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The Role of Dependence in Alcohol Reinforcement: Neurobehavioral Mechanisms

Michal Atzram
Graduate Center, City University of New York

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The Role of Dependence in Alcohol Reinforcement:

Neurobehavioral Mechanisms

by

Michal Atzram

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2015
This manuscript has been read and accepted by the
Graduate Faculty in Psychology in satisfaction of the
dissertation requirements for the degree of Doctor of Philosophy

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THE CITY UNIVERSITY OF NEW YORK

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Abstract

The Role of Dependence in Alcohol Reinforcement: Neurobehavioral Mechanisms

by

Michal Atzram

Adviser: Dr. Michael J. Lewis

The motivation to consume alcohol is complex, though physiological dependence has long been implicated in alcohol reinforcement and alcoholism. The research presented here was conducted to examine the role of alcohol dependence on voluntary alcohol consumption and preference, the validity of the Lieber-DeCarli (LD) liquid diet as a procedure to induce dependence, and the effect of alcohol administration on dopamine and endocannabinoids. While the LD diet has been used extensively to induce alcohol toxicity and teratogenic effects, there has not been research that has examined its use in the development of dependence and alcohol reinforcement. This research utilized varying periods of chronic alcohol administration in three cohorts of C57 mice followed by two-bottle choice procedures to examine the role of dependence in alcohol consumption. Chronic oral intake of the alcohol containing LD diet produced moderate symptoms of physical dependence and increased motivation to consume alcohol. In all three cohorts, there were indications of greater alcohol intake or preference in mice that consumed the alcohol diet compared to the control diet. These differences were greatest in the cohort receiving chronic alcohol for 30 days in comparison to 21 and 52 days. Following behavioral data collection, analyses were performed to explore the role of dopamine and endocannabinoid brain systems in dependent mice. While significant differences were not found in the endocannabinoids, AEA or 2AG, there was a trend toward significance for experimental animals
exhibiting lower levels of dopamine. These data indicate the value of orally administering alcohol chronically via a liquid diet. Utilizing equicaloric alcohol and non-alcohol LD diets permitted nutritional maintenance and caloric control while evaluating the effects chronic alcohol administration. Overall, animals in the experimental groups preferred alcohol to water on more occasions than the control animals. These findings confirm the role of dependence in motivating alcohol drinking and the validity of LD diet as a model for inducing alcohol dependence. Application of these methods will enhance our understanding of the role of dependence in alcohol drinking and in the development of novel treatments for alcohol use disorder.
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### Abbreviations

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<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>2-AG</td>
<td>2-arachidonylglycerol</td>
</tr>
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<td>4-MP</td>
<td>4-methylpyrazole</td>
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<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>ACD</td>
<td>Acetaldehyde</td>
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<tr>
<td>ADH</td>
<td>Alcohol dehydrogenase</td>
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<tr>
<td>AEA</td>
<td>Anandamide</td>
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<tr>
<td>BAL</td>
<td>Blood alcohol level</td>
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<tr>
<td>C57</td>
<td>C57BL/6J mice</td>
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<tr>
<td>CB</td>
<td>Cannabinoid</td>
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<tr>
<td>CRF</td>
<td>Corticotrophin releasing factor</td>
</tr>
<tr>
<td>D2</td>
<td>Dopamine subtype 2 receptor</td>
</tr>
<tr>
<td>DSM-5</td>
<td>Diagnostic and Statistical Manual of Mental Disorders fifth edition</td>
</tr>
<tr>
<td>DOPAC</td>
<td>Dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>FH/Wjd</td>
<td>Fawn-Hooded</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>HAD</td>
<td>High-Alcohol-Drinking</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneally</td>
</tr>
<tr>
<td>LAD</td>
<td>Low-Alcohol-Drinking</td>
</tr>
<tr>
<td>LD</td>
<td>Lieber-DeCarli</td>
</tr>
<tr>
<td>MR/Har</td>
<td>Maudsley Reactive</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reactions monitoring mode</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NP rats</td>
<td>Non-preferring rats</td>
</tr>
<tr>
<td>P rats</td>
<td>Alcohol preferring rats</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>sP</td>
<td>Sardinian alcohol preferring rats</td>
</tr>
<tr>
<td>UPLC/MS/MS</td>
<td>Ultra Performance Liquid Chromatography with Tandem Mass Spectrometric Assays</td>
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<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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Chapter 1: A Re-Evaluation of Need in Motivation: Its Significance in Alcohol Use Disorder

The motivation underlying behaviors that are essential for our survival involves mechanisms that may also play a significant role in behaviors that are detrimental to our health. Mechanisms that control food intake may play a particularly important role in not only survival, but also drug addiction. An essential feature of drug and alcohol intake may be the induction of drive that is produced by the need for nutrients and the resulting production of a cyclic pattern of need and intake. The need for these addictive substances produced by recurrent periods of deprivation may play an essential feature in generation and maintenance of dependence. Recently, the addiction field has focused on external stimuli that direct drug taking behavior. Though external stimuli are quite important, there may also be internal drives which may be equally important in the use of drugs including alcohol. The classic work on drive and need that has been applied to food intake can also be applied to drug intake. Just as the hungry individual is motivated to eat because of a need for nutrients, the dependent drug user is motivated to use drugs because of a need to remediate a physiologically significant drug deficit. Linking food intake and drug use to need and the basic physiological processes will provide a more complete picture of the motivation to use drugs.

The absence of nutritious food is common in many parts of the world. However, we incorrectly assume that it occurs periodically, in poorer areas, with little that can be done to prevent it or administer to the starving because of natural disasters or uncontrollable geopolitical events. Starvation occurs far too frequently, but is rare in comparison to undernutrition from poor food quality for many throughout the world. Malnutrition is probably the single most solvable and preventable problem facing a surprisingly immense percentage of the world’s population,
and the need for food and adequate nutrition is a compelling motivator for many. The pervasive and unremitting state that is produced in starving people, as well as those malnourished, alters individual and collective priorities of daily life. There are a myriad of physiological responses to the complete lack, or insufficient amount, of food. Lacking basic nutrients such as the macronutrients of fat, carbohydrate, and protein and micronutrients including sodium, potassium, and vitamins each produce physiological changes to alleviate the deficits by motivating behaviors to remediate the losses resulting from nutritional deficiencies. Acute effects of severe deficits are unpleasant and compel restless activity and specific behaviors to alleviate the deficiencies. These acute deprivation effects may lead to even more physiological and behavioral disturbances if chronic deprivation is experienced. Chronic intermittent periods of deprivation may cause additional disruptions that could have long-term physiological and behavioral consequences such as predisposing one to substance use disorder and other psychopathologies.

The presence of need has been shown to be a powerful motivator of behavior in appetitive operant tasks (e.g. operant learning and performance under food deprivation) (Skinner, 1935) and of the intensity of Pavlovian conditioned responses (e.g. startle response under food deprivation) (Anderson, Crowell, & Brown, 1985). Likewise, the need for opioid drugs established by chronic administration of powerful opioids enhances intake and operant performance for opioids during withdrawal (Schuster & Thompson, 1969; Lewis, Margules, & Ward, 1975). In dependent individuals, the establishment of need by deprivation of these drugs in such experiments has been shown to increase drug intake resulting in an increase of endogenous opioid levels; later, drug intake decreases after need is remediated by the opioids. Although not all substances of abuse have a strong withdrawal response, those that do exhibit a powerful cyclic pattern of intake driven by the onset of abstinence periods, which is followed by
reduced intake after drug consumption. Illustrating this, during periods of abstinence heroin addicts frequently are heard exclaiming their “need for a fix.” As with food deprivation, the lack of the opioid in bodily and brain tissues of one dependent on opioids results in a powerful motivation to seek remediation of the deficit. Opioid antagonist microinjection into periaqueductal gray, and other sites linked with opioid dependence including the ventral tegmental area (VTA), produces opioid-like withdrawal during the deprivation period and microinjection of opioids into these sites reduces opioid abstinence symptoms (Laschka, Teschemacher, Mehraein, & Herz, 1976; Wise & Bozarth, 1984).

Although the research is less clear than with opioid dependence, this need state during deprivation can be observed with other drugs of abuse including alcohol. When those dependent on alcohol are asked why they drink, it is not uncommon for them to verbalize that they “need a drink” not that they “want” a drink, that a drink would taste good or other more conditional phrases that might suggest they are drinking for reasons other than need. The progression from “I want a drink” to “I need a drink” often highlights the progression from a casual social drinker to someone who meets criteria for alcohol use disorder. It is clear that addictive behaviors are complex and result from multiple aberrant physiological and behavioral processes that motivate drug intake; however, the role of need and the resultant drive for the drug in addictive behaviors have received relatively little attention. Recent approaches have emphasized incentive motivation and the possibility that basic processes are altered to produce motivational mechanisms that lead to the increased importance of drug taking stimuli. Robinson and Berridge (1993) have proposed a theory that emphasizes that incentive salience leads to greater drug wanting. This view highlights important motivational mechanisms that may play an important
role in addiction. It is possible that as with nutrition, the need and the resultant drive play an equally important role in addictive behavior (see Figure 1).

*Figure 1*. Internal and external motivational mechanisms that mediate drug use. The inner circle illustrates the internal changes that contribute to drug use, while the outer circle shows the external changes. When a drug dependent individual lacks the drug in their bodily tissues it results in physiological changes in the central and peripheral nervous systems that include altered neurotransmitter and endocrine mediators, which lead to a need state. The lack of drug in the body also leads the dependent individual to be more sensitive to environmental cues that result in increased incentive for drug administration (wanting). The need state is consistent with Hull’s drive theory while incentive salience and want are as hypothesized by Robinson and Berridge’s (1993) Incentive Motivation Theory. Both the need and want fuel behavioral activation that motivates further drug consumption and when drug levels fall again the cycle repeats itself.

The key to understanding of the role of need in motivation is the realization that the brain and peripheral systems of any individual, with substance use disorder or not, are operating in the same basic way. When food deficits occur, there are resulting unpleasant physiological changes
that indicate the absence of needed substances, and physiological changes also occur when a
drug dependent person is in withdrawal. These similar states of deprivation signal that the body
needs something, whether it is food or drug.

External, in addition to internal, stimuli contribute to the motivation to eat. For example,
when hungry, food is needed and one is motivated by multiple stimuli to eat. Externally, the
smell or sight of food will direct behavior toward the goal of eating it, while at the same time one
experiences multiple internal changes, such as a reduction in blood glucose level, or sensations
from the gastrointestinal tract that also signal it is time to eat. These external, as well as internal,
stimuli both motivate the individual to seek food (see Figure 1). One approach to explain how
internal states affect behavior comes from drive theory. A central idea of drive theory is that
when an organism needs food, a state is produced that is unpleasant and motivates specific and
non-specific changes in behavior directed towards food and its consumption. This state is
characterized as energized activity, some of which is goal directed (e.g. procurement of food or
foraging) (Woodworth, 1918) and other non-specific behaviors (e.g. increased locomotor
activity) (Richter, 1922 as cited in Bindra, 1968). Often, a component of theories of drive state is
homeostatic regulation. Our bodily systems are regulated to maintain homeostasis for food and
vital nutrients. When these are lacking, one experiences increased arousal and is motivated to
engage in specific remediating behaviors, this state is called drive and it serves to restore the
homeostatic balance (Cofer & Appley, 1964, pp. 310-311). Similarly, chronic drug exposure,
especially with opioids and alcohol, results in withdrawal when lacking the drug. This state is
characterized by altered physiological organ and system functioning, is akin to the deprivation
state of food and water, and may be the underlying mechanism for the need for drugs.
Need gives rise to drive and drive directs the organism toward the goal that would satisfy that drive state. Drive is independent of need, the situations that initiate it and conditioning to environmental or internal stimuli. The role of drive in motivation, and specifically its role in reinforcement, was hypothesized by Hull (1943) and reformalized by Miller (1955; 1957). They indicate that drive is an energized state that addresses need; however, it also anticipates remediation of the need permitting the onset of satiety before actual tissue need occurs (Kohn 1951; Berkun, Kessen, & Miller, 1952). Hull (1943) theorized that motivation had two key components: the drive that arose from internal stimuli produced by need and the incentive that was motivated by the appearance of related stimuli, largely external, that had previously been associated with meeting the need. Later work by Spence (1951) and others lead to a much greater emphasis on incentive motivation over drive in behavior and many current theories of addiction emphasize incentive motivation mechanisms.

The focus on drug-related stimuli in addiction has been a feature of several of the leading theories of addiction. These stimuli are conceptualized as discriminative stimuli of drug availability that serve as cues that direct drug taking behavior. The role of sensitization of these stimuli has been hypothesized by Robinson and Berridge (1993). They state that continual drug use leads to sensitization of neural systems that mediate drug cues, which results in drug craving and relapse to drug use. Sensitization of motor effects of many drugs of abuse has been extensively investigated (see Steketee & Kalivas, 2011 for review). Robinson and Berridge (1993) suggest that in addition to motor stimulation, behavioral sensitization of drug-related stimuli occurs and leads to incentive salience to these stimuli resulting in drug wanting, craving and seeking. They further suggest that the process of drug ‘wanting’ is distinct from that of ‘liking’ the hedonic qualities of drugs. The neuronal systems mediating the two are different in
that only wanting is sensitized by repeated drug use, liking is not (Robinson & Berridge, 1993). Furthermore, incentive salience to drug-related stimuli is hypothesized to be mediated by the mesolimbic dopamine system in a manner similar to its role in locomotor sensitization. That is, with chronic drug administration, increased drug-taking behavior is produced by heightened dopamine activity (Robinson & Berridge, 1993). The hedonic qualities of drugs that result in the “liking” do not involve the dopamine systems according to Robinson and Berridge (1993); however, Wise (2013) has found evidence of dopamine mediation of these effects. Although stimulus dependent aspects of drug taking, along with the increase in dopamine activity are clearly important, the internal drive state that is fueled by need for the drug also contributes to the motivation in drug-taking.

Need is defined by Hull (1943), Miller (1955; 1957) and others as requiring something that is necessary and the absence of it will result in discomfort, pathology or harm. In contrast, want is usually attributed to the act of desiring something. We do not tend to attribute a state of want to a situation where the lack of what is wanted will result in a state detrimental to the organism or species. While a person suffering from substance use disorder may want or desire a drug, there may also be a state of need for the drug that is compelling and not related to the process of wanting or liking. Those who are dependent develop both the need and want states in the addiction process and they are both present during withdrawal (when the drug has been metabolized and no longer in the tissues). The wanting component of addiction is the result of the increased salience of external cues associated with drug taking. However, need for the drug to be within the bodily tissues, and the resultant drive, is comprised of the internal physiological changes within the organism generated by multiple systems within the brain and periphery that are mediated by several neurobiological systems (neurotransmitters, hormones and
neuropeptides). As discussed before, this pushes the individual to rectify the imbalance and leads
to a drive state that dramatically energizes and directs the dependent individual to seek and
administer drugs. This need state, in both drug and food deprivation, can be understood in terms

The theoretical perspective of the Opponent Process Theory (Solomon & Corbit, 1974)
would explain the need state as the B-state, when the A-state is produced by the pleasurable
qualities of food or drug intake. The B-state is an aversive mood state that includes withdrawal
symptoms and depression that occur in the absence of the drug (Solomon & Corbit, 1974; Koob
& Le Moal, 1997). The aversiveness is the result of the need for the drug in order to relieve the
withdrawal symptoms and to possibly return to the pleasurable state that is experienced with the
drug. The signals of need for food undoubtedly involve central processing, and the brain systems
that control the regulation of food intake and satiety are complex (Levine, Kotz, & Gosnell,
2003; Schwartz, Woods, Porte, Seeley, & Baskin, 2000). Just as the need for food is a result of
deficiencies in multiple physiological necessities, drug need, specifically for alcohol and opioids,
is a complex interaction of physiological alterations that motivate behavior and produce
compensatory physiological processes. Similarly, several brain systems play a role in the
mediation of the aversive signals of drug withdrawal and need, as well as the pleasurable effects
of the drug during acute and chronic drug administration.

The person suffering from alcohol use disorder may experience a need for alcohol that
has many features of the need for food (see Figure 1). Several researchers have drawn this
connection between the consummatory behaviors of eating and drinking alcohol. Barson,
Morganstern and Leibowitz (2012) summarized multiple neural mechanisms and neurochemicals
that are involved in the consummatory behaviors of both eating and drug taking and they present
research that suggests that these two behaviors affect, and are affected by, changes of the same systems and neurochemicals. Multiple neurochemicals including DA, endogenous opioids, galanin and ghrelin enhance intake of both food and alcohol (Barson et al., 2012). Several findings suggest that fat, one of the three macronutrients of food, may play a significant role in alcohol intake. Fat intake has been shown to interact with alcohol intake and the pathology observed with excess alcohol consumption (Carrillo, Leibowitz, Karatayev, & Hoebel, 2004). Specifically, with respect to the consumption of fat and its similarity to alcohol consumption, different neurochemicals are involved including galanin, endogenous opioids and endocannabinoids (Barson et al., 2012). This overlap further suggests that biological commonalities between these behaviors may make the motivations for them similar. Lewis (2011) suggests that there is a complex interaction between fat recognition and metabolism, involving both peripheral and central nervous system mechanisms that are also involved with alcohol intake. With long-time chronic administration of alcohol in alcoholism, this interaction may become most significant with the chronic deterioration of brain and peripheral motivational and nutritional systems (Lewis, 2011). While multiple systems play a role in drug and food intake, many hormones and neurotransmitters are also involved in these behaviors. One of the most widely studied is DA, with substantial research highlighting its role in the motivation to eat as well as use drugs.

DA’s role in regulating eating, and specifically calorie intake, has been extensively investigated and reviewed (Hoebel 1984, 1985, 1997; Hoebel, Hernandez, Schwartz, Mark, & Hunter, 1989; Hoebel, Rada, Mark, & Hernandez, 1994; de Araujo, Ferreira, Tellez, Ren, & Yeckel, 2012). Mice that were genetically manipulated to be dopamine deficient did not eat enough to survive but feeding was restored when they were treated with L-dopa, which is
converted into dopamine, or when dopamine was produced in the dorsal striatum following viral rescue (Sotak, Hnasko, Robinson, Kremer, & Palmiter, 2005). Geiger and colleagues (2009) found that obese rats, as compared to chow fed normal weight animals, had lower dopamine levels and lower dopamine release in the nucleus accumbens (NAc) in response to chow or amphetamine. When the obese rats were deprived and then offered a high calorie diet, there was also an increase in dopamine. The authors suggest that low levels of dopamine in the mesolimbic system may be associated with obesity (Geiger et al., 2009). To separate out the role of dopamine in oral and gut responses to fat, Ferreira and colleagues (2012) conditioned mice to lick a sipper tube that did not deliver anything while at the same time intragastrically infusing fat. They found that in the dorsal and ventral striatum, dopamine levels increased in response to increased caloric infusions and if dopamine signaling was blocked then calorie intake was disrupted (Ferreira, Tellez, Ren, Yeckel, & de Araujo, 2012). Similarly, dopamine levels were increased centrally when glucose was delivered intragastrically (Ren et al., 2010). The authors suggest that independent of taste or eating behaviors, there is a connection between the gut and brain dopamine that serves to monitor fat intake (Ferreira et al., 2012). These data suggest that dopamine is involved in maintaining the homeostatic balance of calories (de Araujo et al., 2012). That is, via peripheral signals, dopamine may mediate the need for food by indicating it is time to eat when dopamine levels decrease. The link between dopamine and food consumption is similar to its link with drug addiction. Just as dopamine is involved in the need for food, it is also implicated in need for drugs including alcohol.

Extensive research has indicated a major role of dopamine in addiction including both motivational and reward properties (Wise & Bozarth, 1987; Koob & Bloom, 1988; Koob & Le Moal, 2005, 2008). Alcohol increases dopamine levels in the NAc (Di Chiara & Imperato, 1985)
and when a dependent drug user is in withdrawal, dopamine dopamine levels decrease (Weiss et al., 1996). This decrease in dopamine may be one of the manifestations of the user’s need and resulting drive state that motivates alcohol seeking in order to reestablish dopamine levels. A further manifestation of this may be that those with alcohol use disorder have fewer D2 receptors compared to healthy individuals (Volkow et al., 1996). Volkow and colleagues (2007) suggest that other reinforcers may be less effective due to decreased in dopamine levels as well as its receptors, which results in an increased need for alcohol to activate the remaining dopamine system. In addition, similar to the periphery influencing dopamine levels to drive food consumption and maintain homeostasis, it has also been found to be important in alcohol consumption. Alcohol has been found to increase dopamine in the NAc and striatum when it’s delivered via intraperitoneal injections (Di Chiara & Imperato, 1985) and increase it in the striatum when delivered via gavage (Vasconcelos et al., 2003). These data suggest that peripheral changes from not only food but also alcohol, mediate a central dopamine response that contributes to the cyclic pattern of need when in a deprivation state and the absence of that need when satiated.

Administering alcohol via the periphery undoubtedly produces a pleasurable state that is reinforcing to animals and humans; recently it was found that blocking the metabolism of alcohol exclusively in the periphery altered alcohol reinforcement. An experiment suggesting the role of peripheral tissue in alcohol consumption was conducted by administering alcohol or acetaldehyde (ACD), to rats along with 4-methylpyrazole (4-MP), which inhibits alcohol dehydrogenase (ADH) in the periphery (Peana et al., 2008). In addition to alcohol being reinforcing, Peana and colleagues (2008) presented multiple studies suggesting that ACD, which results from the metabolism of alcohol by ADH, may also be reinforcing. The results of this
study were such that conditioned place preference for alcohol was blocked by 4-MP (Peana et al., 2008). This suggests that alcohol’s reinforcing properties may be mediated in part by ACD in the periphery and that tissues beyond the central nervous system are important in the effects of alcohol. Given this, the periphery may also be involved in signaling need when there is an absence of drug in order to motivate further drug use.

Motivation to use another drug of abuse, nicotine, has been suggested to be mediated in part by peripheral signals. Watkins, Stinus, Koob and Markou (2000) subcutaneously injected rats with a dose of chlorisondamine, a nicotinic acetylcholine receptor antagonist that is not centrally active. Administering chlorisondamine to nicotine dependent animals increased the number of physical withdrawal symptoms as compared to nondependent animals (Watkins et al., 2000). As previous research suggests, the physical symptoms of drug withdrawal, including nicotine, do motivate self-administration, though to a lesser degree than emotional withdrawal symptoms (Koob, Markou, Weiss, & Schulteis, 1993; Markou, Kosten, & Koob, 1998; Watkins et al., 2000).

**Conclusions:** It is a complex picture of the multiple neurochemical systems that are involved in both feeding and drug taking (see Figure 1), with dopamine as just one piece of the puzzle. Biological changes signal a need state during deprivation that drives an animal to consume more food or drug. Just as the starving or malnourished person is highly motivated to seek out and consume food, the drug addicted individual seeks to consume drug to satisfy their drug need. In addition to the external stimuli that motivate these similar behaviors, internal need mediated by the central nervous system and peripheral tissues also plays a significant role. The implications for the role of need in drug addiction highlight the importance of rectifying the homeostatic imbalance possibly via pharmacological interventions, as well as addressing the role
of the environmental influence through cognitive behavioral therapy. Ignorance or neglect of the importance of need in both food deprivation and addiction will impede progress of their treatment and remediation.
Chapter 2: Introduction: The Role of Dependence in Alcohol Reinforcement:

Neurobehavioral Mechanisms

**Alcohol Dependence**

Alcoholism, also referred to as alcohol dependence, is a pervasive and costly disease with many deleterious effects to the individual user as well as to society. Considerable advances have been made over the last several decades in understanding the complex causes of the disease. These advances are the result of important new research into the behavioral, neurochemical, and clinical aspects of alcohol’s effects. Alcohol is a complex drug of abuse, affecting and being affected by many neurochemical systems. In addition to its pharmacological properties, which like other drugs of abuse motivate the user to consume it; alcohol is also a food that provides calories. Many of the issues concerning the mechanisms that mediate alcohol effects and intake are shared with other drugs of abuse and with drug addiction or dependence. An understanding of the general issues of the motivation and neurobiology of addiction are also applicable to alcohol abuse and dependence.

**Effects of Chronic Alcohol & Alcohol Dependence: Magnitude of Problem**

Alcoholism is a dangerous and costly disease and chronic alcohol consumption has many harmful effects to the user. These include liver problems such as hepatitis and cirrhosis; neurological damage leading to Wernicke-Korsakoff syndrome, epilepsy and dementia; Cancer of the liver, mouth and throat; reproduction problems, Fetal Alcohol Syndrome and heart disease (McKim, 2007). Alcohol related problems are also costly to society; the United States spends approximately $175.9 billion annually on issues related to alcohol (Rice, 1999).
Recently, researchers in the United Kingdom attempted to evaluate the harm associated with the use of 20 commonly abused drugs. A panel of scientists and specialists scored these drugs on 16 criteria related to the harm they pose to both the individual user and society. Heroin and crack cocaine were rated the most harmful to the individuals who use them and alcohol and heroin were the most harmful to society. When scores of harm to the individual and to society were combined, alcohol ranked most harmful overall with heroin second (Nutt, King, & Phillips, 2010). Such efforts highlight the risk associated with alcohol abuse and dependence, not only to the user but also to society.

**Genetics of Alcohol Dependence**

Alcohol dependence has a genetic component and recently researchers have found 51 small chromosomal regions that have at least three nucleotide differences between alcohol dependent and nondependent humans (Johnson et al., 2000). One of the best known contributions of genes to the development of alcoholism is seen with those genes that code for the enzymes alcohol dehydrogenase and aldehyde dehydrogenase that metabolize alcohol in the liver (Li, 2000). Ethanol is metabolized into acetaldehyde by alcohol dehydrogenase and aldehyde dehydrogenase converts acetaldehyde into acetate (National Institute on Alcohol Abuse and Alcoholism, 2007). People with a deficiency of these genes will not metabolize alcohol completely and there will be a buildup of acetaldehyde that will cause unpleasant symptoms such as flushing and nausea. This is seen in much of the Asian population and the adverse reactions experienced when consuming alcohol may protect them from developing alcoholism (Thomasson et al., 1991; Li, 2000).
Alcoholism has also been found to be genetically inherited in men. The Stockholm Adoption Study found that adopted males whose biological fathers or mothers were alcoholics had a significantly greater probability of being alcoholics themselves, as compared to males whose biological parents were not alcoholics. This study also concluded that when children were adopted by alcoholic parents there was little environmental contribution to developing alcoholism (Schuckit, Li, Cloninger, & Deitrich, 1985; Bohman, Cloninger, Sigvardsson, & von Knorring, 1987). These studies have defined two types of alcoholism that are genetically influenced; type I and type II alcoholism.

Type I alcoholics usually become alcoholics after 25 years of age, exhibit extended binges, show alcohol tolerance, have trouble controlling their intake of alcohol and are more commonly men who have a female alcoholic relative. Type II alcoholics usually develop alcoholism before 25 years old, and are males who have male alcoholic relatives and exhibit behavioral problems including alcohol induced fighting, accidents and arrests. Type I is a result of both genetic and environmental factors in both males and females, while Type II is only genetically inherited in males (Cloninger, 1987; Irwin, Schuckit, & Smith, 1990; Addolorato, Leggio, Abenavoli, & Gasbarrini, 2005).

In addition to human studies, animal experiments indicate genetic factors in alcohol consumption. Inbred mouse strains have been shown to differ significantly in consumption and preference for alcohol. C57 mice show a preference for alcohol while DBA mice do not readily consume alcohol. Selectively bred rat lines have been produced that prefer alcohol (e.g. P rats) and those that do not (e.g. NP rats). The development of these animal models has proven invaluable in investigating the behavioral and neurochemical determinants of alcohol preference, intake and dependence.
As is true for any trait or behavior, a genetic predisposition does not equate to genetic certainty. That is, just because humans or animals are genetically predisposed to prefer or avoid alcohol, those traits may be modified by the environment. Samson, Files, Denning and Marvin (1998) tested alcohol consumption in multiple rat lines that were bred to prefer or avoid alcohol. Animals were trained to consume ethanol by adding, and then gradually fading out, sucrose (see below: Behavioral Models of Ethanol Consumption). While on average, alcohol preferring rats consumed more alcohol than alcohol avoiding (NP) rats, after being presented with alcohol the NP rats that were bred to avoid it still consumed alcohol and were reinforced by it as shown in higher levels of operant responding for alcohol. The genetic predisposition of the rats that were bred to avoid alcohol had been influenced and altered as a result of consuming alcohol (Samson et al., 1998; Camarini & Hodge, 2004).

**Alcohol as a Food**

Alcohol is the only drug of abuse that has nutritive properties and provides 7 kcal/g of energy, second only to fat that has approximately 9 kcal/g, and it is the only drug of abuse that provides calories to the user (Dunford & Doyle, 2008; Lewis, 1996; Lieber, 2003). Long term alcoholics have deteriorating organ systems due to their consumption; the brain, liver, pancreas and gastrointestinal tracts all exhibit drinking pathology. This deterioration leads to a nutritional deficiency that may be a motivating factor in alcohol drinking because the long term alcoholic now needs the alcohol for its high caloric value. When alcohol is consumed in this state, it incorrectly signals the brain and digestive system that nutrition has been provided (Lewis, 1996). It is important to note that up to half of the total calories consumed by alcoholics may be derived from alcohol (Patek, Toth, Saunders, Castro, & Engel, 1975).
Clearly, alcohol provides calories to the drinker, but this is not the sole motivation for alcohol consumption and reinforcement. Though animals that are food deprived will consume increased amounts of alcohol (Meisch & Henningfield, 1977), food deprivation increases intake of other drugs that are reinforcing such as stimulants, depressants and analgesics (Meisch, 1984). These other drugs do not offer added calories as does alcohol, indicating that alcohol is not only consumed for its nutritive properties.

**Concepts of Addiction and Dependence**

Defining addiction can be difficult and the terms drug addiction and drug dependence are often used interchangeably. The Diagnostic and Statistical Manual of Mental Disorders fifth edition (DSM-5) does not define the term addiction, but instead uses the diagnosis of Substance Use Disorder that can be applied to 10 classes of drugs including alcohol, defined as Alcohol Use Disorder. These substance use disorders are marked by the appearance of the following 11 possible diagnostic criteria: taking the drug in larger than intended amounts, unsuccessful attempts to decrease use, spending excessive time procuring and using the drug, strong craving, failure to meet obligations, continued use despite negative social effects, giving up or reducing other important activities, using despite physical hazards, continued use despite knowledge of problems that result from drug use, tolerance and withdrawal (American Psychiatric Association, 2013). The severity of the Substance Use Disorder is then determined by the number of criteria that are met with the severity being mild if the person suffers from two to three symptoms, moderate if they suffer from four to five or severe if they suffer from six or more symptoms. Though the DSM-5 does not use the term addiction, many attempt to define it by encompassing aspects of a person’s life that are affected by long term drug use. A widely cited definition for
addiction by George Koob defines addiction as having three components; seeking and taking a
drug compulsively, losing the ability to control intake of a drug and developing negative
emotions associated with a lack of the drug (Koob & Le Moal, 2005, 2008a). There are two
aspects of addiction, or dependence, which perpetuate the harmful use of a drug: they are the
physiological and the behavioral components. As with other drugs of abuse, the essential features
of alcoholism are both the presence of physical dependence and the consumption of, and
preference for, alcohol.

While many do use the terms addiction and dependence interchangeably, recently there
has been support to distinguish between the two. Prominent scientists in the field of addiction
research discussed that we should instead use the term addiction over dependence (O’Brien,
Volkow, & Li, 2006). In an editorial piece for the American Journal of Psychiatry, they present
that “addiction” encompasses the behavioral components including the compulsive drug seeking
and taking, but that “dependence” only encompasses the physical effects of drug use including
withdrawal and tolerance. They explain that dependence may occur when a person uses many
types of drugs including medications, which do not lead to compulsive use (O’Brien, et al.
2006). The DSM-5 notes that a patient should not be diagnosed as suffering from Substance Use
Disorder if they only meet the criteria of tolerance and withdrawal when undergoing appropriate
treatment with medications that were prescribed, as this is not enough to be considered addiction,
when in fact it is dependence.

Initiation of Self-administration

The factors that lead to the initiation of drug use are varied and probably idiosyncratic to
the individual user. The subjective experience of the pharmacological effects of the drugs is
undoubtedly essential to drug use. Most drugs produce pleasant subjective effects, although they may not be experienced with the initial use. The presence of the pleasant effects provides the basis for positive reinforcement or reward, and these terms are often used interchangeably. The subsequent operant conditioning of the drug self-administration occurs as the operant behavior is reinforced by the pleasurable drug effects. Taking drugs of abuse results in pleasure that motivates the individual or animal to continue to engage in those behaviors that will result in the ability to take more drug, and this is the basis of the positive reinforcement model of addiction. There are many factors that can affect how reinforcing a particular drug is, including the type and dose of the specific drug, genetic differences of the individual, the environment, cues related to the reinforcer, stress, and being deprived of things such as food and water. This relatively simple learning is also not the only reason for drug self-administration, but is certainly an essential motivational component.

In addition to positive reinforcement, negative reinforcement also plays a role in why someone may initially choose to use drugs or alcohol. Negative reinforcement applies to the situations when someone drinks to relive a negative condition, often anxiety, stress or depression. The relief of these aversive states may motivate the user to consume the drug again. In fact, one study found that alcoholic participants rated stressful scenarios as resulting in a higher urge to drink as compared to non-alcoholic participants (Fouquereau, Fernandez, Mullet, & Soru, 2003). This suggests that negative reinforcement of stress relief may be stronger in alcoholics.

When drugs are being used the animal, or human, chooses to consume drugs of abuse (including alcohol) over other options. The field of addiction uses the term “preference” to explain these situations. Generally, when animals are offered the choice between alcohol and
water (see below: Two-Bottle Choice) and reliably consume more alcohol than water they are said to have preference for alcohol and thus be reinforced by it (Martinetti, Andrzejewski, Hineline, & Lewis, 2000). As detailed below, animals are not exhibiting preference for alcohol because of the calories it offers or for the taste; they are reinforced by the pharmacological effects on the central nervous system (Gatto, McBride, Murphy, Lumeng, & Li, 1994).

Dependence

Physical dependence is widely believed to be an essential feature of addiction. It can lead to withdrawal, which may include aversive symptoms caused by abstinence from the drug that can motivate a user to continue to seek and consume the drug to the detriment of most things in their life (McKim, 2007). This is the negative reinforcement model of addiction that theorizes that drug use persists in order to avoid an aversive condition caused by withdrawal (Wise & Bozarth 1987; Robinson & Berridge, 1993). The role of physical dependence in addiction has been questioned. Some drugs of abuse, such as marijuana, may not cause a severe withdrawal like that which is seen with other drugs such as heroin. Abusing drugs such as cocaine, amphetamine and marijuana result in an abstinence syndrome that is qualitatively different and, for most abusers, less severe than that produced by opioids. Supporting the theory that drug addiction is not only a result of negative reinforcement is that occasionally, a few abusers may voluntarily discontinue using a drug even though they experience negative symptoms of withdrawal and some will relapse to drug use even after withdrawal symptoms are no longer experienced (McKim, 2007). While dependence is not a sole motivating factor in drug use, it has been shown to contribute to use. To determine the role of physical dependence in self-administration of opiate drugs, Schuster (1970) implanted catheters that delivered morphine into
the jugular veins of monkeys after they bar pressed. When saline was substituted for high doses of morphine, they increased their responding on the lever to self-administer more morphine. When saline was substituted for low doses of morphine, the monkeys did not increase responding on the lever. Schuster concluded that the different responses to removal of either high or low doses of morphine were because at high doses the animals were physically dependent but at low doses they were not. Even though the animals failed to show physical dependence to the low doses of morphine, they still found it reinforcing and self-administered it when it was available earlier in the study, and the reinforcing properties of morphine were enhanced when the monkeys were in withdrawal (Schuster, 1970). Animals will self-administer opiates even if they are not dependent and have not experienced withdrawal, though producing physical dependence will increase the motivation to consume morphine. This and other studies show that it is not just physical dependence that motivates use and abuse, but dependence will result in an increase in self-administration. If dependence were not a motivating factor, one would expect the monkeys in Shuster’s study (1970) to administer similar amounts of morphine regardless if the dose was high or low.

**Negative Reinforcement and Dependence**

While negative reinforcement may be a factor in the initial decision to use drugs, it may also be a factor in the maintenance of drug use. In 1974, Solomon and Corbit presented their theory of motivation that described different affective reactions that vary in hedonic levels and how they contribute to behavior. This Opponent-Process Theory of Motivation suggests that the central nervous system responds to various pleasurable stimuli and their resultant pleasurable states (A state) with an aversive after reaction (B state), and the more the opponent process (B
state) is exhibited the stronger it may get. This theory has been described in terms of many behaviors including drug use and addiction. For example, when someone takes a drug it results in a pleasurable high (A state) and later they experience aversive withdrawal (B state). As the drug use progresses to addiction and dependence, the A state becomes less pleasurable and the B state more aversive and longer lasting. This B state leads to drug craving in order to avoid the aversive symptoms of withdrawal and re-experience the A state, resulting in the motivation to consume more drug (Solomon & Corbit, 1974; Koob, Markou, Weiss, & Schulteis, 1993).

Later, Koob and Le Moal (1997b) expanded on the Opponent-Process Theory of Motivation and how it relates to drug addiction by applying the process of allostasis to it. Allostasis is different from homeostasis in that allostasis is the response by the whole organism to environmental effects, and the anticipation of environmental effects, to reach a set point that is not normal and may become pathological (Koob & Le Moal, 1997a, 1997b). Like Solomon and Corbit’s Opponent-Process Theory of Motivation (1974), Koob and Le Moal (1997b) suggest that when a drug is taken the drug produces a pleasurable state (A process) that leads to a negative state without the drug (B process). The A process is more pleasurable than the normal state (set point) and the B process is more negative. They explain that when a person becomes addicted to a drug by continuing to take it over time, they enter an allostatic state of the reward systems in the brain. The reward systems respond to the A process with a B process, which over time results in a mood set point that gets progressively more negative. During allostasis the person dependent on drugs experiences a negative state without the drug and taking the drug again will not result in an A process that can overcome the new more negative B process. The motivation to take more drug is now under the control of negative reinforcement, not positive reinforcement (Koob & Le Moal, 1997b).
Craving and Relapse

Craving and relapse are also components of drug addiction that perpetuate the continued use of a drug, often at times well after any physical dependence has been overcome. Craving usually begins before the physiological symptoms of withdrawal and is an intense preoccupation with getting and taking more drug or alcohol. It is usually linked to the memory of the pleasure the drug produces and can be triggered by stimuli that are associated with the drug, obtaining more of it, the withdrawal symptoms it produces or stress. Interestingly, this state of craving has not been found to be correlated with addicts’ relapse to drug taking after periods of abstinence (Koob, 2000; Koob & Le Moal, 2008a). Craving has been broken down into two separate types. One type of craving is termed reward craving and is when the person suffering from Substance Use Disorder is craving the drug for its pleasurable properties, a result of positive reinforcement. The other type is the relief of withdrawal craving, resulting from negative reinforcement (Verheul, Den Brink, & Geerlings 1999; Heinz et al., 2003; Vengeline, Bilbao, Molander, & Spanagel, 2009). Verheul and colleagues (1999) also discuss a third type of craving termed obsessive craving that happens when the person with a drug addiction loses control over obsessive thoughts about drug use. In accordance with this theory, these different cravings will be experienced in different emotional states. Specifically, if alcohol is craved for its rewarding effects it will mostly be craved during positive emotional states, as compared to craving based on relief withdrawal that will be craved during negative emotional states (Verheul et al., 1999). This theory was later tested in detoxified adults using alcohol related inventories and appetitive or aversive responses were assessed via eye blink responses to visual stimuli. It was concluded that drinking during positive situations did correlate with appetitive reactions to alcohol stimuli,
though drinking during negative as well as positive emotional states were correlated with craving for withdrawal relief (Heinz et al., 2003).

Alcohol craving and consumption also involve aspects of conditioning. It has long been known that environmental cues, when paired with a drug of abuse, can elicit a response similar to the drug’s withdrawal symptoms and lead to relapse (Wikler, 1948; Addolorato et al., 2005). These cues can be anything that is reliably paired with the use of the drug, such as specific environments, people, items related to drug use or even moods (O’Brien, Childress, Ehrman, & Robbins, 1998).

**Tolerance**

Drug tolerance can also be subject to conditioning. A classic experiment by Siegel, Hinson, Krank and McCully (1982) showed experimentally how important these conditioned associations are to the drug effects. Rats in this study received heroin in one room and a vehicle injection in a second room, later they were challenged with a high dose of heroin in one of the two experimental rooms. The mortality rates were twice as high for the rats that were challenged with heroin in the room they had received only the vehicle during training, as compared to rats that received the challenge dose and training in the same room. In addition, rats that had different training and challenge rooms had a higher survival rate than a control group that never received heroin during training. These results show that heroin does produce a physiological tolerance response, as seen by comparing the control group and the different treatment group, and also that heroin produces a conditioned tolerance response. The authors comment that this conditioned tolerance is important and a factor in many heroin overdose deaths at times when users inject a
usual dose of drug in a different environment because they have not developed tolerance to the drug in the new environment (Siegel et al., 1982).

**Habit**

Habit is another component to addiction that has been implicated in continual drug and alcohol use. That is, when the action of taking the drug has progressed to the point where the behavior is under almost automatic control lacking conscious thought. This habit of drug taking is thought to be a result of abnormal learning because the drugs affect the neural basis of learning (Tiffany, 1990; Robinson & Berridge, 2003). Robinson and Berridge (2003) do not think habit accounts for addiction because it incorrectly assumes that the automatic behavior of habit is actually compulsion. They point out that automatic behaviors do not necessarily imply that they are compulsive, for example while tying a shoe is automatic; it is not compulsive as is drug taking. In addition, they discuss that in reality; the person suffering from addiction is flexible and compulsive in how he obtains the drug, unlike a true habit that may only explain ritualistic drug taking not flexible drug procuring (Robinson & Berridge, 2003).

Later, Everitt and Robbins (2005) explained the concept of a drug habit in terms of conditioning. They said that a drug habit is a result of a learned association between stimuli and responses and the reinforcing aspect of the drug strengthens this association, but that the reinforcer itself is not the goal that behavior is directed toward. This would explain why a person with Substance Use Disorder would continue to use a drug even after the drug is no longer pleasurable, because the stimuli related to using the drug have been strongly associated with the response of taking it.
Neurobiology of Addiction

Though the positive reinforcement model of addiction has encountered criticism, and is clearly not the sole reason addiction persists, it is regarded as a component to the initiation and maintenance of drug use. Positive reinforcement can be explained in terms of brain mechanisms that are responsible for it. The neurobiological areas and mechanisms involved in the positive reinforcement aspect of drug addiction include the VTA, NAc and the mesolimbic dopamine system, also known as the mesocortical system, which connects these two structures. Cell bodies of mesolimbic dopamine system are in VTA, the axons travel through the medial forebrain bundle and the synapses are located in the limbic system and forebrain in addition to other areas (see Figure 2). The NAc, which is part of the limbic system, receives many synapses from the VTA. In addition to the NAc, other parts of the limbic system receive input from the VTA including the amygdalae and hippocampus (Carlson, 2010). The NAc is also part of the motor loop because it connects to the basal ganglia and when the mesolimbic dopamine system is activated behavior increases. Systems involved with sensation, learning and memory also contribute to addiction. The mesocortical system has cell bodies that are also in the VTA and axons that extend to the prefrontal cortex and drugs of abuse also activate this system (Carlson, 2010; Ettinger, 2011). The mesolimbic dopamine system is heavily involved with reinforcement and the motivation for drugs of abuse as well as natural reinforcers; it produces both pleasure and the desire for more drug via dopamine, which is one neurotransmitter used by this system (McKim, 2007).

Any stimulus that acts as a positive reinforcer will release dopamine in the NAc; these stimuli can be natural reinforcers such as food, water, sex or even sweet tastes such as sucrose (Hajnal & Norgren, 2001). Drugs of abuse are also stimuli that to release dopamine in the NAc;
they work in the same way that natural positive reinforcers do (Carlson, 2010). Wise and Bozarth (1981, 1987) were among the first to suggest the involvement of dopamine in the reinforcing effects of a wide range of drugs of abuse. They said that drugs of abuse both stimulate psychomotor activity and are reinforcing via dopamine. Injections of opiates into the NAc have confirmed its involvement in the reinforcing effects of those drugs, and the dopamine pathway leading to the NAc is involved in the self-administration of stimulants as determined by lesion studies (Wise & Bozarth, 1987). Psychomotor stimulants such as cocaine activate the dopamine system and specifically augment dopamine at the synapses in the NAc and caudate nucleus, which increases locomotion from low doses and stereotyped behavior from higher doses (Creese & Iversen, 1975 and Kelly et al., 1975 as cited in Wise & Bozarth, 1987). Increases of dopamine in the NAc have also been shown to be involved in ethanol administration as discussed in detail below.

Later, it was shown that in vivo exposure to drugs with different mechanisms of action (cocaine, amphetamine, morphine, nicotine and ethanol) all have the same effect on dopamine neurons from the VTA to NAc by increasing the strength of excitatory synapses (Saal, Dong, Bonci, & Malenka, 2003). To confirm that these results were not due to giving any psychoactive substance, psychoactive therapeutic drugs without any abuse potential, such as fluoxetine, were administered in the same way and were not found to have the same effect on the dopamine neurons from the VTA. These data suggested that the effects of different drugs of abuse on the dopamine synapses from the VTA are common features of addiction that increase the motivational salience of drugs (Saal et al., 2003).

The dopamine projections from the VTA extend to a number of regions, such as the NAc, that show augmented activation because of neuronal changes resulting from the drugs of abuse.
The compulsive behaviors of addiction, such as drug seeking, are caused by neuronal changes in the dorsal striatum of the caudate nucleus and putamen and seem to happen after extended long term drug use (Carlson, 2010). There are two components to drug taking, the initial reinforcing effects that are experienced as a result of the pleasurable feelings the drug causes that begin the process of drug taking, and the maintenance of the drug taking behavior over a longer time course that leads to addiction. When maintenance occurs, the drug addicted individual is no longer only taking the drug only for the pleasurable effects. There are two separate mechanisms that are responsible for these components, the ventral striatum mainly the NAc, controls the initial reinforcing effects and the dorsal striatum maintains the addiction (Carlson, 2010).

Many neuroimaging studies in humans have confirmed the involvement of the ventral and dorsal striatum during different components of addiction. Nora Volkow’s (2006) laboratory looked at the increase in dopamine to conditioned stimuli in those addicted to cocaine using positron emission tomography (PET) scans while they viewed neutral or cocaine related videos. The results showed that dopamine levels increased in the dorsal, but not ventral, striatum in the cocaine addicted volunteers when they viewed cocaine related videos. This increase in the dorsal striatum was positively correlated with both the levels of craving and of reported withdrawal symptoms. These results support the fact that dopamine levels in the dorsal striatum contribute to drug craving and addiction, and that craving can be manifested through conditioned stimuli (Volkow et al., 2006). Taking this approach further, and determining the role of the dorsal and ventral striatum in different types of drinkers, Vollstädt-Klein and colleagues (2010) investigated which brain regions are activated when people view images of alcohol. Heavy drinkers and light social drinkers were shown alcohol and neutral images while they were in a functional magnetic resonance imaging (fMRI) machine. When viewing images of alcohol, the dorsal striatum in
heavy drinkers was activated significantly more compared to the social drinkers, who showed significantly more activation in the ventral striatum and prefrontal cortex. They also suggested that the increase in prefrontal cortex activation in social drinkers may be a result of the control they exhibit when exposed to alcohol related cues, which in turn could hinder the progression to heavy drinking (Vollstädt-Klein et al., 2010). The activation of the dorsal striatum in the heavy drinkers suggests that their drinking behavior has progressed to the habitual long term addiction, as compared to the initial reinforcing phase that the social drinkers may be in. There are multiple aspects that contribute to social and heavy drug use, including drug liking and wanting.

Robinson and Berridge (1993) suggested that drug “wanting” is a result of the increase in incentive salience to drug or alcohol cues. Based on various experimental findings involving many drugs of abuse, they suggested that the increase in salience to these cues that causes wanting or craving is controlled by an increase in sensitivity of the dopamine system that projects to the NAc in the ventral striatum. Later, this idea was tested by Heinz and colleagues in 2004 by giving fMRI and PET scans to detoxified alcoholics and healthy men and showing them alcohol related and neutral photos. In this study, fMRI scans revealed that detoxified alcoholics, as compared with healthy men, had fewer dopamine subtype 2 (D2) like receptor availability in the ventral striatum. Strong alcohol craving in the recovering alcoholics was also related to fewer D2 receptors in the NAc of the ventral striatum. The decrease in dopamine receptors in the ventral striatum can contribute to the increase in incentive salience of alcohol related cues which may cause an increase in craving in alcoholics and possible relapse in recovering alcoholics. The decrease in D2 receptors in the ventral striatum may have also caused an increase in activation in the medial prefrontal cortex and anterior cingulate when the subjects were exposed to alcohol related cues (Heinz et al., 2004). Fewer D2 receptors in the caudate, putamen, and ventral...
striatum of alcoholics were later confirmed through the use of PET scans by another laboratory (Martinez et al., 2005).

Alcohol increases dopamine release, and at a higher level in those who are addicted to alcohol, which may cause them to find it to be more reinforcing than healthy individuals. As the addiction progresses, those who are dependent will experience a down regulation in D2 receptors which will contribute to maintaining their cravings for alcohol and their addiction (Martinez et al., 2005). In addition to a decrease in D2 receptors, dopamine release is also decreased in the striatum and NAc in those addicted to alcohol addicted (Volkow, Fowler, Wang, Swanson & Telang, 2007). Volkow and colleagues (2007) suggest that as dopamine levels and receptors decrease, the addicted individual will find natural reinforcers less pleasurable and reinforcing, thus increasing their need for the drug in order to activate the remaining dopamine systems.

The neurobiology of negative reinforcement occurs as a result of the effects of the withdrawal produced from alcohol abstinence. It operates on the same neural systems that positive reinforcement does and as addiction develops, reward is decreased because the brain threshold for it is increased (Koob & Le Moal, 2005, 2008a, 2008b). In addition to a decrease in reward, anxiety also increases during withdrawal because of changes in levels of gamma-aminobutyric acid (GABA), corticotrophin releasing factor (CRF) and neuropeptide Y (NPY); the alcoholic then drinks to avoid this aversive condition that includes anxiety and stress, both of which are lowered by consuming more alcohol (Valdez & Koob, 2004; Clapp, Bhave, & Hoffman, 2008).
Neurobiology of Craving

The three separate types of craving discussed above are each controlled by different neurobiological pathways. Reward craving is most likely a result of dysregulation of dopamine and opioids. The relief of withdrawal type of craving may be a result of dysregulation of GABA or glutamate or problems with signaling of the CRF receptors (Verheul et al., 1999; Heinz, et al., 2003; Vengeline et al., 2009). The obsessive type of craving is thought to be a result of a serotonin deficiency, and it is suggested that if multiple mechanisms account for varying types of craving, then different pharmacotherpaies would be appropriate depending on the type a patient suffers from (Verheul et al., 1999).
To study the brain correlates of drug craving, Childress and colleagues (1999) scanned the brains of detoxified and naïve cocaine users while viewing both cocaine related and neutral videos. They found that while viewing videos relating to cocaine, those participants who had previously been dependent experienced cocaine related craving and increases in cerebral blood flow in the limbic system as compared to naïve participants. The limbic regions that were shown to have increased blood flow were the amygdalae and anterior cingulate. The authors concluded that this increase in limbic activation is due to the cue induced craving that occurred when viewing the cocaine related video and that limbic activation may occur during craving for other drugs of abuse (Childress et al., 1999).

**Neurobiology of Alcohol Dependence**

Unlike other drugs of abuse, alcohol’s effects are not a result of direct influence on one, or even two neurotransmitters or receptor systems. It is a complex interaction involving many neurotransmitters, receptors and brain regions. The physiological effects of alcohol, such as intoxication and tolerance, are thought to result from the fact that it is an indirect agonist at inhibitory GABA\textsubscript{A} receptors and indirect antagonist at excitatory glutamate NMDA receptors (Carlson, 2010; Chandler, Harris & Crews 1998). The reinforcing effects of alcohol, and other drugs of abuse, that result from activity of the mesolimbic dopamine system, are mediated by a number of other neurotransmitters in addition to dopamine, such as glutamate (Clapp et al., 2008). The serotonin (5-HT) system of the dorsal raphe nucleus alters the activity of dopamine in the NAc and VTA. In addition, the VTA is also under the control of GABA, enkephalin, cholinergic and glutamatergic input originating from areas including the NAc, ventral pallidum and prefrontal cortex. Dopamine is also released in the NAc when those neurons are activated in
the VTA by cannabinoids or opioids (Vengeline et al., 2008). While dopamine is the transmitter that contributes most to the reinforcing effects of alcohol, serotonin, glutamate and GABA also mediate its effects.

To determine the neurobiological basis of alcohol consumption, many investigators utilize rat lines and mouse strains that differ in alcohol consumption. McBride and colleagues (1993) looked at the neurobiological differences between alcohol preferring (P) and non-preferring (NP) rats. They delivered ethanol to rats either by an intraperitoneal injection or by perfusion with a microdialysis probe. Both administrations resulted in increased extracellular dopamine and 5-HT in the NAc. The P rats had lower dopamine and 5-HT than the NP rats, and later P rats but not NP rats were found to self-administered ethanol directly into the VTA. The difference in the neurochemistry of these two rat lines are thought to contribute to both the difference in their ethanol administration and ethanol’s rewarding effects (McBride et al., 1993). Other studies have also confirmed that P and NP rats differ in their self-administration of alcohol directly into the VTA. In an operant paradigm, P rats, but not NP rats, self-administered alcohol into the VTA at high levels more than they self-administered artificial cerebrospinal fluid. The P rats also pushed the lever that delivered alcohol to the VTA at significantly higher rate than did the NP rats, while the NP rats pushed the lever that delivered alcohol and the one that did not at the same rate for all concentrations of alcohol (Gatto et al., 1994).

The role of dopamine in alcohol consumption has been widely studied. Di Chiara and Imperato (1985) implanted rats with dialysis probes into the NAc and the dorsal striatum to measure dopamine and its metabolite dihydroxyphenylacetic acid (DOPAC) during alcohol consumption. A low dose of ethanol resulted in animals increasing behaviors such as movement and grooming. Within about twenty minutes this low dose increased dopamine release by
approximately 100% in the NAc, with it returning to normal levels within one hour, DOPAC levels also increased though to a lesser degree. At a higher dose of alcohol that caused sedation and later motor activation, dopamine was increased for up to three hours in the NAc and also the striatum, though for a shorter amount of time (Di Chiara & Imperato, 1985). Another study looked at how ethanol affected synapses of VTA dopamine neurons in vivo of both alcohol preferring (C57) and non-preferring (DBA) mice one day after receiving an injection of ethanol or saline. They found that alcohol increased the release of GABA on VTA dopamine neurons of the DBA and C57 mice but lowered glutamate receptor function in VTA dopamine neurons of DBA mice. This further suggests that the VTA dopamine neurons are affected by alcohol consumption and their changes might also be a factor for consuming alcohol (Wanat et al., 2009).

**Cannabinoid System and Alcoholism**

In addition to dopamine and the other neurotransmitter systems discussed above, the cannabinoid (CB) system has recently been found to be involved in the reinforcing effects of drugs of abuse including alcohol, mainly via the CB1 receptor type (see Figure 3). The CB system operates differently than most other neurotransmitter systems. While other neurotransmitters are released from the presynaptic neuron and bind to receptors on the postsynaptic neuron, the endogenous cannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are released from the postsynaptic neuron and bind to receptor sites on the presynaptic neuron. When endocannabinoids bind to CB1 receptors in the VTA, dopamine neurons are disinhibited, which contributes to the rewarding effects of drugs (Lupica & Riegel, 2005; for review see Covey, Wenzel & Cheer, 2014).
To investigate the function of CB1 receptors in alcohol self-administration and dopamine transmission, some studies have used mice lacking the gene for the CB1 receptor (CB1<sup>−/−</sup>). Confirming that the CB1 receptor is involved with the positively reinforcing effects of alcohol, one study found that the CB1<sup>−/−</sup> mice consumed less alcohol and did not release dopamine in the NAc when they consumed alcohol, as compared to wild type mice that did have the gene for the CB1 receptor (Hungund, Szakall, Adam, Basavarajappa, & Vadasz, 2003). Genetic variations of the CNR1 gene that controls the CB1 receptor in humans have also been found to be associated with drug and alcohol dependence (Zuo, Kranzler, Luo, Covault, & Gelernter, 2007).

Blocking or activating the CB1 receptor will also affect alcohol intake. The CB1 receptor antagonist SR 141716, also known as rimonabant, decreases alcohol and sucrose, but not food or water, consumption in C57 mice (Arnone et al., 1997). Both WIN 55,212-2, a CB1 receptor agonist, and CP 55,940, a CB1 and CB2 agonist, increase alcohol intake in Sardinian alcohol preferring rats (sP) without affecting food, water or sucrose intake. The CB1 receptor antagonist SR 141716 and opioid receptor antagonist naloxone, block this increase in alcohol intake by the receptor agonists, suggesting that the involvement of CB1 receptor system in alcohol intake may be mediated in part by the opioid system (Colombo et al., 2002).

Further evidence that the cannabinoid system is involved in alcohol intake is that not only do the CB1 receptors influence the reinforcing effects of alcohol; endogenous cannabinoids are also affected by alcohol. The levels of AEA and 2-AG were increased in vivo when exposed to alcohol (Basavarajappa & Hungund, 1999; Basavarajappa, Saito, Cooper, & Hungund, 2000). Additionally, administering THC, WIN 55,212-2 and CP 55,940 intravenously to rats increases dopamine neuron firing of A10 neurons in the mesolimbic system that run from the VTA to the
NAc, and the effect is blocked by SR 141716A suggesting that cannabinoids are also involved in dopamine transmission (Gessa, Melis, Muntoni, & Diana, 1998).

\[\text{Figure 3. CB1 receptors in the mouse brain. The distribution of the CB1 cannabinoid receptor in the mouse brain. From “Cannabinoid-hypocretin cross-talk in the central nervous system: what we know so far,” by A. Flores, R. Maldonado, and F. Berrendero, F., 2013, Frontiers in Neuroscience, 7(December), p. 3. 256. Copyright 2013 A. Flores, R. Maldonado, and F. Berrendero, F. Adapted with permission.}\]

**Initiating Alcohol Intake and Dependence**

In order to study alcohol consumption in laboratory animals, scientists must first ensure that animals will consume alcohol. If alcohol is simply placed on the cage, few if any mice or rats will voluntarily consume it. Over the years, many methods have been employed to initiate alcohol consumption in laboratory animals and to induce alcohol dependence (see below: Alcohol Training). These methods pre-expose alcohol to the animals to motivate them to consume it on their own later, sometimes this motivation is a result of withdrawal and negative reinforcement.
Pre-exposure to Alcohol

It has been well documented that pre-exposure to drugs of abuse will result in later preference for those drugs. When rats were pretreated with multiple administrations of morphine, amphetamine, or cocaine, they later showed a preference for the drugs’ effects as indicated by conditioned place preference (Lett, 1989). Alcohol preference also increases when mice are pretreated with it. As compared to saline injected C57 mice, those that were injected once with 2 mg/kg of ethanol showed a five percent increase in ethanol consumption and preference after ten days of the two-bottle choice procedure described below (Melis, Camarini, Ungless, & Bonci, 2002).

In a study by Camarini and Hodge in 2004, C57 and DBA mice were pretreated with either 1 g/kg or 2 g/kg ethanol injections and later tested with the two-bottle choice procedure. Mice received up to five injections of ethanol over ten days in an acute or chronic pretreatment condition. During the two-bottle choice they first received ten percent sucrose combined with five percent ethanol versus sucrose, then five percent ethanol with five percent sucrose versus sucrose and finally five percent ethanol versus water, each for four days. They found that pre-exposure to ethanol resulted in higher levels of self-administration that varied between two strains; DBA mice consumed more when they received higher concentrations or more injections, and regardless of pre-exposure condition, C57 mice that received injections always consumed more ethanol than those without prior exposure. However, both strains consumed more five percent alcohol during self-administration than saline if they received chronic alcohol pretreatment injections of 2 g/kg, even though the DBA mice are known to be alcohol avoiding and the C57 are known to be alcohol preferring. The larger dose of alcohol or chronic treatment both caused the DBA mice to self-administer more ethanol than saline. Both the C57 and DBA
pretreated with 2 g/kg of ethanol also self-administered more during two-bottle choice with the five percent sucrose/ethanol solution than the saline pretreated animals (Carmani & Hodge, 2004). These results suggest that even in mice that normally avoid alcohol, pre-exposure can increase later intake. This study exposed mice to alcohol via injections; the question remains if consuming alcohol orally will also increase later self-administration. It also utilized sucrose during the self-administration procedure; it is possible that the presence of sucrose was a confounding variable as even though the last alcohol solution given during self-administration was sucrose free, it was still preceded by ethanol and sucrose mixtures.

Research has also been done with humans investigating the subjective experience of the first encounter with a drug. It was found that the level of reinforcement individuals experienced and recounted predicted their later drug use for multiple drugs including alcohol. Subjects were more likely to be addicted to alcohol if they experienced drug liking and reinforcement when they first tried it (Haertzen, Kocher, & Miyasato, 1983).

**Withdrawal Mediated Drinking and Negative Reinforcement**

As discussed above, withdrawal is one condition that leads to continued alcohol consumption in order to avoid the negative aspects associated with it. Researchers sometimes bring about the alcohol deprivation effect in laboratory animals by maintaining them on ethanol until they show stable drinking and then removing the alcohol. After this forced abstinence, animals will reliably show increased alcohol consumption when it is available again (Koob, 2000). It has been found that continual cycles of access to alcohol followed by deprivation periods lead to a stronger alcohol deprivation effect where the animals consume large quantities of ethanol. Animals will show irregular intake behaviors where they will consume high

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concentrations of alcohol even at times when their overall activity levels are low, such as during the light phase. This deprivation effect persists after extended periods of abstinence and the alcohol drinking that follows is deemed to be uncontrolled (Spanagel & Holter, 1999).

Differences exist in how rodent strains respond to the alcohol deprivation effect paradigm. There are multiple substrains of the C57 mouse that can be obtained from different suppliers. The C57BL/6J mice are known to be high alcohol drinkers while the C57BL/6NCrl show significantly lower consumption of, and preference for, alcohol (Ramachandra, Phuc, Franco, & Gonzales, 2007). In another experiment, when both of these strains were exposed to one or multiple cycles of alcohol deprivation, it was found that C57BL/6NCrl mice initially consumed less alcohol than C57BL/6J mice, but the C57BL/6J strain showed a lower alcohol deprivation effect by consuming less, and having a decreased preference for, ethanol after one or multiple deprivations. Additionally, when the C57BL/6NCrl mice were allowed to self-administer alcohol chronically for a longer period of time before deprivation, they consumed an even larger amount of alcohol after it was offered again (Khisti, Wolstenholme, Shelton, & Miles, 2006). Allowing animals to consume alcohol chronically is thought to cause them to experience the positively reinforcing effects of alcohol, that later after deprivation cycles, will cause them to consume more alcohol once it is presented again. This effect was observed with C57BL/6J mice that consumed more alcohol after deprivation cycles than they did before they experienced the repeated withdrawals (Becker & Lopez, 2004).

Alcohol Training

Studies that investigate alcohol consumption, dependence and related issues must first introduce alcohol to an animal model. Over the years, many methods for delivering alcohol have
been used; these include the two-bottle choice with or without sucrose fading, alcohol vapor, intraperitoneal injections of alcohol, and forced consumption of alcohol via gavage or the Lieber-DeCarli liquid diet. Multiple procedures have been developed because most animals do not readily consume alcohol because of its aversive taste (Mello, 1973). Issues arise with many of these procedures, making them inappropriate for experiments investigating consumption and dependence.

**Experimenter Administration of Alcohol**

The models utilized to induce a pharmacological dependence on alcohol are helpful in initiating dependence quickly in order to study its effects on the animal during the later stages of dependence, but they do not allow for the study of the progression of dependence and the behavioral aspects of consumption (Mello, 1973).

**Inhalation of Ethanol Vapor** - Delivering ethanol to animals via a vapor chamber is employed to induce physical dependence quickly. In this experimental procedure, animals are housed in a clear box where ethanol vapor is administered into the air. Pyrazole, an alcohol dehydrogenase inhibitor, is sometimes administered once a day to help keep blood alcohol levels constant, though it may cause negative side effects and become toxic (Rogers, Wiener, & Bloom 1979). A vapor chamber is sometimes preferred over intraperitoneal injections because it maintains constant blood levels of alcohol that cannot be achieved via injections due to the fast elimination of ethanol. It is also considered to be less stressful to the animal than intravenous or intraperitoneal administration. Multiple animals can be housed in the box making it possible to experiment on many animals at once (Goldstein, 1971). However, the vapor chamber has been
found to result in lower blood alcohol levels (BAL) over time even when the dose of alcohol remains constant. To avoid this, some have suggested monitoring BAL and moving animals to lower or higher concentrations of ethanol vapor to maintain a constant BAL among all animals and ensure that the animals do not reach a toxic dose (Rogers et al., 1979).

**Intraperitoneal Injections -** Often researchers inject ethanol intraperitoneally (i.p.), directly into the lab animal’s body cavity to induce dependence and later ethanol consumption. Female rats housed singly were found to consume increased amounts of ethanol after ethanol injections though later, the same researchers were not able to get group housed rats to consume ethanol after injections in an operant paradigm (Neill, Domeney, & Costall 1993; Neill, Domeney, & Costall 1994). As stated above, pre-exposure to ethanol via intraperitoneal injections resulted in larger amounts of ethanol consumed during a two-bottle choice procedure (Carmani & Hodge, 2004). These results varied depending on animal strain, injection concentration and number of injections delivered. Although delivering ethanol via i.p. can induce later self-administration of alcohol, it does not give the animal experience with consuming alcohol orally. This removes factors other than the physiological, such as taste, which may influence if and how much alcohol the animal drinks.

**Gavage -** Another method for delivering ethanol to laboratory animals is to introduce it directly into the stomach either via gavage, which puts a steel tube down the animal’s esophagus, or via the direct implantation of a cannula into the stomach. This method is often preferred over i.p. injections because the ethanol is delivered into the digestive system where it metabolized in the same way humans do during consumption, adding to the validity of the model. After a few gavage procedures the animals become acclimated and may suffer less stress than they would from injections.
Like injections, the use of gavage is limiting in that it does not deliver alcohol at a constant rate and blood alcohol levels (BAL) fluctuate. To address this issue, researchers have developed an automated system to deliver continuous high concentrations of ethanol directly into the stomach. Infusing ethanol six times into the stomach this way was not found to keep blood alcohol levels consistently high and increasing the concentration of ethanol only resulted in a higher death rate. Increasing the number of infusions, however, resulted in the death of some animals and stable BAL in others. Also, instead of just ethanol, this experiment infused nutritive ethanol solutions that resulted in high stable BAL and no morbidity, and this overcame the issue that animals often decrease food consumption as BALs rise (Rogers et al., 1979).

While the direct infusion procedures may be helpful in maintaining constant high BAL, they do not model the consumption of alcohol by humans very well. Human drinking patterns, like laboratory animals given free access, are such that they consume alcohol in bouts often going through periods of abstinence where they experience withdrawal effects (McKim, 2007). In this sense, maintaining consistent high BALs is not relevant to human alcohol consumption. As with other methods of forced consumption, gavage and direct infusions remove the option of self-administration, oral consumption and taste.

Lieber-DeCarli Liquid Diet - The Lieber-DeCarli (LD) liquid diet, with and without alcohol, is nutritionally complete with the all of the necessary macro and micronutrients. Both diets provide the same caloric density and thus permit the control of caloric intake as well as nutrition. The diet has proven to be a simple and useful method for studying alcohol related issues including consumption, reinforcement, physiological effects and for inducing fetal alcohol syndrome in rodent pups. It was once thought that vitamin deficiencies in the diet of chronic drinkers caused liver damage, but studies utilizing the LD diet in laboratory animals showed that
it is actually ethanol itself that produces this liver damage in alcoholics, not an inadequate diet (Lieber & DeCarli, 1982). Other LD advantages include the ability to alter the diet to be high or low fat, allowing for the experimentation of many animals and the fact that it is relatively inexpensive (Lieber, DeCarli, & Sorrell, 1989a). In addition to rats, it is easy to get the C57 mice to voluntarily consume a liquid diet (Snell et al., 1996; Anji & Kumari, 2008). When animals are given the LD diet, it is continually available so that they can consume it voluntarily; this results in drinking bouts throughout the day and fluctuations of BAL. Blood alcohol levels in animals consuming the LD diet are more variable compared to animals exposed to ethanol vapor, however, these drinking patterns and BAL fluctuations are more similar to that which is seen with humans (Rogers et al., 1979). Liquid diets have also been found to cause rats to become physically dependent and show tolerance. Dependence is measured as displays of tremors and muscle rigidity and tolerance as determined by treadmill performance after a challenge injection of ethanol (Miller, Goldman, Erickson, & Shorey, 1980). Tolerance has also been shown in rats as measured by seizures produced following a very loud auditory tone during withdrawal, after two to four weeks consuming alcohol (Lieber & DeCarli, 1973).

When alcohol is consumed separately from a chow diet or added to a liquid diet, it results in a decrease of overall food and nutrient intake because the ethanol adds a substantial amount of calories and energy to the animals’ diet. The LD liquid diet allows the researcher to separate out the effects of alcohol alone from the nutritional deficiencies that result from long term alcohol consumption (Lieber et al., 1989a). The standard way to introduce alcohol to animals is to increase the concentration of ethanol in the LD diet so that the animals drink progressively higher concentrations every few days. Lieber and colleagues (1989a) suggested that animals should first consume 3% then 4% each for two days and 5% after; 5% was found to result in a
A high amount of ethanol consumed per kilogram of body weight and at times relatively high BALs such as 100-150 mg/100 mL.

A concern regarding the Lieber-DeCarli diet is that the animals maintained on the control diet may be nutritionally deficient as compared to chow fed animals, which could lead to different experimental findings in behavior and neurochemistry. However, in response to increased potassium extracellularly in an in vivo model, no differences were observed in the control LD fed animals compared to chow fed animals in the release of acetylcholine, dopamine, glutamate or norepinephrine in the cortex or hippocampus (Sabira et al., 2003). Questions remain, however, if the LD diet is a good model for behavioral dependence and if it results in preference for ethanol. Other models of alcohol consumption have not been found to produce behavioral dependence and those that do, such as the alcohol vapor chamber, are not valid models of human alcohol use and cause severe withdrawal symptoms that often result in the death of many experimental animals.

**Behavioral Models of Alcohol Consumption**

The behavioral models of alcohol consumption of operant conditioning and sucrose fading are useful for measuring self-administration of drugs but they fail to initiate dependence in the animal unless the animal is genetically predisposed to consume the drug, such as seen in the P rat. As a result, they are only useful models to measure consumption and not one to bring about dependence for further investigation.

**Operant Conditioning** - Laboratory animals can be trained to self-administer alcohol in an operant paradigm, where access to ethanol is contingent on an operant response such as
pressing a lever. Once the desired operant response has been made, alcohol can be delivered to
the animal intravenously, intragastrically or orally (Meisch, 1984). A point of contention with
operant paradigms that employ an additional response to deliver alcohol, or to allow the animal
access to it, is that simple oral administration can also be seen as an operant response (Martinetti,
et al., 2000). When a laboratory animal licks a sipper tube that extends from a bottle containing
alcohol, each individual lick can be viewed as an operant response that is reinforced by the
alcohol that drips out of the sipper tube when the animal licks the end. This makes the addition
of a lever which must be pressed in order to gain access to alcohol redundant.

**Sucrose Fading** – It is important for lab animals to orally self-administer ethanol so that
any results obtained will be valid when applying them to the problem of alcoholism in humans
(Sanchis-Segura & Spanagel, 2006). The sucrose fading procedure is employed to get animals to
develop a preference for alcohol and later consume it orally during self-administration. Adding
sucrose to ethanol helps initiate self-administration, as the sweet taste makes the alcohol more
palatable for the animal. The procedures employed vary, with some utilizing an operant
paradigm where the animals have to lever press to gain access to the sucrose and ethanol
solution. Generally, the content of the solution is varied over the course of a few weeks
beginning with only sucrose, then every few days the sucrose concentration is decreased and the
ethanol concentration is increased until finally the animal is consuming only ethanol at about ten
percent ethanol by volume. Though this procedure helps to get the animal to consume high levels
of ethanol, it is not sufficient for showing dependence or tolerance (Matthews, Overstreet,
Rezvani, Devaud, & Morrow, 2001). Naturally, problems arise when interpreting the data
obtained via a sucrose fading procedure. It is unclear if animals are consuming sucrose and
alcohol solutions for the sweet taste of the sucrose, the sucrose’s nutritive value or for ethanol’s physiological properties. It has been shown experimentally that animals will consume more of a solution comprised of relatively high sucrose and high ethanol percentages compared to ethanol alone (Samson, Sharpe, & Denning, 1999). It is also unclear if adding sucrose will affect ethanol metabolism and blood alcohol levels (Matthews et al., 2001). To test this, Matthews and colleagues (2001) allowed rats to self-administer various ethanol solutions with added sweeteners and also administered alcohol via gavage; later, they tested self-administration and blood alcohol levels. They found that rats allowed to drink ethanol mixed with sucrose consumed more than those that drank ethanol with saccharin or ethanol alone, and that the rats consuming ethanol with saccharin drank more than the rats consuming only ethanol. While sweeteners increased self-administration, there were no differences observed in blood alcohol levels. This is contrary to results obtained via gavage that showed a lower blood alcohol concentration when animals received 10% sucrose with up to 3 g/kg of ethanol. The authors suggest that this is a result of slower gastric emptying and increased ethanol metabolism (Matthews et al., 2001). Differing BALs as a result of added sweeteners were also seen in an earlier experiment that trained rats to orally self-administer different solutions of ethanol and sucrose or saccharin; this study concluded that adding sucrose lowers BALs in rats as compared to drinking ethanol alone (Roberts, Heyser, & Koob, 1999). These data make using the sucrose fading paradigm questionable for studying alcohol pharmacology and behavior.

**Two-Bottle Choice** - In the two-bottle choice paradigm, animals are given two bottles with stainless steel sipper tubes extending into their cages, one with water and the other with ethanol. Bottles are placed on their home cages for a limited amount of time daily, usually one
hour, and the placement is varied each day to ensure the animals are not simply drinking the bottle that is always on one side. The training procedure involves starting at a low concentration of ethanol and increasing it until the rats are reliably consuming a relevant percentage of alcohol by volume. The steps and methods of increasing alcohol concentration vary by laboratory; some increase alcohol by two percent only when the animal reliably drinks more alcohol than water for a few days, other laboratories increase concentration in small steps regardless of preference. Martinetti and colleagues (2000) increased ethanol concentration if the animal consumed at least three milliliters of the alcohol solution and if that was at least 50% of the total volume of liquid from the water and the ethanol bottles during the choice procedure; this took about one month for animals to reach a 10% alcohol solution. The two-bottle choice procedure produces stable alcohol consumption without adding additional variables, such as sucrose, that may influence the amount of ethanol consumed or how reinforcing it is to the animal (Martinetti et al., 2000). The animals are said to have preference for, or be reinforced by, alcohol if they reliably consume more alcohol than water. This model for reinforcement has been shown to produce standard and consistent results with the C57 mouse strain in multiple laboratories. When comparing results from three different laboratories, Crabbe and colleagues found that only genetic and sex differences accounted for difference in consumption, not site or animal shipping practices, which is not the case with other behavioral tests such as the open field or the elevated plus maze (Crabbe, Wahlsten, & Dudek, 1999).

This paradigm may raise the question of whether the animals are consuming alcohol because they have a preference for its taste or if they are reinforced by the physiological effects it produces. As mentioned above, alcohol preferring rats will self-administer alcohol into the VTA at high levels (Gatto et al., 1994). Since that study delivered alcohol directly into the brain,
bypassing oral consumption, it suggests that the animals are administering alcohol because of a preference for its physiological effects, not only for taste.

**Experimental Animals**

As previously discussed, animals with different alcohol preferences are used for alcohol research. Many lines of rats and strains mice have been developed to aid researchers in determining the neurobiological differences between low and high alcohol drinkers. Rat lines include the Preferring (P) and Non-Preferring (NP) rats, Sardinian alcohol-preferring (sP), High-Alcohol-Drinking (HAD) and Low-Alcohol-Drinking (LAD), Fawn-Hooded (FH/Wjd), Maudsley Reactive (MR/Har) and the Lewis rats. Mouse strains used often are the alcohol preferring C57BL/6J (C57) and alcohol avoiding DBA/2J and BALBc mice. Mouse models of human alcohol reinforcement and neurobiology are considered to be valid because relevant brain structures are similar in both species (Spanagel, 2000).

The C57 mice have a well-documented strong preference for alcohol. In one experiment, 70% to 90% of their total fluid intake over five hours was comprised of a 10% ethanol solution as compared to DBA mice which consumed only 10% of their total fluid from the ethanol solution (George et al., 1995). Studies using the C57 mice have elucidated neurochemical differences that contribute to their alcohol preference. George and colleagues (1995) also investigated dopamine differences in mouse strains and the differences in dopamine as a result of ethanol consumption. The C57 mice had lower levels of dopamine in the olfactory tubercle and hypothalamus compared to the DBA/2J and BALBc alcohol avoiding mice.
Experimental Rationale and Significance

Many variables contribute to the development of addictions including alcoholism. Based on current theories, it seems that addiction begins with positive reinforcement. The drug addicted individual experiences a pleasurable state, or high, when they first consume alcohol. This positive state reinforces behaviors that are associated with obtaining alcohol, and cause the user to engage in those behaviors again in order to drink alcohol and re-experience that positive state. Some have theorized that negative reinforcement also contributes to the development of addiction; this is when a person is consuming alcohol to alleviate a negative condition of anxiety or depression. It does not seem, however, that negative reinforcement greatly contributes to the progression to addiction as anxious people do not abuse valium more than non-anxious people and depressed people do not abuse amphetamine more than non-depressed people (de Wit, McCracken, Uhlenhuth, & Johanson, 1987; de Wit, Uhlenhuth & Johanson, 1987).

Negative reinforcement does play a role in addiction after the person has progressed to physical dependence. When an alcoholic, or drug abuser, is dependent, one factor that contributes to their consumption is the avoidance of withdrawal and the negative symptoms associated with it. They drink to remove the aversive withdrawal symptoms that result from the lack of alcohol. As their addiction progresses they also find other positive reinforcers less appealing, things that used to bring them pleasure are no longer enjoyable. As a result, they continue to drink in attempt to make their mood more positive since other reinforcers are not contributing to mood elevation.

In addition to positive and negative reinforcement, many other variables contribute to the progression to addiction and alcoholism. Conditioning will cause dependent individuals to respond to environmental cues with drug craving or symptoms like those experienced during
withdrawal, this will cause them to seek and consume more drug or alcohol (Wikler, 1948). The body of the alcoholic is also reliant on the calories that alcohol provides the user, also motivating continued consumption (Lewis, 1996). A genetic predisposition for addiction will also cause the user to be more likely to progress to dependence, as will simply drinking sufficient quantities of alcohol. During this complicated progression, the alcoholic’s neurochemistry is changing, dopamine and dopamine receptors are decreasing. This decrease in dopamine binding results in a more negative hedonic state, contributing to the motivation to consume alcohol to reach a more normal positive state. All the above reasons interact and contribute to alcohol dependence.

Though we know a great deal about the progression to alcoholism and the variables that contribute to it, there is still a great deal that we do not yet know. There are a number of neurotransmitters including but not limited to dopamine, GABA, glutamate, opiates and cannabinoids, which are involved in the initiation and maintenance of alcoholism, but it is not clear how they all interact. It is also unknown what role dependence has in the motivation to drink alcohol.

It is assumed that dependence is a motivator for the consumption of drugs including alcohol. The avoidance of withdrawal from other drugs, such as crack cocaine, opiates and even nicotine and caffeine, is a strong motivator. However, patterns of human alcohol consumption would suggest that dependence, and the avoidance of withdrawal symptoms, is not a universally strong motivator for drinking among alcoholics. Those that are physically dependent on drugs such as opiates or nicotine, will often maintain a constant blood level to avoid the negative withdrawal. Alcoholics, however, do not always drink when given the opportunity and also do not maintain a constant blood level of alcohol as would be expected if they were trying to avoid withdrawal. Humans, and nonhuman animals, drink in bouts where they have periods of high
alcohol consumption but also periods of no consumption where they experience negative withdrawal symptoms (McKim, 2007).

The idea of dependence motivating drug use is not always correct. A person can be dependent on medication like antidepressants, or even opiate pain medications, but not show a preference for them or a strong motivation to consume them. The question remains if a human or nonhuman animal can be physically dependent on alcohol and not have a preference for it. It is clear that preference may lead to dependence, but it is not clear if dependence leads to preference.

This experiment will help elucidate the role of dependence in alcohol preference, consumption and ultimately addiction. The Lieber-DeCarli (LD) liquid diet is assumed to be a method for producing severe alcohol dependence. The diet is a well-established method for chronic administration of sufficient quantities of ethanol to induce physical dependence and is the best-known method for inducing dependence while maintaining adequate nutrition. Researchers often maintain animals on this diet to force consumption of alcohol and control the intake of nutrients before conducting behavioral tests of the effects of alcohol. The diet has been used to determine the physiological effects of alcohol on the liver and other organ systems or to examine the teratogenic effects of alcohol in animal models of fetal alcohol syndrome. It clearly produces physical dependence, but it is unknown what behavioral effect it has in terms of the motivation to consume alcohol and the preference for it. In these experiments, the animals’ subsequent motivation to consume alcohol, and preference for it, will be determined by the two-bottle choice procedure after maintenance on the LD liquid diet. When presented with the two-bottle choice, the animals will be physically dependent on alcohol, and I hypothesize that their resulting alcohol consumption will show that they have developed a preference for it. This basic
research, which has not been conducted, will conclude two important things. First, it will
determine the role of alcohol dependence in alcohol reinforcement in the mouse model. Second,
it will validate the LD diet as a model for studying alcoholism in a rodent

To determine the role of alcohol dependence in alcohol reinforcement in mice, I will
compare alcohol consumption via the two-bottle choice procedure between mice that will be
made dependent on alcohol via the LD diet with alcohol with mice that will not consume alcohol
in the LD diet and are not dependent. This will show the difference in how reinforcing alcohol is
between the two groups of mice and if dependence alone is a motivating factor in alcohol
consumption. It will also conclude if the LD diet is a valid model for studying alcoholism and the
motivational aspects of alcohol dependence in mice. In addition, I will also examine the role of
specific brain neurochemicals known to play a role in alcohol reinforcement in dependent
animals. Specifically, I will look at the difference in dopamine and cannabinoids between the
mice that were on the alcoholic LD liquid diet and those that were not. However, during later
rounds of two-bottle choice, both groups of mice will have had experience with alcohol, one
through the alcoholic LD diet and one only via the earlier rounds of two-bottle choice. This will
model two patterns of alcohol consumption in humans, the dependent alcoholic drinker and the
non-dependent social drinker. This study is important for the field of alcohol research and
addiction research in general. By validating the LD liquid diet model, researchers will be able to
apply results obtained by using this diet to the human condition of alcohol consumption and
abuse. Determining the role of dependence in the motivation to drink alcohol will also help to
increase our knowledge of alcoholism and addiction. This could further our understanding of the
causes of the initiation and maintenance of alcohol consumption in humans and potentially lead
to better treatments and therapies for alcoholism.
Specific Aims

1. Investigate the role of alcohol dependence in alcohol reinforcement in mice.

   The effect of inducing alcohol dependence via the LD liquid diet on alcohol reinforcement will be determined by measuring ad libitum consumption of alcohol and the preference for alcohol over water.

2. Determine the value of using the Lieber-DeCarli liquid diet as a model for examining the motivational aspects of dependence.

   The diet is a well-established method for chronic administration of sufficient quantities of ethanol to induce physical dependence. I will determine if physical dependence produced by the Lieber-DeCarli liquid diet is a sufficient condition to motivate voluntary alcohol consumption and induce behavioral dependence in mice.

3. Examine the role of specific brain neurochemicals and regulatory systems known to play a role in alcohol reinforcement in dependent animals.

   I will explore the neurobiological systems that mediate the effects of alcohol consumption by comparing animals that received alcohol to those that did not. Dopamine and cannabinoid differences will be explored.
Chapter 3: Materials and Methods

Experiment 1

Animals

Male C57BL/6J mice ($N = 15$) were purchased from Jackson Laboratories (Bar Harbor, ME) and were approximately four months old during experimentation. The animals were divided into two groups, experimental ($n = 8$) and control ($n = 7$). Animals were weighed and given clean cages once a week. They were housed at the Institute of Comparative Medicine at Columbia University Medical College, which is accredited by the Association of the Assessment and Accreditation of Laboratory Animal Care, International. All animal experimentation and care was in accordance with the criteria established by the National Academy of Science as described in the Guide for the Care and Use of Laboratory Animals (NIH publication 865-112, Bethesda MD) and approved by the Institutional Animal Care and Use Committee of Columbia University.

Apparatus

Mice were individually housed in polypropylene cages with stainless-steel wire mesh tops. Animals were kept at Columbia University Medical Center in an environmentally controlled facility maintained at 22-24°C. Rooms were on a 12 hour alternating light:dark cycle with lights out at 19:00. Before experimentation began animals had free access to water and to a standard chow diet (LabDiet 5001, PMI, St. Louis, MO).

The Lieber-DeCarli Liquid Diet was administered in 50 mL glass liquid feeding tubes; 3 ¾ inches x 1 ¼ inches (BioServ, Frenchtown, NJ) (see Appendix). The tubes have milliliter calibrations printed on the on the side. They were hung from the wire top of the cages with stainless steel holders and were autoclaved every other day.
Alcohol and water for the two-bottle choice and Drinking in the Dark paradigms were administered in 150 mL Nalgene bottles with stainless steel ball bearing sipper tubes through rubber stoppers.

**Lieber-DeCarli Liquid Diet**

The Lieber-DeCarli Liquid Diet ships in powder form and is prepared into liquid using tap water (control: F1259SP, alcohol: F1258SP; Bio-Serv, Frenchtown, NJ). Control and alcohol diets are equicaloric so that once ethanol is added to the alcohol diet both mixtures provide the same number of calories to the animals. The control diet contained 151 kcal/L protein, 359 kcal/L fat and 490 kcal/L carbohydrate. The alcohol diet contained 151 kcal/L protein, 359 kcal/L fat, 135 kcal/L carbohydrate and 355 kcal/L ethanol. All mice were provided control diet for two days to acclimate to eating a liquid diet, at this time solid chow was removed from cages. Tap water was available ad libitum for the entire experiment. The alcohol diet was diluted with the control diet to gradually increase the concentration of alcohol so that the experimental group could adapt to alcohol. Adaptation to ethanol was such that mice in the experimental group received 2.13% v/v alcohol and then 4.27% v/v alcohol each for four days. Following adaptation the experimental animals were maintained on 5.33% v/v alcohol for 21 days, control animals were given the control Lieber-DeCarli Liquid Diet without alcohol for the duration of the study.

**Experimental Paradigm**

**Two-Bottle Choice- Phase 1**

The experimental group was maintained on 5.33% v/v alcohol for 21 days. Following this time both groups were given solid chow diet and the two-bottle choice paradigm. They received
two bottles on their home cages, one containing alcohol and one tap water one hour into the dark phase for one hour (20:00-21:00), on alternating sides each day to avoid animals developing side preference. Bottles were weighed to the nearest tenth of a gram before and after the drinking phase. All animals received the choice between water and 5% v/v alcohol for four days and then received the choice between water and 10% v/v alcohol for four days (see Table 1). When the choice between water and 10% v/v alcohol began, water bottles were removed at 16:00 and returned after the two-bottle choice procedure was completed. Bottle leakage was assessed in all experiments by testing the amount of liquid each bottle spilled when it was placed on the cage. Leakage was calculated by weighing the bottle, placing it on an empty cage on the same side as it was during that day’s choice experiment and weighing it again. The side it was on during experimentation was relevant to possible leakage calculations because bottles tended to leak more when placed on the same side as the food pellets and less when placed on the side that usually had the water bottle.

Drinking in the Dark

Following the two-bottle choice paradigm, animals were given one bottle containing 10% v/v alcohol on their home cages three hours into the dark phase for two hours (22:00-00:00) for three days. Bottles were weighed to the nearest tenth of a gram before and after the drinking phase.

Two-Bottle Choice- Phase 2

After the Drinking in the Dark paradigm animals were once again given the two-bottle Choice procedure with one bottle containing tap water and the other 10% v/v alcohol on their home cages one hour into the dark phase for one hour (20:00-21:00) for two days, alternating sides each day to avoid animals developing side preference. Bottles were weighed to the nearest
tenth of a gram before and after the drinking phase. The following day animals were sacrificed and brains were removed and frozen in preparation for the biochemical assays.

Table 1

*Experimental paradigm for Experiment 1*

<table>
<thead>
<tr>
<th>Day</th>
<th>1</th>
<th>2</th>
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</tr>
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**Ultra Performance Liquid Chromatography with Tandem Mass Spectrometric Assays (UPLC/MS/MS) of Dopamine in Mouse Plasma and Brain**

After the completion of Experiment 1, all mice were sacrificed the following day by decapitation and brains were quickly removed and dissected into two midsagittal brain regions. These were immediately frozen and maintained at -80º C. Prior to neurochemical analysis, the NAc was removed and dissected into two regions for each side of the brain; one that contained largely shell tissue and another largely core tissue.

**Materials.** Dansyl chloride, LC/MS grade formic acid, water, ethyl acetate, acetonitrile and acetone, calibrating standards dopamine were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich (St. Louis, MO, USA). Deuterated internal standards dopamine-d4 were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada).

**Sample Preparation.** The samples were processed on ice or at 4º C under dim light except when otherwise indicated. Fifty microliters of plasma or brain homogenate (containing 10 mg brain tissue in water) were mixed with 50 µL of 0.4 M perchloric acid containing 50 pmol of
dopamine-d4 in a microcentrifuge tube. The sample was vortexed well and centrifuged at 12,000 g for 5 minutes. The clear supernatant was then transferred to another tube and 200 μL of 1 M sodium bicarbonate was added and mixed well. Next 400 μL of 1% (w/v, dissolved in acetone) dansyl chloride was added and the mixture was vortexed and heated at 60 °C for 15 minutes in darkness. After the sample was chilled on ice for 2 minutes, it was spun at 12,000 g for 2 minutes and the supernatant was transferred to another tube. Next, 400 μL ethyl acetate was added to the sample and vortexed again. Then the sample was centrifuged at 12,000 g for 5 minutes and the yellow supernatant was transferred to a glass test tube and blown down under a nitrogen stream at 37 °C. The dryer was re-suspended using 50 μL of acetonitrile and transferred to an Agilent amber screw top micro sampling LC/MS vial (P/N 5184-3554. Agilent Tech, Santa Clara, CA, USA) for assay.

**UPLC/MS/MS.** All assays were carried out on a Waters Xevo TQ MS ACQUITY UPLC system (Waters, Milford, MA, USA). The system was controlled by MassLynx Software 4.1. The sample was maintained at 4°C in the autosampler at a volume of 5 μL and it was loaded onto a Waters ACQUITY UPLC BEH Phenyl column (3 mm inner diameter × 100 mm with 1.7 μm particles. Waters, P/N 186004673), preceded by a 2.1 × 5 mm guard column containing the same packing (Waters, P/N 186003979). The column was maintained at 40°C. The flow rate was 300 μL/min in a binary gradient mode with the following mobile phase gradient: initiated with 50% phase A (water containing 0.1% formic acid) and 50% mobile phase B (acetonitrile containing 0.1% formic acid). Gradient of acetonitrile was increased linearly to 99% over 5 min and maintained till 10 min. Then column was conditioned by using the initial gradient for 2 minutes and the next sample was injected. The flow of first 2.2 minutes was diverted to waste to avoid the high level sodium bicarbonate entering the MS part. Positive ESI-MS/MS with multiple
reaction monitoring (MRM) mode was performed using the following parameters: capillary voltage 4 kV, source temperature 150 °C, desolvation temperature 500 °C, desolvation gas flow 1000 L/hr. Optimized MRM transitions were 853.3 > 170.2 and 857.3 > 170.2 for dopamine and dopamine-d4 respectively. The cone voltage was 46 V and the collision energy was 64 eV. Dopamine and dopamine-d4 were confirmed by comparing the retention times of experimental compounds with those of authentic standards. Concentrations of dopamine in the samples were quantified by comparing integrated peak areas for those of each compound against those of known amounts of purified standards. Loss during extraction was accounted for by adjusting for the recovery of dopamine-d4 added before extraction. The results were then normalized by using plasma volume or brain tissue weight.

UPLC/MS/MS Assays of Endocannabinoids in Mouse Plasma and Brain

Animal sacrifice and tissue dissection were carried out in the same manner as for the dopamine assay (see above).

Materials. All solvents employed for sample extractions and LC/MS analysis were LC/MS grade (or LC grade when LC/MS grade was not available) and were purchased from Fisher Scientific (Pittsburgh, PA, USA). Calibration standards AEA and 2-AG and deuterated internal standards AEA-d8 and 2-AG-d8 were purchased from Cayman Chemical Co. (Ann Arbor, MI).

Extraction Method. Samples were extracted using chloroform:methanol on ice at 4°C under dim light except otherwise indicated. Briefly, 3 mL of chloroform:methanol (vol:vol = 2:1, containing 100 pmol AEA-d8 and 500 pmol 2-AG-d8) was added to 20 μL of plasma or brain homogenate (containing 4 mg brain tissue disrupted in water) in a clean glass test tube. The...
mixture was vortexed well and half of a milliliter of water was added to the mixture to allow for phase separation. The mixture was vortexed again and centrifuged at 3,000g for 10 minutes. The lower organic phase was then transferred to a second clean glass tube using a Pasteur pipette. Two mL of chloroform was added to the residual aqueous phase, followed by vortex mixing and centrifugation again at 3,000g for 10 min to extract any remaining lipids. The lower organic phases were pooled and evaporated under nitrogen at 37 °C. The extracted lipids were reconstituted in 30 μL of acetonitrile and transferred to amber LC/MS autosampler vials (Waters, P/N 600000755CV) for injection.

**LC/MS Conditions.** All experiments were carried out on a Waters Xevo TQ MS ACQUITY UPLC system (Waters, Milford, MA). The system was controlled by Mass Lynx Software version 4. 1. Samples were maintained at 4°C in the autosampler and 5 μL was loaded onto a Waters ACQUITY UPLC HSS C18 column (2.1 mm inner diameter × 100 mm with 1.8 μm particles), preceded by a 2.1 × 5 mm guard column containing the same packing. The column was maintained at 40°C throughout analysis. The UPLC flow rate was continuously 300 μL/min with an isocratic mobile phase composition of 17% solvent A (water containing 0.1% formic acid) and 83% solvent B (acetonitrile containing 0.1% formic acid). The UPLC running time was 4 minutes and endocannabinoids of interest eluted between 2 and 3.5 min. Positive ESI-MS/MS was performed employing the following parameters: capillary voltage, 3.8 kV; source temperature, 150 °C; desolvation temperature, 500 °C; desolvation gas flow, 1000 L/hr; and collision gas flow, 0.15 mL/min. The optimized cone voltage was 22 V, collision energy for multiple reactions monitoring mode (MRM) was 14 eV. For MRM analysis the following transitions were employed: AEA 348→62, AEA-d8 356→63, 2-AG 379→287, 2-AG-d8 387→294. Another AG isomer 1-AG shares the same transition as 2-AG, though 1- and 2-AG
can be separated by liquid chromatography and identified by different retention time. Compounds of interest were confirmed by comparing the retention times of experimental compounds with those of authentic standards. Loss during extraction was accounted for by adjusting for the recovery of internal standards added before extraction. The results were normalized by using plasma volume or brain tissue weight.

**Data Analysis**

Statistical analyses were performed using IBM SPSS Statistics version 21 (SPSS, Chicago, IL) and significance was set as $p < .05$. The experimental design for the research was based on an a priori planned comparisons of experimental and control groups after chronic alcohol administration. Differences between the mean intake of alcohol during two-bottle choice testing and preference testing as well as bodyweight and total fluid intake for the days after chronic intake were evaluated using independent samples t-tests. Other a posteriori comparison used t-test or post hoc procedures.

Animal weights were recorded in grams approximately once a week and were averaged. Independent-samples t-tests were conducted to compare the body weights of the experimental and control groups, the amount of liquid lost from the alcohol and water bottles during the two-bottle choice testing in both groups, and the amount of the Lieber-DeCarli liquid diet consumed by both groups during training.

The amount of alcohol consumed during the two-bottle choice procedure by each group was compared using independent samples t-tests. Since the groups did not differ in the average body weight of the animals, consumption was analyzed using the amount of liquid consumed in grams.
Preference for ethanol over water was analyzed two ways, first, by comparing the percent ethanol consumed between the two groups during the two-bottle choice procedure. Percent ethanol was calculated by dividing the amount of ethanol consumed (in grams) by the amount of total liquid consumed (sum of ethanol and water) (in grams) and conducting independent samples t-tests. The second method preference was calculated was by using paired samples t-tests to determine if the amounts of water and ethanol consumed differed within each group.

The amounts of water and alcohol consumed were also averaged across the four days of receiving the choice between water and 5% ethanol and the four days of receiving the choice between water and 10% ethanol, and paired samples t-test were conducted to determine any differences within each group.

Brain levels of dopamine, AEA and 2AG were averaged for the left and right sides of the brain in the NAc core, shell and whole brain. Independent samples t-tests compared levels between the control and experimental groups.

Data are expressed as mean and standard error of the mean (SEM) and were graphed using GraphPad Prism version 6 (GraphPad, La Jolla, CA).

**Experiment 2**

**Animals**

Two cohorts of male C57BL/6J mice were purchased from Charles River Laboratories (Kingston, NY); both were approximately four months of age when experimentation began. Each cohort was further divided into control and experimental groups; in Cohort 1 there were 7 control and 8 experimental animals and in Cohort 2 there were 9 control and 11 experimental animals. Animals were weighed every other day and given clean cages once a week. They were housed in
the Hunter College Animal Facilities, which is accredited by the Association of the Assessment and Accreditation of Laboratory Animal Care, International. All animal experimentation and care was in accordance with the criteria established by the National Academy of Science as described the Guide for the Care and Use of Laboratory Animals (NIH publication 865-112, Bethesda MD) and approved by the Institutional Animal Care and Use Committee of Hunter College.

**Apparatus**

Mice were individually housed in polypropylene cages with stainless-steel wire mesh tops. Animals were kept at Hunter College in an environmentally controlled facility maintained at 22-24°C. Rooms were on a 12 hour alternating light:dark cycle with lights out at 19:00. Before experimentation began animals had free access to water and to a standard chow diet (LabDiet 5001, PMI, St. Louis, MO).

The Lieber-DeCarli Liquid Diet was administered in 50 mL glass liquid feeding tubes, 3 ⅜ inches x 1 ¼ inches (BioServ, Frenchtown, NJ). They were hung from the wire top of the cages with stainless steel holders and were washed every other day.

Alcohol and water for the two-bottle choice was administered in 150 mL Nalgene bottles with stainless steel sipper tubes through rubber stoppers.

**Lieber-DeCarli Liquid Diet**

The Lieber-DeCarli Liquid Diet was the prepared in the same way as it was for Experiment 1 (see above). Adaptation to ethanol was such that mice in the experimental groups received the control diet then 2.13% v/v alcohol and then 4.27% v/v alcohol each for four days
(Cohort 1) or for two days (Cohort 2). Following adaptation, the experimental animals were maintained on 5.33% v/v alcohol for 30 days (Cohort 1) or 52 days (Cohort 2), control animals were fed the control Lieber-DeCarli Liquid Diet without alcohol for the duration of the study.

**Experimental Paradigm**

**Two-Bottle Choice**

Following maintenance on the Lieber-DeCarli Liquid Diet, both the control and experimental groups were given solid chow diet and water in glass bottles with stainless steel sipper tubes through rubber stoppers. When the two-bottle choice paradigm was administered, the water bottles were removed and all animals received two Nalgene bottles on their home cages, one containing alcohol and one tap water, three hours into the dark phase (22:00) for two or three hours, on alternating sides each day to avoid animals developing side preference. Bottles were weighed to the nearest tenth of a gram before and after the drinking phase. There were three testing rounds separated by days without experiential testing during which all animals continued to receive chow and water (see Tables 1 and 2). Bottle leakage was assessed using the same method as in Experiment 1 (see above).

### Table 2

**Experimental paradigm for Experiment 2, Cohort 1**

<table>
<thead>
<tr>
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<td>4%</td>
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<td></td>
<td>2%</td>
<td>5%</td>
</tr>
</tbody>
</table>

*Note. Two-bottle Choice lasted for two hours for the first two days, after which I extended it to last three hours. On day 8, the animal room lights did not shut off at the proper time (20:00) and I manually shut them off at 22:00, because of this I re-administered 4% alcohol v/v the next day.*

65
Table 3

*Experimental paradigm for Experiment 2, Cohort 2*

<table>
<thead>
<tr>
<th>Day</th>
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<tbody>
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<td>2BC 4%</td>
<td>2BC 5%</td>
<td>Chow</td>
<td>10% 24hr</td>
<td>20% 24hr</td>
<td></td>
</tr>
</tbody>
</table>

**Data Analysis**

Statistical analyses were performed using IBM SPSS Statistics version 21 (SPSS, Chicago, IL) and significance was set as $p < .05$. Individual cases were excluded if there was excessive bottle leakage. The same statistical methods and tests used for Experiment 1 were used for Experiment 2 for body weight, bottle loss, Lieber-DeCarli consumption and preference calculations (see above).

For Cohort 1, the control and experimental groups did not differ in the average body weight of the animals (see Results), therefore, body weight was not considered during analysis and the amount of alcohol and water consumed was reported in grams for both groups. For Cohort 2, the control group weighed significantly more than the experimental group (see Results), therefore, consumption of alcohol and water in all analyses corrected for body weight by calculating grams of alcohol consumed by kilograms of body weight of each animal (g/kg).

In addition to analyzing daily differences, the amounts of alcohol consumed were averaged for each of the three rounds and for each of the three concentrations across the three rounds. A mean of ethanol consumption over all rounds and for all concentrations was also calculated. Independent samples t-tests were conducted to compare consumption between the groups and paired-samples t-tests were conducted to compare consumption within the groups for all calculations. For Cohort 2, the amounts of 10% and 20% alcohol consumed in grams per
kilogram of body weight were compared between the groups using independent samples t-tests. Data are expressed as mean and SEM and were graphed using GraphPad Prism version 6 (GraphPad, La Jolla, CA).
Chapter 4: Drinking Behavior Results: Consumption and Preference

Experiment 1

Animal Weights

There was no significant difference between the body weights measured in grams of the alcohol consuming ($n = 8$) ($M = 27.76, SD = 2.04$) and control ($n = 7$) ($M = 29.08, SD = 3.24$) groups; $t(13) = -0.96, p = .352$. Levene’s test of equal variances was not significant and equal variances were assumed. These results suggest that any differences in consumption between the two groups are not attributed to body weight.

Bottle Loss

There was no significant difference between the mean water lost measured in grams from the experimental group bottles ($M = 0.63, SD = 0.17$) and control group bottles ($M = 0.66, SD = 0.09$), $t(13) = -0.36, p = .725$, Levene’s test was not significant and equal variances were assumed. For the mean amount of ethanol lost, Levene’s test was not significant and equal variances were assumed. There was no significant difference between the mean grams of ethanol lost from the experimental group bottles ($M = 0.86, SD = 0.17$) and control group bottles ($M = 0.97, SD = 0.27$), $t(13) = -1.02, p = .327$. These data suggest that there were no differences in grams in the amount of liquid lost between the two groups and that any differences in consumption or preference were not a result of leakage. As a result, further analyses do not consider liquid lost from the bottles.
Lieber-DeCarli Liquid Diet

There was no difference in the mean consumption of the LD diet measured in grams between the experimental group ($M = 27.71$, $SD = 2.35$) consumed compared to the control group ($M = 29.95$, $SD = 2.82$), $t(13) = -1.68$, $p = .117$. This ensures that the differences observed during testing were not due to the diet or calories consumed during training.

Alcohol Consumption

There were no differences in grams of ethanol consumed each day by the control and experimental groups (see Figure 4). In addition, there were no differences in ethanol consumption (grams) between the groups when the amount of 5% ethanol consumed was averaged over the four days $t(13) = 0.41$, $p = .688$, nor for the grams of 10% ethanol consumed when averaged over the four days $t(13) = -0.61$, $p = .554$.

![Figure 4: Data represent mean (± SEM) alcohol consumption (g) across the eight days of two-bottle choice for Experiment 1.](image-url)
Alcohol Preference

On the first day of the eight days of two-bottle choice, the experimental group ($M = 0.50$ g, $SD = .08$ g) had a significantly higher preference for ethanol than the control group ($M = 0.40$ g, $SD = 0.07$ g), $t(13)= 2.45$, $p < .05$. Of the total volume of liquid each group consumed, the experimental group drank 50% as ethanol as compared to the control group that drank 40% as ethanol (see Figure 5). Independent samples t-tests of the remaining seven days did not result in significant differences in preference between the alcohol consuming and control groups. In addition, there were no significant differences in the percent of ethanol consumed between the experimental group and control group when the percent of 5% ethanol consumed was averaged over the four days $t(13) = 0.06$, $p = .956$, nor for the percent of 10% ethanol consumed when it was averaged over the four days $t(13) = -0.38$, $p = .713$.

*Figure 5: For Experiment 1, these data represent alcohol preference as mean ($\pm$ SEM) percent alcohol consumed of total liquid intake for day 1 of 5% alcohol.*
The control group drank significantly less 5% ethanol ($M = 0.83 \text{ g}, SD = 0.21 \text{ g}$) than water ($M = 1.24 \text{ g}, SD = 0.22 \text{ g}$) on the first day, $t(6) = -3.63, p < .05$. Of the remaining seven days, there were no significant differences on the second through fifth days; however, the control group consumed significantly more 10% ethanol than water on days six, seven and eight. On the sixth day the control group drank more ethanol ($M = 2.04 \text{ g}, SD = 0.57 \text{ g}$) than water ($M = 1.29 \text{ g}, SD = 0.41 \text{ g}$), $t(6) = 3.31, p < .05$. Day seven they drank more ethanol ($M = 1.59 \text{ g}, SD = 0.26 \text{ g}$) than water ($M = 0.99 \text{ g}, SD = 0.23 \text{ g}$), $t(6) = 5.29, p < .05$, and day eight they drank more ethanol ($M = 1.60 \text{ g}, SD = 0.50 \text{ g}$) than water ($M = 0.94 \text{ g}, SD = 0.18 \text{ g}$), $t(6) = 2.72, p < .05$ (see Figure 6). In addition, in the control group, there was no difference in the average amount of water and 5% ethanol consumed over four days, $t(6) = 1.27, p = .252$. However, the control group did consume significantly more 10% ethanol over four days ($M = 1.64 \text{ g}, SD = 0.32 \text{ g}$) than water ($M = 1.08 \text{ g}, SD = 0.15 \text{ g}$), $t(6) = 4.11, p < .05$.

**Figure 6:** For the control animals in Experiment 1, these data represent mean (± SEM) alcohol and water intake (g) over the eight days of two-bottle choice.
With the experimental group, there were no significant consumption differences on the first through fifth days; however, they consumed significantly more 10% ethanol than water on days six, seven and eight. On day six they drank more ethanol (g) ($M = 1.68, SD = 0.48$) than water (g) ($M = 0.99, SD = 0.28$), $t(7) = 3.36, p < .05$. On day seven they drank more ethanol (g) ($M = 1.46, SD = 0.24$) than water (g) ($M = 1.01, SD = 0.36$), $t(7) = 3.67, p < .05$, and day eight they drank more ethanol (g) ($M = 1.74, SD = 0.46$) than water (g) ($M = 0.98, SD = 0.26$), $t(7) = 3.83, p < .05$ (see Figure 7). In the experimental group, there was no difference in the average amount of water and 5% ethanol consumed over the four days, $t(7) = 1.40, p = .204$. However, they consumed significantly more 10% ethanol (g) over four days ($M = 1.54, SD = 0.28$) than water (g) ($M = 1.07, SD = 0.16$), $t(7) = 4.34, p < .05$.

*Figure 7: For the experimental animals in Experiment 1, these data represent mean (± SEM) alcohol and water intake (g) over the eight days of two-bottle choice.*
Drinking in the Dark

There were no significant differences in ethanol consumption measured in grams between the control group and experimental group for the drinking in the dark procedure. Leakages (g) from the ethanol bottles were not significantly different between groups, $t(13) = 0.50, p = .628$, therefore, leakage was not considered during analyses. There were no differences between the groups on day one $t(13) = -1.05, p = .311$, day two $t(9.39) = -0.86, p = .411$ or day three $t(13) = -0.55, p = .589$.

Two-Bottle Choice - Round Two

Consumption

When comparing the experimental group to the control group, there were no differences in amount of alcohol consumed in grams or in percent of alcohol consumed of the total liquid.

Preference

However, when comparing within the groups, both the experimental and control groups consumed more alcohol than water on both days. The experimental group drank more alcohol (g) ($M = 1.95, SD = 0.28$) than water (g) ($M = 0.93, SD = 0.18$) on day one $t(7) = 7.26, p < .05$ and more alcohol (g) ($M = 2.03, SD = 0.22$) than water (g) ($M = 1.18, SD = 0.60$) on day two $t(7) = 3.38, p < .05$. In addition, the control group drank more alcohol (g) ($M = 2.44, SD = 0.58$) than water (g) ($M = 1.03, SD = 0.25$) on day one $t(6) = 6.06, p < .05$ and more alcohol (g) ($M = 1.89, SD = 0.66$) than water (g) ($M = 1.13, SD = 0.44$) on day two $t(6) = 2.58, p < .05$. 
Experiment 2 – Cohort 1

Animal Weights

An independent-samples t-test was conducted to compare the body weights of the experimental group (n = 8) to the control group (n = 7). Animal weights that were recorded in grams approximately twice a week were averaged. Levene’s test of equal variance was not significant and equal variances were assumed. There was no significant difference between the body weights in grams of the experimental ($M = 34.67$, $SD = 2.68$) and control ($M = 33.43$, $SD = 1.30$) groups; $t(13) = -1.12, p = .283$. These results suggest that any differences in consumption between the two groups are not attributed to body weight.

Bottle Loss

There was no significant difference in leakage lost in grams from the experimental and control group’s alcohol bottles during the one bottle challenge, $t(13) = -1.81, p = .094$ (experimental, $M = 0.34$, $SD = 0.09$; control, $M = 0.26$, $SD = 0.08$). During the two-bottle choice, for the average amount of both water and alcohol lost over all rounds and for all concentrations, Levene’s tests were not significant and equal variances were assumed. There was no significant difference between the average amount of water lost in grams from the experimental group bottles ($M = 0.38$, $SD = 0.04$) and control group bottles ($M = 0.33$, $SD = 0.06$), $t(13) = -1.75, p = .103$. There was no significant difference between the average amount of ethanol lost from the experimental group bottles ($M = 0.44$ g, $SD = 0.07$ g) and control group bottles ($M = 0.42$ g, $SD = 0.05$ g), $t(13) = -0.583, p = .570$. These data suggest that there were no differences in the amount of liquid lost between the two groups and that any differences in consumption or
preference were not a result of leakage. As a result, further analyses do not take into consideration the amount of liquid lost from the bottles.

**Lieber-DeCarli Liquid Diet**

The control group consumed significantly more LD liquid diet in grams \( M = 33.62, SD = 1.20 \) than the experimental group \( M = 30.36, SD = 1.99 \), \( t(13) = 3.76, p < .05 \).

**Alcohol Consumption**

During the one bottle challenge, no differences were observed in the average grams of ethanol consumed by the control and experimental groups, \( t(13) = -0.28, p = .783 \).

For each of the three rounds, the experimental group drank significantly more ethanol than the control group. For the Round 1 the Levene’s test was significant and equal variance was not assumed, the experimental group \( M = 1.53, SD = 0.34 \) consumed significantly more ethanol in grams than the control group \( M = 1.24, SD = 0.12 \), \( t(8.812) = -2.28, p < .05 \). For Round 2 the experimental group \( M = 1.46, SD = 0.32 \) drank more alcohol in grams than the control \( M = 1.08, SD = 0.15 \), \( t(13) = -2.88, p < .05 \) and for Round 3 the experimental group \( M = 1.33, SD = 0.23 \) drank more alcohol measured in grams than the control group \( M = 0.98, SD = 0.08 \), \( t(13) = -3.84, p < .05 \) (see Figure 8).

For each concentration averaged across the three rounds, the experimental group drank significantly more alcohol (g) than the control group. At 2% (average consumed during rounds 1, 2 and 3), the experimental group \( M = 1.35, SD = 0.26 \) drank more alcohol (g) than the control group \( M = 1.01, SD = 0.17 \), \( t(13) = -2.97, p < .05 \). For 4% Levene’s test was significant and equal variance was not assumed, the experimental group \( M = 1.63, SD = 0.32 \) consumed more
alcohol (g) than the control ($M = 1.30, SD = 0.06$), $t(7.59) = -2.81, p < .05$. For 5% the experimental ($M = 1.34, SD = 0.17$) drank more alcohol (g) than the control group ($M = 0.98, SD = 0.09$), $t(13) = -5.11, p < .05$ (see Figure 9). In addition, for the average ethanol consumption over all rounds for all concentrations, the experimental group ($M = 1.44, SD = 0.20$) drank significantly more alcohol (g) than the control group ($M = 1.10, SD = 0.06$), $t(13) = -4.37, p < .05$ (see Figure 10).

Figure 8: Experiment 2, Cohort 1 mean (± SEM) alcohol consumption (g) of experimental and control animals. Consumption values are the means of all concentrations of alcohol averaged together for each round of two-bottle choice.
Figure 9: Experiment 2, Cohort 1 mean (± SEM) alcohol consumption (g) of experimental and control animals. Consumption values are the means of all rounds of two-bottle choice averaged together for each concentration of alcohol.

Figure 10: Experiment 2, Cohort 1 mean (± SEM) alcohol consumption (g) of experimental and control animals. Consumption values are the means of all rounds and concentrations of two-bottle choice averaged together.
Alcohol Preference

No differences were observed between the groups for the average preference for alcohol (in percent alcohol consumed) during Round 1 or Round 2 (all concentrations) and no differences were observed between the groups for 2%, 4% or 5% (all rounds). For Round 3 the experimental group drank a significantly higher percentage of alcohol of their total liquid consumed ($M = 0.62, SD = 0.07$) than the control group ($M = 0.53, SD = 0.06$), $t(13) = -2.52, p < .05$. In addition, when percent alcohol consumed was averaged across all rounds and all concentrations, the experimental group preferred alcohol ($M = 0.60, SD = 0.03$) significantly more than the control group ($M = 0.55, SD = 0.03$), $t(12) = -2.69, p < .05$ (see Figure 11).

![Figure 11: Experiment 2, Cohort 1 mean (± SEM) alcohol preference (%) of experimental and control animals. Consumption values are the means for all rounds and concentrations of two-bottle choice averaged together for the percent alcohol of the total volume consumed.](image-url)
For individual days, the control group did not consume more alcohol or water, in grams, on the following days: Round 1-2%, Round 1-5%, Round 2-2%, Round 2-5%, Round 3-2% or Round 3-4%. However, the control group drank more alcohol (g) on Round 1-4%, \( t(6) = 11.53, p < .05 \) (alcohol: \( M = 1.64, SD = 0.20 \); water: \( M = 0.96, SD = 0.15 \)), Round 2-4%, \( t(6) = 7.75, p < .05 \) (alcohol: \( M = 1.31, SD = 0.23 \); water: \( M = 0.87, SD = 0.16 \)) and Round 3-5%, \( t(6) = 3.65, p < .05 \) alcohol (\( M = 0.96, SD = 0.18 \)) water (\( M = 0.77, SD = 0.11 \)).

For individual days, the experimental group did not have a preference for alcohol or water on the following days: Round 1-5%, Round 2-2%, Round 2-5%, Round 3-2% and Round 3-4%. For Round 1-2% the experimental group drank significantly more alcohol (g) (\( M = 1.33, SD = 0.36 \)) than water (g) (\( M = 0.51, SD = 0.54 \)), \( t(7) = 2.86, p < .05 \) (see Figure 12). They also drank significantly more alcohol (g) on Round 1-4%, \( t(7) = 4.41, p < .05 \) (alcohol: \( M = 2.09, SD = 0.79 \); water: \( M = 1.08, SD = 0.26 \)), Round 2-4% \( t(7) = 6.06, p < .05 \) (alcohol: \( M = 1.68, SD = 0.34 \); water: \( M = 0.74, SD = 0.33 \)) and Round 3-5%, \( t(7) = 6.26, p < .05 \) (alcohol: \( M = 1.58, SD = 0.39 \); water: \( M = 0.54, SD = 0.23 \)).
Figure 12: Intake of alcohol and water for the experimental and control animals of Experiment 2, Cohort 1 for day 1 of two-bottle choice. Intake of liquid is expressed as mean (± SEM) alcohol or water consumed.

When the amounts of alcohol and water consumed were averaged across rounds and for each concentration, the control group did not drink more alcohol in grams for Round 3, for 2% ethanol or 5% ethanol. However, there was a trend toward significance for Round 1 when the control group drank more alcohol (g) ($M = 1.24, SD = 0.12$) than water (g) ($M = 0.96, SD = 0.26$), $t(6) = 2.44, p = .051$. During Round 2, they drank significantly more alcohol (g) ($M = 1.08, SD = 0.15$) than water (g) ($M = 0.87, SD = 0.18$), $t(6) = 3.08, p < .05$. They also drank more 4% alcohol (g) across the three rounds $t(6) = 6.33, p < .05$ (alcohol: $M = 1.30, SD = 0.06$; water: $M = 0.89, SD = 0.16$). When the consumptions were averaged across all three rounds and for all concentrations the control group drank more alcohol (g) ($M = 1.10, SD = 0.06$) than water (g) ($M = 0.91, SD = 0.15$), $t(6) = 4.03, p < .05$ (see Figure 13).
Figure 13: Experiment 2, Cohort 1 control animals’ mean alcohol and water intake (g) (± SEM) by round and by concentration alcohol during two-bottle choice. The control animals preferred alcohol on three of six occasions.

The experimental group, however, drank significantly more alcohol than water for all three rounds and at all concentrations. They drank more alcohol than water, measured in grams, for each of the three rounds: Round 1, $t(7) = 4.89, p < .05$ (alcohol: $M = 1.53, SD = 0.34$; water $M = 0.97, SD = 0.21$), Round 2, $t(7) = 2.79, p < .05$ (alcohol: $M = 1.46, SD = 0.32$; water: $M = 1.10, SD = 0.08$) and Round 3, $t(7) = 4.32, p < .05$ (alcohol: $M = 1.33, SD = 0.23$; water: $M = 0.83, SD = 0.13$). The experimental group also drank more alcohol than water, measured in grams, for each concentration: 2%, $t(7) = 2.77, p < .05$ (alcohol: $M = 1.35, SD = 0.26$; water $M = 0.93, SD = 0.23$), 4%, $t(7) = 6.77, p < .05$ (alcohol: $M = 1.63, SD = 0.32$; water $M = 0.95, SD = 0.16$) and 5%, $t(7) = 4.16, p < .05$ (alcohol: $M = 1.34, SD = 0.17$; water: $M = 1.01, SD = 0.14$).

In addition, when consumptions were averaged across all rounds and for all concentrations the
The experimental group drank significantly more alcohol (g) ($M = 1.44, SD = 0.20$) than water (g) ($M = 0.97, SD = 0.06$), $t(7) = 6.67, p < .05$ (see Figure 14).

*Figure 14:* Experiment 2, Cohort 1 experimental animals’ mean alcohol and water intake (g) (± SEM) by round and by concentration alcohol during two-bottle choice. The experimental animals preferred alcohol on six of six occasions.
Experiment 2 – Cohort 2

Animal Weights

The experimental \((n = 11)\) \((M = 30.71 \text{ g}, SD = 2.42 \text{ g})\) group weighed significantly less than the control \((n = 9)\) \((M = 33.78 \text{ g}, SD = 2.34 \text{ g})\) group; \(t(18) = 2.86, p < .05\), Levene’s test of equal variance was not significant and equal variances were assumed.

Bottle Loss

For the average amount of both water and alcohol lost over all rounds and for all concentrations, Levene’s tests were not significant and equal variances were assumed. There was no significant difference between the average grams of water lost from the experimental group bottles \((M = 0.45, SD = 0.07)\) and control group bottles \((M = 0.44, SD = 0.05)\), \(t(18) = -0.27, p = .790\). There was no significant difference between the average amount of ethanol lost from the experimental group bottles \((g)\) \((M = 0.57, SD = 0.13)\) and control group bottles \((g)\) \((M = 0.57, SD = 0.08)\), \(t(18) = -0.12, p = .907\). These data suggest that there were no differences in the amount of liquid lost between the two groups and that any differences in consumption or preference were not a result of leakage. As a result, further analyses do not take into consideration the amount of liquid lost from the bottles.

Lieber-DeCarli Liquid Diet

The control group consumed significantly more LD liquid diet in grams \((M = 33.60, SD = 1.80)\) than the experimental group \((M = 29.61, SD = 1.39)\), \(t(19) = 5.72, p < .05\).
Alcohol Consumption

There were no significant differences between the groups for Round 1 or Round 2. However, for Round 3 the control group drank significantly more alcohol (g/kg) ($M = 38.97$, $SD = 7.32$) than the experimental group ($M = 30.48$, $SD = 4.32$), $t(17) = 3.18$, $p = .006$ (see Figure 15). There were no significant differences in alcohol consumption between the groups for 2%, 4% or 5% ethanol or when consumption was averaged across all rounds for all concentrations (see Figure 16).

Figure 15: Experiment 2, Cohort 2 mean (± SEM) alcohol consumption (g/kg) of experimental and control animals. Consumption values are the means of all concentrations of alcohol averaged together for each round of two-bottle choice.
**Figure 16:** Experiment 2, Cohort 2 mean (± SEM) alcohol consumption (g/kg) of experimental and control animals. Consumption values are the means of all rounds of two-bottle choice averaged together for each concentration of alcohol.

**Alcohol Preference**

No differences were observed between the groups for the average preference for Round 2 or Round 3 (all concentrations) and no differences were observed between the groups for 2% or 4% (all rounds). For Round 1 the experimental group drank a significantly higher percentage of alcohol of their total liquid consumed ($M = 0.61, SD = 0.05$) than the control group ($M = 0.56, SD = 0.03$), $t(16) = -2.47, p < .05$. For 5% (across all rounds), the experimental group ($M = 0.64, SD = 0.06$) preferred alcohol more than the control group ($M = 0.57, SD = 0.03$), $t(18) = -2.99, p < .05$. In addition, when percent alcohol consumed was averaged across all rounds and all concentrations, there was a trend toward significance for the experimental group preferring alcohol ($M = 0.60, SD = 0.03$) more than the control group ($M = 0.57, SD = 0.02$), $t(16) = -2.06, p = .057$. 

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For the individual days, the control group did not consume more alcohol or water on the following days: Round 1-2%, Round 1-5%, Round 2-4%, Round 3-2% and Round 3-5%.

However, the control group drank more alcohol (g/kg) than water (g/kg) during Round 1-4%, \( t(7) = 7.60, p < .05 \) (alcohol: \( M = 55.66, SD = 9.76 \); water: \( M = 33.90, SD = 11.92 \)), Round 2-2%, \( t(8) = 3.32, p < .05 \) (alcohol: \( M = 49.36, SD = 12.38 \); water: \( M = 37.23, SD = 11.96 \)), Round 2-5%, \( t(7) = 7.13, p < .05 \) (alcohol: \( M = 45.79, SD = 7.99 \); water: \( M = 21.69, SD = 8.78 \)) and Round 3-4%, \( t(8) = 3.82, p < .05 \) alcohol (\( M = 45.86, SD = 14.00 \)) water (\( M = 27.46, SD = 7.12 \)) (see Figure 17).

**Figure 17**: Experiment 2, Cohort 2 control animals’ mean (± SEM) alcohol and water intake (g/kg). The control animals preferred alcohol to water every other day on four of the nine two-bottle choice days.
For individual days, the experimental drank significantly more alcohol (g/kg) than water (g/kg) on all of the days: Round 1-2%, $t(10) = 2.62$, $p < .05$ (alcohol: $M = 51.54$, $SD = 9.37$; water: $M = 41.01$, $SD = 7.95$), Round 1-4%, $t(9) = 5.22$, $p < .05$ (alcohol: $M = 50.97$, $SD = 10.67$; water: $M = 28.20$, $SD = 5.93$), Round 1-5%, $t(10) = 6.43$, $p < .05$ (alcohol: $M = 48.11$, $SD = 5.65$; water: $M = 30.66$, $SD = 5.75$), Round 2-2%, $t(10) = 3.75$, $p < .05$ (alcohol: $M = 39.44$, $SD = 5.37$; water: $M = 31.14$, $SD = 5.11$), Round 2-4% $t(10) = 2.54$, $p < .05$, (alcohol: $M = 33.68$, $SD = 7.75$; water: $M = 23.70$, $SD = 6.17$), Round 2-5%, $t(10) = 4.38$, $p < .05$ (alcohol: $M = 41.76$, $SD = 9.72$; water: $M = 19.92$, $SD = 10.11$), Round 3-2%, $t(9) = 3.18$, $p < .05$ (alcohol: $M = 41.76$, $SD = 9.72$; water: $M = 19.92$, $SD = 10.11$), Round 3-4%, $t(10) = 2.89$, $p < .05$ (alcohol: $M = 27.72$, $SD = 4.47$; water: $M = 20.74$, $SD = 6.06$) and Round 3-5%, $t(10) = 3.16$, $p < .05$ (alcohol: $M = 30.87$, $SD = 8.30$; water: $M = 19.83$, $SD = 7.51$) (see Figure 18).
Figure 18: Experiment 2, Cohort 2 experimental animals’ mean (± SEM) alcohol and water intake (g/kg). The experimental animals preferred alcohol to water every day on nine of the nine two-bottle choice days.

When consumption was averaged across rounds and concentrations, the control group drank more alcohol than water in grams per kilogram of body weight for all comparisons. The control group drank significantly more alcohol (g/kg) during all three rounds: Round 1, \( t(7) = 4.79, p < .05 \) (alcohol: \( M = 46.83, SD = 9.43 \); water: \( M = 36.12, SD = 5.87 \)), Round 2, \( t(7) = 7.50, p < .05 \) (alcohol: \( M = 42.30, SD = 6.82 \); water: \( M = 31.01, SD = 7.77 \)) and Round 3, \( t(8) = 2.80, p < .05 \) (alcohol: \( M = 38.97, SD = 7.33 \); water: \( M = 33.48, SD = 8.00 \)). They also drank significantly more alcohol (g/kg) than water (g/kg) for each concentration: 2%, \( t(8) = 3.95, p < .05 \) (alcohol: \( M = 41.47, SD = 6.77 \); water: \( M = 35.18, SD = 8.02 \)), 4%, \( t(7) = 5.11, p < .05 \) (alcohol: \( M = 45.40, SD = 9.91 \); water: \( M = 31.54, SD = 9.72 \)) and 5%, \( t(7) = 5.63, p < .05 \)
(alcohol: $M = 42.69$, $SD = 7.47$; water: $M = 34.00$, $SD = 5.82$). In addition, when all rounds and all concentrations were averaged, they drank more alcohol (g/kg) ($M = 43.58$, $SD = 7.49$) than water (g/kg) ($M = 33.02$, $SD = 7.22$), $t(6) = 17.06$, $p < .05$.

When consumption was averaged for each round the experimental group drank more alcohol (g/kg) than water (g/kg) for all three rounds: Round 1, $t(9) = 6.83$, $p < .05$ (alcohol: $M = 51.12$, $SD = 6.70$; water: $M = 32.72$, $SD = 4.49$), Round 2, $t(10) = 5.85$, $p < .05$ (alcohol: $M = 38.29$, $SD = 4.96$; water: $M = 24.92$, $SD = 4.12$) and Round 3, $t(9) = 4.41$, $p < .05$ (alcohol: $M = 30.48$, $SD = 4.32$; water: $M = 22.12$, $SD = 4.73$). When consumption was averaged for each concentration across all rounds, the experimental group drank more alcohol (g/kg) than water (g/kg) for all concentrations: 2%, $t(9) = 5.13$, $p < .05$ (alcohol: $M = 40.77$, $SD = 4.34$; water: $M = 32.07$, $SD = 3.81$), 4%, $t(9) = 8.22$, $p < .05$ (alcohol: $M = 37.33$, $SD = 4.86$; water: $M = 24.07$, $SD = 2.55$) and 5%, $t(8) = 10.12$, $p < .05$ (alcohol: $M = 39.62$, $SD = 4.24$; water: $M = 26.17$, $SD = 2.15$). In addition, when consumption was averaged across all rounds and all concentrations the experimental group drank more alcohol (g/kg) ($M = 39.19$, $SD = 4.22$) than water (g/kg) ($M = 26.34$, $SD = 2.10$), $t(9) = 9.62$, $p < .05$.

**Two-Bottle Choice – 10% and 20% ethanol**

**Consumption**

There was no difference in amount of 10% ethanol consumed by the experimental and control groups, $t(18) = -0.36$, $p = .724$. The control group drank significantly more ($M = 293.21$, $SD = 92.18$) 20% ethanol (g/kg) than the experimental group ($M = 199.28$, $SD = 77.40$), $t(18) = 2.48$, $p < .05$. 

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Preference

Both the control group and experimental groups drank more alcohol than water in grams per kilogram of body weight, at both 10% and 20% ethanol. The control group drank significantly more 10% ethanol (g/kg) ($M = 132.01, SD = 39.29$) than water (g/kg) ($M = 71.12, SD = 21.14$), $t(8) = 3.36, p < .05$ and they drank significantly more 20% ethanol (g/kg) ($M = 293.21, SD = 99.91$) than water (g/kg) ($M = 92.18, SD = 45.37$), $t(8) = 7.56, p < .05$. The experimental group drank significantly more 10% ethanol (g/kg) ($M = 139.04, SD = 46.83$) than water (g/kg) ($M = 79.55, SD = 34.46$), $t(10) = 2.55, p < .05$ and they drank significantly more 20% ethanol (g/kg) ($M = 199.28, SD = 77.40$) than water (g/kg) ($M = 65.39, SD = 37.21$), $t(10) = 4.66, p < .05$.

Additionally, both the control and experimental group preferred 20% to 10%. The control group drank significantly more 20% alcohol ($M = 0.75, SD = 0.07$) than 10% ($M = 0.64, SD = 0.12$) of the total volume of liquid consumed, $t(8) = -2.53, p < .05$ and when consumption was calculated in grams per kilogram of body weight they also drank significantly more 20% ethanol ($M = 293.21, SD = 92.18$) than 10% ($M = 132.01, SD = 39.29$), $t(8) = -6.29, p < .05$. Similarly, the experimental group drank significantly more 20% alcohol ($M = 0.74, SD = 0.17$) than 10% ($M = 0.62, SD = 0.17$) of the total volume of liquid consumed, $t(10) = -3.15, p < .05$ and when consumption was calculated in grams per kilogram of body weight they also drank significantly more 20% ethanol ($M = 199.28, SD = 77.40$) than 10% ($M = 139.04, SD = 46.83$), $t(10) = -3.56, p < .05$. 

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Discussion – Drinking Behavior: Consumption and Preference

Consumption

The effects of chronically consuming alcohol via the LD liquid diet on the behaviors of alcohol consumption and preference were analyzed by using the two-bottle choice procedures. To determine if alcohol dependence would result in increased motivation to consume alcohol, consumption and preference were compared between the mice chronically consuming the alcohol LD diet and those consuming the non-alcohol LD diet. The data show that the mice that chronically consumed alcohol drank more alcohol or preferred alcohol to water more than mice that did not chronically consume alcohol. The differences between the two groups were small in some of the testing and the levels of consumption were not as great as expected. The data suggest that the alcohol dependence produced by chronic oral intake is sufficient to induce dependence and motivate alcohol preference and intake. They also suggest that the paradigm is a valid method for investigation of the reinforcing effects of alcohol. It stands in contrast to other methods that produce dependence without substantial chronic administration and not by the oral route of administration. The LD procedure also permits the control of variables such as alcohol derived calories and nutritional intake. The testing occurred in three cohorts and explored the length of chronic alcohol exposure in the development of dependence. The data suggest that longer periods produce larger differences; however, the advantage is not as simple as anticipated.

To investigate the effect of length of chronic administration on alcohol consumption and preference the three cohorts were maintained on the diets for varying periods of time. Experiment 1 mice received the LD diet for 21 days, Experiment 2, Cohort 1 received it for 30 days and Experiment 2, Cohort 2 received it for 52 days. The experimental animals from Experiment 2, Cohort 1 showed the most robust differences in consumption as compared to the
control group. This group consumed more alcohol than the control group for all instances; all three replications, all three concentrations and when all rounds and all concentrations were combined. The first group that received the LD diet for only 21 days did not show a higher consumption in comparison to the control mice. Interestingly, extending chronic intake of the LD to 52 days in Experiment 2, Cohort 2, did not produce greater consumption differences over 30 days of chronic intake. Additionally, alcohol consuming mice of Experiment 2, Cohort 1 drank more alcohol than water on more occasions than the other two cohorts. These data suggests that the chronic intake of alcohol LD diet should be for longer than 21 days, but the optimal motivational effects may not be with intake greater than 52 days. Determining the duration of chronic administration that produces the maximum intake and preference is key to understanding the motivational effects of dependence. It appears from these data on total intake that exposure to alcohol for more than 30 days may alter dependence and the reinforcing properties of alcohol.

Clugston et al. (2011) showed that after five hours of fasting, blood alcohol concentrations were on average \( 0.1 \pm 0.05 \% \) in mice that received the LD diet for three weeks in concentrations from 2.2% to 6.7%. The research presented here maintained mice for approximately three, four or seven weeks at 5% alcohol v/v, more than a sufficient concentration and length of time to keep mice at clinically significant blood alcohol levels. Additionally, many experiments chronically administer alcohol for at least four to six weeks and in studies that are designed to mirror human alcohol abuse, animals are given alcohol for four to 20 weeks (D’Souza El-Guindy et al., 2010). D’Souza El-Guindy and colleagues (2010) suggested that those in the alcohol field should agree that chronic alcohol intake for at least a month, if not longer, would be sufficient to define chronic administration. Thus, these data suggest that four weeks of alcohol intake, as with the LD diet in this research, is sufficient to produce dependence.
and to provide the degree of alcohol exposure for maximal motivation to consume alcohol. This could explain why there were fewer differences observed in alcohol consumption between the experimental and control animals with only three weeks of chronic alcohol intake.

When the quantity of alcohol consumed during the two-bottle choice was compared between the mice that received the alcohol LD diet and those that received the control diet results were unexpected for Experiment 2, Cohort 2. These animals, that received the alcoholic LD diet for 52 days, did not consume more alcohol than the controls. While consumption differences were not found for the last cohort, preferences differences were observed, they did exhibited greater preference when data were analyzed for individual days of the two-bottle choice.

Preference

Preference was analyzed in two ways; first, using a preference score calculated as percent alcohol of total liquid consumed and second, by comparing the amount of alcohol consumed to water. The analysis of alcohol preference showed that for Experiment 1 the mice that received the alcohol LD diet drank a higher percentage of alcohol of their total liquid on the first day. In Experiment 2, Cohort 1 the experimental group consumed a higher percentage of alcohol during round three and overall when all rounds and all concentrations were combined. The experimental animals from Experiment 2, Cohort 2 also preferred alcohol during round three. Preference differences were more pronounced when comparing the amount of alcohol animals consumed to water within each experimental group.

When preference was analyzed within the experimental and control groups, different patterns emerged in each cohort. For Experiment 1, on day one, the experimental group preferred alcohol over water while the control group drank more water than 5% alcohol. Both drank more
alcohol versus water during the two-bottle choice on days six, seven and eight, when alcohol was at the highest concentration (10%). This suggests that with five days of experience drinking alcohol, animals prefer it to water in the same manner as animals that received alcohol chronically for 21 days. Additionally, the control group may have found the alcohol aversive at first but with additional exposure for a few days they developed a preference for it. In support of this hypothesis that a few days of alcohol exposure during the two-bottle choice procedure was sufficient to motivate alcohol preference, studies using alcohol preferring rats and mice also found brief prior alcohol exposure increases later alcohol preference. One study using male Maudsley reactive rats, found that one day of access to 10% alcohol was sufficient to produce alcohol preference (Adams, Campbell, & Mitchell, 2003). Increased preference for alcohol that was observed in alcohol preferring rats after only one day of alcohol access may partially explain why the control mice in the current experiment preferred alcohol during the two-bottle choice. It is possible that for these alcohol preferring rodents, a few days of alcohol exposure during two-bottle choice is sufficient to motivate increased preference during the later days of the two-bottle choice procedure. These unexpected results lead us to extend chronic alcohol administration in the other experiments and as a result, similar to consumption, preference differences were more pronounced in Experiment 2, Cohort 1.

In Experiment 2, Cohort 1 the experimental group drank more alcohol than water during all three rounds and all three concentrations, six of the six instances, while the control group only drank more alcohol during rounds one and two and at 4%, three of six instances. The data show that chronic intake of alcohol results in later alcohol preference, although the last cohort showed a different pattern of alcohol preference.
In Experiment 2, Cohort 2, when individual days of the two-bottle choice were compared, the experimental group drank more alcohol on all nine of the days while the control group only drank more alcohol on four of the nine two-bottle choice days. Interestingly, the control group showed a pattern of preference for alcohol where they consumed more alcohol than water every other test day, where one day they would drink significantly more alcohol but the following test day there was no difference in alcohol and water consumption. This suggests that in non-dependent animals, heavy drinking one day is followed by a loss of alcohol preference the next. It is possible that the alternating days that the control group did not prefer alcohol were because high alcohol consumption resulted in an aversive state. The aversion may have been similar to the well-known hangover that many non-dependent drinkers experience when consuming too much. The control group in this study may be similar to social drinkers in regards to their drinking patterns of one day of alcohol preference followed by a day of no alcohol preference to recover from alcohol’s effects. However, the experimental group may have shown drinking patterns more similar to alcoholics in that they consistently preferred alcohol and responded to the demands of alcohol dependence and other motives associated with chronic intake that override such aversions.

When analyzing preference data among the three cohorts, as with consumption, it appears that chronic alcohol administration for 30 days produces greater preference for alcohol than the other two intervals. Preference for alcohol over water was greatest in Experiment 2, Cohort 1, where the experimental animals preferred alcohol on all six instances while the control group preferred alcohol on only three of the six. In Experiment 2, Cohort 2, after 52 days of chronic administration, experimental animals preferred nine out of nine days as compared to the control that preferred only four of nine days. In Experiment 1 there was only a difference in preference
between the groups on the first day with the mice chronically drinking alcohol showing increased preference. Further research on the limitation of the interval of chronic administration is necessary.

An important finding that was common among all three cohorts was that the experimental mice from all three cohorts drank more alcohol than water on the first day of the two-bottle choice, whereas the control mice did not. This suggests that the chronic access to alcohol via the liquid diet motivates later alcohol preference. Consuming the alcohol would be expected to alleviate the withdrawal that these animals may have been experiencing that was produced by the chronic alcohol. Also, the chronic intake may have diminished any initial aversive effects of alcohol that control mice experience with their first access to alcohol.
**Chapter 5: Brain Biochemistry Results**

**Experiment 1**

**Dopamine levels in nucleus accumbens**

When dopamine levels (nmol/g) were compared between the experimental and control groups of Experiment 1, no differences were found in the core of the NAc, $t(13) = -0.19, p = .851$, experimental ($M = 2.56, SD = 1.15$), control ($M = 2.69, SD = 1.53$). For the shell, the Levene’s test was significant and equal variances were not assumed, there was a trend toward significance in differences in dopamine levels (nmol/g) between the experimental group ($M = 1.18, SD = 0.90$) and the control group ($M = 3.31, SD = 2.53$), $t(7.33) = -2.12, p = .07$. When dopamine amounts (nmol/g) were averaged for core and shell, no differences were observed between the groups, $t(13) = -1.60, p = .13$ (experimental, $M = 1.87, SD = 0.88$; control, $M = 3.00, SD = 1.77$) (see Figure 19).

![Graph](image)

**Figure 19:** Data represent mean (± SEM) dopamine levels (nmol/g) of experimental and control animals (Experiment 1) in the NAc core, shell, and the mean of the two.
Cannabinoid levels in nucleus accumbens

**AEA**

When AEA levels (pmol/g) were compared in the NAc of experimental and control animals, no differences were observed in the core, \( t(13) = 0.09, p = .931 \) (experimental, \( M = 21.14, SD = 2.09 \); control, \( M = 21.04, SD = 2.64 \)) or shell, \( t(8.80) = -2.07, p = .07 \) (experimental, \( M = 17.98, SD = 3.08 \); control, \( M = 20.37, SD = 1.05 \)). Additionally, no differences were observed in AEA levels (pmol/g) when the core and shell were averaged together, \( t(13) = -1.88, p = .083 \) (experimental, \( M = 19.56, SD = 1.26 \); control, \( M = 20.70, SD = 1.07 \)) (see Figure 20).

![Figure 20: Data represent mean (± SEM) AEA levels (pmol/g) of experimental and control animals (Experiment 1) in the NAc core, shell, and the mean of the two.](image-url)

Figure 20: Data represent mean (± SEM) AEA levels (pmol/g) of experimental and control animals (Experiment 1) in the NAc core, shell, and the mean of the two.
2AG

When 2AG levels (pmol/g) were compared in the NAc of experimental and control animal no differences were observed in the core, $t(13) = 0.94, p = .367$ (experimental, $M = 53.70$, $SD = 13.90$; control, $M = 47.01$, $SD = 13.76$) or shell, $t(13) = -0.86, p = .405$ (experimental, $M = 28.11$, $SD = 12.47$; control, $M = 32.75$, $SD = 7.33$). Additionally, no differences were observed in 2AG levels (pmol/g) when the core and shell were averaged together, $t(13) = 0.38, p = .711$ (experimental, $M = 40.90$, $SD = 5.38$; control, $M = 39.88$, $SD = 5.04$) (see Figure 21).

![Figure 21](image_url)

*Figure 21:* Data represent mean (± SEM) 2AG levels (pmol/g) of experimental and control animals (Experiment 1) in the NAc core, shell, and the mean of the two.
Brain Biochemistry Discussion

In addition to behavioral data on alcohol consumption and preference, dopamine and endocannabinoid levels were measured in Experiment 1. In this experiment, brains were removed one day after the final one hour two-bottle choice. The brains were placed on ice and then quickly frozen for storage. Later, the NAc core and shell were removed for analysis. At time of sacrifice, animals would have metabolized any alcohol they consumed the previous day. In mice, blood alcohol concentration reached zero after i.p. doses ranging from two to five g/kg of body weight within 16 hours (Haseba, Kameyama, Mashimo, & Ohno, 2012). Small animals metabolize alcohol faster than larger ones and mice metabolize alcohol five times faster than humans (Cederbaum, 2012). In our experiment, a one hour drinking bout would not result in detectable blood alcohol levels the following day in mice.

While Experiment 1 did not find significant differences in dopamine and cannabinoid levels between the experimental and control groups, there was a trend toward a reduction of dopamine in the shell of the NAc as compared to the control animals. This reduction in dopamine in the NAc of animals maintained on an alcoholic liquid diet is consistent with previous literature. Weiss and colleagues (1996) found that accumbens dopamine was reduced over eight hours of alcohol withdrawal in Wistar rats that consumed an alcoholic liquid diet for several weeks (Weiss et al., 1996). With additional time on the alcoholic LD diet, it is expected that animals in this study would have also shown significantly lower levels of dopamine as compared to control mice.

Similar to the dopamine findings, there were no significant differences between the control and experimental groups in the levels of the endocannabinoids AEA and 2AG one day after the last alcohol exposure. Previous studies have focused on the variation of the levels of
these neurochemicals during alcohol exposure more so than during alcohol withdrawal. One study using mice found that dependence produced by three days of alcohol vapor increased AEA levels in the cortex during exposure and AEA levels returned to baseline levels 24 hours after alcohol administration (Vinod, Yalamanchili, Xie, Cooper, & Hungund, 2006). Although with the present experiment, alcohol was administered for a longer period than the Vinod (2006) study, it is possible that AEA levels return to baseline levels quickly following alcohol exposure. Ceccarini and colleagues found no differences in AEA levels in the NAc after consumption and also two weeks after alcohol cessation (Ceccarini, Casteels, Koole, Bormans, & Van Laere, 2013). Similar to AEA, 2AG levels were not found to differ in the accumbens of our mice. In a 2002 study, after chronic alcohol administration for 15 days, rats showed no differences in AEA or 2AG levels in the striatum, including the NAc (González et al., 2002). It is possible that differences in these cannabinoids would be found after administering alcohol longer or at a higher concentration. Given the behavioral data presented here with oral alcohol intake, exploration of these neurochemical systems after 30 days or more of the liquid diets would be appropriate.
Chapter 6: General Discussion and Conclusions

The data presented here shows that alcohol dependence is an important factor in the motivation to consume alcohol and contributes, along with rewarding effects of alcohol (euphoria and anxiolytic effects) to the escalation and maintenance of alcohol seeking that occurs with alcohol use disorder (AUD). The research employed chronic oral intake of a nutritionally complete alcohol-containing liquid diet to produce dependence. Chronic alcohol effects were evaluated while controlling caloric intake and nutrition using the LD alcohol and non-alcohol equicaloric liquid diets. Three cohorts of experimental (alcohol consuming) and control (non-alcohol consuming) mice, with increasing periods of LD exposure, allowed for replications of methods to study the basic effects of chronic alcohol and to evaluate the effect of varied alcohol exposure. In addition, two key neurochemical systems in alcohol reinforcement, dopamine and endogenous cannabinoids, were evaluated in dependent animals.

Overall, the findings indicate that dependence induced by oral intake of an alcoholic LD diet motivates increased consumption and/or preference for alcohol. As measured in the three separate cohorts, in comparison to C57 mice that chronically consumed non-alcohol liquid diets, mice that chronically consumed the alcohol-containing liquid diet subsequently showed increase alcohol consumption and/or preference as measured by the widely used two-bottle choice procedure. Consumption differences between the groups were most pronounced in Experiment 2, Cohort 1. Additionally, with respect to preference between the experimental and control groups, all cohorts showed that the experimental group preferred alcohol on the first day of the two-bottle choice and generally on more days as compared to the control group. Interestingly, the group that had the alcohol LD diet the longest, 52 days, did not show an increased consumption as compared to the control group, and on average both groups preferred alcohol to water though
differences emerged when daily preferences were analyzed. More research of these data are necessary to determine the role of dependence and the LD diet in motivating further alcohol consumption and preference.

Previous research on the effects of prior alcohol exposure via different methods also shows that mice subsequently preferred alcohol. Cunningham and colleagues (Fidler et al., 2011) found passive intragastric infusion of 10% alcohol (v/v) administered along with a flavored oral solution for five days increased later preference for the flavored solutions that were paired with alcohol infusions. The effects were the same in both high drinking and low drinking strains (Fidler et al., 2011). Another study by Camarini and Hodge (2004) showed that i.p. injections of alcohol to C57 and DBA mice for either one day or five days produced higher levels of consumption of 5% alcohol in the two-bottle choice procedure. While intragastric infusions and i.p. injections increased subsequent alcohol intake, neither procedure produced alcohol dependence. Additionally, intraperitoneal injections bypass the digestive system and while intragastric infusions are still metabolized by the liver and stomach, they are passively administered and avoid the influence of taste and other oral factors. The results presented here are comparable with the previous studies that administered alcohol via other, less relevant methods and validate the LD diet as an appropriate an effective experimental procedure to motivate alcohol consumption and preference and induce alcohol dependence. It is only with the LD diet that alcohol is presented in a clinically relevant procedure that also produces physical dependence.

Determining dependence objectively in laboratory rodents is quite difficult and although it is a challenge with many drugs of abuse, it is particularly challenging with alcohol. The behaviors that comprise the alcohol withdrawal syndrome are subtle and often difficult to
observe, and they are qualitative and not easily quantified. Whereas some significant symptoms are quantifiable with opiates (e.g. wet-dog shakes), they are not with alcohol. Seizure, a well-known and life-threatening clinical symptom of withdrawal in humans, can also be observed in dependent laboratory animals. It must be induced for reliable laboratory observation and is not linearly quantifiable. Additionally, it is often terminal and is disruptive of behavioral patterns including alcohol preference and intake. While it is difficult to determine dependence and the resulting withdrawal, many studies have in fact concluded that alcohol withdrawal symptoms are not correlated with preference for alcohol in mice (Allen, Fantom & Wilson, 1982; Lopez, Grahame & Becker, 2011). Cunningham and colleagues (Fidler et al., 2011) found that withdrawal produced by passive alcohol intragastric infusion was not related to the tendency to self-infuse alcohol and that C57 mice scored lower on measures of withdrawal than a mouse strain that consumes less alcohol. Though the method of intragastric self-administration is not relevant to oral intake, it highlights the lack of a relationship between alcohol preference and withdrawal symptoms. Other symptoms of alcohol dependence that can be observed in laboratory animals such as altered posture, unsteady ambulation, abnormal tail position and sniffing behaviors are not easily quantified and their relationship to alcohol intake or concentration consumed is unclear. It is also possible to measure chronic intoxication symptoms such as increased hind limb splaying and falling backwards during rearing, as compared to the control animals, which are indications of dependence when observed daily.

In these experiments we did not observe seizures and in fact we endeavored not to produce seizure for the reasons discussed above; however, dependence was apparent in several of the behaviors that the alcohol consuming mice exhibited in their home cages. First, in confirming dependence, was the fact that the mice consumed high amounts of alcohol during the
prolonged exposure to the alcohol containing LD. Second, because of the nature of withdrawal as
discussed above, it was expected that they would show the more subtle withdrawal signs of
dependence. While we did not observe seizures, selected mice were observed for more subtle
signs of alcohol intoxication and withdrawal. Measures of intoxication have confirmed that
incidence of hind leg splaying in mice is associated with high blood alcohol level (Metten et al.,
2004). In our experiment, selected mice from alcohol LD group were observed and exhibited
increased abnormal postures including increased hind leg splaying, falling backwards during
rearing, rigid tail posture (held strait or erect) and tremulousness as compared to animals on the
control diet. Based upon these observations and data, we concluded that our experimental
animals were physically dependent on alcohol.

The implications of our data for addiction research are that the LD diet, when
administered for extended periods of time (one month or more is preferable), is sufficient to
produce alcohol dependence. This conclusion is important to the field because it is the only
method of chronic alcohol administration where animals orally self-administer while controlling
for nutrition. The use of the LD diet ensures that experimental animals consume adequate
nutrition and the use of the nutritionally similar control diet, as in this experiment, ensures that
caloric intake and nutrition are controlled. Other experimental methods used to produce
dependence do not allow animals to self-administer (e.g. i.p. injections), do not allow for normal
taste or smell (e.g. gavage), or result in decreased food consumption and nutritional deficiencies
(e.g. replacing water with alcohol while maintaining a chow diet). None of these paradigms
control for the alcohol calories (7 kcal/g) or basic nutritional need. Research on alcohol
dependence that controls for these essential factors will lead to results that are due to the alcohol
administration and not to these possible confounding and uncontrolled variables.
The data presented here not only have implications for alcohol research on dependence, but also for clinical research and treatment. While dependence did motivate increased drinking as compared to mice that were not dependent, in Experiment 2, Cohort 2 dependent mice did not consume or prefer more alcohol on average than control animals. It is important for clinicians to consider that while dependent humans may drink alcohol often and in large quantities, nondependent patients may consume equally large quantities occasionally. Dependence does motivate drinking; however, it is not necessary for alcohol consumption or preference. This is evident in that the average social alcohol drinker who is not physically dependent may consume excessive alcohol and may not be overtly distinguishable from the dependent drinker in many ways. This is cautionary in the larger picture of treatment of the alcoholic, simply looking at alcohol intake may mask the important health consequences associated with alcoholism as seen in the morbidity and mortality statistics of the disease.

Potential limitations of this study include the use of C57 mice, an alcohol preferring strain. A study that investigated alcohol preference among multiple mouse strains concluded that the C57 strain consumed the highest quantities of 10% alcohol of 22 strains tested (Yoneyama, Crabbe, Ford, Murillo, & Finn, 2008). It is possible that more robust differences between the control and experimental groups were not observed because the C57 are alcohol preferring mice. A future direction would involve using the same procedures with a low alcohol preferring strain such as the DBA, which might result in a stronger influence of prior alcohol consumption via the LD diet. Other studies have found that pre-exposure to alcohol increased later preference for it at different levels in different mouse strains. One study found that passive intragastric alcohol administration increased later self-infusions of alcohol in both C57 and DBA mice, but there were larger increases in alcohol infused by the DBA mice than the C57 (Fidler et al., 2011).
While the Fidler (2011) study administered alcohol intragastrically, the prior exposure has a stronger influence on low preferring mice. Similarly, it might be expected that if alcohol was administered to DBA via the LD diet, larger increases in consumption might be observed as compared to this experiment with the high preferring C57 mice.

In addition to running the same protocol with low alcohol preferring mice, changing the administration of alcohol during the LD training to intermittent access instead of continuous might improve preference later. One study found that C57 mice that had intermittent access to 20% alcohol for 16 weeks consumed and preferred 20% alcohol more than mice that had continuous access during training (Hwa et al., 2011). They concluded that intermittent access to alcohol every other day results in marked dependence and increased alcohol preference. However, it should be noted that employing these methods could not be easily accomplished while controlling for caloric and nutritional variables. Additionally, increasing the interval between the alcohol training and the two-bottle choice rounds might also be explored. The current procedure used a one week interval between training and choice testing and the motivation to drink may be greater during a longer period of abstinence. In rats the use of longer abstinence has been observed using the alcohol deprivation effect. These data indicate that durations of two weeks may produce increased intake. After an extensive review of animal methods in alcohol research, Spanagel and Hölter (1999) concluded that two conditions need to be met in order to observe a strong alcohol deprivation effect. First animals must receive alcohol daily for longer than a month, and second they must be deprived of alcohol for at least 14 days before choice procedures. While these guidelines were developed using rat models, investigating them in mouse strains (preferring and non-preferring) would be a logical direction for future research. The research presented here established that alcohol should be chronically administered
orally for an extended period of time (perhaps a month or more) for alcohol consumption and preference, the addition of a prolonged abstinence period may further enhance these measures.

**Conclusion**

The experiments presented here indicate that C57 mice made dependent by chronically consuming alcohol via a nutritionally complete liquid diet will develop stronger preference for alcohol as compared to control animals exposed to a non-alcohol diet, as determined using the two-bottle choice procedure. Physical dependence does increases alcohol reinforcement as indicated by the increased preference for alcohol over water on more occasions, although alcohol reinforcement can occur without it. The experiments indicate that chronic intake of a month or more appears important to see dependence motivated consumption and preference. Additionally, these results support the use of the LD liquid diet as a valid model for studying alcoholism and the motivational aspects of alcohol dependence in mice. Future research directions included determining a better experimental protocol to enhance subsequent alcohol consumption and preference. Alcohol drinking is a complex behavior mediated by multiple neurochemical systems involving numerous brain regions. It is clear that there are known environmental and genetic factors that contribute to alcohol consumption, abuse and dependence. Although we have made great progress in our understanding of these factors, there is much more to do. Improving experimental methods for studying alcohol drinking and dependence will undoubtedly lead to enhanced understanding of, and better treatments for those suffering from, Alcohol Use Disorder.
Appendix

BioServ Liquid Feeding Tubes
References


Basavarajappa, B. S., Saito, M., Cooper, T. B., & Hungund, B. L. (2000). Stimulation of cannabinoid receptor agonist 2-arachidonylglycerol by chronic ethanol and its modulation by specific neuromodulators in cerebellar granule neurons. *Biochim Biophys Acta, 1535*(1), 78-86.


Haseba, T., Kameyama, K., Mashimo, K., & Ohno, Y. (2012). Dose-Dependent Change in Elimination Kinetics of Ethanol due to Shift of Dominant Metabolizing Enzyme from ADH 1 (Class I) to ADH 3 (Class III) in Mouse. *International Journal of Hepatology, 2012*(Class III), 408190. doi:10.1155/2012/408190


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