 mg/kg. Dwoskin (2008) showed that for locomotor activity, acute administration of mecamylamine and dihydro-β-erythroidine inhibited locomotor activity. However this result was observed after repeated nicotine treatment. At a dose of 5.8 μmol/kg, bPiDI significantly reduced locomotor stimulant effects of nicotine relative to saline. Wooters (2011) and colleagues found that at a 5.8 μmol/kg dose of bPiDI, nicotine self-administration was decreased over a seven day treatment in male Sprague-Dawley rats. In the study done by Madsen (2014), a 10 mg/kg bPiDI dose showed no effect on baseline locomotor activity over a three consecutive day period in WT mice. The nicotine results showed that a 5 mg/kg dose of bPiDI impaired

acquisition of nicotine self-adminstration and reduced established nicotine self-administration in WT mice. It should be noted that the same pattern of reduction was observed for a 10 mg/kg dose of bPiDI in α4-S248F mice although they were backcrossed on a C57BL/6J background. Also, this study tested only two dosages of bPiDI: 5 mg/kg and 10 mg/kg. Kamens (2010) showed that varenicline, a partial agonist at α4β2 nAChRs, had depressant effects on baseline locomotor activity in female mice at the 2 mg/kg dose in comparison to 1 mg/kg and saline. In males this depressant effect was shorter. This outcome is similar to the present results in that bPiDI seems to cause initial depressant effects on its own in both male and female mice. It should be noted that bPiDI dosages that influenced alcohol-induced behaviors are higher than the dosages of varenicline that influence nicotine behaviors. Although these results do not agree with the results of baseline effects in rats (Zaniewska et al., 2008), it has been shown that mice commonly experience locomotor depression (Marks et al., 1989; Tritto et al., 2004). Prior studies using nicotine show that α6* nAChRs are involved in regulating nicotine's reinforcing properties (le Novere et al., 1999; Pons et al., 2008; Brunzell et al., 2009; Tuesta et al, 2011).

We were not able to detect any differences in ethanol metabolism between dose and time after the locomotor experiment. However we did find a significant interaction of time and sex. When data was separated by sex, BECs at the 60 minute time point were slightly higher for female mice that were given the 20 mg/kg dose bPiDI (Fig 6). The inability of detecting differences in metabolism through the genes coding for α6 had been shown previously in a study using *Chrna6* and *Chrnb3* mice (Kamens et al., 2012). BECs showed a trend of decrease over time following the ethanol injection. This outcome along with the current results agree that α6* nAChRs are not responsible for the metabolism of ethanol. Further work on the baseline expectations of this drug will have to be done to make a better comparison of what to expect in alcohol-induced responses as this drug was originally established for studying nicotine-induced responses. This data combined with the outcomes of the locomotor experiment suggest that bPiDI may influence locomotor activity depending on time and dose with no influence on EtOH metabolism.

We were able to show that bPiDI influences ethanol and tastant consumption depending on time and dose. These behavioral outcomes are not due to the initial decreases

observed in the locomotor activity study as that study was done to gauge possible times for bPiDI pre-treatments. Results of these experiments showed that bPiDI significantly reduced ethanol consumption in a time and dose dependent manner where reduction was observed at the 30 minute and one hour time point for the highest bPiDI dose given (Fig 7). Around the 2 hour time point, the effects of bPiDI were no longer observed. What this result suggests is that α6β2* subunit containing receptors may influence initial ethanol consumption depending on the time and dose of the drug. For tastant consumption, bPiDI showed initial decreases at the 30 minute and one hour time points for the highest dose of bPiDI given. Because the mesolimbic dopamine pathway is involved in the rewarding effects of alcohol (Aistrup et al., 1999; Blomqvist et al., 1993; Steensland et al., 2007) as well as the location of α6β2* nAChRs (Champtiaux et al., 2003; Larsson et al., 2004; Grady et al., 2007; Drenan et al., 2008), the effects of bPiDI may be due to switches in DA transmission to other brain regions. These results on consumption agree with there being involvement of α6* containing nAChRs in modulating alcohol consumption.

The α6β2*selective antagonist bPiDI did not influence alcohol-induced motor incoordination as measured by the balance beam method (Fig 8). There are three known studies that examined nAChRs role using motor incoordination models. Data from studies done by Taslim et al., 2008 and Kamens et al., 2010 showed that with the selective α4β2* agonist (RJR-2403) and the partial agonist varenacline, respectively, motor incoordination may be modulated by α4β2* containing nAChRs. The results reported here agree with the results of Kamens et al., 2012 suggesting that α6*-containing nAChRs are not involved in ethanol-induced ataxia. It is known that ataxia is a complex behavior and requires more than one behavioral measure for valid results (Crabbe et al., 2005). In addition, pretreatments of bPiDI did not influence the sedative effects of EtOH during LORR (Fig 9). These data are not in agreement with the results of Kamens et al., 2012 who showed that there were significant effects of knocking out the *Chrna6* gene on ethanol-induced LORR.

Taken together, the data presented here complement the existing human genetic association studies that are in support of α6* nAChRs being involved in the modulation of alcohol reward responses (Ehringer et al., 2007; Schlapfer et al., 2008; Wang et al., 2008;

Zeiger et al., 2008; Hoft et al., 2009; Landgren et al., 2009; Liu et al., 2013). Genetic animal behavioral studies also echo the support of α6* nAChRs in these responses (Kamens et al., 2012; Powers et al., 2013). The present study was the first to use the α6β2* selective antagonist bPiDI as a pharmacological blockade to examine α6* nAChRs in modulating alcohol-induced behavioral responses in adolescent mice. Due to known increases in risk-taking behaviors during adolescent development in humans, it is crucial that efforts be made to deduce where alcohol is truly interacting in the brain to further progress towards mechanisms of action. These findings add to the growing possibility of a mechanistic approach to nAChR targeted treatment for alcohol abuse through the use of subunit selective ligands.

Future studies related to the α6β2* selective antagonist bPiDI would be helpful in better characterization of its effects on EtOH related responses. Repeats of all experiments presented in this thesis would be a necessary start. Some possible studies would be to evaluate ataxic modulation by using other mouse behavioral models such as the fixed-speed rotarod test, the dowel test and or the parallel floor rod test. A two-bottle choice drinking model as well as studies with genetically modified mice would be great to analyze the potential of bPiDI effects being specific to EtOH-mediated responding. It would also be useful in showing if bPiDI may interact with other nAChR subunits. Because most studies using this drug have been focused on nicotine, any alcohol-induced behavioral studies would break new ground to further characterization of α6* nAChRs in EtOH-mediated behavioral responses and eventual clinical applications using drugs selective for these receptors.

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