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Exploring Pharmacological and Behavioral Mechanisms Involved in Alcohol Dependence During  
Adolescence

A dissertation submitted in partial fulfillment of the requirements for the degree of M.S. Program  
at Virginia Commonwealth University.

By

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## List of Abbreviations and Symbols

ADH	Alcohol Dehydrogenase
ALDH	Aldehyde Dehydrogenase
ACh	Acetylcholine
AUD	Alcohol Use Disorders
AWS	Alcohol withdrawal symptoms
B6	C57Bl/6 mice
BAL	Blood Alcohol Level
BEC	Blood Ethanol Concentration
CA	Continuous Access
CPP	Conditioned Place Preference
CTA	Conditioned taste aversion
D2	DBA/2 mice
DA	Dopamine
DID	Drinking-in-the-Dark
EDE	Ethanol Deprivation Effect
EPM	Elevated Plus Maze
EtOH	Ethanol (Alcohol)
GABA	Glutamate, $\gamma$ -aminobutyric acid
I.p.	Intraperitoneal
IA	Intermittent Access
LORR	Loss Of Righting Reflex

L/D Boxes	Light and Dark Boxes
MRI	Magnetic resonance imaging
MDS	Mesolimbic Dopamine System
NMDARs	N-methyl-D-aspartate receptors
NCADD	National Council on Alcoholism and Drug Dependence
NAC	Nucleus Accumbens
nAChR	Nicotinic Acetylcholine Receptor
NIC	Nicotine
OVV	Out-of –the vivarium space
QTL	Quantitative Trait Loci
PND	Postnatal day
PFC	Prefrontal Cortex
2-BC	Two Bottle Choice
SAL	Saline
SEM	Standard error of the mean
S.C.	Subcutaneous
VTA	Ventral Tegmental Area
WHO	World Health Organization

## Abstract

Exploring Pharmacological and Behavioral Mechanisms Involved in Alcohol Dependence During Adolescence

By: Rabha M. Younis

A dissertation submitted in partial fulfillment of the requirements for the degree of M.S. Program at Virginia Commonwealth University.

Virginia Commonwealth University, 2016

Director: M. Imad Damaj, PhD  
Professor, Department of Pharmacology and Toxicology

Alcoholism is a serious illness that is marked by uncontrollable drinking and physical dependence to alcohol. Long-term alcoholism has been linked to many health concerns such as cirrhosis of the liver and cardiovascular disease. Alcohol is one of the most commonly used drugs among adolescent populations. Given that adolescence is a unique developmental stage during which alcohol has long-term effects on future drug-taking behavior; it is essential to understand how early exposure to alcohol during adolescence may affect the abuse liability of the drug later in life. Indeed, most alcohol users start during adolescence suggesting that exposure to alcohol during adolescence increases the risk of alcohol abuse in adulthood. Our studies focus on identifying behavioral mechanisms involved in alcohol dependence during adolescence by using well-established mouse models of alcohol drinking. Our hypothesis is that exposure to alcohol during early adolescence will increase alcohol intake later in adulthood. We investigated the impact of alcohol drinking in male and female early adolescent C57BL/6J mice using the Drinking In the Dark (DID) model. Our results showed that exposure to alcohol during early adolescence enhanced ethanol intake later in adulthood in the DID and the 2-bottle choice

drinking paradigms. In contrast, adult exposure of alcohol did not enhance later alcohol intake. Our data illustrates that enhanced alcohol intake are affected by the duration, age of exposure, and mouse genotype. In addition, we conducted behavioral studies to elucidate in part the mechanisms underlining the relationship between adolescent ethanol exposures and enhance alcohol intake in adult mice. We hypothesized that these changes in alcohol intake are due to an alteration between ethanol aversive and rewarding properties. Our results showed that ethanol exposure during adolescence induces a less aversive state (less withdrawal intensity in the FST test) later in adulthood. In contrast, our results showed that ethanol exposure in adolescence enhanced the rewarding properties of ethanol in the CPP test and alcohol intoxication (as measured by the LORR test). Overall, our results suggested that this increase in the alcohol intake was correlated with an increase in alcohol rewarding properties and a reduction of alcohol aversive effects. Further research will be required in order to more fully examine the mechanisms of action for the observed changes in alcohol intake.

# Chapter one: General introduction to alcohol dependence

## 1.1 Background and Significance

Alcoholism, also called dependence on alcohol is characterized by the compulsive and uncontrolled consumption of alcohol. It's a global problem, that results in the death of 2.5 million people in the world annually and causes illness and injury to millions more (The National Council on Alcoholism and Drug Dependence (NCADD), 2013). Alcohol abuse claims more lives than HIV and costs up to 3.3% of the gross domestic product in Western societies (World Health Organization (WHO), 2010). Moreover, long-term alcoholism has been linked to many health concerns such as cirrhosis of the liver and cardiovascular disease. It also affects many individuals from different ethnic and racial backgrounds. All over the world, almost in every family, there will be someone who has suffered, directly or indirectly, from alcohol abuse (Global Status Report on Alcohol and Drugs of Abuse, 2004). Euphoria is one of the tentative effects of alcohol, and it can be either mild like in a case of relief from stress or intense like in a case of uncontrollable happiness. This artificial feeling what keeps alcohol being the most widely used drug in the western societies. In the United States, 4% of the population is affected by the estimated economic burden exceeding \$366 billion a year (Chatterjee et al. 2010). Interestingly, most alcohol users start consuming alcoholic beverages as teenagers. Adolescence is a developmental period characterized by significant transformations that are highly similar across species. Adolescence is also linked with maturation of cognitive capacities, personalities and frontal cortical executive functions (Crews et al., 2007; Gong et al., 2012; Spear, 2000). In addition to neural and hormonal changes, increases in social activity, novelty seeking, impulsivity, and risk-taking are

frequently reported during this transitional phase (Spear, 2000, 2010). Teenagers engage in more risky behaviors than adults, and they are more likely to experiment with alcohol and illicit drugs. It has been shown that human as well as rodent brains demonstrate high sensitivity to alcohol during adolescence (Crews et al., 2000; Slawecki et al., 2004; Spear et al., 2005; White et al., 2004). Collectively, these findings suggest that the teenager brain may possess a unique vulnerability to alcohol, leading to an increase risk for alcohol dependence in adulthood.

The goal of this thesis is to explore in animal models the pharmacological and behavioral mechanisms involved in alcohol dependence in adolescence in the hope of identifying novel mechanisms underlying that lead in an increase in alcohol vulnerability later in life. The following sections of the introduction will provide an overview of the general pharmacology of alcohol, different targets of alcohol, and mechanisms involved in alcohol dependence as well as the pharmacotherapies for alcohol use disorders (AUD). After that, will discuss the animal models of alcohol dependence in general and what do they offer for science. The introduction will then be concluded by reviewing the current approach taken to model the genetic and behavioral aspects of AUDs in humans and animals, followed by a brief summary of the questions that remain in the field and the experimental approach we intend to take to explore these issues.



## 1.2. General pharmacology of alcohol

### ***1.2.1 Different targets of alcohol***

Alcohol (Ethanol = EtOH) is a small highly membrane-permeable mediator that does not have a defined site of action. EtOH yields a wide-range of behavioral and physiological effects in the body. However, it is poorly understood how exactly EtOH acts to produce these effects. EtOH affects multiple targets, changing neurotransmitter systems such as glutamate,  $\gamma$ -aminobutyric acid (GABA), and adenosine; as well as activating the dopaminergic reward pathway. Since EtOH does not have a defined pharmacological site of action, it is unlikely that these interactions involve binding in a classical pharmacological sense to specific ligand site(s). Alcohol may also affect cholinergic neurotransmission. For instance, it enhances the function of several nicotinic acetylcholinergic receptor (nAChR) subtypes, including the  $\alpha 4\beta 2$ - nAChR, while inhibiting the function of others, such as the  $\alpha 7$ -subtype (Davis and de Fiebre 2006). Moreover, alcohol's reinforcing effects may additionally depend on interactions with numerous neurotransmitter systems that are known to modulate neuronal signaling in the reward pathway, involving the opioid, cannabinoid, serotonergic, and histaminergic systems (Chastain 2006). Although drugs of abuse act on different receptor systems, they activate common downstream sequences of events that lead to compulsive drug taking, craving, and relapse which underlie characteristic behavioral phenotypes and these behaviors can be modeled in rodents.

### ***1.2.2 Mechanisms involved in Alcohol Dependence***

Although, historically, alcohol was thought to be a relatively unspecific pharmacological agent, intense study over the past two decades revealed that this drug has a few known primary targets. 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 exposure of ethanol on specific molecular targets followed by numerous indirect effects on a variety of neurotransmitter/peptide systems (Vengeliene et al., 2008). 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 EtOH mediates its action by membrane disruption (lipid theory), evidence has shifted from this view to one that asserts that EtOH has primary targets (protein theory). Specifically, these are ligand-gated ion channels including glutamate (NMDA), GABAA, glycine, 5-HT<sub>3</sub> and nAChR receptors. Studies show that each of these receptors is 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 (> 300 mM). Generally, the receptor class and subtype direct the degree of modulation. In a concentration range sufficient to produce intoxication, ethanol generally inhibits NMDA receptors and L-Type Ca<sup>2+</sup> channels, while potentiating GABAA, glycine, 5-HT<sub>3</sub>, and nicotinic receptors (nAChRs) receptors (Lovinger et al. 1989, 1991; Mihic et al. 1997; Crawford et al. 2007; Narahasi et al.1999). Additionally, the sensitivity of each receptor type to EtOH depends on both subunit composition and EtOH concentration. 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 EtOH 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). The mesolimbic dopamine system (MDS) in particular has been characterized as a neurochemical substrate for reinforcement, with A10 dopamine (DA) neurons identified as a critical component of this effect (Schultz 2007, Wise et al. 1989, 2004). A myriad of animal studies strongly support a significant effect of EtOH on the MDS. In addition to the DA-mediated processes previously mentioned, EtOH triggers the release of cannabinoids and opioids, which have been shown to be important to ethanol's rewarding effects. Furthermore, the mechanisms through which these actions are mediated include processes that are both dependent and independent of the MDS (Koob et al. 2010). The complexities of all these interactions are compounded by the fact that EtOH causes both acute and lasting changes in gene expression due to repeated exposure to the drug. There are countless changes in signal transduction caused by EtOH exposure, but a primary pathway worth highlighting is the cAMP-PKA pathway that involves the activation of cAMP response element binding protein (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, and metabolism (Lonze & Ginty 2002). There are also CREB-independent genes as well as epigenetic effects induced by ethanol. All these aforementioned changes may occur in a regionally specific manner or in the brain as a whole.

### 1.3. Pharmacotherapies for alcohol use disorders

The treatment of alcoholism mostly focused on altering the reinforcing effects of alcohol. Medication development has concentrated on several neurotransmitter systems that interact with the MDS pathway, which can mediate reinforcement. Currently, there are few pharmacological treatments for Alcohol Use Disorders (AUDs) that are approved by the United States Food and Drug Association (FDA) (Mason and Heyser 2010). The oldest, disulfiram, (Antabuse®), is a drug discovered in the 1920s that works by inhibiting the enzyme acetaldehyde dehydrogenase to prevent metabolism of the toxic alcohol metabolite acetaldehyde. Therefore, alcohol consumption while using disulfiram leads to severely aversive acetaldehyde-induced symptoms, such as headache, nausea and vomiting (Chastain 2006). Despite its long track record, scientific evidence for disulfiram's efficacy is restricted. Its relapse-preventing effects are limited and due to disulfiram's aversive effects patient compliance is low (Williams 2005). The second FDA approved medication for the treatment of alcohol-dependence is naltrexone (ReVia®, Depade®, Vivitrol®). It's an opioid receptor antagonist and the main use of naltrexone is for the treatment of alcohol dependence. It has been shown to decrease heavy drinking. Naltrexone decreases the reinforcing effects of alcohol, most likely by modulating DA signaling in the mesolimbic pathway (Chastain 2006; Tambour and Quertemont 2007; Williams 2005). Although naltrexone's long-term effectiveness is much debated, in combination with behavioral counseling it has been shown to have positive effects on drinking outcome (Anton et al. 2006). Furthermore, naltrexone helps patients overcome opioid addiction by blocking the drugs' euphoric effects. Acamprosate (Campral®) is the final and most widely prescribed FDA approved medication for AUDs in the USA (Mason and Heyser 2010). Its mechanism of action is poorly understood, but it is believed to normalize alcohol-induced dysregulation of glutamatergic and GABAergic signalling (Anton et al. 2006; Mason and Heyser 2010). Acamprosate is well tolerated together with additional

medication. In combination with behavioural therapy, it was shown to reduce intake and to prolong abstinence (Mason and Heyser 2010; Williams 2005). However, these medications are effective but they available to cause unpleasant side effects and relapse following treatment (Bouza et al., 2004; Whitworth et al., 1996; Wright and Moore 1990). These issues suggest a need for more effective therapeutic approaches. A new therapy was inspired from the significant interest interaction of nicotine and alcohol. It has been shown that heavy alcohol and nicotine use commonly occur in the same individual (Toneatto et al. 1995). Smoking is more prevalent in heavy drinkers (Mello et al. 1987; Room 2004) and alcohol potentiates nicotine reward (Rose et al. 2004) and self-administration (Mitchell et al. 1995). Furthermore, animal studies indicate that neuronal nicotinic acetylcholine receptors (nAChRs), also contribute to the rewarding effects of alcohol (Bito-Onon et al. 2011). Thus, varenicline (Chanix) a nicotine receptor (nAChR  $\alpha 4\beta 2$  subunit) partial agonist has been approved by the FDA for smoking cessation. It reduces both nicotine reward and ethanol seeking and consumption in rodent models (Chatterjee et al. 2011; Steensland et al. 2007). Consistently, recent human laboratory and open-label studies inform that varenicline can reduce alcohol self-administration in heavy-drinking smokers (Fucito et al. 2011; McKee et al. 2009).

In summary, different pharmacotherapies have been explored for the treatment AUDs have only managed up to a 30% success rate (Spanagel et al. 2009). Thus, there is a clear need for more effective pharmacotherapies in dealing with these disorders. While previous treatments have targeted many of the brain neurotransmitter and neural system 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. However, the common goal of these medications is to reduce the positive effects of alcohol-

intake, ameliorating craving or restoring homeostasis, often by modulating the function of specific receptors or enzymes.

#### 1.4 Animal models of alcohol behaviors

Animal models are extremely useful tools to study genetic and neurobiological mechanisms underlying alcohol behavioral effects. They allow researchers to apply methods that cannot be used in human subject because of the ethical concerns and experimental challenges. However, there is no single animal model that can capture all aspects of alcohol abuse and dependence. Therefore, different models were developed to measure the various aspects of alcohol abuse and dependence from the initial effects of alcohol, acute behavior effects, alcohol drinking, alcohol tolerance, alcohol withdrawal, and physical dependence. Because of the obvious limitation of animal models (variability when considering differences in species or strains of animal used, the method of alcohol administration), results have to be interpreted with caution.

#### 1.5 Animal models of alcohol drinking and alcoholism

The main objective of alcoholism and alcohol abuse research is to investigate why some people drink more than others and why some people who drink develop problems, whereas others do not. It is therefore necessary that animal models of alcoholism satisfy certain criteria in order to investigate these issues. An excellent review by (Cicero, 1979) has proposed the following criteria for animal models of alcohol abuse and dependence: “(1) the animal should orally self-administer EtOH; (2) the amount of EtOH consumed should result in pharmacologically relevant blood EtOH levels; (3) EtOH should be consumed for its post-ingestive pharmacological effects, and not strictly for its caloric value or taste; (4) EtOH should

be positively reinforcing, or in other words, the animals must be willing to work for EtOH; (5) chronic EtOH consumption should lead to the expression of metabolic and functional tolerance; and (6) chronic consumption of EtOH should lead to dependence, as indicated by withdrawal symptoms after access to EtOH is terminated". More recently, a 7th criterion has been added that states an animal model of alcoholism should also display characteristics associated with relapse (McBride and Li, 1998) because alcoholics generally go through episodes of abstinence and relapse. Rodents have been the most widely used animals for studying mechanisms of alcohol drinking. The most common procedure of ethanol intake used in research is self-administration. There are two categories of self-administration models; operant or non-operant procedures. Operant self administration is considered the most direct procedure to evaluate the reinforcing of a substance in which animals are enclosed in an "operant box" consisting of devices that deliver the drug reinforcer (in general, this means to lever press) 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. Operant self-administration has been described by Weeks (1962) as a technique for intravenous self-administration of morphine in the rat. Since then, it has been shown for different types of drug of abuse including heroin, cocaine, amphetamine, nicotine, and ethanol. 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. In this thesis, we have conducted studies with two drinking paradigms: Drinking-in-the-Dark (DID) and two-bottle choice (2-BC) tests. 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 for a few hours (we performed the procedure for 4 hr) into the dark cycle since this is a window of high activity in rodents (Rhodes et al. 2005). Studies show that subjects under these conditions, particularly mice selectively bred to prefer ethanol solutions, will reliably self-administer ethanol to achieve BEC in excess of 1.0 mg/ml, enough to cause measurable behavioral intoxication (Rhodes et al. 2005,2007). On the other hand, in the two-bottle choice paradigm the subject is presented with one water bottle and one ethanol bottle at different concentrations (usually 10, 15 and 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.

### 1.6 Animal models of relapse

As was mentioned above in the criteria for an animal model of alcohol abuse and dependence (Cicero, 1979), animals with a chronic history of alcohol intake should demonstrate relapse of alcohol drinking following a prolonged period of abstinence. The alcohol deprivation effect (ADE) is a phenomenon in laboratory animals that models a relapse – like conditioned drinking behavior. ADE is 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). ADE also, has been described as a temporary increase in alcohol drinking seen after short (24h or less) or long (1 week and longer) intervals of alcohol deprivation (Sinclair and Li 1989). The ADE has been observed across many animal species including rats, mice, monkeys, and humans (Khisti et al. 2006). In ADE 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).

### 1.7 Animal models of Alcohol withdrawal

Alcohol withdrawal is an important aspect of alcohol dependence. In humans and rodents, withdrawal from alcohol results in multiple manifestations that indicate physical dependence and affective signs such as anxiety, depression, craving, and aversion. In humans, alcohol withdrawal symptoms (AWS) can begin within two hours after the last drink. The pathophysiology of AWS involves the disruption of homeostasis between inhibitory and excitatory actions in the central nervous system. Alcohol enhances the inhibitory tone of GABA<sub>A</sub> receptor complexes and inhibits excitatory effects from glutamate on NMDA receptors (Kosten and O'connor, 2003; Kattimani and Bharadwaj, 2013; and Hoffman et al., 1992). As was mentioned in the different targets of alcohol section (sec.1.2.2), GABA is the major inhibitory neurotransmitter of the brain. Chronic consumption of ethanol leads to a decrease in endogenous neuronal GABA release, a down-regulation of GABA<sub>A</sub> receptors, an up-regulation of NMDA receptors, and increased production of glutamate. The abrupt cessation of alcohol results in a net excitatory state and the clinical signs and symptoms of AWS (Kosten and O'connor, 2003; Kattimani and Bharadwaj, 2013; Sarff and Gold, 2010; and Trevisan et al., 1998). AWS usually develops within several hours to a few days of cessation. Some withdrawal symptoms such as insomnia, mild anxiety and tremors can happen while the individual still has a measurable blood alcohol level, yet most occur after alcohol has left the body. Depending on the severity of the level of alcoholism, withdrawal symptoms from alcohol generally last from several days to several weeks and in some cases months.

Rodent models of alcohol withdrawal have been extremely useful for investigating the factors that influence both risk and severity of alcohol withdrawal.

### 1.8 The mouse as an animal model to study alcohol behaviors

Choosing the appropriate animal model is an important factor to consider when employing the methods described above. The advantage 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). Therefore, C57BL/6 (B6) and DBA/2 (D2) mice are particularly highlighted in alcohol studies and 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 and Loss of Righting Reflex (LORR) making them good models of ethanol sensitivity (Phillips et al. 1995, Metten & Crabbe, 1994, Crabbe et al. 2003, 2006, Cunningham et al. 1992).

For our studies, we chose the C57BL/6J model for several reasons. First, highly backcrossed mice from the C57BL/6J background are, genetically, nearly homogenous and there is much phenotypic data available for wild-type C57BL/6J mice (Lim et al. 2012). Second, mice from this background display high preference for EtOH solutions and have long been documented to readily self-administer relatively high quantities of EtOH (McClearn et al. 1959).

A third important advantage of using C57BL/6J mice is the ability to use gene-targeting methods. We also employed DBA/2J in chapter3 of this thesis in which we were investigating the effect of the strain difference in the enhancement of alcohol consumption. By testing two different strains (C57BL/6J and DBA/2J) under standardized environmental conditions, our observation suggests that genetic factors may be regulating this enhancement.

Building on all of that, there are still several unanswered questions remaining in the field that we are investigating in the following chapters of this thesis such as, is the phenomena of enhancement of alcohol intake unique to adolescence period of life, and does the exposure patterns (continuous access “CA, daily” vs. intermittent access “IA, every other day”) of ethanol will have an impact later on life.

## **CHAPTER TWO: Adolescence and Alcohol use disorders**

### 2.1 Adolescence and binge drinking

Adolescence is the period of physical and psychological development from the beginning of puberty to complete growth maturation. It is characterized by behavioral, hormonal and neural changes in humans as well as nonhuman mammals (see Spear, 2000 for a review). The behaviors characteristic of both human adolescents as well as adolescent rodents is increasing in risk-taking and social interactions (Primus and Kellogg, 1989; Spear et al., 1980; Vanderschuren et al.,

1997). Thus, adolescence is a period of high vulnerability to alcohol use and abuse.

## 2.2 Overview of brain development

Brain development is a mingle of growth and recession interactions. Understanding of adolescent brain development continues to escalate rapidly. Imaging technologies such as Magnetic resonance imaging (MRI) have proved valuable for detailing the brain size (Lenroot and Giedd, 2006; Shaw et al., 2006) at different ages. Studies using this technology indicate that cortical development correlates with cognitive development. During childhood, sensory systems mature before association cortices, which are responsible for language skills and spatial attention, while higher-order association areas, including the prefrontal cortex (PFC), continue to develop into adolescence (Casey et al. 2005; 2008). Over the past decades, studies showed that adolescence is a crucial period of morphological, biological and functional changes. (Spear, 2010). Although the human brain and behavior are more complex than other species, the relevance of studies using simple mammalian models of adolescence is assisted with considerable of similarities in behavior and biology have seen across species. However, the basics concepts of brain structure and function have been conserved (Allman, 2000). By the time we reach adolescence, the total volume of our brain changes little and significant morphological changes are ongoing. Grey matter volume develops according to an inverted U-shape, with peak values at the beginning of adolescence, followed by a gradual decline into adulthood (Bava and Tapert 2010; Casey et al. 2005; 2008). These changes, which are seen in humans and rodents, are an indication of ongoing remodeling of neuronal connections throughout the PFC, limbic and hippocampal regions. These brain regions are involved in reward processing and development of emotional. Indeed, the brain is one of the major target organs of

alcohol. Excessive alcohol exposure can lead to structural and functional changes of the brain (Oscar and Marinkovic, 2007; Lebel et al., 2011; Zahr et al., 2011; Schulte et al., 2012), resulting in disorder of the central nerve system (CNS) activity, as well as deleterious influence of body function (Gao and Bataller, 2011; George and Figueredo, 2011; Giacosa et al., 2012).

### 2.3 Impact of early alcohol abuse

Binge drink has been well documented among adolescent humans more than any other age group. This early alcohol use has been shown to increase the risk for alcohol- related problems later in life. Indeed, large longitudinal population studies find that the earlier the age of drinking onset the greater the risk of lifetime alcohol use disorder (Zhu et al., 2010) and lifetime alcohol related violence and injuries (Hingson and Winter, 2003; White et al., 2011).

Previous studies focused on some risk factors associated with increased alcohol drinking and alcohol dependence in adulthood after being exposed to alcohol during adolescence. 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 of developing alcoholism in the future (Grant & Dawson 1998, Crews et al.2000, Slawewski et al. 2004, Spear et al. 2005). As mentioned above, teenagers are most engaged in binge drinking, which is the most common manner of alcohol consumption among teenagers who drink alcohol.

Binge drinking is defined as a pattern of drinking alcohol that brings blood alcohol content (BAC) to 0.08g percent or above (or 80 mg%). For the typical adult, this pattern corresponds to consuming "five or more drinks (male), or four or more drinks (female), in about 2 hours"(see Spear, 2000 for a review). Binge drinkers are more likely than non-binge drinkers

also to experiment with illicit drug use. In 2009, there was 41.8% among high school students was consuming alcohol, and 24.2% of them were reported in binge drinking (Vital Signs, 2010). Recent work has indicated that binge drinking during the adolescent years may enhance the risks for negative outcomes in adulthood that include health problems, low educational achievement, and social difficulties (Norstrom and Pape, 2012). Major concern is that adolescents who are binge drinking might set the stage for unhealthy drinking patterns later in life.

#### 2.4 Rodent models of adolescent alcohol exposure

Despite their limitations (such as modeling the social, psychological and economic factors in human behaviors), animal models allow the possibility of exploring and investigating biological and brain mechanisms without the ethical constraints of human studies. The term “adolescence” in rodents is used to describe animals aged from weaning postnatal day (PND21) to adulthood (PND60); animals that are no longer infants, but that are not yet adults (Laviola et al., 2003). The period of adolescence also subdivided into more into early (PND21– 34), middle (PND34–46), and late (PND46–59) adolescence (Spear, 2000). Many of the behaviors in adolescent rodent models believed to be trademarks of human adolescence, such as elevated levels of social play and affiliative behaviors (Meaney and Stewart, 1981; Panksepp, 1981). These behaviors peak at PND32 and exhibit an ontogenetic shift to more aggressive and competitive behaviors (Terranova et al., 1993; 1998). In this thesis, we used mice models to study the impact of adolescent alcohol exposure and how it may contribute to the study of alcohol intake and behaviors later in adulthood.

#### 2.5 Previous studies: adolescence and alcohol intake

Epidemiological studies elucidate that adolescence is a developmental period and specially vulnerable to alcohol use and abuse (Yamaguchi and Kandel, 1984; Robins and Przybeck, 1985; Deykin, 1987; Grant and Dawson, 1997; Hawkins, 1997). Alcohol consumption during adolescence predominantly involves heavy drinking patterns such as binge drinking (Johnston et al., 2006). High averages of alcohol consumption have been reported for human and rodent adolescents in comparison with adults (Bates and Labouive, 1997 and Doremus et al., 2005). The majority of studies in laboratory animals looking at EtOH exposure during adolescence and later EtOH drinking in adulthood have found an enhancement in alcohol consumption. Male rats were found to be sensitive to passive social influences that mediate voluntary EtOH consumption and early EtOH exposure induced long-term changes in EtOH responses later in life (Antoniette et al., 2009). Other studies examining free-choice ethanol exposure during adolescence and later operant self-administration of EtOH in P rats, reported that P rats exposed during adolescence to alcohol acquired EtOH faster in adulthood and showed more ethanol-seeking behavior as adults than naive P rats (Rodd-Henricks et al., 2002a). In a companion and control study, adult P rats given free-choice pre-exposure beginning in adulthood and tested later did not show increased ethanol seeking in an operant paradigm (Rodd-Henricks et al., 2002b). In another study comparing 24-hour drinking access of adolescent and adult P rats aged of 4-week, adolescent male rats showed an increase in ethanol licking behavior across weeks than adult (Bell et al., 2006). However, forced administration of ethanol vapor or forced consumption during adolescence did not show enhancement of the intake in adult Sprague–Dawley rats (Slawecki and Betancourt, 2002; Tolliver and Samson, 1991).

Based on all of that, in our studies we characterized the impact of alcohol in C57BL/6J adolescent mice on the overall intake and consumption. We tested if the sex of animals (male vs.

female), as well as pattern of exposure (continuous access “CA, daily” vs. intermittent access “IA, every other day”) by using DID procedure, will impact alcohol consumption later in life. We also investigated the impact of adolescence exposure to alcohol on alcohol drinking later in life using the 2-bottle choice procedure (2-BC). Furthermore, we studied the alcohol deprivation effect (ADE) by testing the impact of alcohol exposure during adolescence on alcohol intake in adulthood after one week of deprivation. Lastly, we investigated possible pharmacological and behavioral mechanisms that could be possibly involved in the increase in alcohol intake later in life by measuring the aversive state in the withdrawal test such as Forced Swim Test (FST) as well as measure the rewarding effect of alcohol using Conditioned Place Preference (CPP) later in adulthood

## 2.6 Dissertation hypothesis

We hypothesize that repeated exposure to alcohol in adolescent mice will enhance alcohol intake later in adulthood and this enhancement is unique to adolescence period.

## 2.7 Dissertation significance and objectives

The research in this thesis focuses on the impact of adolescent alcohol exposure on the subsequent behavioral responses by using C57BL/6J. Based on our preliminary data and previous literature, we hypothesized that exposure of adolescent mice to alcohol will lead to increased alcohol intake later in life and that phenomena of enhancement is unique to adolescence phase. Our first specific aim was to determine if exposure to alcohol during early adolescence will increase alcohol intake later in adulthood using well-established mouse models of alcohol drinking (DID). The second specific aim was to determine if this enhancement of



alcohol intake is unique to adolescence period by using the same administration model. The third specific aim was to examine whether the enhancement of alcohol is strain dependence. The fourth aim was to determine if exposure to alcohol will increase alcohol intake using 2-bottle choice paradigm. Our fifth and final aim was to elucidate the changes in ethanol sensitivity (negative and positive) effects of ethanol.

Using experimental mice behavioral models, the overall objective of this project is to extend the current knowledge on this area by approaching alcohol abuse from two different angles. One set of experiments explores adolescent vulnerability to alcohol exposure. Investigating how a history of alcohol-use in adolescent mice model affects performance of mice behavioral in alcohol taking and seeking in later adulthood. Employing the same behavioral models, a second set of experiments is to study the changes in alcohol sensitivity under conditions in which our experiments were performed to explore the rewarding and positive effects as well as aversive and negative effects of alcohol. We assessed two patterns (continuous access “CA, daily” and intermittent access “IA, every other day”) of binge drinking behavior in adolescent mice using limited access ethanol drinking in the dark (DID) that mimics the behavior of binge drinking in which some human adolescence engage, as well as models of voluntary ethanol consumption, 24-h two bottle choice preference, with choice between ethanol and water in male and female mice. We also conducted testes in these mice in ethanol induced hypnosis such as Loss Of Righting Reflex (LORR), anxiety such as Elevated Plus Maze (EPM) and Light and Dark Boxes (L/D boxes), and lastly, measure the EtOH rewarding effect using Conditioned Place Preference (CPP).

## **CHAPTER THREE: Impact of ethanol in adolescent mice on alcohol intake later in life**

### 3.1 Introduction

Adolescence is a stage of elevated alcohol consumption in humans as well as in animal models. It is the time that most individuals first experience alcohol exposure. Binge drinking is common during adolescence. Adolescence is a crucial time period of cognitive, emotional, and social maturations and ethanol exposure during this period may affect these complex processes. There are major changes that occur during adolescence in the brain morphology including the selective removal of ~40-50% of the synapses in cortical and subcortical brain regions (Johnston, 1995; Lidow et al., 1991; Seeman, 1999, Van Eden, 1990), continued myelination of cortical regions (Giedd et al., 1996; Sowell et al., 1999), and alterations in receptor levels and sensitivity in various neurotransmitter systems during this time (Gould et al., 1991; Lidow et al., 1991; Hamano et al., 1996; Nunez et al., 2000). The similarities in biological and behavioral features across adolescents of different species aid scientists to use adolescent animal models when examining neural and environmental contributors to adolescent characteristic functioning. Undoubtedly, the full complexity of human brain and behavioral function during adolescence or at any other stage in life cannot be completely modeled in other species. Therefore, validity of any adolescent model demands cautious consideration and the results have to be interpreted carefully. Previous studies have shown that adolescent rodents consume more ethanol compared to adults. For instance, a study have been done by Vetter et al., (2007) showed that Sprague–Dawley rats consume approximately 2 times more ethanol on a g/kg basis than adult animals in a 2- bottle choice free-access paradigm. Another study by Holestein et al., (2011) using B6 mice, showed a significant increase in ethanol intake compared to adults animals in the DID paradigm.

The purpose of our studies is to determine the impact of alcohol intake during adolescence on alcohol consumption later in adulthood. We also determined if the pattern of alcohol intake as well as the sex differences had an impact of long- lasting effect of alcohol during adolescence exposure.

### 3.2 Materials and Methods

#### *Animals*

Adolescent and adult male and female C57BL/6J mice from Jackson Laboratories (Bar Harbor, ME, USA), age three weeks (adolescent, PND28) averaged weight 13g, and nine weeks (adult, PND70) averaged weight 25g at the beginning of the experiments. We controlled for the litter effect by ordering mice from different litters, which were further divided so that each testing group is comprised of mice from different litters. The animals arrived at our laboratory at the age of 3 weeks old (PND21). We house the animals in an out-of-the-vivarium (OOV) space individually in standard Plexiglas cages with corn-cob bedding and food and water available ad libitum, except where noted in individual experiments. Cages also contained a small cardboard tubes and a nestled for the purpose of environmental enrichment. We keep the animals in the OOV space under a 12-hour inverted light/dark cycle in which animals are exposed to light from 6 PM to 6 AM, and then darkness from 6 AM to 6 PM. The exact time could be adjusted due to lab personnel schedules, but 12 hours is still maintained once the study begins. All experiments were performed during three hours into the dark cycle at which mice will be given only ethanol to consume for 4 hours. At the end of this period, the ethanol tubes are replaced with water tubes and the volume of ethanol consumed is recorded. Institutional Animal Care and Use Committee of Virginia Commonwealth University approved the study. All studies were carried out in

accordance with the National Institute of Health Guide for the Care and Use of Laboratory animals.

### ***Drugs***

Ethanol (200 proof) was diluted with tap water and prepared as a 20% (v/v) solution. The ethanol bottle was purchased from Fisher Scientific Laboratory supplies and equipment.

### **3.3 Drinking in the dark (DID) procedure**

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%). We employed a modified version of the standard DID procedure, which results in alcohol intakes between 6.42-7.85 g/kg alcohol in a 4-h period and yields blood ethanol concentration (BEC) more than 80%g (Rhodes et al., 2005). For this procedure, mice were housed individually with 25ml sipper tubes containing tap water for one week of a habituation in a temperature ( $23 \pm 2$  °C) and humidity controlled room under a 12-hour light/dark cycle, 6:00 a.m.-6:00 p.m. as was described above. This inverted light cycle is managed by using an external timer that powers an incandescent light for the room. We used a separate red light for visibility when we worked with the animals during the dark phase. Subjects were never fluid restricted, and water and food were freely available. The procedure was a modification of the experiment described by Rhodes et al., 2005, with mice having either continuous access (CA, daily) or intermittent access (IA, every other day) of 20% EtOH in a 4-h period for nine days. Three hours after light out, home cage water 25ml tubes were replaced with sipper tubes containing a 20% (v/v) EtOH in tap water, and mice were allowed to drink for 4h. Each day, initial volumes were recorded, and 4h later the final volumes were recorded. Mice were weighed after recording the final volume of alcohol intake to avoid

stressing the animal before offering ethanol. Control mice received an identical sipper tube of tap water.

### 3.4 Two bottle choice (2-BC) procedure

Mice were never fluid restricted, and water and food were freely available. Subjects were acclimated to the test room with cages containing two 25-ml pipettes filled with tap water. At the end of the acclimation week, one water tube was replaced with 5% (w/v) EtOH with 24-hr access for three days. To determine the effects of ethanol concentrations on adult mice intake and preference, the ethanol concentration was increased incrementally by 5%(w/v): 5%(w/v) for 3 days, 10% (w/v) the next 3 days and then 15% (w/v) the last 3 days. Each day, initial volume was recorded, and then 24h later the final volume was recorded as well. After recording the final volume of alcohol intake, mice were weighed to avoid stressing the animal and to accurately calculate ethanol intake. The position of the water and ethanol tubes was switched every day to avoid side preference. Intake was reported as g/kg ethanol consumed while the preference was calculated as the ratio between ethanol consumed divided by the total ethanol and water intake.

### 3.5 Blood Ethanol Concentration (BEC) Analysis

35-50µl-blood sample was taken via cheek bleeding using 5mm Lancets (Medipoint, Inc., Minenola, NY). Ethically, blood collection will not exceed 20% of total blood volume and collections will be after day nine of alcohol exposure during the adolescence period (PND36). The blood was stored in BD microtainers and analyzed using Gas Chromatography similar to a previously described procedure (Gallaher et al. 1996). Animals received food/water ad libitum after blood collection and injected with saline according to their weights to avoid hypovolemic.

### 3.6 Experimental design

#### ***DID for adolescents C57BL/6J mice***

To determine if exposure to alcohol during early adolescence will increase alcohol intake later in adulthood using DID procedure. Male and female B6 at PND28 were exposed to 20% alcohol in the DID procedure for nine days with either continuous access (CA, daily) or intermittent access (IA, every other day). There were a total of eight groups with n=7 per group. The first four groups were all male C57BL/6J mice and the last four were all female C57BL/6J mice. They were divided as follows: Group 1 was given 20% alcohol in the DID procedure for nine days CA at PND28, then the same procedure was repeated under the same conditions at PND72 (36 days of abstinence). Group 2 (control adult group) was given 20% alcohol in the DID procedure for nine days CA at PND72 and then tested at PND108 (36 days of abstinence) n the same procedure. Group 3 was given 20% alcohol in the DID procedure for nine days IA at PND28 and then repeated under the same conditions at PND72 (36days of absence) Group 4 (control group) was given 20% alcohol in the DID procedure for nine days IA at PND72 only. Group 5 was given 20% alcohol in the DID procedure for nine days CA at PND28 and then repeated under the same conditions at PND72 (36 days of abstinence). Group 6 (control group) was given 20% alcohol in the DID procedure for nine days CA at PND72 only. Group 7 was given 20% alcohol in the DID procedure for nine days IA at PND28 and then repeated under the same conditions at PND72 (36 days of abstinence). Group 8 (control group) was given 20% alcohol in the DID procedure for nine days IA at PND72 only (table1).

Mice were allowed to drink for 4h and then the alcohol tubes were removed and the home cage water tubes were replaced. Subsequently, after these nine days of exposure animals were kept in their home cages individually for 36 days with access ad libitum to food and water until they reached adult period (PND 72) and then all groups were exposed to 20% g/kg EtOH under the same conditions. In the case of control group, mice were only exposed during adult period (PND72) to 20%EtOH in both patterns (CA/IA) as well as sex (male/female). A one –way repeated measures ANOVA with post hoc analysis were applied. Further, analysis of average exposure using two- way repeated measures ANOVA (treatment × access) was applied.

**Table (1):** Experimental groups of ethanol intake during first and second phases of ethanol exposure.

Sex	First Exposure	Second Exposure
Male	CA at PND28	CA at PND72
Female	CA at PND28	CA at PND72
Male	IA at PND28	IA at PND72
Female	IA at PND28	IA at PND72

***DID for adult /adult control mice***

To determine if the enhancement of alcohol intake is unique to adolescence period using DID procedure. Male and female B6 at PND 73 were exposed to 20% alcohol in the DID procedure for nine days with continuous access pattern (CA, daily). There were a total of four groups with n=7 per group. The first two groups were male C57BL/6J mice and the last two were Female B6 mice. They were divided as follows: Group 1 was given 20% alcohol in the DID

procedure for nine days CA at PND73, and then repeated under the same conditions at PND117 (after 36 days of absence). Group 2 (control group) was given 20% alcohol in the DID procedure for nine days CA at PND117 only. Group 3 was given 20% alcohol in the DID procedure for nine days CA at PND73 and then repeated under the same conditions at PND117 (after 36 days of abstinence) Group 4 (control group) was given 20% alcohol in the DID procedure for nine days in continuous access pattern (CA) at PND117 only.

Mice were allowed to drink for 4h and then the alcohol tubes were removed and the home cage water tubes were replaced. Subsequently, after these nine days of exposure animals were kept in their home cages individually for 36 days with access ad libitum to food and water until they reached PND117 and then all groups were exposed to 20% g/kg EtOH under the same conditions. In the case of control groups, mice were only exposed during PND117 to 20%EtOH in CA.

### ***Two bottle choice (2-BC)***

To determine if exposure to alcohol in adolescence will increase alcohol intake using two bottle choice (2-BC) procedure. The 2-BC paradigm is a voluntary procedure at which the subjects are not forced to drink alcohol. Male C57BL/6J were housed individually (procedure described 3.4 section in details). At PND72 mice had 24h free access to both ethanol and water. The EtOH concentration was ramped up as 5, 10 and 15%. Each concentration was performed for three consecutive days. In each day after recording the measurement the tubes were switched to avoid side preference. Followed by one week of deprivation, mice had a free access to 15% EtOH and water for 24h for 7days continuously. EtOH intake was reported as g/kg and EtOH preference was reported as the ratio between EtOH consumption divided by the total of EtOH



and water fluid intake. Subjects were weighted daily to accurately calculate ethanol intake.

### 3.7 Statistical Analysis

Data were analyzed using prism (6). An unpaired t-test (two-tailed) or a two –way repeated measures ANOVA with Bonferron’s post hoc multiple comparison test were applied (time ×sex) /(treatment × access). However, due to the fact that prism does not determine the progression time effects, the effect of time showed in figures 1, 3, 7 and 8 should be interpreted cautiously.

### 3.8. Results

#### ***DID for adolescents C57BL/6J data***

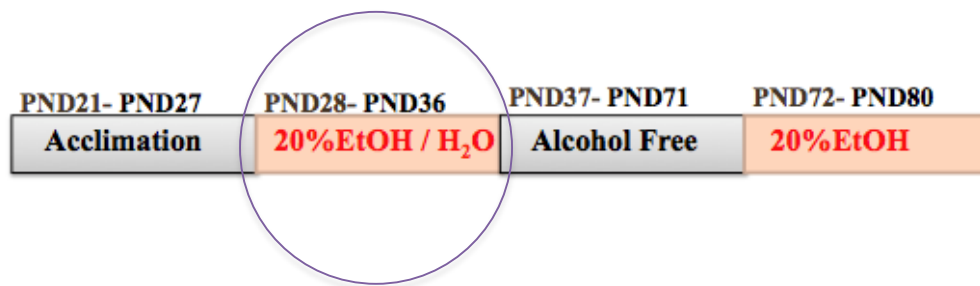
The first experiment was to determine if exposure to alcohol during early adolescence will increase alcohol intake later in life by testing two patterns of exposure (CA/IA) during adolescence (from PND28 to PND36) in male and female mice using DID procedure. In continuous access (Figure 1A) a two way repeated measures ANOVA was performed and revealed significant effect of time [F (8, 96) = 3.641, p = 0.0010], sex [F (1, 12) = 10.78, p = 0.0065, Bonferron’s post-hoc =0.01] and no significant interaction [F (8, 96) = 1.207 p = 0.3032]. In intermittent access (figure 1B) a two way repeated measures ANOVA was performed and revealed significant effect of time [F (4, 48) = 8.958, p< 0.0001], but no significant effect of sex [F (1, 12) = 3.677, p = 0.0793] or interaction [F (4, 48) = 2.161, p = 0.0876].

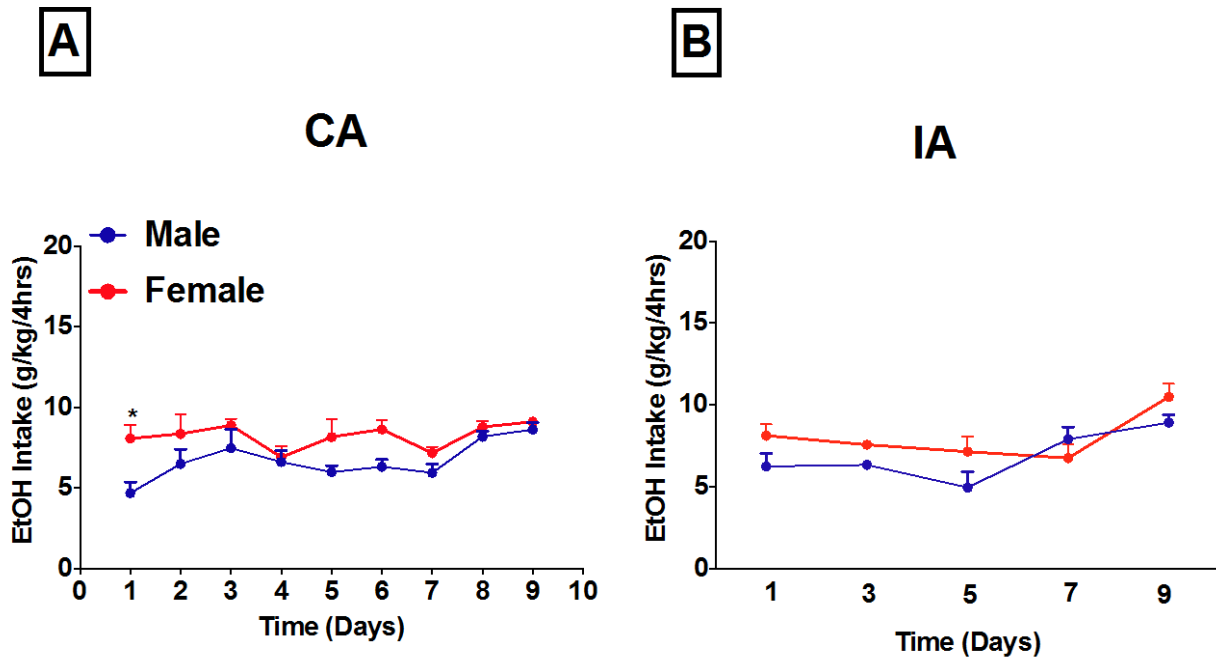
For the average daily intake for all days in CA, we found that adolescent female consume was significantly higher alcohol than male (figure 2A; p= 0.0096) which was reported previous studies for this procedure (Metten,et al.; 2011). However, for the average daily intake for all days in IA, there were no sex differences (Figure 2B; p=0.2530).

Subsequently, in the second phase of the exposure (from PND72 to PND80) the CA pattern in male showed higher intake of alcohol for the group that was exposed to alcohol during early adolescence (Figure 3A). A two way repeated measures ANOVA was performed and revealed significant effect of time [F (8, 104) = 4.753, P < 0.0001] treatment [F (1, 13) = 19.51, P = 0.0007] and with no significant effect of interaction [F (8, 104) = 1.745, P = 0.0966]. Bonferron's Post- hoc analysis revealed a significant difference of treatment on all days except for day 3 (p<0.05). In the IA pattern in males (Figure 3B), a two way repeated measures ANOVA was performed and revealed no significant effect of time [F (4, 52) = 0.4615, P = 0.7636] but there was a significant effect of the treatment [F(1,13) = 13.44, P = 0.0028] and interaction [F (4, 52) = 4.714, P = 0.0025]. Bonferron's Post- hoc analysis revealed a significant difference of treatment on only day 3 and 5 (p<0.001). In the CA pattern in female showed higher intake of alcohol for the group that was exposed to alcohol during early adolescence (Figure 3C). A two way repeated measures ANOVA was performed and revealed significant effect of time [F (8, 104) = 4.299, P = 0.0002] treatment [F (1, 13) = 29.13, P = 0.0001] and no interaction [F (8, 104) = 1.242, P = 0.2822]. Bonferron's post hoc analysis revealed a significant effect of treatment on all days except for day 3 and 9 (p<0.05). In the IA pattern in females (Figure 3D), a two way repeated measures ANOVA was performed and revealed significant effect of time [F (4, 48) = 3.379, P = 0.0163], treatment [F (1, 12) = 7.549, P = 0.0177] and interaction [F (4, 48) = 4.835, P = 0.0023]. Bonferron's post- hoc analysis revealed a significant difference of treatment on only day 5 (p<0.01).

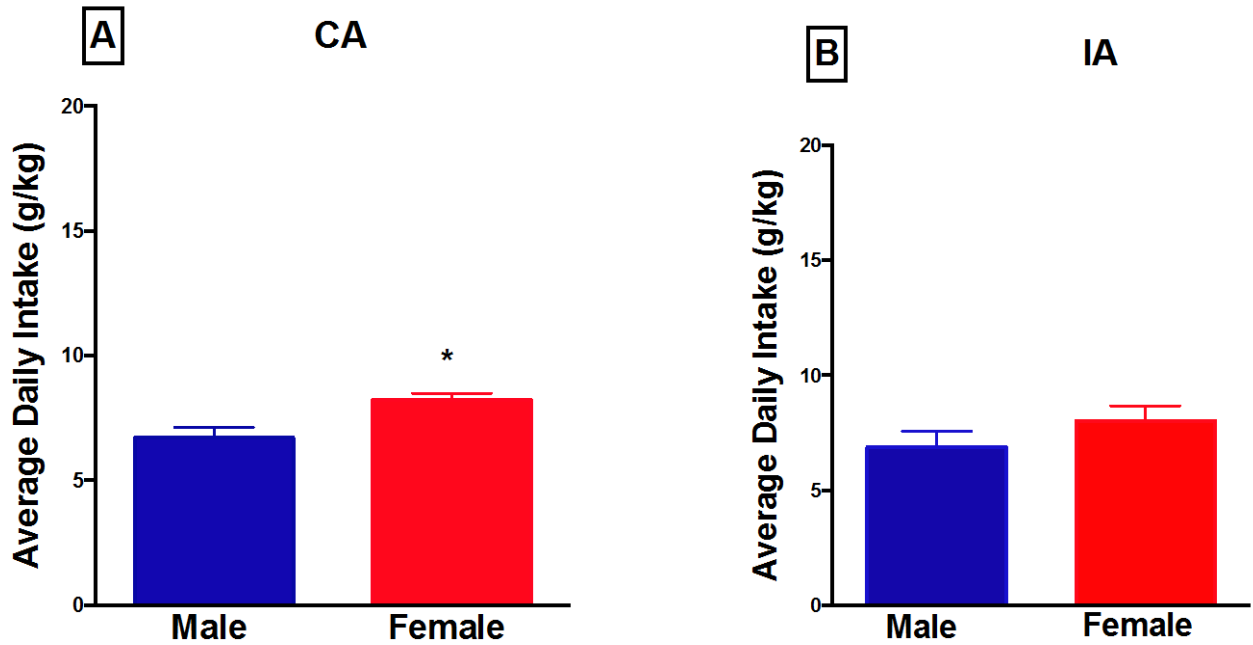
In fact, when we calculate the Area Under the Curve (AUC), we used unpaired t-test (two-tailed) and we found that exposure to alcohol during adolescence enhances alcohol intake later in adulthood. T-Test was performed and both male and female results showed animals that

were exposed to alcohol (EE) during adolescence are significantly different from the animals that were only exposed to alcohol during adult. (Figure 4).

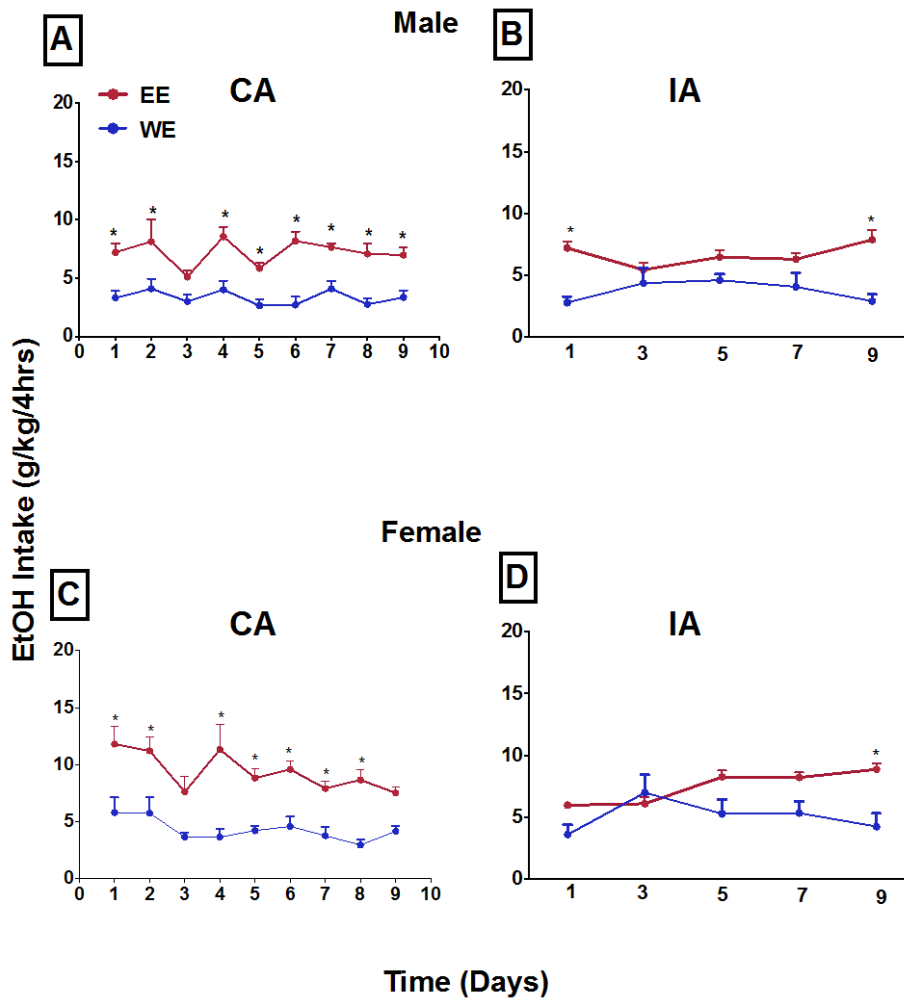
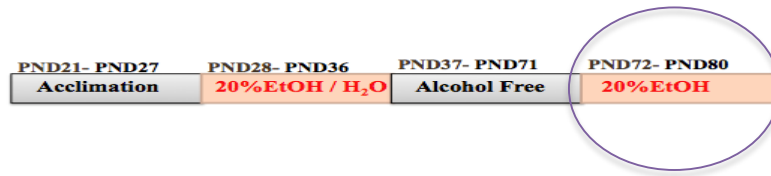




**Figure (1):** Daily intake profile of EtOH in the first exposure (PND28 to PND36) to 20% EtOH during adolescence using DID procedure for 9 days. EtOH consumption in both male and female is relevantly stable with trend to increase later on in both patterns. Shown are mean EtOH intake values  $\pm$  SEM. Panel (A) depicts the ethanol intake in g/kg in 4hrs in CA pattern while panel (B) depicts the EtOH intake in g/kg in 4 hrs. in IA pattern (N=7 per group).

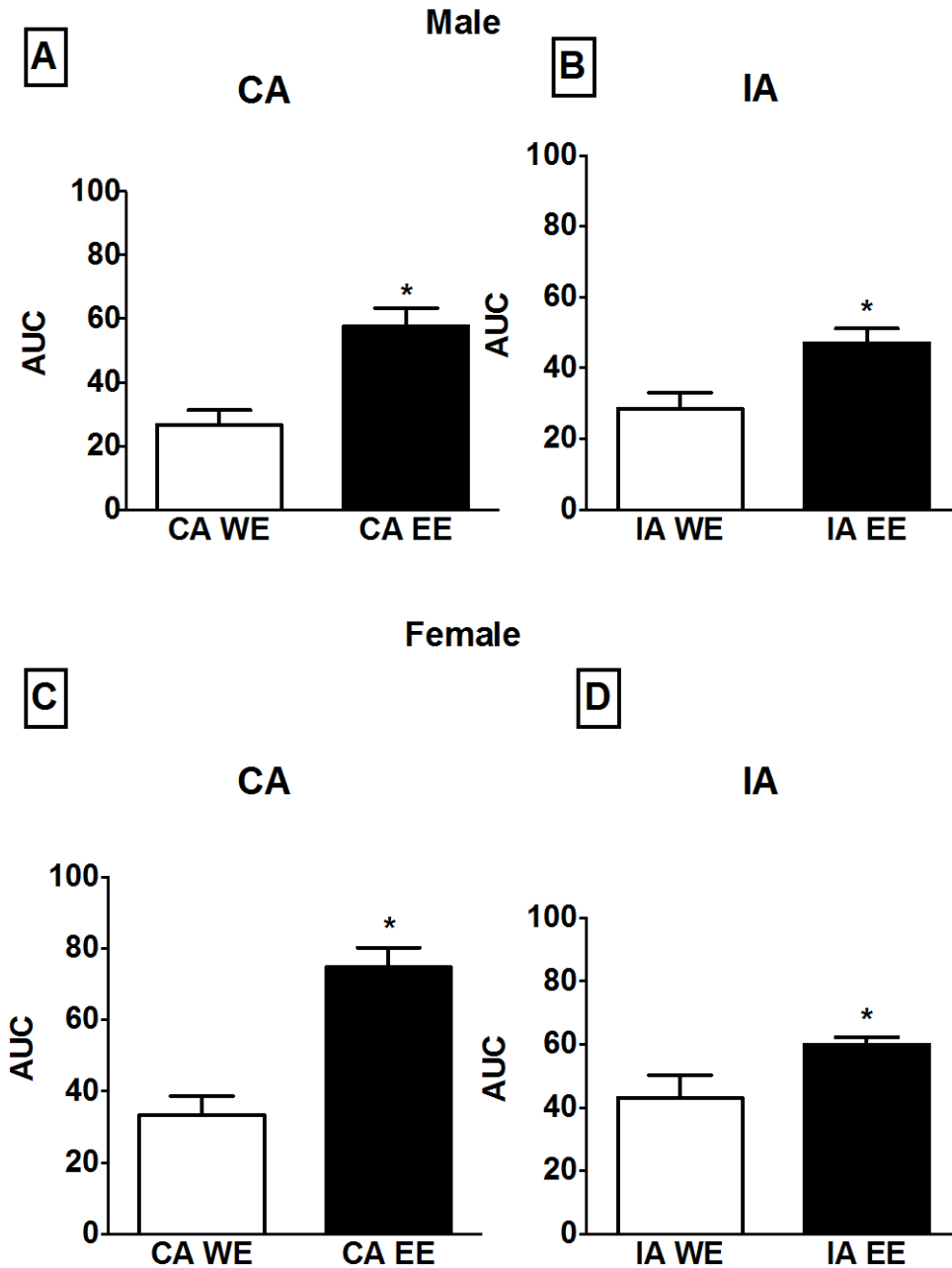


**Figure (2):** Average Intake profile of EtOH in the first exposure from PND28 to PND36 exposure to EtOH during adolescence. Shown are mean EtOH intake values  $\pm$  SEM. Panel (A) depicts female consume significantly higher ethanol than male in the CA pattern whereas Panel (B) depicts female mice have tendency to consume more alcohol than male. (N=7 per group).



**Figure (3):** Daily intake profile of EtOH in the second phase of exposure (PND72 to PND80) to 20% EtOH during adolescence. Shown are mean EtOH intake values  $\pm$  SEM. Panels A and C depict the ethanol intake in g/kg in 4hrs of CA using DID procedure for 9 days in male and female B6 respectively, whereas Panel B and D depict the ethanol intake in g/kg in 4hrs of

intermittent IA using DID procedure for 9 days in male and female C57B/6J, respectively. (N=7 per group).



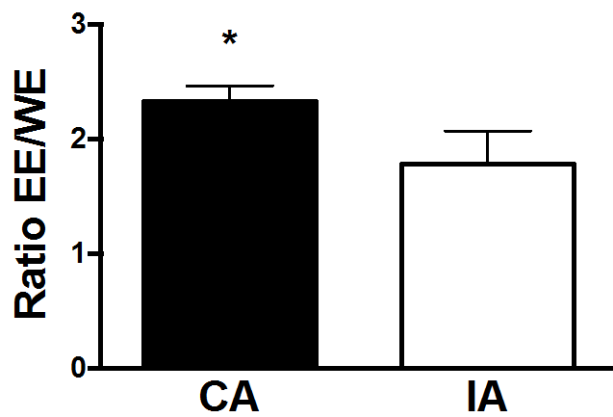
**Figure (4):** Area Under the Curve (AUC) for EtOH exposure during adult. As depicted in the figures A, B, C, and D, animals that were exposed to alcohol (EE) during adolescence are

significantly different from the animals that were only exposed to alcohol during adult. Shown are mean values  $\pm$  SEM. Panels (A and B) depicts male group whereas Panels (C and D) depicts female group. (N=7 per group).

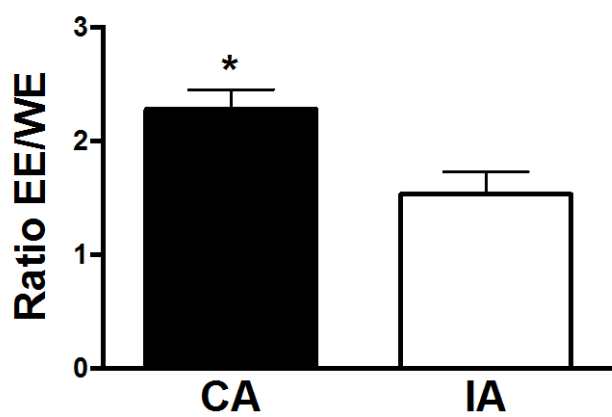
Further analyses were performed using unpaired t-test (two-tailed) with equal SD to investigate the impact of patterns (CA vs. IA) of alcohol exposure as well as the sex differences (male vs. female) during adolescence. We determined the ratio of second exposure verses first exposure for each day; we found that adolescent male mice in CA consume significantly higher alcohol comparing with male mice in the IA (Figure 5A; P= 0.0424). Also, female mice consumed significantly higher alcohol in CA comparing with IA (Figure 5B; P= 0.0176). However, there were no sex differences within the CA (Figure 6A; p= 0.8356) as well as within the IA (Figure 6B; p= 0.4945).



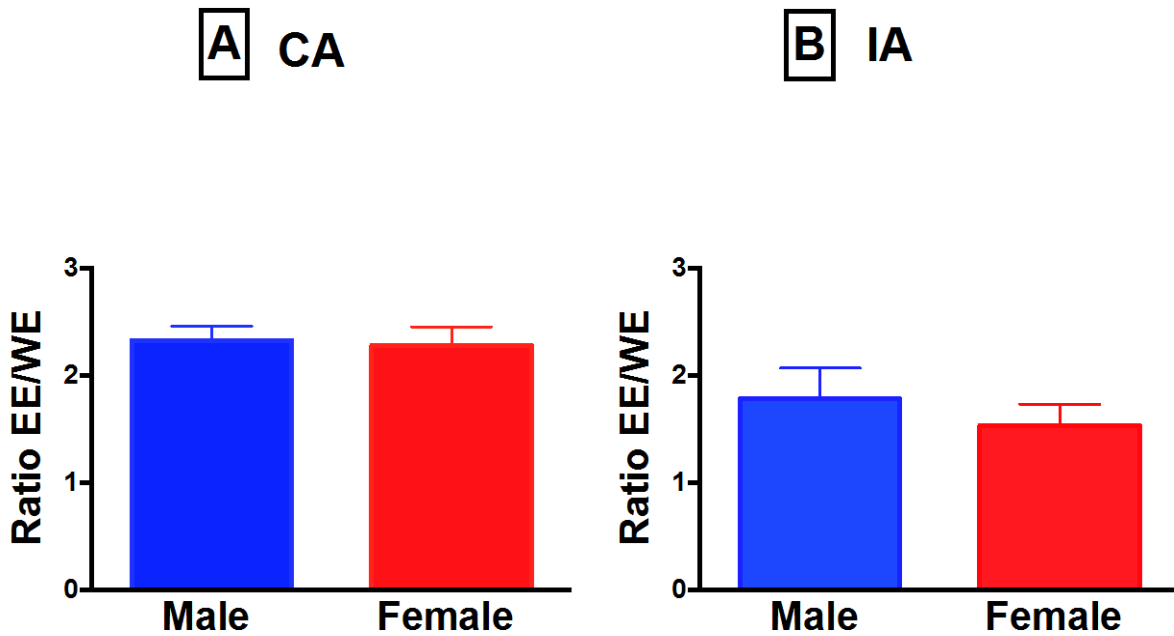
**A** Male Mice



**B** Female Mice



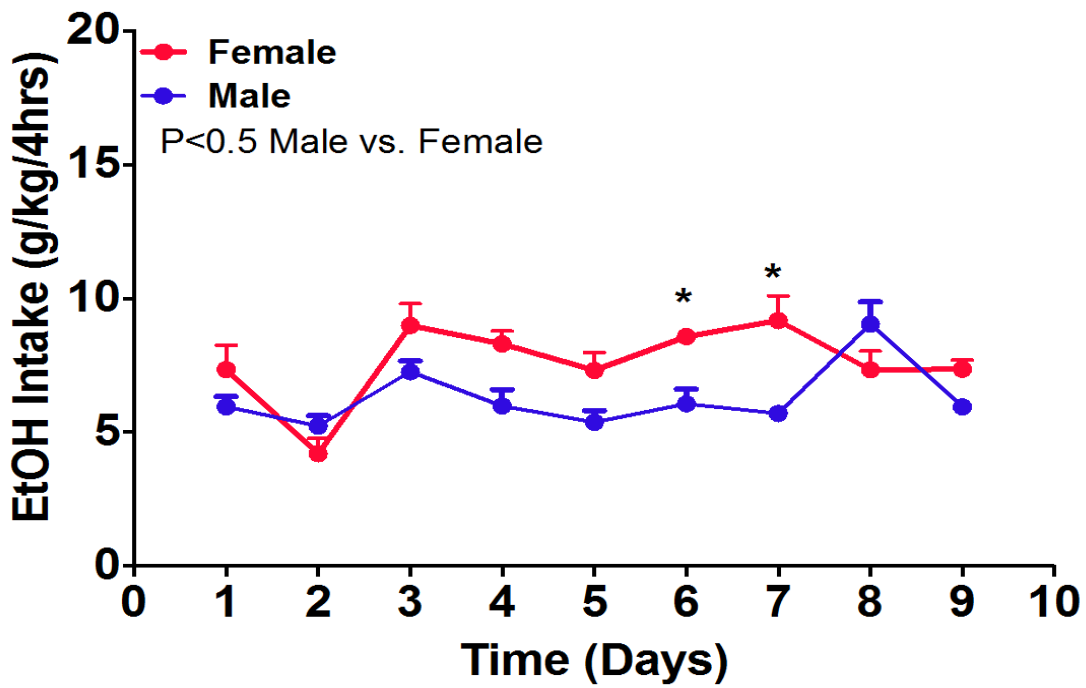
**Figure (5):** Patterns (CA/IA) of alcohol exposure has an impact on the enhancement of alcohol intake Shown are mean ratio values  $\pm$  SEM. In both panels (A, male) and (Female) the CA pattern is significantly higher than IA pattern. (N=7 per group).



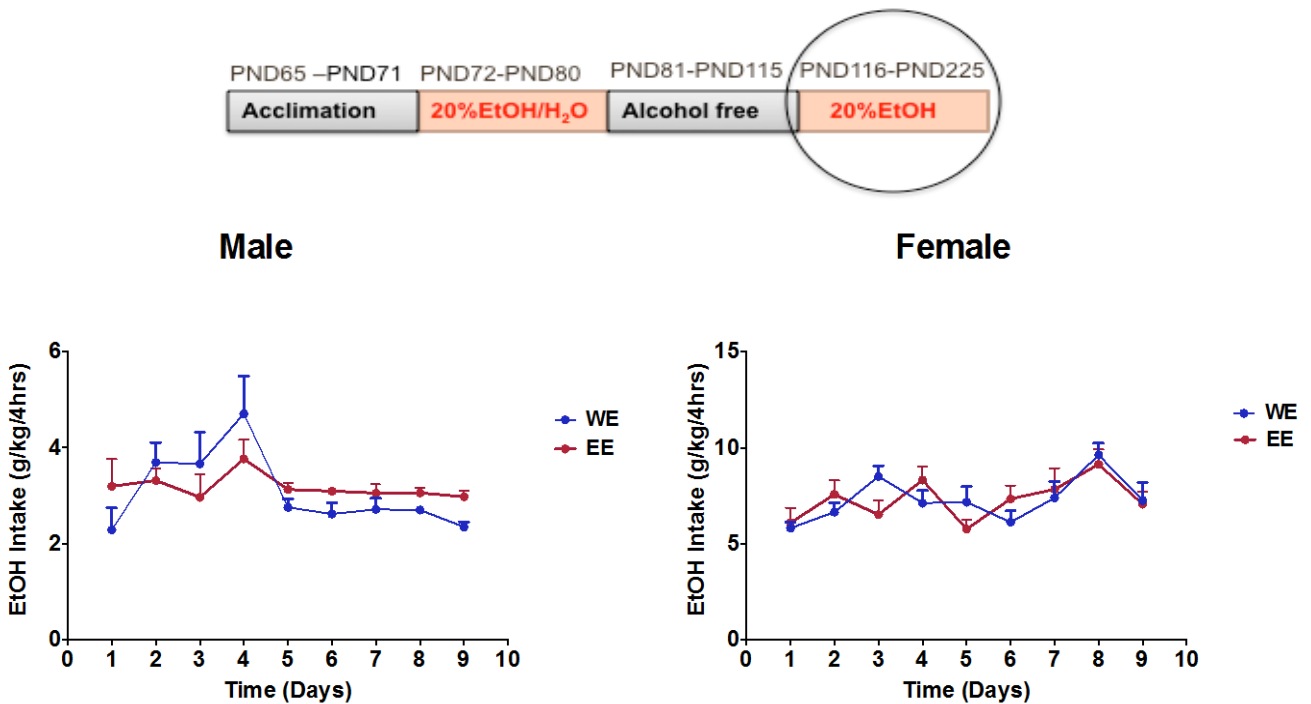
**Figure (6):** No sex differences in the ethanol intake were observed in adulthood. Shown are mean ratio values  $\pm$  SEM. Panel (A) depicts CA groups whereas Panel (B) depicts IA groups. (N=7 per group).

### ***DID for adult /adult control mice data***

The second experiment that we performed was to study if the ethanol exposure in adulthood had similar effects comparing with ethanol exposure in adolescence. In the first phase of alcohol exposure, our data showed that female consume more alcohol than male (Figure 7). A two way repeated measures ANOVA was performed and revealed significant effect of time [F (8, 96) = 7.428,  $p < 0.0001$ ] sex [F (1, 12) = 11.91,  $p = 0.0048$ ] and interaction [F (8, 96) = 4.665,  $p < 0.0001$ ]. Bonferron's post- hoc analysis revealed a significant difference of treatment on only day 6 and 7 ( $p < 0.05$ ). However, when the animals were exposed to the ethanol in the second phase neither male (Figure 8A) time [F (8, 96) = 3.914,  $P = 0.0005$ ] treatment [F (1, 12) = 0.3167,  $P = 0.5840$ ] and interaction [F (8, 96) = 1.573,  $P = 0.1430$ ]; nor female (Figure 8B) time [F (8, 88) = 3.573,  $P = 0.0012$ ] treatment [F (1, 11) = 4.545e-005,  $P = 0.9947$ ] and interaction [F (8, 88) = 1.213,  $P = 0.3009$ ] showed an enhancement of ethanol intake. Furthermore, when we determined the average daily intake for these animals, it was clear that the intake did not differ between the water exposed and alcohol exposed animals in both sexes (Figure 9;  $p < 0.05$ ).

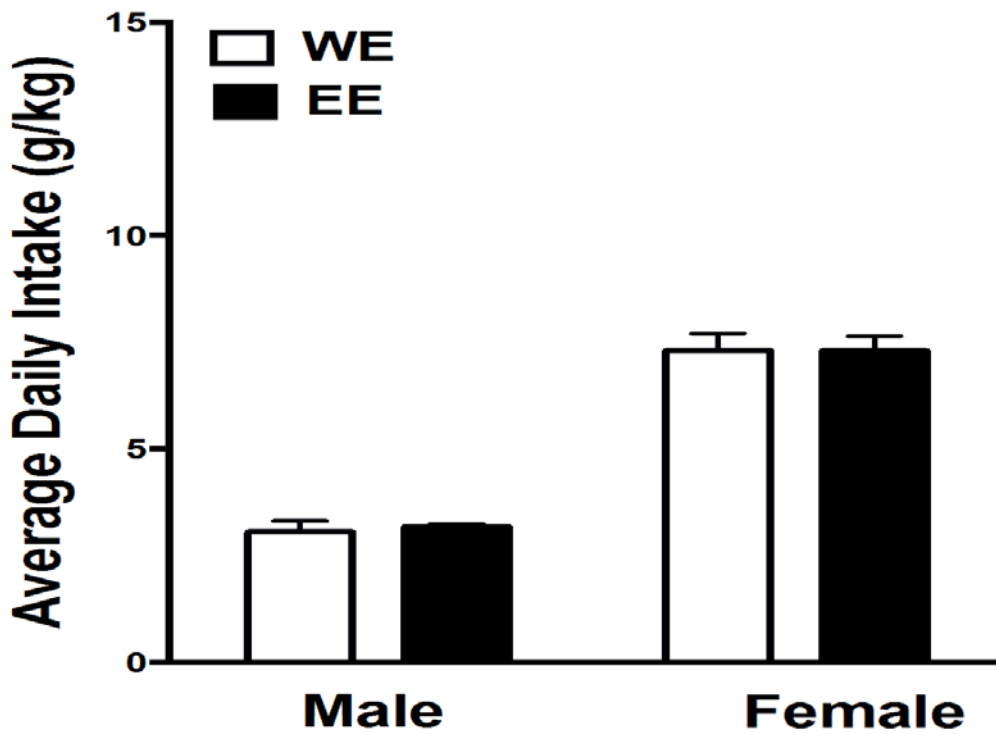


**Figure (7):** Daily intake profile of first phase of EtOH exposure (PND72 to PND117) for adult/adult control male and female C57B/6J mice. Female consume significantly higher ethanol than male in day 6 and 7. Data are expressed as mean EtOH intake values  $\pm$  SEM. (N=8 per group).



**Figure (8):** Daily intake profile of second phase of EtOH exposure for adult/adult control male and female C57BL/6J mice. In both panels A (male) and B (female) there is no different between

the adult group that were exposed to alcohol twice (EE) compare to the animals that were exposed only one time to alcohol. Data are expressed as mean EtOH intake values  $\pm$  SEM. (N=8 per group).



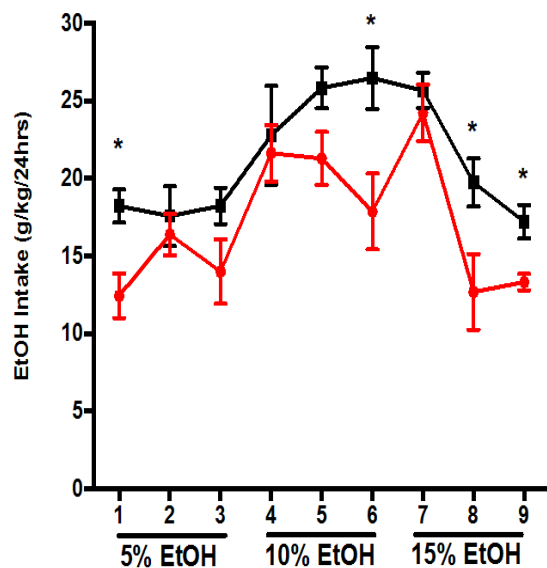
**Figure (9):** Average daily intake profile of second phase of alcohol exposure for adult/adult control male and female C57BL/6J mice. In both male and female there is no different between the adult group that were exposed to alcohol twice (EE) compare to the animals that were exposed only one time to alcohol. Data are expressed as mean EtOH intake values  $\pm$  SEM. (N=8 per group).

*Experiment Three: two bottle choice (2-BC) data*

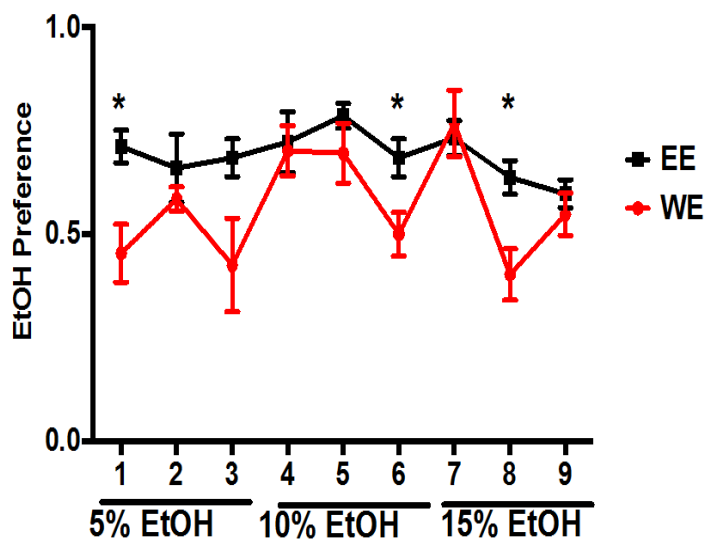
Our third experiment was to determine if exposure to alcohol would increase alcohol intake using two bottle choice (2-BC) procedure. Two-way repeated measures ANOVA followed by Bonferroni's post hoc revealed main significant effects of concentration [ $F(8, 97) = 10.01, P < 0.0001$ ] treatment [ $F(1, 97) = 25.26, P < 0.0001$ ] but no Interaction [ $F(8, 97) = 1.119, P = 0.3577$ ] (Figure 10A). The results for ethanol preference were similar to those of intake as two-way repeated measures ANOVA revealed main significant effects of concentration [ $F(8, 94) = 4.108, P = 0.0003$ ] treatment [ $F(1, 94) = 19.78, P < 0.0001$ ] but no interaction [ $F(8, 94) = 1.694, P = 0.1098$ ] (Figure 10B). After a week of deprivation, mice were exposed to 15% EtOH for one week using the 2-BC procedure. Not surprisingly, only the first day of exposure after ADE showed a significantly higher in ethanol intake. (Figure 11;  $p = 0.0038$ ).



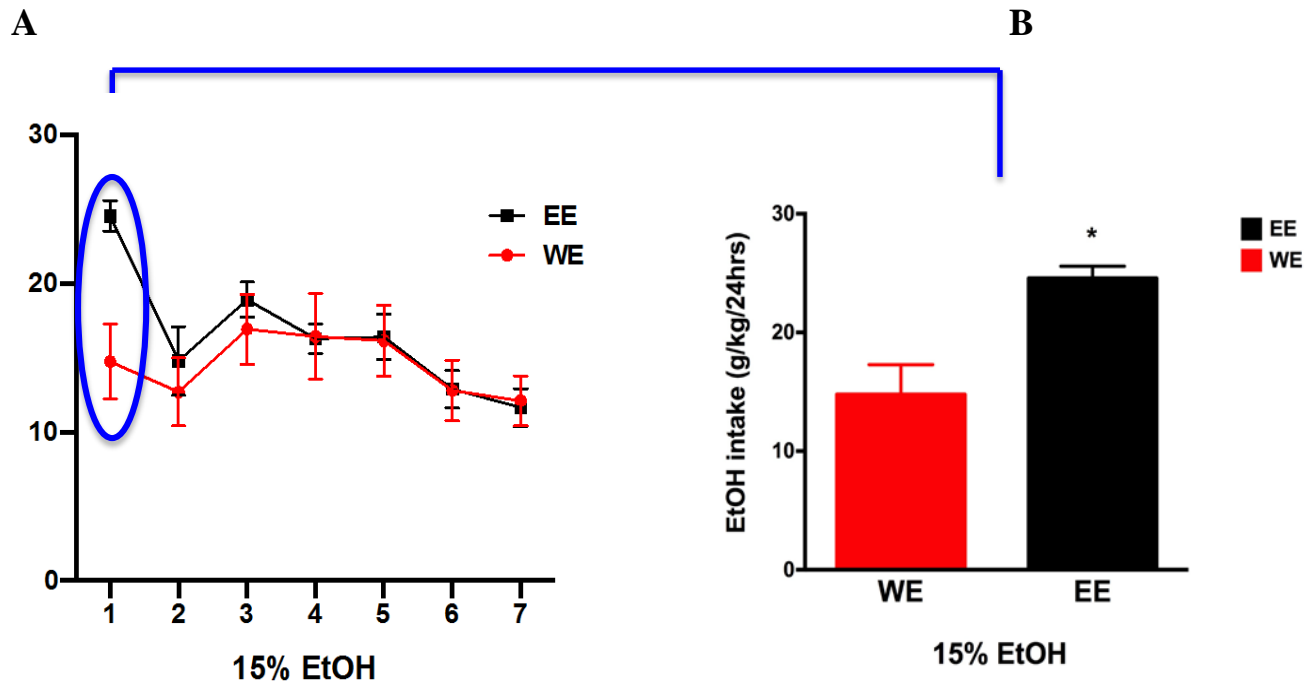
A



B



**Figure (10):** Ethanol consumption in g/kg per day for three ethanol concentrations, 5%, 10% and 15% (v/v) for three consecutive days respectively. Shown are mean EtOH intake values  $\pm$  SEM. Panel (A) depicts ethanol intake, whereas Panel (B) depicts ethanol preference. In both panels animals that were exposed to ethanol during adolescence (EE) consume more than the animals that were only exposed during adult (WE). (N=7 per group).



**Figure (11):** Ethanol consumption in g/kg/24hr for 15% (v/v) EtOH after one week of deprivation. Shown are mean EtOH intake values  $\pm$  SEM. Panel (A) depicts ethanol intake for 7days. Comparing EE to WE, only the first day shows the significance different. Panel (B) depicts the average intake for the first significant day only. (N=7 per group).

*Blood Ethanol Concentration (BEC) data*

Male and female C57BL/6J were tested for 9 days continually for 4h using DID procedure. Blood samples were collected right after the end of the session. BEC were measured using gas chromatography method. Our data showed that both sexes reached a BEC higher than 1 mg/ml.

**Table (2):** Blood ethanol concentration measurements

Sex	BEC (mg/ml) mean $\pm$ SEM	# Of animals
Male	1.070 $\pm$ 0.06	7
Female	1.100 $\pm$ 0.08	7

### 3.9 Summary

The primary objectives of these experiments were to investigate how a history of adolescent alcohol exposure affects alcohol intake later in adult. C57BL/6J mice were used. We employed limited access ethanol drinking in the dark (DID) procedure as a model of binge drinking. The procedure was a modification of the experiment described by Rhodes et al., 2005, with mice having either continuous access (daily) or intermittent access (every other day) of 20% EtOH in a 4-hr period for nine days. We also performed the 24-h two-bottle choice (as 2-BC) procedure as a model of voluntary ethanol consumption, with mice having a choice between ethanol and tap water. In this experiment, the concentration of ethanol given was increased from 5% to 10% to 15% over a period of 9 days consecutively for 24 hrs, while each concentration was given in 3 consecutive days. Following the escalation period, Mice were out of alcohol exposure for one week and then they had access to 15% EtOH and tap water for a week.

Consistence with previous studies our data showed that exposure to alcohol during

adolescence result in an increase of alcohol intake later in adulthood in DID as well as 2-BC paradigm. Data illustrated in Figures 5 and 6 using DID while 12 using 2-BC. Regarding the DID procedures, no sex differences were observed (Figure 7) therefore, we continued the rest of the experiments with using only male mice. Regarding the patterns of alcohol access, Continuous access is significantly higher than intermittent access (Figure 5). Also, for the same reason, we only performed continuous access the rest of the studies.

Not surprisingly, our 2-BC data showed that adolescence alcohol exposure results in an enhancement of alcohol deprivation effect (ADE) at only one-time point of exposure to 15% EtOH (Figure 11). Moreover, in contrast to adolescence, alcohol exposure in adulthood did not result in an enhancement of alcohol intake later in life. Therefore, we demonstrated that the enhancement of later alcohol intake is unique to adolescence period using DID procedure (Figures 4).

## CHAPTER FOUR: Strain Differences

### 4.1. Introduction

Differences sensitivity to alcohol dependence among individuals are most likely due to the combined effects of multiple genes each applying a small individual effect with major contributions from gene–gene interactions and gene–environment interactions. The complexity and heterogeneity of alcoholism makes identification of the relevant genes difficult, but knowing genes and pathways that affect vulnerability to this common and devastating disease may be critical to the development of improved therapy. Alcohol use often begins during the adolescence, a sensitive period during which a number of neurobiological, hormonal, and behavioral changes occur. Such alcohol use during adolescence may have profound effects on brain development and may even influence the propensity to use the drug in later life. However, little is known about the genes and genetic pathways mediating this age vulnerability to alcohol. One of our long-term goals is to use the DID procedure in adolescence to study the genetic basis of enhanced ethanol drinking in mice. For that, we initiated our efforts by the use of C57BL/6J (B6) and DBA/2J (D2) (Rhodes et al. 2005), two inbred strains that show large difference in alcohol intake.

Indeed, the C57BL/6J ethanol consuming and DBA/2J ethanol avoiding inbred mouse strains differ widely in their relative behavioral sensitivities to ethanol. McClearn and Rodgers (1959) demonstrated that whereas C57BL/6J mice will readily consume ethanol in a standard two-bottle choice paradigm, DBA/2J mice avoid the solution. In addition, C57BL/6J animals are less sensitive to ethanol's locomotor stimulant (Phillips et al., 1994), withdrawal (Metten and Crabbe, 1994), and reinforcing actions (Cunningham et al., 1992) than DBA/2J mice. Because the ethanol behavioral sensitivities of adult C57BL/6J and DBA/2J mice are so extensively

characterized, examination of adolescent sensitivity in these strains may provide a useful picture of how ethanol behavioral sensitivity during adolescence relates to that of adulthood. Interestingly, one recent study examined ethanol intake in adolescent and adult B6 and DBA/2J mice using DID found that adolescent B6 mice consume more ethanol than adult counterparts, while there was no difference between adolescent and adult DBA/2J mice (Moore et al., 2010). One important caveat to note in this study is that because ethanol intakes differed between adolescent C57BL/6J and DBA/2J mice (where C57BL/6J mice consumed significantly more ethanol than DBA/2J mice) it is not possible to know if DBA/2J mice provided with the same amount of ethanol exposure during adolescence would increase subsequent ethanol consumption in adulthood. Our study comparing ethanol consumption of adolescence C57BL/6J and DBA/2J will avoid this caveat by giving the same amount of ethanol concentration in the same conditions using DID paradigm.

#### 4.2 Material and method

##### *Animals*

Male C57BL/6J and DBA/2J inbred mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in the OOV space individually in standard Plexiglas cages with corn-cob bedding and food and water available ad libitum, except where noted in individual experiments. Cages also contained a small cardboard tubes and a nestled for the purpose of environmental enrichment. Mice were three weeks old at the time of shipping and were allowed to acclimate to the OOV space for 1week before starting the experiments. Therefore, the mice were tested at the age of PND28. Mice were maintained on a reverse 12-hour light–dark cycle at which lights were out at 6 am and on at 6 pm with the temperature and

humidity of the vivarium kept at approximately 20-23°C and 40-70%, respectively. The exact times may vary due to lab personnel schedules, but 12 hours is still maintained once the study begins. All experiments were performed during three hours into the dark cycle at which mice will be given only ethanol to consume for 4 hours. Due to the fact that C57BL/6J mice were reported in various studies to drink 3-5-fold higher than DBA/2J mice, they were exposed to 5% of ethanol while DBA/2J mice were exposed to 20% of ethanol in order ensure that the two inbred strain mice are drinking similar amount of ethanol. At the end of this period, the ethanol pipettes are replaced with water pipettes and the volume of ethanol consumed is recorded. Institutional Animal Care and Use Committee of Virginia Commonwealth University approved the study. All studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory animals. Red incandescent lamps were used when needed so that experimenters could navigate the procedure room after lights out.

### ***Drugs***

Ethanol (200 proof) was diluted with tap water and prepared as either a 5% (v/v) or 20% (v/v) solutions. The ethanol bottle was purchased from Fisher Scientific Laboratory supplies and equipment.

#### **4.3 Drinking in the Dark DID procedure**

Limited-access ethanol intake model of acute binge drinking behavior in adolescent mice was assessed using DID procedures; n of 8 to 10 per genotype was performed. Our procedure is similar to that of Rhodes et al., (2005). In this procedure, mice were housed individually with 25ml sipper tubes containing tap water for one week of a habituation in temperature ( $23 \pm 2$  °C)

and humidity controlled room under a 12-hour light/dark cycle, 6:00 a.m. 6:00 p.m. as was described above. This inverted light cycle is managed by using an external timer that powers an incandescent light for the room. We used a separate red light for visibility when we work with the animals during the dark phase. Subjects were never fluid restricted, and water and food were freely available. At PND28 water bottle is replaced with a single bottle containing an either 5% ethanol (for C57BL/6J) or 20% (for BDA/2J) ethanol solutions. Mice were given a 4-hour access to these ethanol solutions 3 hours after lights out (beginning at 9am). Each day, initial volumes were recorded, and 4h later the final volumes were recorded. Mice weights were taken after recording the final volume of alcohol intake avoiding stress the animal before offering the ethanol along the study.

#### 4.4 Blood ethanol concentration determination (BEC)

35-50µl-blood sample was taken via cheek punch using 5mm Lancets (Medipoint, Inc., Minenola, NY). Blood collection will not exceed 20% of total blood volume and collections will be after day ninth of alcohol exposure during adolescence period which is PND36 .The blood stored in BD microtainers and analyzed using Gas Chromatography similar to a previously described procedure (Gallaher et al. 1996). Animals will receive food/water ad lib after blood collection they also were injected according to their weights with saline to avoid hypovolemia. We have collected the blood from C57BL/6J (5%EtOH) and DBA/2J (20%) at the day ninth of exposure during adolescence period which is exactly PND36 but data are not shown in thesis because the blood level was undetectable level in the most of the samples.

#### 4.5 Experimental design



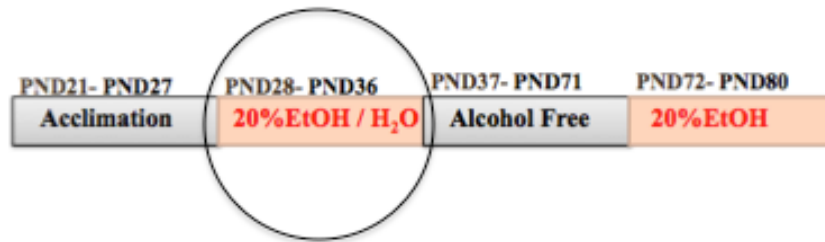
To determine to what extent strain difference play a role in ethanol enhancement later in life using animals have been exposed to ethanol during adolescence using DID procedure. Male C57BL/6J and DBA/2J mice at PND 28 were exposed to either 5% or 20% alcohol respectively in the DID procedure for nine consecutive days. There were two groups with n=7-8 per group. First group was C57BL/6J mice at which they were given its 5% alcohol in the DID procedure for nine days continuously for 4-h access period at PND28 and then the subjects went through 36days of withdrawal. After these nine days of exposure animals were kept in their home cages individually for 36 day with access ad libitum to food and water until they reached adult period (PND 72) and then mice were exposed back to the same procedure under the same conditions after 36days of absences. The second group was DBA/2J mice at which they were given its 20% alcohol in the DID procedure for nine days continuously for 4-h access period at PND28 and then the subjects went through 36days of withdrawal. After these nine days of exposure animals were kept in their home cages individually for 36 day with access ad libitum to food and water until they reached adult period (PND 72) mice were exposed back to the same procedure under the same conditions after 36days of absences.

#### 4.6 Statistical Analysis

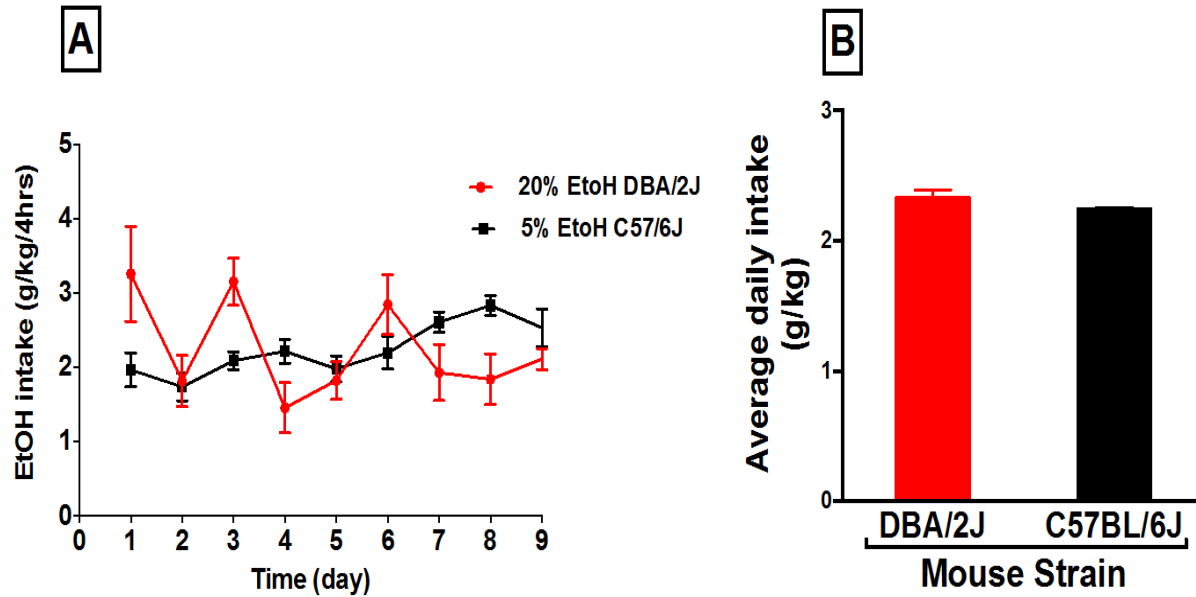
Data were analyzed using prism (6). A two –way repeated measures ANOVA with Bonferron’s post hoc multiple comparison test analysis was applied (treatment × access). However, due to the fact that prism does not determine the progress time effects, the effect of time showed in figures 12(A) and 13(A) should be interpreted cautiously.

#### 4.7. Results

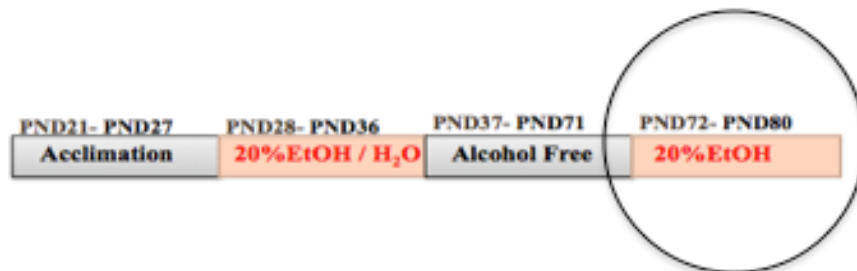
Daily as well as average daily limited access of ethanol intake for adolescent (PND28-36) C57BL/6J and DBA/2J mice can be seen in figure 12 A and B respectively. In the daily intake, C57BL/6J seems to be stable over the time with tendency to increase slightly over time, while DBA/2J intake was variable in the first 2-3 days (Figure 12A). A two way repeated measures ANOVA was performed and revealed significant effect of time [ $F(8, 125) = 2.501, P = 0.0149$ ] and interaction [ $F(8, 125) = 3.901, P = 0.0004$ ] but no strains [ $F(1, 125) = 0.003319, P = 0.9542$ ]. Bonferroni's post-hoc analysis revealed a significant difference of time on only day one ( $p < 0.05$ ). In addition, the average intake for the 9 days for both C57B/6J and DBA/2J mice was not statistically different (figure 13B,  $p = 0.2022$ ). However, when alcohol intake of these two strains was assessed on PND 72, C57BL/6J mice consumed more ethanol than DBA/2J mice (figure 13A). A two way repeated measures ANOVA was performed and revealed significant effect of time [ $F(7, 112) = 4.341, P = 0.0003$ ], strains [ $F(1, 112) = 101.8, P < 0.0001$ ] and interaction [ $F(7, 112) = 4.372, P = 0.0003$ ]. Bonferroni's post-hoc analysis revealed a significant difference of time for all days except for days 6, 8, and 9 ( $p < 0.05$ ). Indeed, when we averaged all the days together during the second phase of exposure to ethanol (during adulthood) we found that the C57B/6J mice consumed significantly more ethanol than their DBA/2J counterparts (figure 15B;  $p < 0.0001$ ).



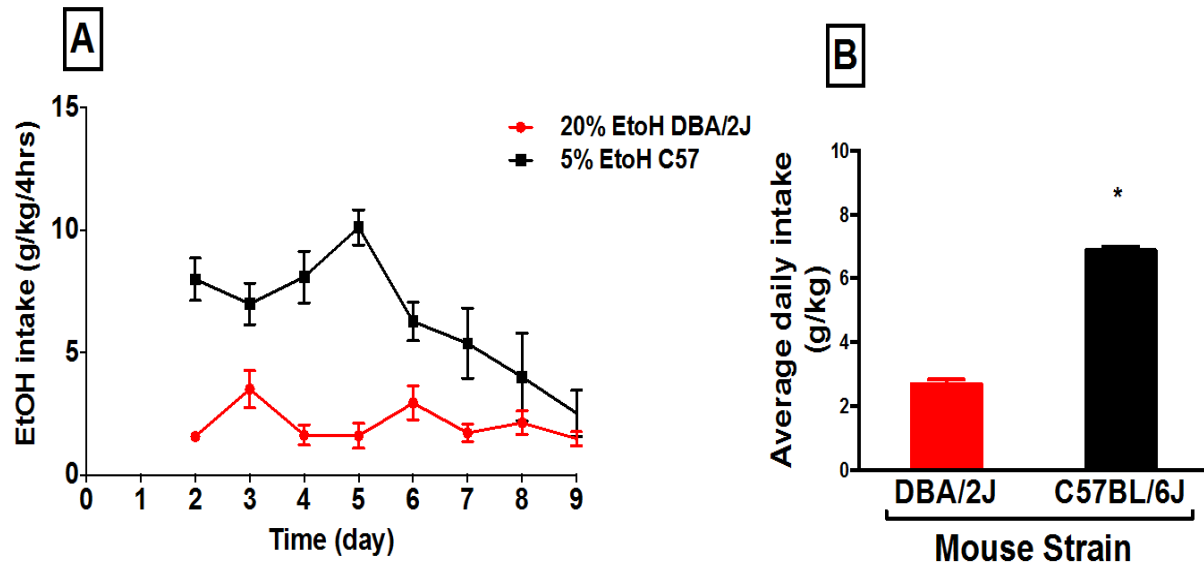
## PND28



**Figure (12):** First phase of alcohol exposure during adolescence in C57BL/6J and DBA/2J mice. Shown are mean EtOH intake values  $\pm$  SEM. Panel (A) depicts ethanol intake of C57BL/6J and DBA/2J for 9 days continuously using DID procedure. Panel (B) depicts the average daily intake. There is no significant difference in intake observed in both panels. (N=8 per group).



## PND72



**Figure (13):** Second phase of alcohol exposure during adolescence in C57BL/6J (5%EtOH) and DBA/2J (20%EtOH) mice. In the second phase of exposure C57BL/6J mice consumed more ethanol than DBA/2J. Shown are mean EtOH intake values  $\pm$  SEM. Panel (A) depicts ethanol intake for 9 days continuously using DID procedure. Panel (B) depicts the average daily intake. C57BL/6J consume significantly higher ethanol intake than DBA/2J. (N=8 per group).

### 4.7. Summary

Ethanol intake during adolescence increases subsequent ethanol intake during adulthood in the C57BL/6J but not DBA/2J mice that were exposed to similar amount of alcohol during adolescence. Ethanol sensitivity is likely dependent upon both age and genotype. Importantly, the present data suggests that genotype is an important factor in the expression of not only adolescent ethanol intake but also subsequent adult ethanol intakes. The current data opens the

door to do more genetic (QTL mapping studies) and genomic studies by using BXD recombinant panel because these 2 stains are the progenitors of this genetic panel.

## **CHAPTER FIVE: Changes in alcohol sensitivity**

### 5.1 Introduction

The mechanism(s) underlying the effect of “binge drinking” experience during adolescence to increase subsequent ethanol intake during adulthood are not known. It is possible

that these age- and genotype-related differences in ethanol sensitivity may underlie the observed differences in ethanol drinking.

In general, adolescents are less sensitive than adults to EtOH effects. For instance, they are less sensitive than adults to the social impairing effects that emerge at higher doses of EtOH (Varlinskaya & Spear, 2002), with this insensitivity being particularly marked in young rather than older adolescents (Varlinskaya & Spear, 2006a). Adolescents are also relatively insensitive to the aversive effects of EtOH, requiring higher EtOH doses to develop conditioned taste aversion (CTA), an aversion to a novel taste paired with an injection of EtOH (Anderson et al, 2010; Vetter-O'Hagen et al, 2009). Ethanol also serves as a less effective discriminative cue for adolescents than for adults, suggesting that adolescents are less able to detect the interoceptive effects of EtOH than are adults (Anderson & Spear, in press). Moreover, adolescents are less sensitive than adults to EtOH's motor impairing, anxiolytic, and sedative effects (e.g., Sliveri & Spear, 1998; Varlinskaya & Spear, 2002; Ramirez & Spear, 2010a). Adolescent rats have even been found to exhibit attenuated sensitivity to some withdrawal effects during the recovery period following a large dose of EtOH (Doremus et al, 2003).

The aim of these studies was to examine whether exposure to alcohol during early adolescence induces long-term changes to ethanol sensitivity, namely its aversive effects and its positive effects. Therefore, we performed a series of tests measuring the initial response and acute ethanol administration in adult C57BL/6J mice. Namely, we performed light /dark boxes (L/D boxes) and elevated plus maze (EPM) to measure ethanol's anxiety effects as well as measuring the sedative/intoxicating effect of ethanol by performing loss of righting reflex (LORR) response as well as measuring alcohol withdrawal signs by performing Forced swimming test (FST). Furthermore, we determined the ethanol rewarding effects by performing

conditional place preference (CPP).

## 5.2 Protocols and experiment designs:

### ***5.2.1 Forced swimming test (FST) protocol***

Among all animal models, the FST remains one of the most used tools for assessing depression-like behaviors. It is currently a popular model, due to the low cost of the experiments and because it is arguably the most reliable model available (Holmes, 2003). Moreover, it has been reported to be reliable across laboratories (Borsini and Meli, 1988). The test is simple and can be performed rapidly. Twenty-four hours after the last alcohol intake, mice were placed into a cylinder filled with warm water (25°C) for 6 min. However, only the last four minutes of the test was analyzed. This is due to the fact that most mice are very active at the beginning of the test, and the potential effects of the treatment can be obscured during the first two minutes. The total amount of mobility (swimming and climbing) time was determined and then subtracted from the 240 seconds of test time (6mins).

We performed FST to determine the impact of alcohol exposure during early adolescence on alcohol withdrawal later in adulthood. Male and female C57BL/6J at PND28 were exposed to 20% alcohol in the DID procedure for nine days with continuous access (CA, daily). The test was performed using same cohort of mice in chapter three except for EW group was added currently with a new group of only male mice. There were a total of four groups (WW, WE, EW, EE) with n=7 per group. The groups were divided as follows: WW was given only water in both adolescence and adult periods. WE was given 20% EtOH in the DID procedure for nine days in the adult period (from PND72 until PND 80). EW was given 20% alcohol in the DID procedure for nine days CA at PND28 until PND36. EE was given 20% alcohol in the DID procedure for



nine days CA at PND28 until PND36 and then repeated under the same conditions at PND72 until PND80. The FST was performed at PND 82 for all groups. The total amount of swimming time was determined and then subtracted from the 240 seconds of test time (6mins) as outcome measurements.

### **5.2.2 Loss of Righting Reflex (LORR)**

The sedative-hypnotic effects of ethanol were measured using the loss of righting reflex assay (LORR). Male C57BL/6J at PND28 were exposed to 20% alcohol in the DID procedure for nine days with continuous access (CA, daily). There were two groups (WE and EE) with n=7 per group. Mice in the pretreatment groups were exposed either to 20% EtOH during adolescence for nine days (PND28 to PND36) period of 4h each day or just tap water. Later during adult (PND72) both groups were injected with 3.5g/kg (20%v/v) EtOH i.p. Time started immediately after the ethanol injection and mice 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 sec. Mice taking longer than 5 min to experience LORR were eliminated from the study due to the possibility of misplaced injection. Two LORR scores were reported for each subject. The first was the total time from ethanol injection until initial LORR, which was reported as the LORR onset time. The second was total time required for the subject to right itself three times within 30 sec from the onset of LORR, which was reported as LORR Duration. Mice taking longer than 5 min to experience LORR were eliminated from the study due to the possibility of misplaced injection. Data (mean  $\pm$  SEM) were expressed as length of drug onset in seconds and as LORR Duration in min.

### **5.2.3 Elevated Plus Maze (EPM)**

The elevated plus maze apparatus (EPM) was used to assess the anxiety-like behavior induced by acute ethanol. The apparatus 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. There were a total of four groups of male C57BL/6J with  $n=8$  per group and they were divided as follows: Group 1 (W-saline) was only given tap water during adolescence and then at PND72 mice were i.p injected with saline. Group 2 (E-saline) was given 20% EtOH in the DID procedure for nine days of 4hr /CA at PND28 until PND36, and then at PND72 mice were i.p injected with saline. Group 3 (W-2g/kg EtOH) was only given tap water during adolescence and then mice were injected with 2g/kg (20%v/v) EtOH i.p. at PND72. Last group 4(E-2g/kg EtOH) was given 20% EtOH in the DID procedure for nine days of 4hr /CA at PND28 until PND36, and then at PND72 mice were injected with 2g/kg (20%v/v) EtOH i.p.

Subsequently, after these nine days of exposure during adolescence animals were kept in their home cages individually for 36 days with access ad libitum to food and water until they reached adult period (PND 72). Mice were given at least 30min to acclimate to the testing room. Then, mice in the pretreatment groups were injected with either 2 g/kg (20%v/v) EtOH or saline i.p. Subjects were then returned to their home cage for 15 min. Each subject was then placed gently in a plastic container and transferred to the center of the maze. The subject was then allowed to freely explore the apparatus for 5 min, as soon as place it in the center of the maze. Data (mean  $\pm$  SEM) were expressed as the total time spent in the open arms in sec. The number of crossovers was also recorded. Two –way repeated measures ANOVA with post hoc analysis were applied.

#### **5.2.4 Light /dark boxes**

The L/D box is an additional test used in the assessment of anxiety-like behaviors. It assesses the spontaneous exploratory behavior of rodents when confronted with stressors such as light and a new environment. It models a conflict of interest between exploring and removing oneself from anxious situations. The test apparatus consisted of two boxes; one of these boxes was darkened. A desk lamp placed above the lit box to provide the room illumination. An opaque plastic tunnel separated the two boxes. The apparatus was equipped with infrared beams for recording. There were a total of four groups of male C57BL/6J with n=8 per group. They were divided as follows: Group 1 (W-saline) was only given tap water during adolescence and then at PND72 mice were i.p injected with saline. Group 2 (E-saline) was given 20% EtOH in the DID procedure for nine days of 4hr /CA at PND28 until PND36, and then at PND72 mice were i.p injected with saline. Group 3 (W-2g/kg EtOH) was only given tap water during adolescence and then mice were i.p injected with 2g/kg (20% v/v) EtOH at PND72. Group 4(E-2g/kg EtOH) was given 20% EtOH in the DID procedure for nine days of 4hr /CA at PND28 until PND36, and then at PND72 mice were i.p injected with 2g/kg (20% v/v) EtOH.

Subsequently, after these nine days of exposure during adolescence animals were kept in their home cages individually for 36 days with access ad libitum to food and water until they reached adult period (PND 72). Mice were given at least 30min to acclimate to the testing room. Then, mice in the pretreatment groups were injected with either 2 g/kg (20% v/v) EtOH or saline i.p. Subjects were then returned to their home cage for 15 min. Each subject was then placed gently in the lit box facing opposite to the tunnel. The subject was allowed to freely explore the apparatus for 5 min, with time starting immediately. Time spent by mice in the lit box and number of light side entries was recorded during a 5-min period. Two –way repeated

measures ANOVA with post hoc analysis were applied.

### ***5.2.5 Conditioned place preference (CPP)***

An unbiased CPP paradigm was used in this study to assess rewarding-like properties 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 1g/kg (20% v/v) EtOH (subcutaneously) 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 was recorded via photosensors using interface and software (MED Associates, St. Albans, VT). Data were expressed as time spent on drug-paired side minus time spent on saline-paired side. A positive number indicated a preference for the drug-paired side.

### **5.3 Statistical Analysis**

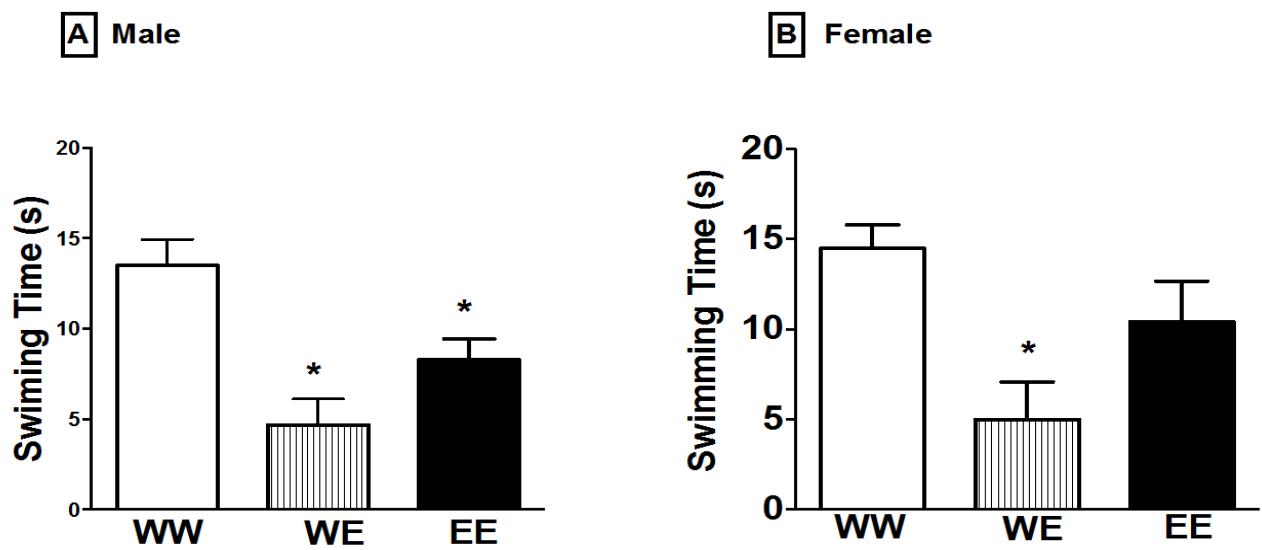
Data were analyzed using prism (6). An unpaired t-test (two-tailed) or a two –way repeated measures ANOVA with Bonferron’s post hoc multiple comparison tests were applied whenever are appropriate. For FST the primary dependent variable was swimming time. Primary dependent variables for LORR were LORR onset and LORR duration. Primary dependent variables for EPM were time spent in open arms and number of crossovers. Primary dependent variables for light/dark boxes were latency to the light side entries and time spend in light side

For CPP, the primary dependent variable was the preference score. Each analysis was followed by Bonferron's post-hoc tests to further analyze significant data with the alpha level set at 0.05.

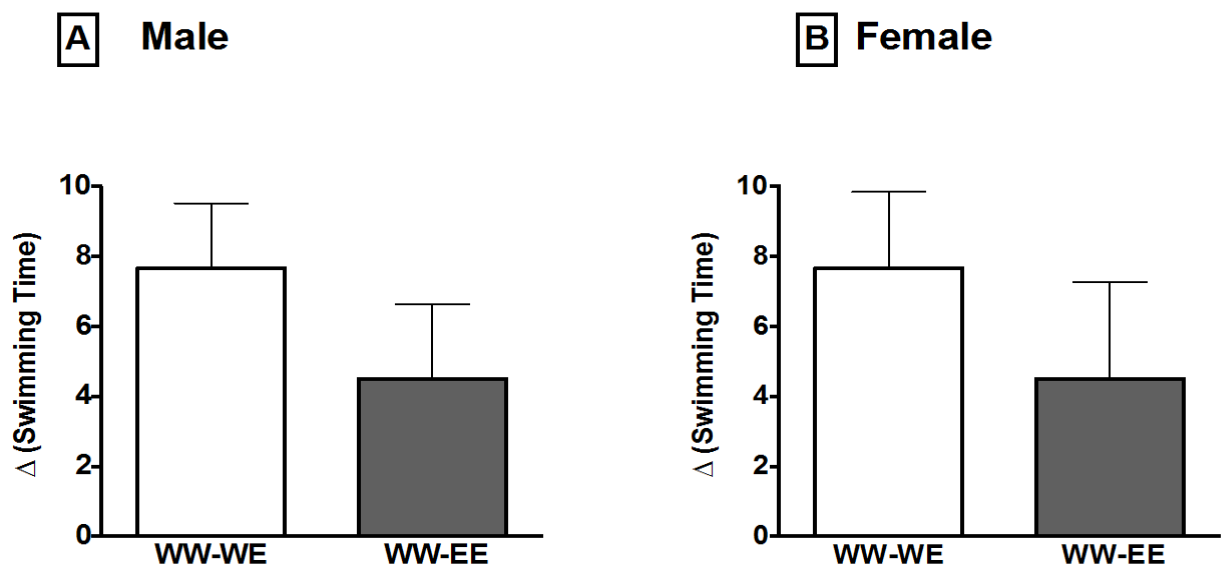
#### 5.4. Results

##### ***5.4.1. Alcohol withdrawal in the FST test:***

Our results showed that male groups that got water during adolescence (WE) as well as those who were giving alcohol during adolescence (EE) swim significantly less than the control group (WW) [Figure 14A,  $p \leq 0.01$ ]. However, female group that got water during adolescence (WE) swim less than control group (WW) [Figure 14B,  $p \leq 0.01$ ] and there was no significances difference when we compared those that got alcohol during adolescence (EE) to the control group.



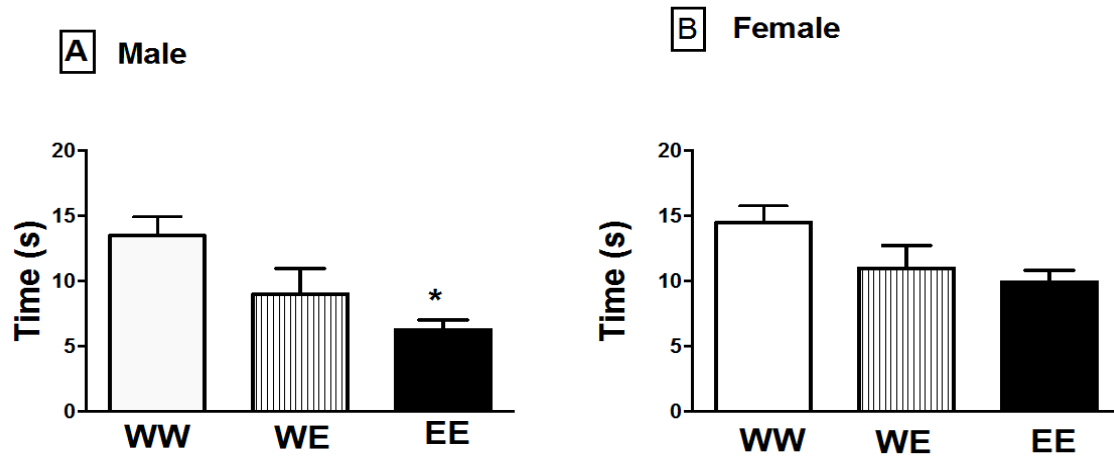
**Figure (14):** Alcohol exposure during adolescence alters alcohol withdrawal later in life in forced swim test. Shown are mean swimming time values  $\pm$  SEM. Panel (A) depicts male groups whereas Panel (B) depicts female groups. (N=7 per group).



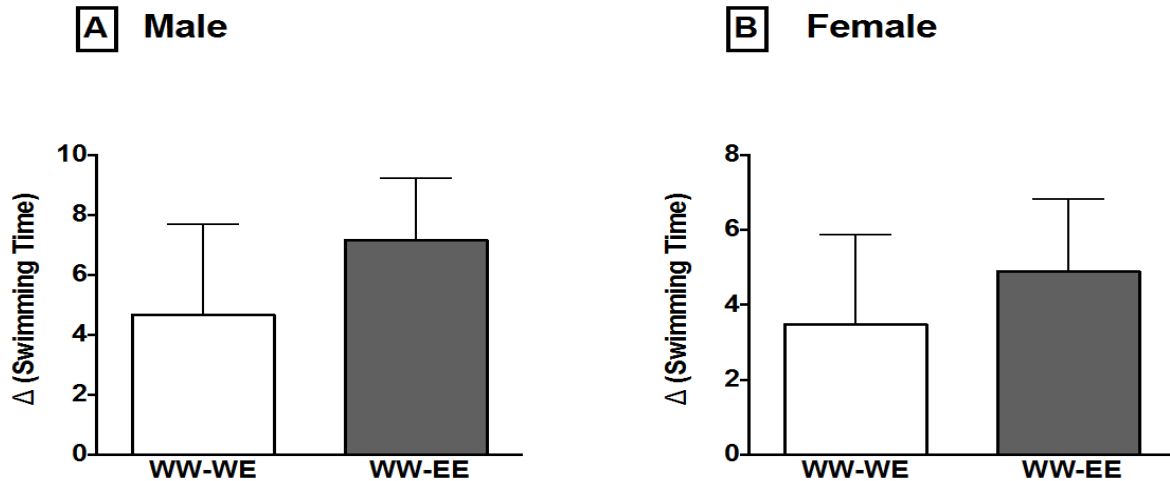
**Figure (15):** Time difference ( $\Delta$ ) between second and first phase of alcohol exposure during adolescence in the forced swim test. Shown are mean  $\Delta$  values  $\pm$ SEM. Panel (A) depicts male groups whereas Panel (B) depicts female groups. (N=7 per group).

Similar to our study of alcohol intake during adolescence, we also conducted alcohol withdrawal in adult animals our data have shown that comparing with the control group (WW) there is an decrease in the swimming time in the animals that received ethanol only during adult (WE). This decrease was even significantly less in the animals that received ethanol during adolescence (EE) [Figure 16A;  $p \leq 0.01$ ]. Our conclusion is that in contrast to adolescence, EtoH exposure in adulthood enhances alcohol withdrawal in FST. However, no significance difference was observed in females among these groups [Figure 16B;  $P=0.8027$ ].





**Figure (16):** Alcohol Exposure Only in Adulthood Enhances Alcohol Withdrawal in FST. Shown are mean swimming time values  $\pm$  SEM. Panel (A) depicts male groups whereas Panel (B) depicts female groups. (N=7 per group).

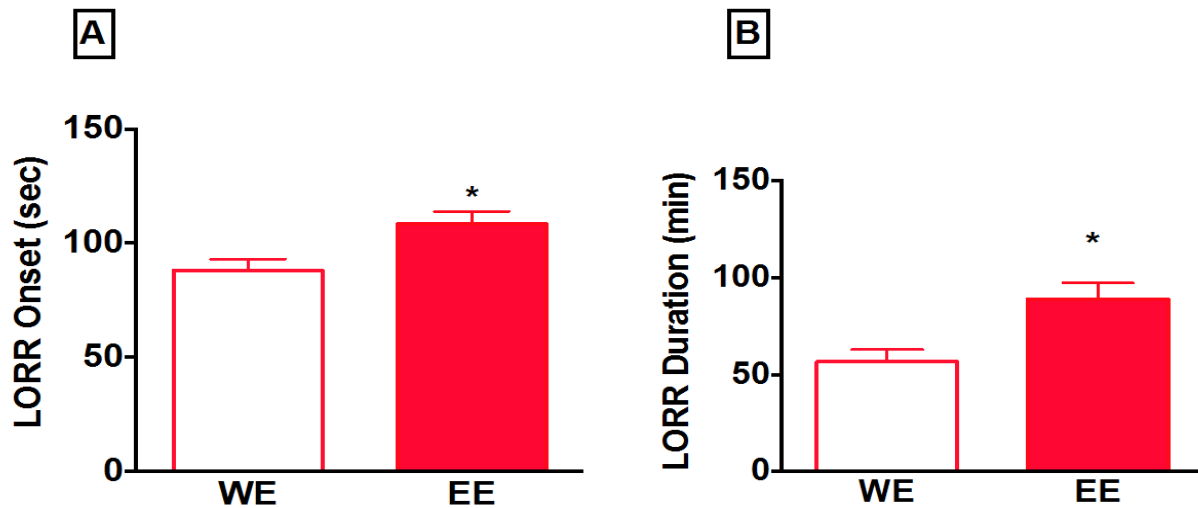


**Figure (17):** Time difference ( $\Delta$ ) between second and first phase of alcohol exposure during adult in forced swim test. Shown are mean  $\Delta$  values  $\pm$  SEM. Panel (A) depicts male groups whereas Panel (B) depicts female groups. (N=7 per group).

#### 5.4.2. LORR Results:

We assessed the impact of ethanol exposure during adolescence on ethanol-induced LORR in adulthood by measuring the onset of LORR in sec and the duration of LORR in min. The length of drug onset means the effect of the drug and it usually takes place within 5 min. A dose of 3.5 g/kg ethanol had the intended effect of inducing LORR (Figure 17). Has shown in figure 17A ( $p \leq 0.01$ ) LORR duration as well as figure 17B ( $p \leq 0.01$ ) LORR onset, we observed

that animals that received ethanol during adolescences and then injected with alcohol at 3.5g/kg during adulthood (EE) significantly different as compare to the group that received water (WE).

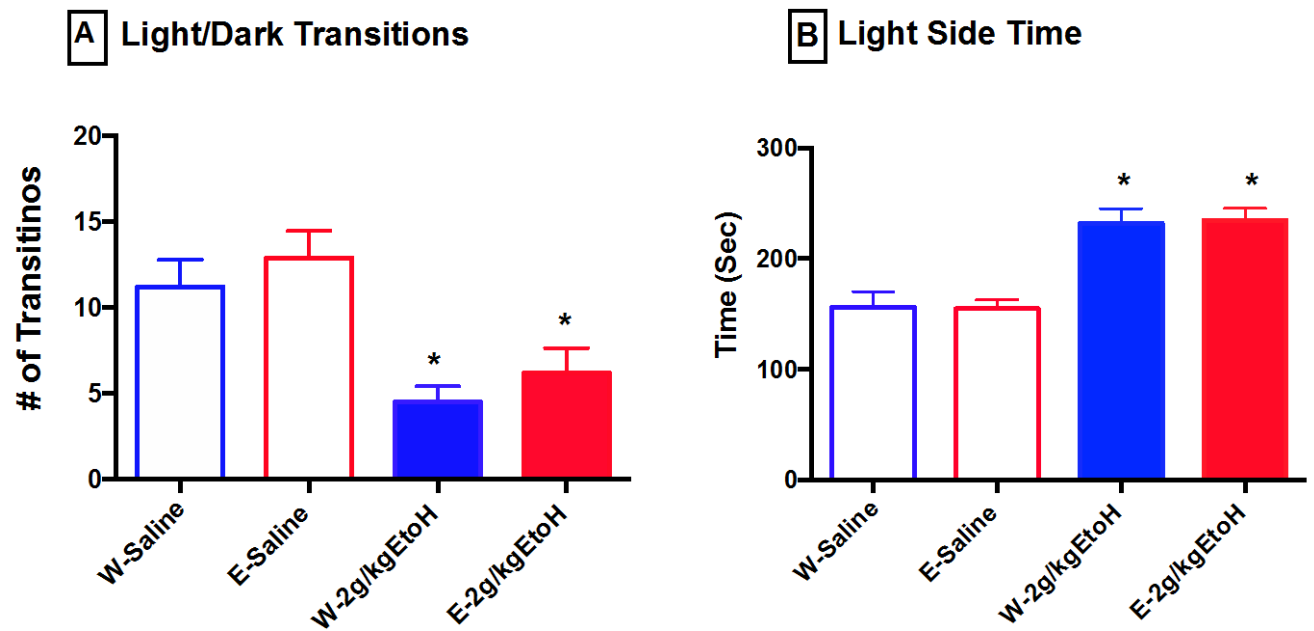


**Figure (18):** Hypnotic sensitivity to ethanol in the adult C57BL/6J mice. Shown are mean values  $\pm$  SEM. Panel (A) depicts length of drug onset in sec whereas Panel (B) depicts LORR duration in min. (N=10 per group).

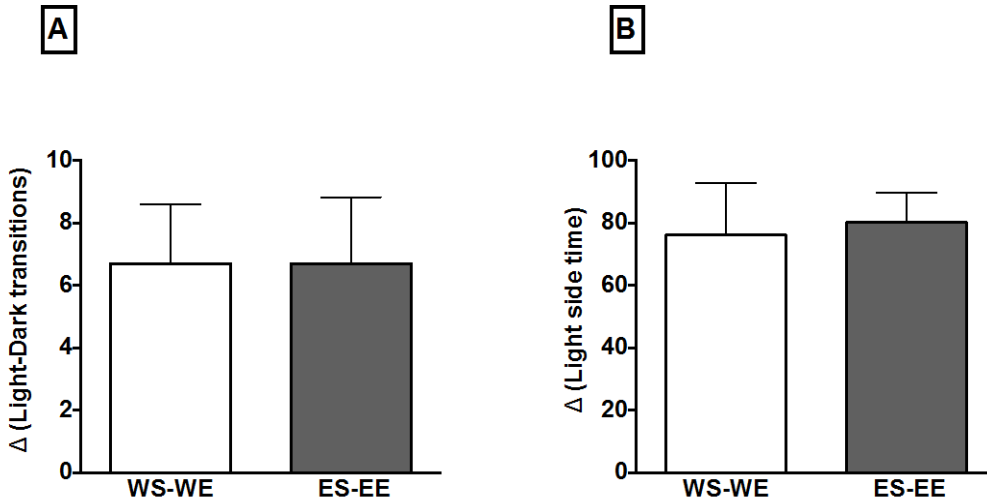
#### 5.4.3. Light/Dark boxes Results:

No significant differences were observed in the time spent in the light side when we compared the group that received either tap water or 20% EtOH during adolescences and then were injected with saline during adulthood. However, when the groups that received either tap water or 20%EtOH during adolescences and then were injected with 2g/kg (20% v/v) dose of ethanol, we observed a significant increase in the time spent in the light side in both groups

(figure 19;  $p \leq 0.01$ ). This ethanol-induced anxiolytic effect was similar between the two groups.



**Figure (19):** Anxiolytic affect in the adult C57BL/6J mice using light and dark boxes. Shown are mean values  $\pm$  SEM. Panel (A) depicts number of transitions whereas Panel (B) depicts time spent in light side. (N=8 per group).

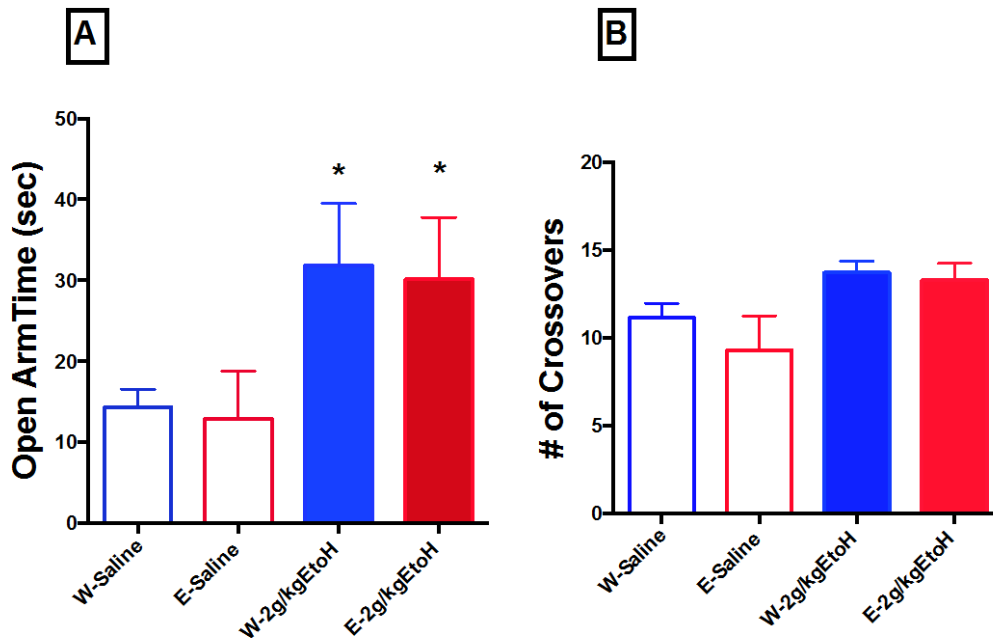


**Figure (20):** Time difference of second and first treatment of alcohol during adult in light and dark boxes. Shown are mean  $\Delta$  values  $\pm$  SEM. Panel (A) depicts delta of light/dark transitions whereas Panel (B) depicts delta time spent in light side. (N=8 per group).

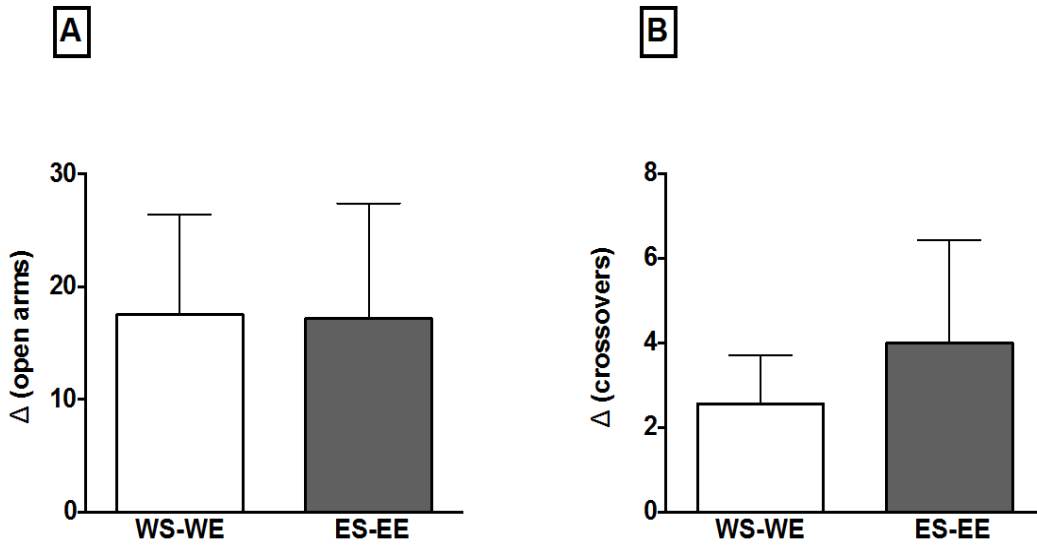
#### 5.4.4. Elevated plus maze results:

No significant differences were observed in the time spent in the open arms when we compared the group that received either tap water or 20% EtOH during adolescences and then

were injected with saline during adulthood. However, when the groups that received either tap water or 20%EtOH during adolescences and then were injected with 2g/kg (20% v/v) dose of ethanol, we observed a significant increase in the time spent in the open arms in both groups (Figure 21A;  $p \leq 0.01$ ). However, there were no significant differences in the number of crossovers (Figure 21B). This ethanol-induced anxiolytic effect was similar between the two groups.



**Figure (21):** Anxiolytic affect in the adult C57BL/6J mice using elevated plus maze. Shown are mean values  $\pm$  SEM. Panel (A) depicts time spent in the open arms whereas Panel (B) depicts number of crossovers. (N=8 per group).

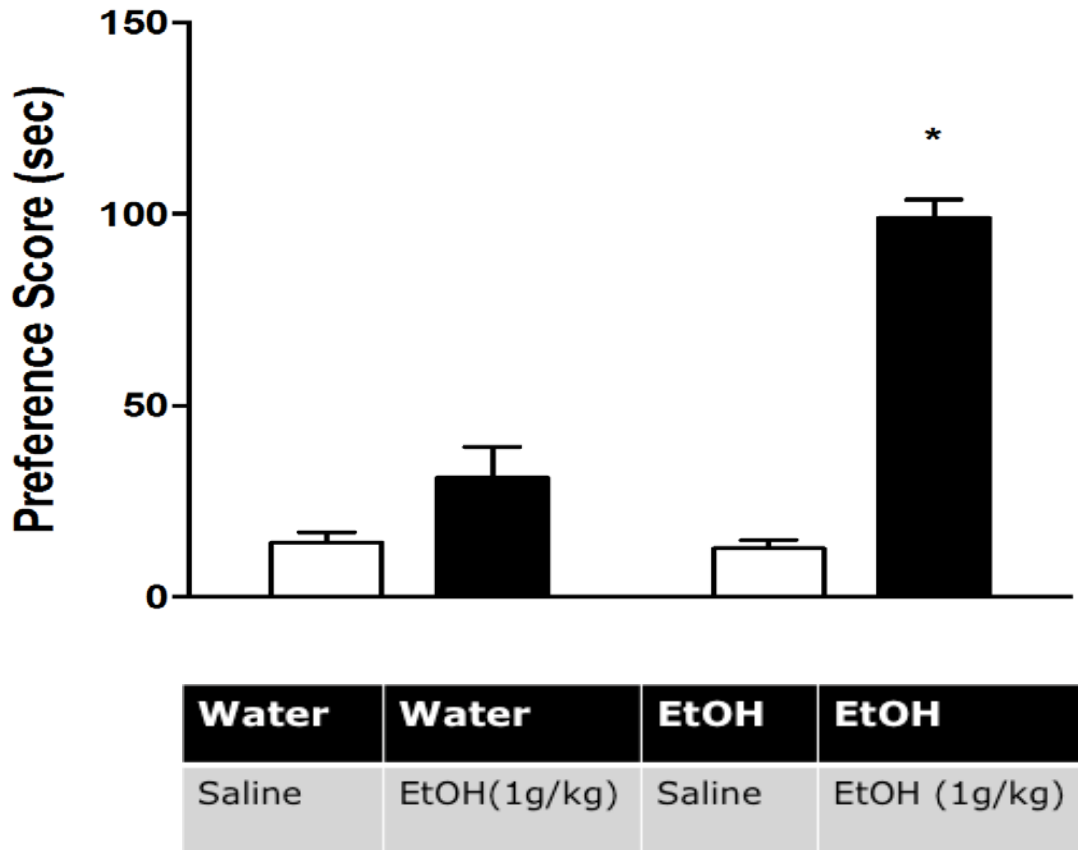


**Figure (22):** Time difference of second and first treatment of alcohol during adult in EPM. Shown are mean  $\Delta$  values  $\pm$  SEM. Panel (A) depicts delta of open arms whereas Panel (B) depicts delta of crossovers. (N=8 per group).

#### 5.4.5. CPP results:

Ethanol exposure in adolescence increases the sensitivity to ethanol in CPP. The animals that received the ethanol during CPP showed no significant preference after conditioning with a low dose of alcohol (1 g/kg, i.p.). However, when animals were exposed to ethanol during adolescence, ethanol induced a significant preference in the CPP test. Has shown in figure 23, we observed that animals that received water during adolescences and then conditioned with alcohol at 1g/kg during adulthood did not show significance preference compared to the saline group. In contrast, animals that were received ethanol during adolescence did show a significance preference.





**Figure (23):** Ethanol exposure during adolescence induced rewarding effect in the CPP test. Preference score in second was plotted against drug conditioning. Shown are mean preference score values  $\pm$  SEM. Mice that received ethanol during adolescence were more sensitive to the rewarding of ethanol. (N=8 per group)

### 5.5. Summary

Our results demonstrated that the enhancement of alcohol intake of C57BL/6 which pre-exposed to 20% EtOH during adolescence induced important changes in ethanol sensitivity later in life. For instance, mice that received ethanol during adolescence are more sensitive to the intoxicating effects (LORR test) of alcohol. Our LORR data showed that ethanol exposure during adolescence increases EtOH induced sleep duration later in adult in the LORR test indicating an increase in ethanol-induced sedation in adulthood. In addition, mice that received ethanol during adolescence were more sensitive to the rewarding effect of ethanol in the CPP test. On the other hand, when we measured the ethanol's anxiolytic effect in the L/D boxes and EPM tests, no changes in the sensitivity to the drug were found. In addition, alcohol exposure during adolescence induced changes in alcohol withdrawal intensity as measured in the FST. Notably, mice that received ethanol during adolescence exhibit lower withdrawal signs in the FST compared to control mice.

## CHAPTER SIX: General discussion and Conclusion

### 6.1 Rationale and summary of overall hypothesis

Alcohol is one of the most commonly used drugs within the adolescent population. Adolescent alcohol dependency is a complex disease. Social, environmental, and genetic factors along with a biological basis all play significant roles in initiation of alcohol use and contribute to progression from use to addiction and/or abuse. As discussed in the Introduction section, alcohol dependence still represents one of the major challenges to the future of public health.

Adolescence is the final developmental period leading to adulthood. It occurs approximately between 12 to 18 years old in humans and between 28 to 60 postnatal days in rodents (Spear 2000; Laviola et al. 2003). During this critical period, the brain is undergoing major developmental changes in addition to other various biological, hormonal, behavioral, etc. changes (for review Spear 2000). These changes may contribute to individuals participating in activities such as risk-taking, novelty seeking, increased social interactions and increased vulnerability to abuse of alcohol as well as other drugs of abuse.

The majority of the literature links tobacco use in human adolescence and subsequent developmental problems in adulthood including effects on later tobacco, alcohol, cocaine, and other illicit drug use and mental health (Anthony and Petronis 1995; Clark et al. 1998). Given the propensity for drug use later in life, it is vital to understand the behavioral and neurochemical processes that occur when adolescents are exposed to alcohol. Exposure to ethanol may have distinctive effects during adolescence, making this time more of a vulnerable period to long-term effects than adults, and contributing to increased risk to developing drug dependence in adulthood. Indeed, studies over the last decade demonstrated that sensitivity and tolerance to ethanol's pharmacological effects varies between adolescents and adult, across species (See

Spear, 2000 for review). This valuable information suggests that there are major differences in the mechanisms that drive alcohol use and abuse in adolescents and adults.

Despite the important contribution of understanding the impact of adolescent alcohol abuse on drug dependence behavioral outcomes in adulthood, few studies assess the effect of ethanol exposure during adolescent on drugs of abused behavioral response occurring in adulthood. Additionally, the studies which address the mechanisms underlying alcohol priming effects are lacking; therefore, the aim of this study was to focus on the impact of adolescent alcohol exposure on the subsequent behavioral response toward alcohol. While it is difficult to explore and understand the causes for this age-related vulnerability in human subjects, the use of animal models allows the exploration of the various behavioral and molecular mechanisms involved.

We hypothesized that adolescent exposed to “binge drinking” alcohol would demonstrate increased sensitivity to ethanol when compared to adults who are first exposed to alcohol in adulthood. To this aim, our studies have focused on establishing and characterizing a mouse model to study the effects of alcohol in adolescence. Secondly, our studies concentrated on identifying the behavioral impact of adolescent ethanol exposure on alcohol intake and drinking in adult. Finally, we conducted behavioral studies to elucidate in part the mechanism underlining the relationship between adolescent ethanol exposure and enhance alcohol intake in adult mice. We hypothesized that these changes in alcohol intake are due to an alteration between ethanol aversive and rewarding properties. The work in this dissertation aims to gain a better understanding of the specific behavioral differences between adolescent and adult brains underlying the effects of alcohol. The benefit of this type of research is that it could advance the understanding of the adolescent period to aid in the development of better preventions and

intervention approaches and policies. Specific drug-dosing regimens tailored to adolescents, or even specific compounds with superior efficacy will be critical in the development of pharmacological treatments in younger alcoholics.

### 6.2 Establishment of alcohol exposure in adolescence: Impact of pattern of drinking, sex and genotype.

Our studies sought to further explore the work done in rodents, by characterizing the impact of alcohol in adolescence on the overall intake and consumption of the drug later in adulthood using two paradigms of drinking (forced = DID and voluntary = 2-BC). For that, we used ethanol continuous access (CA, daily) versus intermittent access (IA, every other day). Consistent with previous data in adolescent mice (Chester et al., 2008; Tambour et al., 2008; Strong et al., 2010; Moore et al., 2010), we found that adolescent C57BL/6J mice given ethanol in “binge drinking” pattern (using the DID model) consume more ethanol when they become adults than do C57BL/6J naïve mice first exposed as adults (Fig 3 and Fig 5). Interestingly, our data is consistent with recent reports on voluntary EtOH drinking during adolescence using the two-bottle choice procedure. EtOH drinking during adolescence has been shown to enhance adult EtOH consumption later in life. (Walker and Ehlers, 2009; Strong et al., 2010; O’Tousa et al., 2013; Jamie E. Toalston et al., 2014). However, when we calculate the ratio of alcohol intake for each single day of adolescence second exposure versus first exposure to investigate the impact of patterns of exposure, we found that CA is significantly higher than IA. Although most of the studies showed that female were more vulnerable to the effect binge drinking, our data with female adolescent mice in the first phase of exposure showed a trend to consume more ethanol than male but no statistical significance was observed.

In addition, the pre-exposure to ethanol in adolescent mice elevated the subsequent consumption of ethanol in a limited access procedure (DID) as well as in an unlimited access procedure (2-BC) when the animals were tested as adults, when compared to ethanol intake in naïve adult mice. Our results using two bottle choice (2-BC) procedure in the daily intake profile of alcohol exposure at PND72, we did not see an enhancement in the alcohol consumption using 2-BC comparing to what we saw in the DID paradigm. However, after one week of deprivation, mice were exposed 15% EtOH for one week using the 2-BC procedure. Only the first day of ethanol exposure showed the significance different between the animals that were exposed to ethanol during adolescence.

Furthermore, our study comparing ethanol consumption of in adolescent C57BL/6J and DBA/2J by giving the same amount of ethanol concentration in the same conditions to avoid caveat that B6 is high ethanol consumer compared with DBA/2J using the DID paradigm. Interestingly, our results are in agreement with a recent study that examined ethanol intake in adolescent C57BL/6J and DBA/2J mice. Using DID model; the authors found that adolescent C57BL/6J mice consume more ethanol than adult counterparts, while there was no difference between adolescent and adult DBA/2J mice (Moore et al., 2010). However, One important caveat to note in this study is that because ethanol intakes differed between adolescent C57BL/6J and DBA/2J mice (where C57BL/6J mice consumed significantly more ethanol than DBA/2J mice) it is not possible to know if DBA/2J mice provided with the same amount of ethanol exposure during adolescence would increase subsequent ethanol consumption in adulthood.

### 6.3 Early adolescence presents a unique period of vulnerability to alcohol effects

Importantly, our results demonstrated for the first time that the enhancement of ethanol

intake after early exposure to ethanol is a unique phenomenon to adolescence period. When we tested adult naïve mice using DID procedure, we did not observe an enhancement of their consumption later in life. To our knowledge, this is the first demonstration that initial “binge drinking” experience selectively increases subsequent ethanol intake during adulthood when the initial exposure occurred during the adolescent period of development. Our results suggest also that in the same condition, the sex of the animals is not a significant factor in the enhancement of alcohol consumption later in life.

#### 6.4 Pharmacological and behavioral mechanisms involved in alcohol priming effect.

Finally, exposure to alcohol during early adolescence enhanced sensitization to alcohol during adulthood. We demonstrated that ethanol exposure during adolescence induces a less aversive state (less withdrawal intensity in the FST test) later in adulthood. In addition, ethanol’s sedative effects were enhanced in mice exposed to ethanol in adolescence, suggesting the enhancement of the intoxicating effects of alcohol later in life. In contrast, our results showed that ethanol exposure in adolescence enhanced the rewarding properties of ethanol in the CPP test. The animals that received the ethanol during CPP showed no significant preference after conditioning with a low dose of alcohol (1 g/kg, i.p.).

One possibility for adolescent ethanol sensitivities is that adolescents may differ from adults in how quickly ethanol is absorbed, distributed, and metabolized in the body. However, it seems unlikely that age differences in pharmacokinetics alone could be responsible for both adolescent-typical attenuated and accentuated ethanol sensitivities. Consistent with the generally faster metabolic rate of adolescents compared to adults, thereby resulting in slightly but sometimes significantly lower levels of ethanol in adolescents than adults at some time points

after ethanol challenge. In addition, no differences were found with ethanol-induced anxiolysis in the EPM and LDB tests. Therefore, we believe that age differences in acute sensitivities to ethanol are thought to reflect pharmacodynamic rather than pharmacokinetic factors (Spear, 2007). For instance, when given a sedative dose of ethanol, adolescents not only generally recover their righting response about twice as rapidly as adults but, importantly, recover with significantly higher levels of ethanol in their brains compelling evidence that their brains are less sensitive to ethanol's sedative properties than are the brains of adults (Silveri & Spear, 1998).

The second possibility is that increases in the release of gonadal hormones at puberty may act on hormone-sensitive brain regions to alter ethanol responsiveness, thereby exerting an organizational role for the emergence of adult-typical ethanol sensitivities. There is little evidence for a notable contribution of puberty-related increases in gonadal hormones to the emergence of adult-typical ethanol sensitivities.

Other possibility contributors to adolescent ethanol sensitivities are related to developmental changes that occur in the neural substrates underlying ethanol's effects. Ethanol affects a variety of neural systems, including GABA, dopaminergic, serotonergic, cholinergic and opioid systems (Eckardt et al, 1998), with many of these neural systems undergoing sometimes marked developmental change during adolescence (Spear, 2000, for review). For instance, NMDA-R associated with the major excitatory neurotransmitter system in the brain – the glutamatergic system – exhibit developmentally enhanced activity during adolescence in certain brain regions (e.g., Kasanetz & Manzoni, 2009), whereas various components of the primary inhibitory neurotransmitter in brain – the GABA system – are still developmentally immature in adolescents (e.g., Brooks-Kayal et al, 2001; Yu et al, 2006). Given that ethanol's effects are mediated in large part by NMDA-R antagonistic and GABA stimulatory actions,



developmental changes in these systems could play a critical role in influencing adolescent responsiveness to ethanol.

### 6.5 Conclusion

Adolescence is a unique developmental stage during which alcohol has long-term effects on future drug-taking behavior. We demonstrate that early adolescent mice exposed to alcohol increased alcohol intake in different schedules in adulthood, which was affected by the dose, duration, and pattern of administration, age of exposure, and mice strain. This increase in the alcohol intake was correlated with an increase in alcohol rewarding properties and a reduction of alcohol aversive effects. This gives insight into the mechanisms at play that increase the likelihood that adolescents who are exposed ethanol will move on to alcohol abuse later in life. Further research will be required in order to more fully examine the mechanisms of action for the observed changes in alcohol reward.

Finally, our data strongly suggest that alcohol intake during adolescence may induce long-term behavioral impact which suggest a possible role for altered gene transcription in the brain. Further research will be required in order to more fully examine the mechanisms of action for the observed changes induced by alcohol. Adolescent alcohol users may be particularly vulnerable to the risk of alcohol abuse. Understanding how these variables relate in individual animals may provide us with a better functional understanding of our results.

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