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Exploring the Alcohol Deprivation Effect in Withdrawal-Seizure Prone and Withdrawal-Seizure Resistant Mice

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EXPLORING THE ALCOHOL DEPRIVATION EFFECT IN WITHDRAWAL-SEIZURE PRONE AND WITHDRAWAL-SEIZURE RESISTANT MICE

by

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Abstract

The alcohol deprivation effect (ADE) refers to a temporary increase in alcohol intake following a period of alcohol deprivation. Repeated ADE studies (Sinclair & Senter, 1968, Melendez, 2006) have shown that there is an innate tendency to increase consumption when access to alcohol is limited, and the ADE is considered to be an animal model for relapse drinking. The present study is the first to examine the ADE in mice selectively bred for high and low susceptibility for withdrawal seizures, withdrawal-seizure prone (WSP) and withdrawal-seizure resistant (WSR) mice, and the purpose of it was to determine the presence or absence of a correlation between the genetic mechanisms that code for alcohol withdrawal severity and the tendency to increase alcohol consumption with limited access to alcohol. The two different genotypes demonstrated the ADE in two different deprivation schedules and across two different environmental conditions. Because no significant difference in response to intermittent access was found between the WSP and WSR mouse lines, it can be concluded that the phenotypic relapse-like increased drinking when access to alcohol is intermittent (i.e. the ADE) is a result of genetic mechanisms separate from the underlying genetics that code for withdrawal severity.

This thesis is dedicated to Leonard Duffy

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Introduction

Consequences of Alcohol

There are over 130 million alcohol users in the United States. 17.6 million Americans abuse alcohol or are dependent on it (NIAAA, 2012). The prevalence of alcohol consumption is overwhelmingly high especially considering society's highly prejudiced and derogatory outlook towards victims of alcoholism (Carr, 2011). Throughout time people suffering from alcoholism, or alcohol addiction, have been scrutinized as miscreants who intentionally choose to behave in harmful ways. The irony of this misguided stigma is that alcohol used in social events doesn't have the same ill repute of the dangerous and addictive drug that it is. This could be due in part to its legality, but also because children grow up around it and learn that recreational alcohol use is not only tolerated, but encouraged by family, peers, and the media. Its immediate and short-term rewarding effects distract users from the numerous long-term health consequences, both psychological and physiological. In fact, heavy drinking (defined by having five or more drinks on one occasion at least once per week for males, and four or more drinks at one time at least once per week for females) contributes to the development of the three leading causes of death in the United states: heart disease, cancer, and stroke (RSA, 2011; Kochanek, et al.). It's true that social lubrication, behavioral disinhibition, and psychological ease and forgetfulness are desirable features of a social night spent with friends, and while these actions of alcohol are well known throughout the public, there is little education regarding the serious dangers of excessive drinking. An unfortunately high percentage of people throughout the world experience neurological adaptations (to be described in more detail later) that lead to alcohol

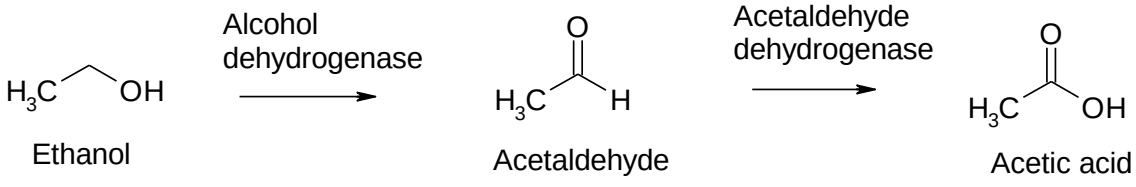
dependence and alcoholism; a disease that is extremely difficult to overcome. It can safely be said that nearly every American has been affected by an alcohol-use disorder, be it through a family member, close friend, or the massive economical impact of alcohol abuse (RSA, 2011). Anybody who has witnessed someone suffering from or been a victim of alcoholism knows that it is an extremely taxing and distressing disease. Often the question is, why? Why do you keep drinking even though you know it hurts your loved ones around you? Why do you continue to buy alcohol while you're sober? Frequently, people close to an alcoholic will take the behavior personally (an understandable response), and act aggressively towards the patient. Unfortunately, this is a very unhealthy behavior because although people suffering from alcoholism feel extreme guilt, they do not have the answers to those questions either. They are suffering from an out-of-control drinking problem, and have little ability to evade the compulsive cravings caused by legitimate neurological deficiencies. Addiction is a severe and unfortunate form of learned behavior, and causes a plethora of complex neural adaptations that cause an alcoholic to know only one way to cope: the bottle.

The widespread recreational use of alcohol is interesting considering the multitudinous harsh consequences following alcohol abuse. David Nutt (Nutt, et al., 2010) concluded that alcohol is the single most harmful drug of abuse when considering social harm to others as well as individual harm to the consumer. While heroin and cocaine were close behind, the fact that the prevalence rate of alcohol use is far greater than other drugs (aside from caffeine) leads the majority of drug researchers to agree with Nutt's conclusion. The popularity of alcohol despite its deleterious effects can be partly credited to poor public education. For example, terms surrounding alcohol-related

problems are defined inconsistently. The Diagnostic Statistical Manual of Mental Disorders, Forth Edition (DSM-IV), which is used as a guideline for the diagnosis of psychiatric disorders, defines alcohol abuse solely by social criteria, such as continued alcohol use despite legal, interpersonal, or role-fulfilling consequences (i.e. missing work). Alcohol dependence, considered more severe than alcohol abuse (by the DSM-IV), is defined mostly by behavioral criteria. A diagnosis of alcohol dependence requires experiencing at least three of the following symptoms within a period of one year: continuing drinking even with a desire or attempts to stop, drinking more than initially intended, sacrificing social activities because of drinking, and continuing drinking despite an awareness of the problems it causes. Its only physiological criteria are the presence of tolerance and withdrawal, and further indicators of quantity or frequency of drinking are not employed in the diagnosis (American Psychiatric Association, 2000). An important term not present in the DSM-IV is addiction. On the contrary, the term addiction (different than dependence) is traditionally used to imply intense psychological craving and loss of control over drinking. The word alcoholism is also not found in the DSM-IV, although very prominent organizations in alcohol research such as the National Institute on Alcohol Abuse and Alcoholism (NIAAA) and the Research Society on Alcoholism (RSA), as well as Alcoholics Anonymous (AA) incorporate the term into their name. It can be difficult to label an individual an alcoholic, because some people engage in "binge-drinking", heavy drinking in a short period of time resulting in elevation of blood-alcohol concentration (BAC) to at least 0.08 gram percent (Crabbe, Harris, & Koob, Preclinical studies of alcohol binge drinking, 2011) on a regular basis, and are physically dependent on alcohol (i.e. would experience signs of withdrawal), but don't experience

the subjective behavioral effects listed in the DSM-IV. [Additionally, many people (not uncommon among college students) engage in binge-drinking and are not dependent.] Thus, the upcoming fifth edition of the DSM will most likely differentiate between alcohol dependence and addiction (alcoholism). Nevertheless, binge-drinking indubitably has detrimental health effects and provokes permanent adaptations in the brain. Alcohol diversely affects many separate regions of the brain, and chronic heavy drinking evokes neuroplasticity, which is a phenomenon in which the brain permanently changes its structure and function due to adaptations to environmental conditions. It is not surprising that alcohol affects people in different ways, and the likelihood of developing an alcohol-use disorder as a result of chronic exposure varies between individuals. Alcoholism is a multifaceted disease, and one's experience with the drug is dependent upon many interacting psychological, biological, and sociocultural factors. Studies have shown that genetic and environmental factors equally contribute to the development or avoidance of alcoholism (Crabbe, et al., 2010; Enoch, et al., 2003), and genetic differences in biological factors such as pharmacodynamics and pharmacokinetics (the rate at which alcohol is metabolized), as well as genetic variations underlying personality (i.e. psychological) traits such as impulsivity and anxiety play a major role in one's risk factor for alcoholism (Kreek, et al., 2005).

Neurochemistry of Alcohol



The alcohol consumed in beverages is ethyl alcohol, or ethanol. Our bodies have an enzyme called alcohol dehydrogenase (ADH) that converts ethyl alcohol to acetaldehyde, which is a molecule that causes significant discomfort and toxicity if it's not quickly metabolized by the enzyme acetaldehyde dehydrogenase (ALDH) into acetic acid (demonstrated in the figure above). The final step of ethanol metabolism includes oxidation reactions that convert acetic acid into carbon dioxide and water. [It is important to know that isopropyl alcohol and methyl alcohol (methanol), while similar in structure to ethanol and just as easily available, are no substitutes for ethanol, as the metabolism of these alcohols produce fatally toxic substances.] The presence and quantity of ADH and ALDH, modulated by the genes coding for the synthesis of these enzymes, are one of many factors that play a role in determining one's risk factor for developing an alcohol-use disorder. For example, in Japan, approximately half of the population is lacking sufficient ALDH activity in the liver, causing a build-up of acetaldehyde and a considerably unpleasant drinking experience. As a result, the prevalence of alcoholism in Japan is relatively low compared to other parts of the world where this lowered ALDH activity is uncommon, such as in Europe and North America (Crabb, et al., 1988). Thus, genetic polymorphism exists in the ADH and ALDH genes, which is just one genetic variable that contributes to the complex network of genetic and environmental interactions that can lead to alcoholism.

Another factor that affects the digestion and metabolism of alcohol is the rate of absorption of alcohol into the blood. Ethanol is soluble in water, which is responsible for its ability to cross the blood-brain barrier and directly affect neurons, but also is significant in that it can be absorbed by food in the stomach (bread, for example). Thus,

when alcohol is consumed along with food, less alcohol is absorbed into the blood. Furthermore, females absorb more alcohol into the blood per total alcohol ingested, and they also have less body water than men, so the absorption results in a higher BAC. For this reason, the NIAAA and RSOA distinguish between males and females when defining "heavy drinking," and is why females require a smaller amount of alcohol before they experience symptoms of intoxication. Variability in absorption is one reason why epidemiological studies are difficult in alcohol research, and another difficulty of such study is that different cultures and ages have varying tendencies to drink with an empty or a full stomach. For example, Italy has a low occurrence of alcoholism even though the total alcohol consumption for people in the country ranks higher than average (World Health Organization, 2011); Italians normally drink while they eat. Therefore, the quantity of alcohol consumed does not directly translate to an increased quantity of alcohol in the bloodstream and affecting the brain, and especially the total amount of alcohol consumed is not directly related to prevalence of alcoholism.

Neurological Effects of Alcohol

Once ethanol is absorbed through the intestines and enters the blood, it complexly affects many neural processes and pathways, including the prefrontal cortex (impulse control and judgment), the cerebellum (motor control), the amygdala (anxiety), the preoptic area (sedation and sleep), and the hippocampus (memory). These multiple cerebral processes that are directly altered by alcohol respond differently from each other, as variables such as temporal factors and quantity of alcohol intake have various impacts on the specific regions. Overall, however, alcohol is considered a central nervous system (CNS) depressant, although a small amount of alcohol has been shown to increase

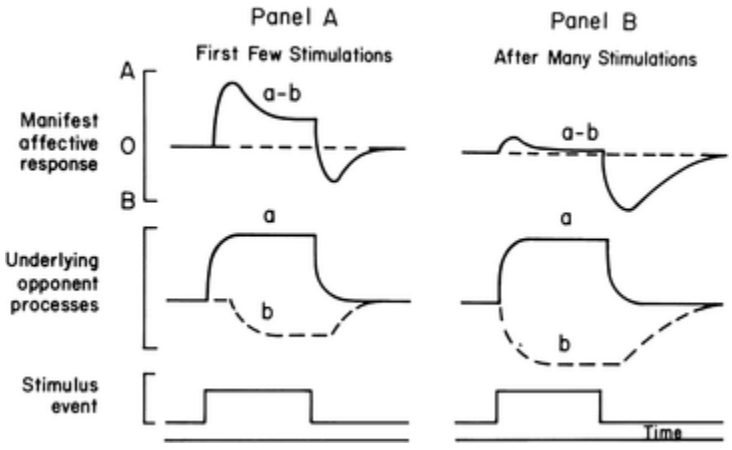
locomotive activity in fruit flies, rodents, and humans (Phillips & Shen, 1996). Alcohol affects the above brain regions indirectly, as well, by altering the most ubiquitous neurotransmitters in the brain: GABA and Glutamate, which executively regulate neural activity. While ethanol is present, it acutely reduces the presynaptic release of Glutamate (which underlies excitatory postsynaptic potentials) and is a Glutamate receptor antagonist, which results in a down-regulation of the excitatory system. Additionally, alcohol acutely potentiates inhibitory neural signaling by enhancing GABA release (which underlies inhibitory postsynaptic potentials), and activation of the $GABA_A$ receptor (Becker, 1998). This combined increase in inhibitory neurotransmission and decrease in excitatory neurotransmission results in alcohol's overall sedative effects, but with chronic exposure to alcohol, the brain undergoes neuroplasticity and counteracts alcohol's impact, resulting in functional tolerance. These combined excitatory responses that are prolonged after alcohol consumption is discontinued leads to the well-known signs of the alcohol withdrawal syndrome. Signs of alcohol withdrawal are (listed in increasing severity): anxiety, sleeplessness, delirium tremens, seizures, excitotoxicity (tissue death due to over-excitation) and brain damage (Clapp, et al., 2008). In summary, when alcohol is present in the brain, it causes overall depressive effects by both enhancing the principal inhibitory neurotransmitter GABA, and inhibiting the principal excitatory neurotransmitter Glutamate. After abstinence, the brain continues to correct for these depressive effects on the CNS, although there's no alcohol to counteract the neuroadaptations, and this leads to a problematic hyper-excitatory state and withdrawal symptoms.

Again, alcohol also acts on multiple regions in the brain, some of which are involved in the acute impairment experienced with alcohol, and others that are influential in more long-term withdrawal symptoms that are present after long periods of abstinence from alcohol in dependent people. A critical pathway in the chronicity of alcohol addiction is the so-called reward system and its major neurotransmitter, dopamine (DA). The DA reward pathway involved in virtually all addictive diseases primarily within the Ventral Tegmental Area (VTA) and Nucleus Accumbens (NAcc), overlaps and interacts with the neural substrates that modulate many fundamental behaviors including reward-seeking, positive reinforcement, goal-oriented behavior, and reproductive behaviors such as pair-bonding, sexual behavior, and maternal behavior. DA is a powerful neurotransmitter that has been considered from an evolutionary standpoint as a guiding chemical for optimal evolution and prolonged existence. Thus, it is not surprising that the powerful motivational effects of DA (i.e. in addiction) can be quite difficult to overcome.

Addiction is an unfortunate form of learning, which is fundamentally regulated by DA and the reward pathway. Alcohol acutely increases DA release, to which the brain reacts with neuroadaptive down-regulation of DA activity, leading to desensitization of one's ability to experience pleasure and reward. Altogether, chronic exposure of alcohol's stimulating effects on dopaminergic systems in the brain results in a permanent adaptation that results in an increased threshold of DA required to experience reward, as well as an overall decrease in DA reward-causing mechanisms (Koob, 2008). Simply put, chronic alcohol use blunts the reward system, which is an occurrence of neuroplasticity involving motivation that has enduring, detrimental consequences on one's overall hedonic state (happiness). The AA community often classifies alcoholism as an incurable

disease, as although one can indeed become an abstinent alcoholic, the repercussions never fully disappear. Extensive research exists regarding this phenomenon of alcohol-induced neuroplasticity causing a blunted reward system, much of which derives from a very influential model known as the opponent-process model of motivation, developed by Solomon and Corbett in the 1970s.

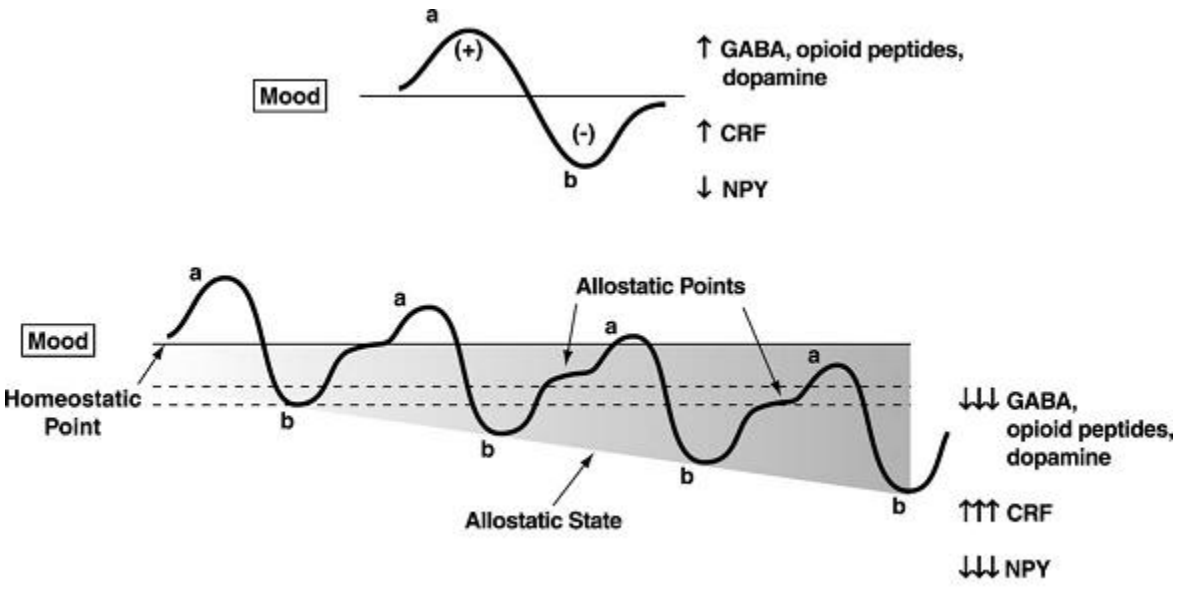
Opponent-Process Model of Motivation



The opponent-process model of motivation (Solomon & Corbit, 1974) shows that with exposure to a drug, there is both a euphoric and a dysphoric response caused by a combination of the drug's actions on the brain. The euphoric response is termed the A-process, and the dysphoric one is the B-process. Specifically, an increase in euphoria (the A-process) is due to an increase in activity of GABA, DA, and homogenous opioid peptides. The dysphoric B-process is due to an elevation of Cortico-releasing Hormone (CRF), which is a hormone that plays an important role in stress response, and a decrease in neuropeptide Y.

The model proposes that when a drug is taken, both the A-process and B-process occur simultaneously. The subjective experience is a summation of the two opponent

processes, which is a sensation that is different with recreational drug use than with chronic drug abuse. Regarding one's hedonic state, initial drug use propagates a considerably positive combined effect, followed by a mildly negative one. In contrast, the sum of the two underlying processes after repeated exposure results in tolerance to the A-process and sensitization to the B-process, an overall effect that causes one to feel marginally better, followed by much greater dysphoria. This is due not to a decrease to the rewarding A-process, but rather to an increase in the severity of the undesirable B-process.



A modification of the opponent-process model of motivation, proposed by Koob and Moal (2001), shows that rather than a decrease in the A-process and an increase in the B-process with chronic exposure to a drug, a steady decline of baseline mood state occurs when drugs are chronically administered. As one's normal mood state continuously declines, the A-process continues to result in a relatively euphoric sensation, but over time this process doesn't even reach the original homeostatic point

(what used to be the baseline level of happiness). Furthermore, the B-process (following metabolism of the drug) delves deeper into a more negative state of depression following repeated drug use. With chronic exposure to a drug, the receptors that are initially up-regulated to cause a positive hedonic state become down-regulated, and the chemical changes responsible for the negative hedonic state are exaggerated, resulting in a downward shift in baseline mood state that has been referred to as "hedonic allostasis." This is a frightening trend for those addicted to a drug, and it explains why drug-addicts continue to consume drugs despite known negative consequences, and even though they give self-reports that they don't enjoy the drug's effects anymore. For these unfortunate victims, the drug has neurologically altered the learning pathway so that they crave the drug because they have "learned" that it will help to relieve the dysphoria associated with absence of the drug. It also explains why people impulsively start taking drugs before they have a drug-problem: it causes a rewarding state of euphoria.

An extension of these models is that drug seeking in individuals who are dependent on a drug is motivated by a different mechanism than in drug-seeking behavior for non-dependent users. The transition from recreational drug use to drug abuse and addiction is due to a shift from control by positive reinforcement to control by negative reinforcement. Before one has experienced chronic exposure to a drug, one is motivated by the rewarding effects of the drug; positive reinforcement. But once repeated exposure to a drug leads to addiction, the motivational influences switch from positive reinforcement to negative reinforcement; meaning that the drug is ingested as an attempt to counteract the negative effects that exist without it. Especially concerning alcohol, the subjective experiences that a dependent drinker seeks to counteract via increased alcohol

intake are not the negative effects of acute withdrawal. Instead, relapse is not uncommon long after the two-to-three day detoxification period, and people often relapse after alcohol has not been present in their body for years. The opponent-process model of motivation attempts to explain this phenomenon of relapse after long periods of abstinence by implying that chronic exposure to a drug causes a state very similar to, if not identical to, chronic depression. In conjugation with knowledge of alcohol's acute stimulating effects on the VTA and NAcc learning pathway that cause chronic desensitization of dopaminergic reward thresholds and down-regulation of DA activity, this appears to be a valid model of the long-term effects of alcohol on one's general psychological state. An understanding that alcohol, no different from other addictive drugs, dramatically alters the brain and its basic pathways underlying happiness and motivation in a permanent way is imperative for having compassion for a victim of its severe consequences. Furthermore, because the brain is an organ, and drugs physically alter the pathways and chemicals that exist within it, it could be argued that there should be no distinction between psychological and physiological diseases (Carr, 2011). The term "psychologically addicted" has commonly not been perceived as a legitimate issue by the general public, but because addictive drugs alter the structure and function of important regions of the most complex organ in the body, psychological effects should not be overlooked. In fact, psychological craving is no less difficult to overcome than physiological withdrawal symptoms.

The transition from casual drinking to the problematic drinking only occurs in some people (approximately 14% of alcohol users, which although is a small percentage, it's nonetheless an alarmingly high number of people). For many years, researchers have

been searching for a definitive answer to the following question: What causes users to become addicts? While independent factors that increase the likelihood of developing a drinking problem have been identified, alcoholism is a very complex disease that results from multiple genetic and environmental factors (Silva, et al., 2007). It is clear that heredity plays a critical role in one's susceptibility for alcoholism, as children with alcoholic parents are four times more likely to become alcoholics than children with nonalcoholic parents (AACAP, 2002; NIAAA, 2012). But a person with genetic traits that increase the risk for succumbing to alcohol dependence is by no means doomed to alcoholism. Environmental factors such as lifestyle, peers, and awareness of the risk (Haller & Chassin, 2010) have been repeatedly shown to influence the onset of the illness. Researchers are currently attempting to identify the specific genes involved in the familial transference of alcoholism, and although a negative correlation between the ALDH2 gene (which blocks the synthesis of ALDH) and alcoholism in Japan have been identified (Rothhammer, et al., 1994), and genes that code for specific personality traits have been shown to influence one's risk factor for alcoholism, specific genes in humans that directly result in alcoholism susceptibility have yet to be identified. A common tool that researchers have used for over 50 years in the study of individual components of alcoholism is the use of rodents as animal models. Certainly there are limitations to this method, because alcoholism is a human-specific disease; only humans can be diagnosed with it due to its subjective behavioral and social criteria. But there are also multiple advantages to animal models, notably the validity of studying one individual component of the complex disease, which is impossible to do among the human population.

Animal Research on Alcohol

The use of animals to study the effects of alcohol on the brain and to study predisposed risk factors for vulnerability to an alcohol use disorder has greatly enhanced our knowledge of alcoholism in a way that would be impossible by using only humans. Although it is true that alcoholism is a human-specific disease, human studies pose a difficult situation of broad genetic heterogeneity, multitudinous and uncontrolled environmental variables, and extensive ethical constraints. Additionally, non-human animal experiments are not conducted as an alternative to human experiments simply to avoid inducing unpleasant and permanent consequences in human participants, but animals frequently serve as a superior model when studying separate aspects of alcoholism. Selective breeding techniques (controlling for genetics) and experimental manipulations (controlling for gene-environment interactions), which are possible only in animals, allow for extraneous variables to be controlled for, which increases the face-validity of any result by making the component of alcoholism in question be the only dependent variable. Thus, phenotypic differences observed when comparing strains of inbred or selectively bred animals (to be discussed later) employed in controlled environmental conditions can be confidently attributed to the genetics underlying that specific phenotype (Crabbe, 2008). But, as previously mentioned, alcoholism is a disease specific to humans only. Staying home to drink instead of attending a social event, obsessive thoughts about obtaining alcohol, and drinking even while aware of adverse consequences are behaviors not applicable to animals, which is an obvious limitation to the usefulness of such studies (McClearn, 1979; Lovinger & Crabbe, 2005). These incomplete animal models (animal models that can only study part of the disease), most

frequently using rodents, are often superior to humans when studying one isolated component of alcoholism, such as alcohol withdrawal, alterations in consumption as a function of different schedules of alcohol availability, a specific neurological pathway involved in dependence, etc. Their ability to have superfluous variables controlled has proven to be a useful research tactic, and many different animal models representing different components of human problem-drinking have been created.

Genetic Manipulations

Mice and rats especially have served as prime organisms to be studied in order to gain knowledge about humans. Mice are desirable to use as models for human research because its genome has been well characterized, and a close relationship (greater than an 85% similarity) has been established between the mouse and human genomes (Lovinger & Crabbe, 2005; Tabakoff & Hoffman, 2000). The first genetic animal model in mice research was an inbred mouse strain created by Dr. Clarence Cook Little in 1909: the DBA strain (Staats, 1966). Inbreeding is a method in which siblings are mated together for 20 or more generations to produce genetically identical animals that not only share identical genes, but also have two identical alleles of each gene (i.e. are homozygous), making them genetically more similar than identical twins. Another useful genetic animal model, which has been practiced since the dawn of agriculture and is the original technique used in behavioral genetics (Crabbe, 2008) is known as selective breeding. Selective breeding was first performed for the sake of alcohol research in a laboratory setting in the 1940s, when Jorge Mardones bred rats for high ethanol-intake preference (Mardones & Segovia-Riquelme, 1983). It is an approach used to create animal models that have a high or low affinity for a specific trait. The process involves selecting animals

from a heterogeneous population that display high or low sensitivity to a particular phenotype, and breeding them with other animals that exhibit similarly high or low sensitivity for that same trait. After several generations of the selective breeding process, the gene that codes for the desired trait becomes more and more prevalent (frequent in the genome) throughout the offspring. Eventually, all of the selectively bred animals share common genes that code for a high or low sensitivity to a certain phenotype. Sometimes these animals are compared to their genetically heterogeneous progenitor (Crabbe, et al., 2009), but sometimes the selective breeding is a bidirectional process that results in two complementary lines with opposite severity for the same trait (McSwigan, et al., 1984). The major advantage of selective breeding is that if the two selectively bred lines differ in any traits beyond the attribute for which it was selected, the differences are theoretically a result of genetic linkages between the phenotypes. Breeding techniques are classic approaches to functional genomics, and their great stability allows them to be very important in aiding more modern techniques of molecular biology used in the rapidly progressing field of alcohol research today.

Environmental Manipulations

In conjunction with breeding techniques, a predominant approach to studying behavioral aspects associated with alcohol use has been to manipulate the availability of alcohol and observe the subsequent findings such as dependence, tolerance, or withdrawal. But a major limitation with rodent studies is that rodents very infrequently drink enough alcohol to reach levels of intoxication when they have 24-hour access to both alcohol and water, even when they're genetically predisposed to have high alcohol preference levels. Thus, environmental manipulations have been employed in order to

provoke a pharmacologically significant BAC, normally 0.08 or above. It is important for researchers to overcome the obstacle of limited free-choice drinking by rodents because problem drinking in humans certainly involves excessive intake. Thus, in order to study the biological, neurological, and behavioral effects of chronic exposure to pharmacologically significant levels of alcohol in rodents, researchers must provoke elevated intake levels. Various methods attempting to overcome this limitation (reviewed in Crabbe, et al., 2011) include forced administration, food and/or water deprivation, limiting access to alcohol, sweetening the solution, and fading procedures in which the concentration of ethanol in the solution is gradually increased over time. The classic method used to engender dependence for over 50 years (Goldstein & Pal, 1971) has been to use ethanol vapor which forces the animals to ingest airborne ethanol through breathing. Numerous studies have incorporated a food and water deprivation protocol in which animals are forced to drink water or eat ethanol-infused food in order to satisfy their nutritional requirements. Sweetening ethanol solutions and fading procedures have been used as methods to evade taste-aversion effects of ethanol. But while these methods do successfully increase BACs, they do not imitate the motivational factors that drive humans to drink. One of the simplest ways to increase ingestive motivation is to present alcohol on an intermittent basis, which has repeatedly been shown to increase voluntary drinking (Breese, et al., 2005; Sanchis-Segura & Spanagel, 2006; Crabbe, et al., 2011). Voluntary, free-choice preference drinking is an important criterion for an animal study investigating motivational factors behind increased drinking, as it is the only method in which humans drink. While these animal models cannot fully represent the human situation, Cicero (1980) proposed a set of criteria that a non-human model should meet

for the study to most completely represent it. They are as follows: 1) ethanol should be self-administered orally; 2) the amount of ethanol consumed should elevate BACs to pharmacologically significant levels; 3) ethanol should be consumed primarily for its pharmacological effects, rather than calories, taste or smell; 4) ethanol should be positively reinforcing; 5) chronic ethanol consumption should produce metabolic and functional tolerance; and 6) chronic ethanol consumption should produce signs of physical dependence (Rhodes, et al., 2005). While it is extremely difficult to create an experiment that meets all of these criteria, various intermittent access schedules have been developed in an attempt to most-closely resemble human alcoholic drinking. The original intermittent-access protocol [presenting animals with a full week of deprivation following multiple weeks of constant access (Sinclair and Senter, 1968)], the drinking in the dark (DID) protocol (in which animals are presented with alcohol only during a few hours during the night, their active phase), developed by John Crabbe (Crabbe, et al., 2009), every-other-day access to ethanol developed by Wise (1973) (and frequently referred to as the "Wise protocol"), and one-day per week access are all intermittent access protocols aimed at engendering elevated voluntary alcohol drinking (Crabbe, 2008). Sinclair and Senter (1968) pioneered the first intermittent-access schedule almost half a century ago using rats, who have continued to consistently show an increase in drinking upon presentation of alcohol after it has been unavailable for a period of time; a phenomenon that has been termed the alcohol deprivation effect (ADE). The ADE is a model of binge-like relapse drinking characteristic of alcohol-dependent humans (Sparta, et al., 2009). It represents compulsive drinking after a period of abstinence, which is an all-too familiar behavior for those directly affected by alcoholism. Again, this is not a

complete representation of relapse drinking, but it has repeatedly shown an innate tendency to voluntarily increase the amount of alcohol consumed as a result of intermittent access (which is a frequent drinking-pattern of human alcoholics) in many different strains of rats (Wise, 1973; Simms, et al., 2008; Hargreaves, et al., 2009; Loi, et al., 2010), mice (Melendez, et al., 2006; Sparta, et al., 2009; Melendez, 2011; Hwa, et al., 2011), and monkeys (Kornet, et al., 1990; Sinclair, 1971). Furthermore, our lab has previously demonstrated a robust ADE under two different deprivation schedules in four different mouse genotypes: C57BL/6, C3H/HeJ, HDID-1, and HS/Npt. Employing different selectively bred genotypes in intermittent-access schedules is a method aimed at identifying genetic predispositions that may alter the expression of the ADE, therefore helping to identify genes associated with binge-like relapse drinking. The present study incorporates intermittent-access methods using mice selectively bred for their susceptibility of withdrawal-seizures and examines the resulting ADE.

The Present Experiment

In the present study, the ADE was explored in selectively bred withdrawal-seizure prone (WSP) and withdrawal-seizure resistant (WSR) mice. The experiment involves monitoring voluntary intake of both water and 10% ethanol solution during six-week periods of varying alcohol availability (constant access to ethanol, 1-day per week access to ethanol, and 3-days per week ethanol access, with constant water availability), and analyzing for both ethanol intake (adjusted by weight) and preference. By using mice selectively bred for differences in alcohol withdrawal severity, this study tested the hypothesis that the gene(s) that influence withdrawal severity partially overlap with those that promote relapse drinking, therefore helping to identify the genetic risk profile for

alcohol abuse. Previous literature (Kosobud, et al., 1987; Metten, et al., 1998; Crabbe, 2002) has shown that WSR mice consume more alcohol than WSPs, indicating a negative genetic correlation between alcohol withdrawal severity and voluntary alcohol consumption. Therefore, the factors that determine the severity of alcohol withdrawal symptoms and alcohol preference appear genetically codetermined, at least partially. Other aspects of alcoholism that have been studied using the WSP and WSR selectively bred lines include neurosensitivity to alcohol and tolerance, neither of which differ between lines (Crabbe & Kosobud, 1986). WSP and WSR mice didn't show different responses to either ethanol-induced hypothermia or their loss of righting reflex due to alcohol. Further, when WSP and WSR mice are given equal doses of ethanol, their BACs raise to an equal level (McSwigan, et al., 1984) indicating that the genes coding for the differences in withdrawal severity are separate from the genes that code for the pharmacological metabolism of alcohol. Also in the McSwigan, et al. (1984) experiment, a negative genetic correlation between withdrawal sensitivity and sensitivity to ethanol's depressive effects of activity in the CNS was found. The WSP mice have an increased susceptibility of developing dependence for alcohol (increased susceptibility of withdrawal symptoms upon alcohol withdrawal); yet have less depressive effects present in the CNS. Conversely, WSR mice demonstrate a greater CNS depressive effect from alcohol. Therefore, there is a negative correlation between the gene causing withdrawal symptoms and the gene causing acute depression of CNS activity due to alcohol. It is important to note that it was also found that the two lines differing greatly in their susceptibility for alcohol withdrawal-induced seizures did not differ in their sensitivity to seizures produced by non-ethanol agents, other than benzodiazepine, which also

potentiate the $GABA_A$ receptor (Crabbe & Kosobud, 1986). In summary, the genes coding for neurosensitivity, tolerance, and metabolism of alcohol are independent from the genes causing sensitivity or resistance to alcohol withdrawal seizures, while some genes underlying alcohol preference drinking and sensitivity to alcohol-induced CNS depression are hypothesized to be responsible for the sensitivity to alcohol withdrawal.

Given that the different component phenotypes associated with alcoholism are due to different, partially overlapping sets of genes, an experiment aimed at identifying the presence or absence of a correlation between sensitivity to alcohol withdrawal and the display of excessive drinking during relapse-like conditions will be valuable to the field of alcohol research. Employing the classical functional genomics approach of selectively bred animals to compare this genetic relationship could possibly lead to identifying the specific genetic risk factors for the human onset of alcoholism.

Methods

8 male WSP-2 and 10 male WSR-2 mice were obtained from the breeding facilities at the Oregon Health and Science University at 8 weeks of age and were housed in commercial use cages equipped with running wheels upon arrival (wheel diameter of 11.5cm Coulbourn Instr., Whitehall, PA). 4 WSP mice and 5 WSR mice had their running wheels mechanically locked, to test the effects of exercise on intake. Cages were housed 3 per shelf in light and sound constricting cabinets with fluorescent tube lights atop each shelf. Wheel-turns were monitored via microswitch and stored in 1-minute bins using the ClockLab computer interface system (Coulbourn Instr., Whitehall, PA). The ClockLab computer interface system also controlled the 12:12 light dark cycle. Food (Prolab RMH 3000) and water (plain tap water) were continuously available ad-libido

throughout the experiment, and 10% v/v ethanol solution was provided intermittently, as described below. Fluids were provided in separate bottles equipped with standard ball-point sipper tubes.

The mice had a 2-week acclimation period, and in the first phase of the experiment referred to as the baseline phase, the mice were then given continuous access to water and ethanol (in separate bottles). After 6 weeks of free-choice continuous preference drinking, the second 6-week phase consisted of intermittent 1-day per week access to ethanol (the IA1 phase). The ethanol bottle was removed and mice were presented with two separate bottles of tap water for 6 days, and one day per week the ethanol bottle replaced one water bottle, allowing for free-choice preference drinking for 24 hours. Water and ethanol intakes were recorded for each of the 6 total free-choice drinking days. The mice were subsequently returned to continuous access to both ethanol and water for the third 6-week phase of the experiment (the continuous-access-2 phase), and measurements were taken as described for the baseline phase. This was followed by an additional intermittent access phase in which the mice had three separate 24-hour free-choice drinking days each week (conducted identically to the one day of ethanol access in the IA1 phase), beginning each Monday, Wednesday, and Friday (the IA3 phase). In the fifth and final phase of the experiment (the final continuous-access phase), one bottle of ethanol and one of water were constantly available for 6 weeks. Throughout the 30 weeks of the experiment, body weights were measured each week, and bottle positions were switched after each data measurement. For the 3 continuous access phases, ethanol and water intakes were recorded once per week, and for the 2 intermittent access phases, ethanol and water intakes were recorded following each 24 hour free-access period.

Additionally, the mice were subjected to preliminary testing for handling-induced convulsions (HIC). No baseline tests were taken while these animals were alcohol-naïve, due to a late decision to include this testing into the experimental design, or after the baseline phase, but on the final day of each phase, alcohol was unavailable for a 6-hour period, causing the alcohol to be metabolized from the animals' systems. After 6 hours of alcohol deprivation, the HIC testing was conducted by lifting each mouse (independently) by their tail, observing, and then gently rotating them and again observing for signs of convulsions. The convulsions elicited by handling were scored using the scoring system described by Goldstein (1972) on a 0 to 4 scale: 0 = No withdrawal signs; 1 = Tonic convulsion when the mouse is lifted and given a gentle 180 turn; 2 = Tonic-clonic convulsion elicited by the gentle spin, or tonic convulsion when lifted without turning; 3 = Tonic-clonic convulsion not requiring any spin; 4 = Violent tonic-clonic convulsion, often continuing after release of the mouse. Scores were applied to each mouse simultaneously by 2 scorers silently and independently, ignorant of the other's score, and the line of mouse was unknown, to avoid bias. For data analysis, the average score assigned by the 2 scorers was analyzed.

Ethanol intakes were expressed as grams of ethanol per kg of body weight per day, and water intakes were expressed as ml per mouse per day. Ethanol preference ratios were calculated as the volume of 10% ethanol divided by the total fluid consumed (ethanol plus water). Data were analyzed using a 3-way mixed factorial ANOVA, with strain and running-wheel access as between-groups factors. The experimental phase (baseline, IA1, continuous access-2, IA3, and final continuous access) was the within-

groups factor. Follow-up pairwise comparisons between experimental phases within strains were performed using Bonferroni-protected dependent-samples t-tests.

Results

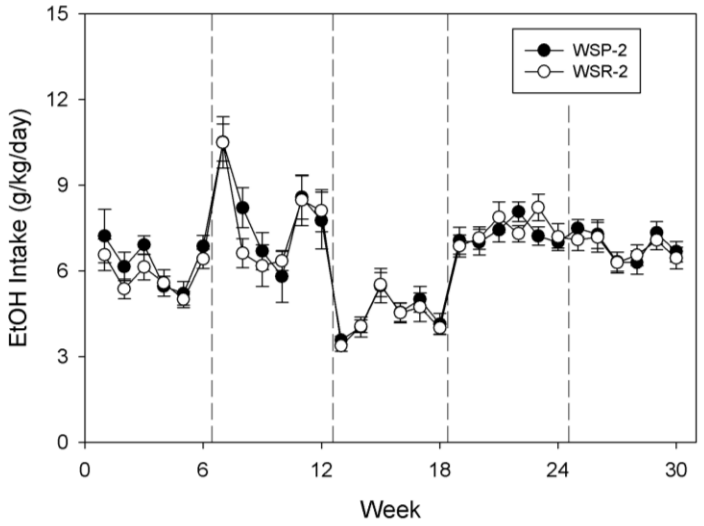


Figure 1. Ethanol intake at weekly intervals, separated by breeding line.

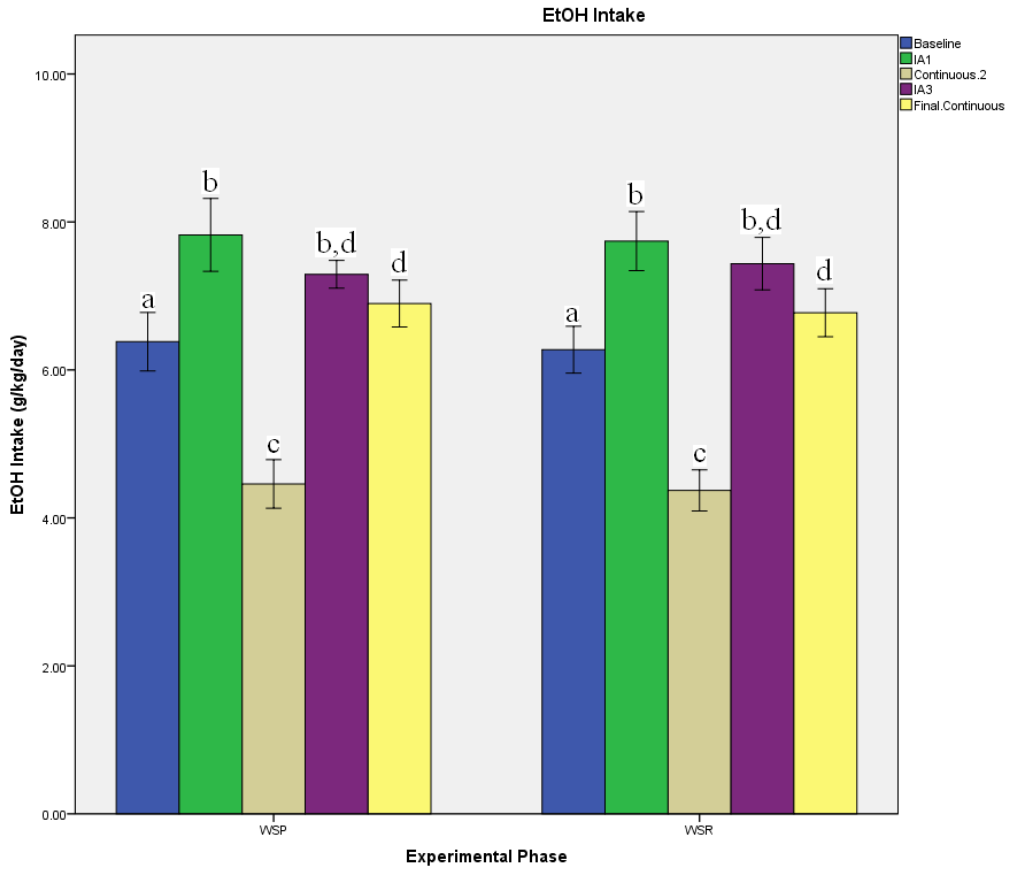


Figure 2. Ethanol intake during each experimental phase separated by breeding line. Significant differences are indicated by dissimilar alpha designators.

Contrary to our initial hypothesis, both the WSP and WSR mice showed nearly identical ethanol intake levels throughout the experiment. Both lines significantly increased their ethanol consumption during the intermittent-access periods compared to the preceding continuous-access periods (Figs 1, 2). As shown in Figure 2, ethanol intake increased approximately 30% from the baseline phase to the first intermittent (IA1) phase, then dropped below baseline levels during the second continuous-access phase, and escalated again during the following intermittent-access (IA3) phase. Notably, a persistent elevated ethanol intake level during the final continuous-access condition was

exhibited. The mice significantly altered their alcohol consumption during each experimental phase [i.e. analysis of ANOVA showed that ethanol intake significantly differed across the experimental phases ($F(4,72) = 45.996$, $p < 0.001$)], and there were no significant differences of intake between the two breeding lines or running-wheel access conditions. Ethanol intake was lowest during the second continuous-access phase, and the highest intake levels were found in the IA1 phase. No difference was detected between the two intermittent-access conditions, or between the IA3 phase and the subsequent final continuous-access phase.

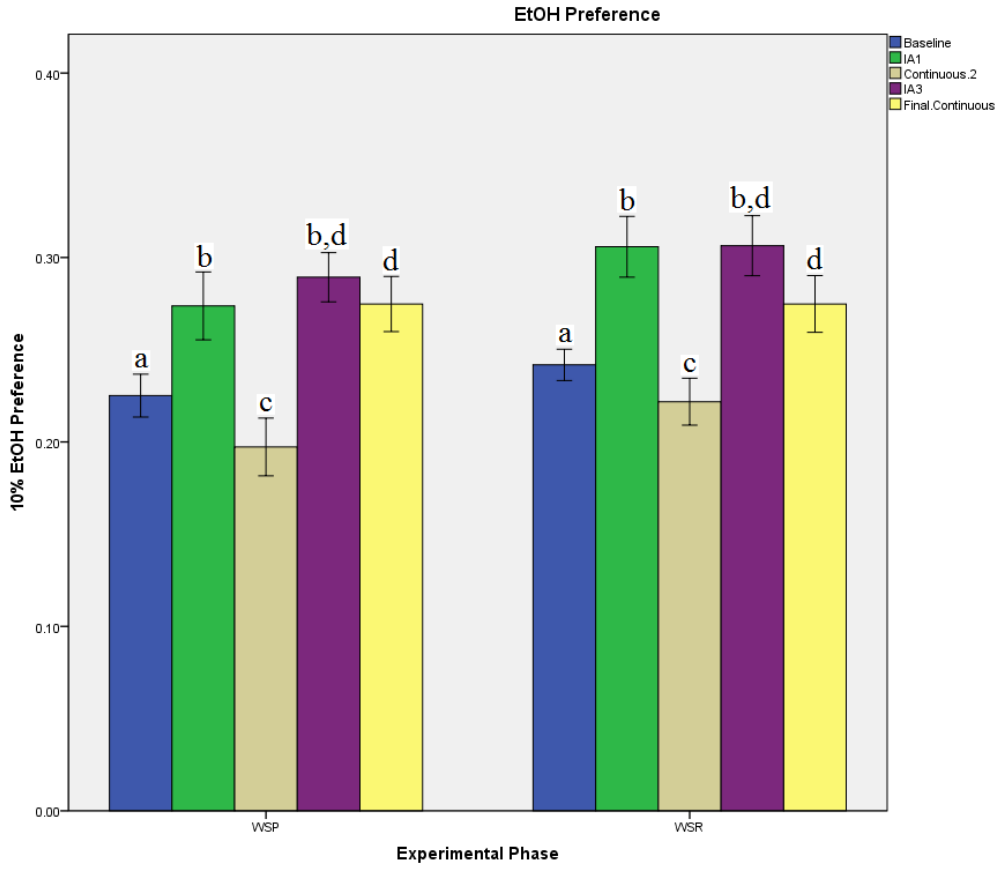


Figure 3. Ethanol preference during each experimental phase separated by breeding line. Significantly differences are indicated by dissimilar alpha designators

Analysis of ethanol preference (Figure 3) is very similar to the analysis of ethanol intake. Thus, there was a main effect on experimental phase, indicating that preference significantly differed across experimental phases ($F(4,72) = 30.050$, $p < 0.001$), and pairwise comparisons indicated that ethanol preference was significantly different between each phase other than between the IA1 and IA3 phases as well as between the IA1 and the final continuous-access phases (no different than ethanol intake pairwise comparisons).

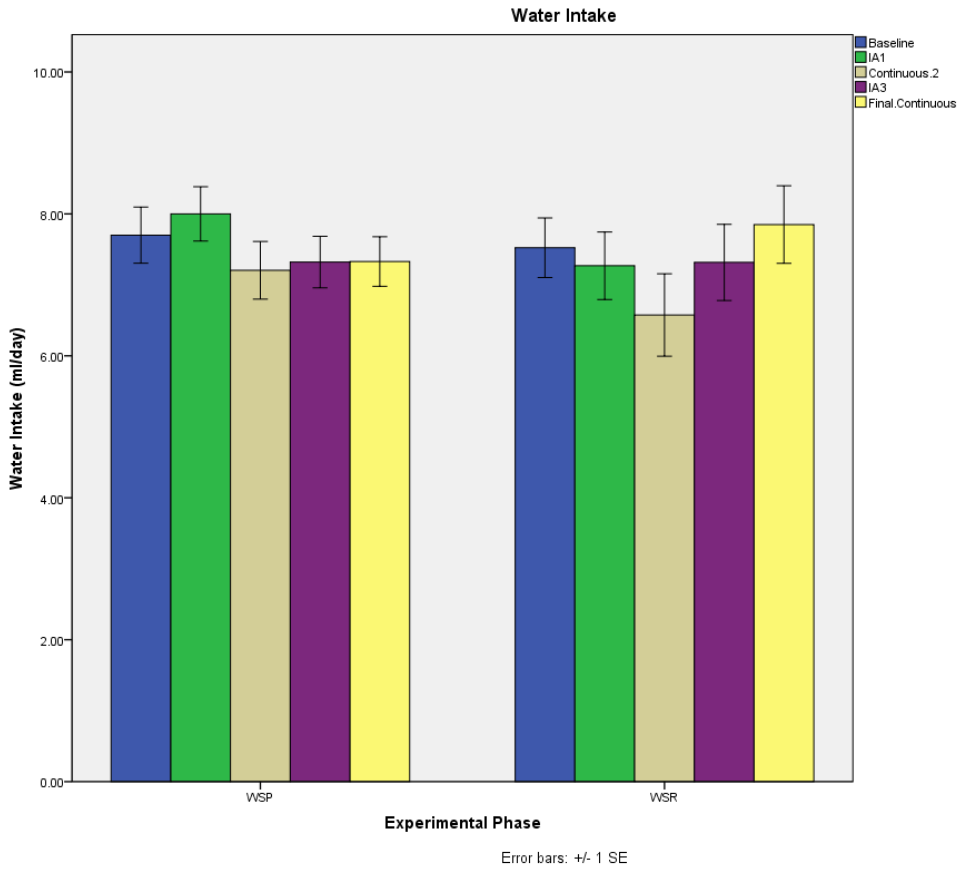
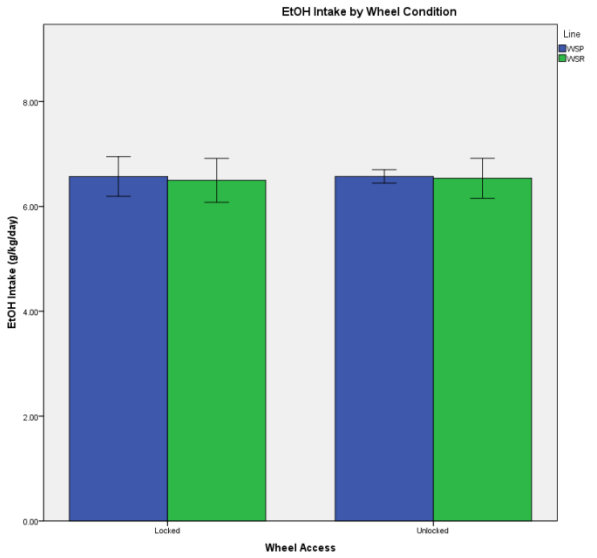


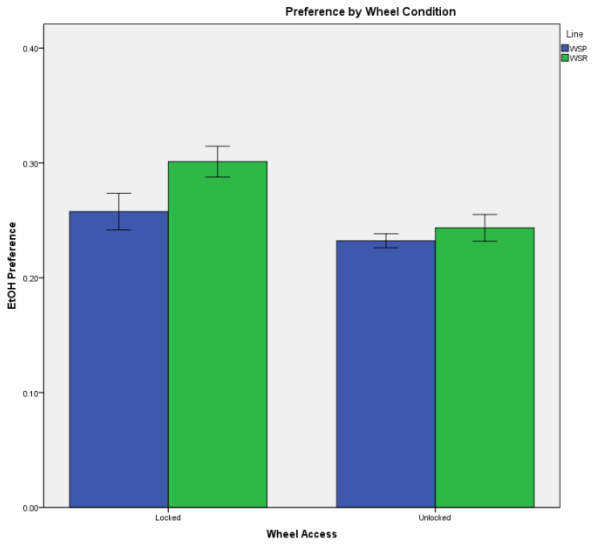
Figure 4. Water intake during each experimental phase separated by breeding line.

Water intake (Figure 4) also showed a main effect of experimental phase ($F(4,72) = 2.916$, $p = 0.027$), and there was also a phase by breeding line interaction, indicating

that the two lines showed different pattern of responses of water intake to the different alcohol-availability schedules throughout the experiment. However, there were no significant differences in water intake between WSP and WSR mice for any of the 5 experimental phases. Pairwise comparisons showed that water intake differed significantly between the continuous access-2 phase and both intermittent-access phases, as well as the final continuous-access phase.

Both genotypes with access to running wheels showed significantly lower ethanol preference ($F(4,72) = 12.204$, $p = 0.003$) than animals with locked wheels, which is most likely due to the significantly higher water intake ($F(1,18) = 5.969$, $p = 0.025$) exhibited in mice with wheel access, because ethanol intake levels did not differ between wheel-access conditions (Figure 5).





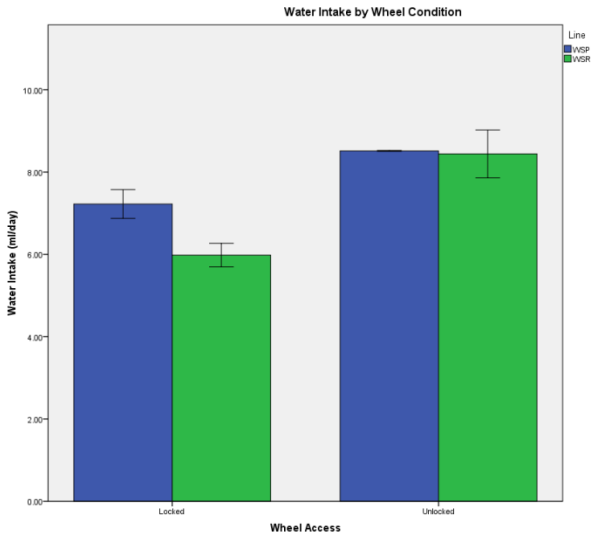


Figure 5. Ethanol intake, ethanol preference, and water intake, separated by running-wheel condition. Error bars are +/- 1 SE.

Analysis of HIC testing indicated a main effect of line, showing that WSP mice scored significantly higher than their WSR counterparts ($F(1,18) = 9.348$, $p = .007$), and there was no main effect of wheel-access condition. WSR mice showed a different trend of HIC scores following different schedules of alcohol availability than WSP mice, as only the WSR line showed a tendency to decrease their convulsion ratings as the experiment progressed, but the difference was not quite significant ($p=.074$).

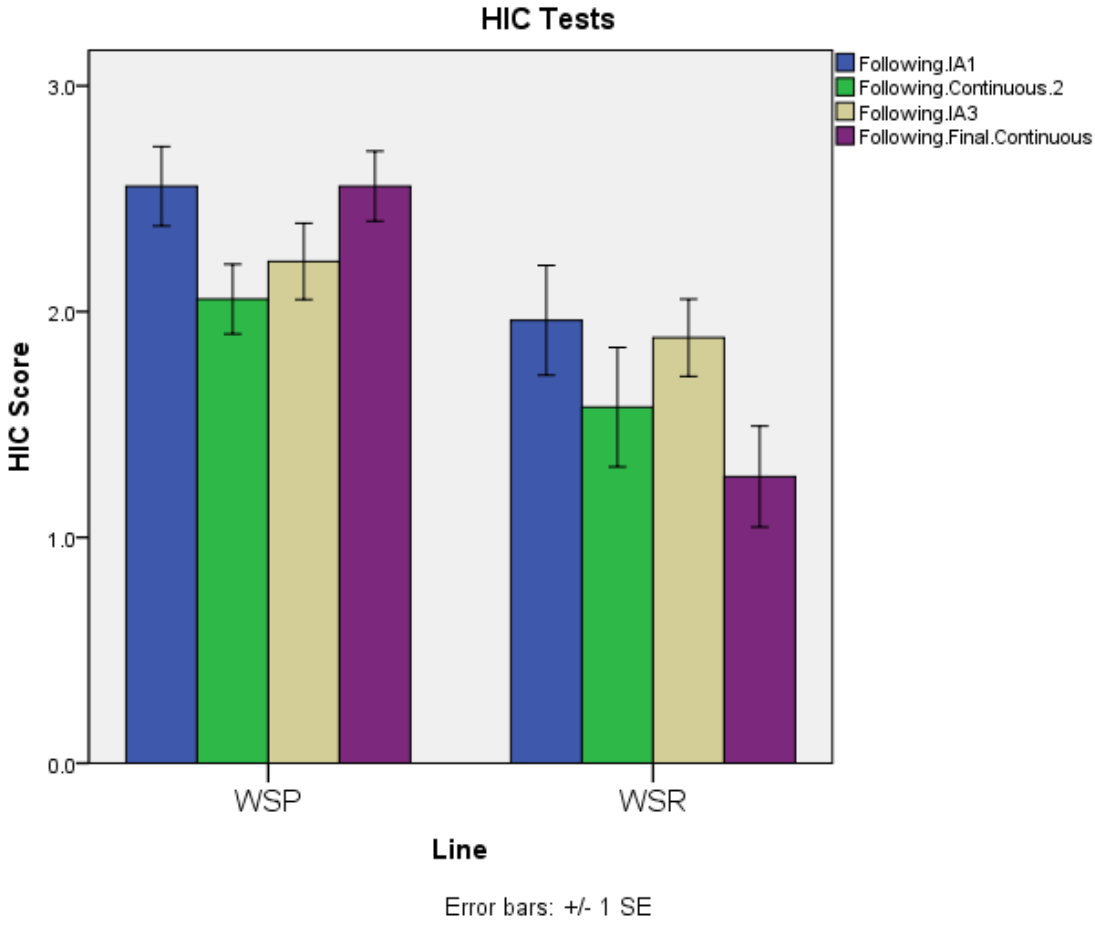


Figure 6. HIC scores following a 6-hour alcohol deprivation period following each of the last four experimental phases.

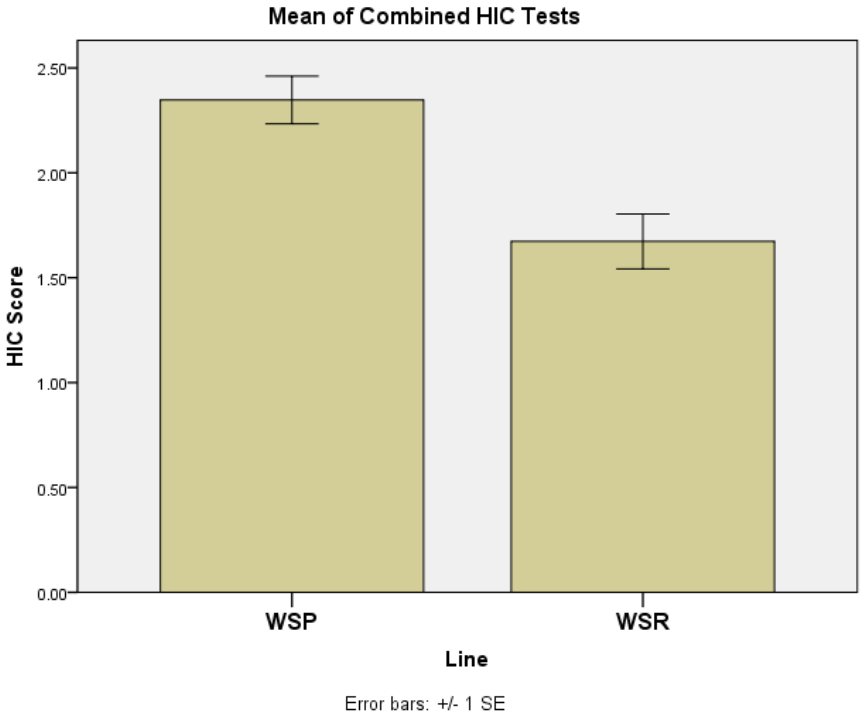


Figure 7. Mean HIC scores of each line. Bars are significantly different (p=.007)

Discussion

Both the WSP and WSR lines increased their consumption of ethanol, thus displayed the ADE, in equal magnitudes under intermittent access to alcohol, both during one-day per week intermittent-access and three-days per week intermittent-access, as well as under locked and unlocked running-wheel conditions. This furthers the generality of the ADE and indicates that the genetic and physiological mechanisms underlying high or low withdrawal severity are largely separate from those controlling the innate behavioral tendency to increase drinking when alcohol access is limited. The two lines responded equally to each alcohol-availability schedule, and showed a significant increase in both intake and preference when the alcohol was only available intermittently after it had been continuously available. It is important to note the similar increases in

preference data because that reflects an increased motivation for alcohol consumption, and is not simply a response of increased total drinking activity. Furthermore, a persistent elevated intake occurred during the final continuous-access period, as intake levels were similar to those during the intermittent-access phases. Excessive consumption induced by intermittent-alcohol exposure has been shown as a model of compulsive drug use (Ashmed & Koob, 1998), and is a hallmark of the transition from drug use to dependence. If replicated, this would be the first experimental demonstration that repeated experience with intermittent drinking results in persistently elevated drinking under free-choice conditions with these mouse genotypes.

The previous study conducted in the Rosenwasser lab (in revision), in which HDID and HS/Npt mice were employed in identical alcohol-availability protocols, is of direct relevance to the present study. Although both genotypes across both studies showed an ADE, the magnitude of the ADE was much greater in the previous experiment relative to the present one, as each line had similar continuous-access intake levels but HDID and HS/Ntp mice showed a much greater increase during intermittency. This is somewhat surprising, given that 6 of the 8 inbred strains used as stocks for the selective breeding process were shared among the HDID and WSP/WSR lines. Thus, our study supports previous observations that preference drinking (and other alcohol-related phenotypes) is controlled by genetic mechanism beyond those controlling free-choice preference drinking, withdrawal severity and high drinking under the DID conditions. The two genotypes tested in the present experiment combined with the four genotypes previously tested in our lab have collaboratively shown that binge-like drinking in response to alcohol deprivation is a robust phenomenon that is due to complex, indirect

genetic factors that are non-overlapping among various phenotypes. The presence of the ADE in multiple studies involving multiple genotypes of different rodents suggests that binge-like drinking episodes may not be attributed to one trait; rather it's a result of the interaction between many different factors. Further, because intermittent-access protocols consistently simulate a pattern of voluntary binge-like drinking similar to that of a human alcoholic's relapse drinking, future studies can help researchers investigate the neurological changes and adaptations that are due to a voluntary self-administrated increase in alcohol preference; an altered intake due to a motivational change in alcohol preference rather than nutritional deprivation. Human problem drinkers certainly have free-choice alcohol access, so increasing voluntary free-choice drinking in animals by influencing drinking motivation can produce more clinically relevant results when studying neurological mechanisms associated with the ADE.

Of course, there are limitations to this study. While human alcoholics do in essence provide themselves with intermittent-access to alcohol by repeatedly quitting and relapsing, this laboratory experiment doesn't perfectly represent that because although many measures were taken to most closely resemble motivational factors behind human drinking (i.e. allowing for purely voluntary drinking when alcohol is present), the paradigm employed forced restriction of alcohol during the deprivation period. Furthermore, there are a multitude of complex motivational factors that are incorporated exclusively in human drinking motivation (that could not be replicated in the present study), ranging from a stressful day at work to an appearance in a wedding. Additionally, a fundamental limitation with animal models is that they generally don't drink levels of alcohol sufficient enough to generate intoxication. While intermittent access escalates the

amount of alcohol consumed, it must be assumed that the animals in this study never reached intoxicating BACs. BACs were not measured in the present study, because measuring the BAC includes taking a blood sample, and a weekly episode with a needle could be stressful enough to confound the results. Another limitation to the present study is one shared by all animal studies that investigate human phenomena: Are the observed results capable of being translated to human clinical use? In the case of this study, do the mechanisms underlying the occurrence of the ADE reflect similar mechanisms that would exist in humans under the same conditions? If so, these identified mechanisms would be relevant to the treatment of alcoholism as they could lead to the development of pharmaceutical agents that block the pathways underlying binge-like drinking under intermittent availability, posing the possibility of reducing the likelihood of relapse drinking in humans.

Unfortunately, however, the mechanism for the ADE demonstrated in this study is not entirely known, but because both high and low withdrawing mice demonstrated equivalent responses to limited access, the ADE was probably not due to the animals' attempt to counteract withdrawal effects. But this does not mean that pharmacological consequences don't occur, and one theory that has been considered a possible cause of the ADE and has been recently supported by Sparta (2009), is that when the alcohol is taken away, the environmental change imposed on the mice is a stressor. When the alcohol is reinstated, the mice drink greater quantities of alcohol due to their state of heightened stress, which is a state due at least in part to the cessation of alcohol acting on the brain. In fact, when receptors for Cortico-releasing Hormone (CRH), a very important stress hormone that increases in activity while a stressor is experienced, are inhibited, the

ADE does not occur (Sparta, et. al., 2009). Therefore, a stress response that releases CRH is a critical component that modulates the phenomenon of increased drinking during intermittent-access, and abstinence of alcohol is responsible for this stress response, even when the animals do not experience dependence. This theory is relevant to intermittent problem drinking in humans, as cessation of alcohol intake after chronic alcohol use results in a depressive, stressful state. Therefore, many humans (driven by negative reinforcement) return to alcohol drinking, and frequently in elevated quantities. CRH has also been shown to modulate excessive drinking in animal studies that provoke dependence, and pharmaceutical CRH antagonists are under investigation in human clinical trials.

Alcohol drinking is extremely complex. For example, in two different preference studies, each incorporating HDID mice and their heterogeneous HS/Npt controls, the two genotypes show a huge difference in John Crabbe's DID protocol (Rhodes, et al., 2005) in which alcohol is limitedly available only for a few hours during their most active period of the day, but no difference in the magnitude of the ADE expressed when alcohol is available intermittently but for 24-hour periods (Rosenwasser, et al., in revision). Both of these are preference studies (although under the DID protocol, water is not available while alcohol is present), so although the binge-drinking tendency for the HDID mice in the DID condition clearly has a genetic influence, that genetic influence is separate from the tendency to show an ADE when alcohol is available for the course of a full day. This illustrates how mechanisms of drinking behavior come from complex, indirect, non-overlapping genetic factors. Nevertheless, some human attributes have been shown to be associated with a heightened risk factor for the onset of an alcohol-use disease, such as

impulsive, risk-taking behavior (Kreek, et al., 2005), parental alcoholism (Haller & Chassin, 2010), and low alcohol sensitivity (Meyer & Quenzer, 2005). While no specific genes that contribute to an increased risk factor for alcoholism have been identified with studies involving the ADE, and animals incorporated in these studies can never become alcoholic, the broad generality of this effect is nonetheless an important finding in alcohol research, as it shows an innate tendency for relapse-like drinking.

The HIC testing data shows that WSP mice, which are selectively bred for high withdrawal-seizures after dependence is induced by forced alcohol vapor, also have a tendency for higher HICs after mild alcohol withdrawal without dependence than WSR mice. Unfortunately, though, these data are incomplete, due to the failure to obtain baseline data before the mice were exposed to alcohol and after the initial continuous-access phase. Thus, critical information required for analyzing a trend is missing. However, the limited results obtained are curious, and although the researchers were inexperienced in the HIC-scoring exercise, the scoring system used is very unambiguous, as there was very little variation in HIC scores assigned by each scorer, with no scores deviating by more than 1 unit. The major contribution of this trial HIC testing is the evidence that it would be interesting and worth-while for researchers to investigate trends in HICs in future studies that explore the ADE in rodents.

In summary, alcohol research aimed at identifying specific causes of the unfortunate development of alcoholism is important in helping healthcare professionals better treat and prevent this harmful disease. The ADE is an animal model of intermittent drinking, a drinking pattern commonly seen in human alcoholics, and the present study demonstrated that there is no correlation between the genetic mechanisms that code for

withdrawal severity and the behavioral phenotype of increasing alcohol intake following periods of deprivation, which is a robust phenomenon among various rat and mouse genotypes.

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Appendix

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Author's Biography

Peter C. Brooks was born in Austin, Texas on February 8, 1990. He was raised in Austin for 13 years before moving to Bremen, Maine, and graduated from Lincoln Academy in 2008. Double-majoring in psychology and Spanish, Peter has a minor in pre-medicine and is a member of Phi Beta Kappa. Upon graduation, Peter plans to study in Argentina before working on a medical degree and ultimately becoming a practicing physician.