ASSESSING THE REWARD VALUE OF
HIGH CONCENTRATIONS OF ALCOHOL IN RATS

By

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>alc</td>
<td>alcohol</td>
</tr>
<tr>
<td>ABV</td>
<td>alcohol by volume</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>AUD</td>
<td>alcohol use disorder</td>
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<td>BAC</td>
<td>blood alcohol concentration</td>
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<tr>
<td>BP</td>
<td>breakpoint</td>
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<tr>
<td>cm</td>
<td>centimeter</td>
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<tr>
<td>CDP</td>
<td>chlordiazepoxide</td>
</tr>
<tr>
<td>CRF</td>
<td>continuous reinforcement</td>
</tr>
<tr>
<td>cSNC</td>
<td>consummatory successive negative contrast</td>
</tr>
<tr>
<td>dB</td>
<td>decibel</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>EEG</td>
<td>electroencephalogram</td>
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<tr>
<td>ERP</td>
<td>event related potential</td>
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<tr>
<td>ESM</td>
<td>emotional self-medication</td>
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<td>fl oz</td>
<td>fluid ounces</td>
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<tr>
<td>fMRI</td>
<td>functional magnetic resonance imaging</td>
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<td>FR</td>
<td>fixed-ratio</td>
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<td>h</td>
<td>hour</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>ITI</td>
<td>inter-trial interval</td>
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<td>g</td>
<td>grams</td>
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<td>Abbreviation</td>
<td>Full Term</td>
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<tr>
<td>LH</td>
<td>lateral hypothalamus</td>
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<tr>
<td>NAc</td>
<td>nucleus accumbens</td>
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<td>nMol</td>
<td>nanomoles</td>
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<tr>
<td>µL</td>
<td>microliter</td>
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<td>µM</td>
<td>micromolar</td>
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<td>mg</td>
<td>milligram</td>
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<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>PR</td>
<td>progressive ratio</td>
</tr>
<tr>
<td>PND</td>
<td>postnatal day</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SUD</td>
<td>substance use disorder</td>
</tr>
<tr>
<td>VEH</td>
<td>vehicle</td>
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<tr>
<td>vol</td>
<td>volume</td>
</tr>
<tr>
<td>VR</td>
<td>variable-ratio</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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<td>wat</td>
<td>water</td>
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</table>
Overview of Alcohol Use Disorder

The abuse of substances with addictive potential such as alcohol, prescription anxiolytics, and psychoactive drugs is classified in the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5; American Psychiatric Association, 2013) under the broad heading of substance use disorders (SUDs). Severe abuse of alcohol alone without the co-occurrence of another SUD is classified as an alcohol use disorder (AUD). The addictive behaviors associated with AUD include a subset of 11 main symptoms such as excessive consumption, craving, tolerance, and withdrawal with a diagnosis requiring a minimum of two symptoms within the past 12 consecutive months. An AUD is evaluated on a spectrum and the diagnostic degree of severity depends on the number of symptoms a person is currently experiencing and can range from mild (two or three), to moderate (four or five), and to severe (six or more) impairment in functioning.

In 2017, an estimated 16 million Americans were diagnosed with an AUD contributing to 88,000 alcohol-related deaths and causing alcohol to become the third leading cause of preventable death (National Institute on Alcohol Abuse and Alcoholism, 2017a; NIAAA). Men are more likely to receive an AUD diagnosis than women, and less than 1.6 million people who are diagnosed will seek treatment. Despite the low degree of treatment seeking behavior, the Centers for Disease Control and Prevention (2018) reported an economic burden exceeding $249 billion per year related to AUDs.

Alcohol use in humans

Consuming the occasional alcoholic beverage does not always progress into a pattern of addictive behavior. According to a survey conducted by the NIAAA (2018), only around 2% of adults who engage in regular alcohol consumption will develop an AUD. This regular, or low-
risk drinking is defined as no more than three alcoholic beverages per day for women and no more than four per day for men. Once alcohol consumption surpasses the regular limits, it is considered atypical or high-risk drinking, and is defined as exceeding the low-risk daily totals of more than seven alcoholic beverages per week for women and more than fourteen per week for men. Around 25% of high-risk drinkers are diagnosed with an AUD.

**Standard drink sizes.** A standard drink is quantified as any alcoholic beverage that contains 14 g of alcohol (NIAAA, 2017b). The serving size of alcoholic beverages varies by type. For example, a standard serving of regular beer is 355 ml (12 fl oz) and contains between 5-7% alcohol by volume (ABV), whereas a standard serving of a distilled spirit (e.g., vodka or tequila) is 44 ml (1.5 fl oz) and contains at least 40% ABV. While the standard serving size of the two drinks in ml varies considerably, on average they both contain 14 g of alcohol. As shown in Experiment 1, below, rats consume comparable levels of alcohol in free-choice tests, confirming the relevance of animal models of voluntary alcohol consumption to AUD.

**Animal models of alcohol use**

Many laboratory studies use mice or rats to test addictive behaviors that parallel symptoms from the DSM-5 criteria. These animal models typically involve diluted ethyl alcohol which is commonly shortened to *alcohol* or *ethanol*. Researchers have used intravenous and intraperitoneal injections of alcohol to test the neurobiological effects of the substance (Gass & Olive, 2007; Le & Kalant, 2017; Nelson et al., 2016). More relevant to this proposal are studies involving voluntary, oral consumption of alcohol. A major limitation of many rodent studies is the difficulty researchers face when trying to establish pharmacologically relevant levels of alcohol drinking. This difficulty has led to the development of several procedures aimed at encouraging rodents to consume alcohol, as described in the following sections.
**Alcohol deprivation effect.** One method for generating high levels of alcohol intake is the alcohol deprivation effect (ADE) developed by Sinclair and Senter (1967). Non-food-deprived rats were given four weeks of continuous access (24 h/day) to 7% alcohol and water followed by a period of alcohol deprivation before resuming access. Alcohol consumption increased following the period of deprivation. The ADE prompts the increased consumption of alcohol for a limited time frame after the deprivation period and thus is a transient effect.

Laboratory tests of the ADE resemble human relapse behavior, but as with humans, relapse behavior has a strong genetic component and not all rodents will engage in relapse-like increases in alcohol consumption following periods of alcohol deprivation. Vengeliene, Bilbao, and Spanagel (2014) tested the ADE in male C57BL/6 mice and male Wistar rats over the course of 20 weeks using alcohol concentrations ranging from 5 to 20% and including three, 2-week periods of deprivation during the study. The results revealed that while Wistar rats developed compulsive drinking with intake levels around 3 g/kg/day and achieved high BAC levels following periods of alcohol deprivation, but the C57BL/6 mice demonstrated a significant decline in alcohol intake.

**Intermittent access.** Another established method for inducing high levels of alcohol consumption is an intermittent access paradigm using a two-bottle choice test. Wise (1973) exposed groups of male Wistar rats to 20% alcohol by either gradually increasing the concentration of alcohol over sessions or abruptly introducing the 20% solution upon initial presentation. Some animals were provided continuous access throughout the experiment while others received intermittent access to the alcohol solution. Additionally, some animals were provided with alcohol and water (free-choice) and others with alcohol in both bottles (forced-choice). Animals that received intermittent access to alcohol, regardless of initial presentation
method (gradual vs. abrupt) consumed more alcohol (~5 g/day) than rats receiving continuous access (~4 g/day), which assessing for body weight, is equivalent to around 9 g/kg/day over the course of the experiment. These data suggested the usefulness of the intermittent access method for studying alcohol use in the rat model.

**Taste fading.** The taste of alcohol can be inherently aversive. Sucrose-fading methods were developed to abate this aversiveness and initiate alcohol consumption (Grant & Samson, 1985, Samson, 1986; Tolliver, Sadeghi, & Samson, 1988). Tolliver, Sadeghi, and Samson (1988) exposed rats to a period of preference testing with 10% alcohol and water in their home cages, and separated them into groups based on their initial preference for alcohol. Low-preferring rats exhibited preference levels lower than 25% while moderate-preferring rats showed 25-50% preference levels. Both groups were then trained to lever press using 20% sucrose as a reinforcer. Water deprivation was used to promote learning of the operant response. Once rats began lever pressing consistently, water deprivation ceased, and the sucrose reinforcement schedule was changed from a continuous reinforcement schedule (fixed ratio 1; FR1) to a FR4 schedule—every fourth response was reinforced. The alcohol concentration in the sucrose solution was gradually increased from 5% to 20%. After the self-administration training ended, animals were reintroduced to 10% alcohol preference testing in their home cages. The low-preferring rats exhibited a marked increase in alcohol preference following the sucrose-fading relative to the moderate-preferring rats. However, though sucrose-fading methods are effective, they introduce an additional source of calories. To alleviate the added caloric content from sucrose-fading methods, saccharin can be substituted as it still provides an appetitive taste, but is free from calories (Puaud et al., 2018).
While ADE, intermittent-access, and sucrose/saccharin-fading procedures are able to induce voluntary, oral alcohol consumption, these methods all involve an alcohol initiation period before animals begin to consume relevant levels of alcohol. Simms et al. (2008) developed a modified intermittent-access paradigm without the use of an alcohol initiation period. Both Long-Evans and Wistar rats were used. Animals were exposed to a 24-h intermittent-access 2-bottle free-choice preference test between 20% alcohol and water for 20 drinking sessions. These animals exhibited increased alcohol intake over the first 5-6 sessions before reaching a stable level of intake around 6 g/kg/day.

**Consumption without initiation methods.** Simms et al. (2010) attempted to extend these high alcohol intake findings to an operant self-administration paradigm without the use of any alcohol initiation procedures (i.e., no sucrose/saccharin fading or gradual alcohol introduction). One group of rats was trained to lever press during intermittent 14-h overnight sessions on an FR1 schedule for 0.1 ml, 20% alcohol reinforcement conducted three times per week for four weeks. Next, the session length was reduced to 45-min three times per week for two weeks. Finally, the response requirement was increased to FR3 and session length was further reduced to 30-min three times per week for six weeks. A second group of rats was trained to lever press for 20% alcohol using continuous access sessions with 12 overnight FR1 sessions, six 45-min FR1 sessions, and twenty 30-min FR3 sessions for comparison. Animals in the intermittent-access group self-administered significantly more alcohol than continuous-access animals suggesting that intermittent-access methods without sucrose/saccharin fading are successful in generating high levels of voluntary alcohol consumption.

Augier et al. (2014) and Augier, Dulman, Singley, and Hellig (2017) reported on methods to establish operant self-administration of alcohol without the use of water deprivation,
sucrose/saccharin fading, or intermittent/extended access training. In one study they exposed two groups of rats to a 20% alcohol self-administration paradigm under a continuous reinforcement schedule for 30 min per day. One group of rats was water deprived and the other was not. Researchers found a small difference in lever pressing between the water deprived and non-deprived groups during the first week of testing, with both groups responding within 20 lever presses of each other on average. An extension of this study was conducted by Puaud, Ossowska, Barnard, and Milton (2018) that attempted to replicate the finding that initiation methods are unnecessary in establishing alcohol self-administration. In this case, one group of rats was trained under conditions of saccharin fading lasting 14 days and a second group of rats was not saccharin-faded before self-administration of 10% alcohol. During the 14 days of saccharin fading, rats in the saccharin fading group were transported into a separate room from the colony and presented with a bottle containing saccharin and alcohol for 1 h/day. The concentration of alcohol within the saccharin solution increased daily. Rats in the non-saccharin fading group simply remained in their home cages for 14 days prior to the start of self-administration with 10% alcohol. Similar to Augier et al. (2014), they found that both groups of rats acquired alcohol self-administration regardless of the use of fading procedures.

Sex differences. In human clinical studies, men are more likely to abuse alcohol than women (NIAAA, 2017a). However, preclinical studies comparing male and female non-human animals are scarce (Priddy et al., 2017; Randall, Stewart, & Besheer, 2017). Previously, female rats were thought to be more variable due to their estrous cycle. A meta-analysis conducted by Becker, McClellan, and Reed (2011) showed that male and female rats perform consistently across a wide-range of behavioral manipulations. Priddy et al. (2017) tested sex differences in alcohol consumption using Long-Evans and Wistar rats across two-bottle and operant self-
administration tests, and found that females consume more alcohol during two-bottle tests, but consume equal amounts as males in operant tests. Randall et al. (2017) also explored sex differences using male and female Long-Evans rats in operant tests and found that male rats engaged in more responses for alcohol than female rats, but that their levels of alcohol intake in g/kg were roughly equivalent. Taken together, these data suggest the importance of including both males and females in preclinical studies involving alcohol.

**Alcohol concentrations**

Animal models of alcohol administration typically involve the use of low concentrations of alcohol and report that rodents struggle to consume concentrations higher than 20% (Gilpin et al., 2009; Becker, McClellan, & Reed, 2017). However, considering the clinical relevance that animal models of high concentration alcohol consumption can yield, it is important to evaluate higher concentrations at least up to 70%. Alcohol concentrations greater than 70% (vol/vol) yield lethal levels of toxicity and should not be used (Centers for Disease Control and Prevention, 2018b; Wiberg, Trenholm & Coldwell, 1970).

Carnicella, Yowell, and Ron (2011) studied both voluntary alcohol consumption and operant oral self-administration in rats testing a range of concentrations between 2.5 and 60% alcohol. Researchers found a standard inverted U-shaped dose-response curve asserting that rats preferred 10 and 20% concentrations to the lower and higher range of concentrations. These data suggest that alcohol consumption is motivated, in part, by efforts to maintain a certain pharmacological effect. Achieving the same pharmacological effect from low concentration alcohol (i.e., lower than 10%) that would be achieved by a higher concentration is possible. However, it would take the consumption of a greater amount of low-concentration alcohol solution (ml) to achieve an equivalent level of pharmacological effect than a smaller amount (ml)
of a higher concentration solution due to the amount of alcohol contained within each individual ml of the concentration solutions. The same amount of response effort is required to consume 1 ml of any solution; therefore, it can be argued that to consume a pharmacologically relevant amount of a low concentration alcohol solution requires more response effort than would be required for a high concentration alcohol solution. Furthermore, while rodents may find reward value in many concentrations, a potential explanation for the greater consumption of higher concentrations lies within the pharmacological effects associated with these concentrations.

**Motivation to consume**

**Reduction of negative emotions.** Many theoretical frameworks focus exclusively on the positive and rewarding properties of substances with addictive potential and suggest that the initiation into consumption is motivated by their pleasurable effects (Koob, 2009; Koob & Volkow, 2010). An alternative framework asserts that the consumption of substances with addictive potential is motivated by their ability to reduce negative emotions. This emotional self-medication (ESM) hypothesis of addiction suggests that organisms voluntarily consume alcohol because it attenuates negative emotions (Khantzian, 1985, 2013; Torres & Papini, 2016). Frustrative nonreward is a source of negative emotion that has been explored in the context of the ESM hypothesis (Torres & Papini, 2016). A common method of establishing frustrative nonreward in a laboratory setting is through reward downshift in a consummatory successive negative contrast (cSNC) situation. In a typical cSNC experiment, animals are first trained to expect a high-value 32% sucrose reward for ten sessions before being downshifted to a low-value 4% sucrose reward for five sessions. In this situation a marked decrease in sucrose consumption is observed on the first downshifted session (Session 11) relative to the preshift consumption and to an unshifted control group always receiving 4% sucrose. This decrease is
attributed to frustration, a negative emotion, and is transient; thus, the reduction in consumption dissipates by Session 15.

Reward downshift involves the unexpected (i.e., surprising) omission or devaluation of a reward that induces a negative emotion—frustration (Amsel, 1992; Papini, Fuchs, & Torres, 2015). This frustrative nonreward is characterized by an aversive emotional state which produces a transient reduction in the consumption of the solution presented in the postshift phase. Consistent with this framework, pharmacological manipulations using anxiolytics, such as benzodiazepines and alcohol, have been shown to attenuate the cSNC effect (Flaherty, 1996). Reward downshift has also been shown to activate the hypothalamic-pituitary-adrenal axis and promote the release of the stress hormones adrenocorticotropic hormone and corticosterone (Pecoraro et al., 2009). Furthermore, lesions of the centromedial and basolateral amygdala attenuated the cSNC effect (Kawasaki, Glueck, Annicchiarico, & Papini, 2015; Kawasaki, Annicchiarico, Glueck, Moron, & Papini, 2017). These results, among others, suggest that reward downshift induces a negative emotion (Papini et al., 2015).

Tests of ESM involve inducing a negative emotion and then providing the opportunity to voluntarily consume alcohol (e.g., Manzo et al., 2014; Manzo, Donaire et al., 2015). Such tests have shown transient changes in alcohol consumption during periods in which animals are subjected to reward downshifts. These transient changes in alcohol consumption occur during the same sessions that provide behavioral evidence of frustration in the induction task involving reward devaluation or omission. As the negative emotion is attenuated, alcohol consumption in ESM experiments also decreases.

**Reducing motivation pharmacologically.** Motivation to consume natural rewards and substances with addictive potential stems from many systems including the orexin system (also
called hypocretin system), primarily known for its role in feeding, reward, and addiction. More specifically, orexin neuropeptides expressed in the lateral hypothalamic region of the rat brain have motivational and reward seeking functions and those in the dorsomedial hypothalamic and perifornical areas of the rat brain play a role in stress and arousal (Hervieu et al., 2001). Two orexin neuropeptide receptors have been identified: orexin-1 and orexin-2. These G-protein coupled orexin neuropeptide receptors have widespread distribution in the brain with projections to cholinergic neurons in the spinal cord and reticular formation, to the thalamus, basal ganglia, basal forebrain, amygdala, as well as to dopaminergic neurons in the suprachiasmatic nucleus and locus coeruleus (Ebrahim et al., 2002). Considering the projection of these orexin neuropeptide receptors to dopaminergic neurons, there is a posited role of excitatory dopamine neurotransmission. Dopamine plays a critical role in the rewarding properties of many drugs of abuse with increased expression of dopaminergic cells in the ventral tegmental area and nucleus accumbens (Volkow et al., 2011). Baimel et al., (2015) confirmed the projection of orexin neurons from the lateral hypothalamus to the ventral tegmental area and further suggested an implication of the orexin system in reward processing. The orexin signaling in this brain area affects the underlying function of dopaminergic neurons contained within the ventral tegmental area.

Lawrence et al. (2006) explored the relationship between the orexin system and alcohol reward motivation by training rats in a cue-induced alcohol-seeking paradigm with 10% alcohol and water. After extinction was implemented, rats were treated with the orexin-1 antagonist, SB-334867, or vehicle to test the effects on cue-induced reinstatement. The results revealed that orexin-1 antagonism decreased alcohol-seeking behavior, but had no effect on responding for water. Antagonists of the orexin-1 receptor such as SB-334867 have also been shown to reduce
voluntary alcohol consumption and alcohol self-administration. For example, rats trained to voluntarily consume 10% and 20% alcohol under intermittent-access conditions in their home cages were administered 30 mg/kg SB-334867 i.p. and found to decrease their consumption following drug administration (Moorman et al., 2009). Anderson et al. (2014) also tested the effects of SB-334867 on home cage drinking in mice using 15% alcohol, finding similarly that alcohol intake was attenuated. Jupp, Krivdic, Krstew, and Lawrence (2011) compared the effects of SB-334867 on responding for 10% alcohol versus responding for 0.2-0.7% sucrose under both fixed and progressive ratio schedules of operant reinforcement. In a progressive ratio schedule, the number of responses required to obtain successive rewards increases according to a fixed step (e.g., 1, 5, 10, 15, 20, and so on). Antagonizing the orexin-1 system significantly reduced responding for both alcohol and sucrose under fixed ratio and PR conditions. However, only responding for alcohol was affected. In a similar test, Moorman et al. (2017) administered SB-334867 to rats receiving 20% alcohol self-administration and alcohol reinstatement paradigms and found that orexin-1 antagonism decreased the motivation to respond for alcohol, even in the case of reinstatement. An interesting question would be whether orexin-1 antagonism can also decrease motivation for higher concentrations of alcohol. The orexin system is a potentially clinically relevant target for future AUD drug treatments.

**Aims of the Current Research**

The present dissertation has relevance for both translational and theoretical problems in three areas. First, with alcohol rising to the third leading cause of preventable death in the United States, there is a fundamental need to understand the mechanisms leading to the transition from regular drinking behavior to addictive behavior. An animal model that explores the initiation into problematic, intoxicating drinking provides important insight for translational research as well as
attempts at treatment. In this vein, the present research aimed to achieve pharmacologically high levels of alcohol consumption in the rat as a model of translational value.

Second, this research aimed at exploring the rewarding properties of high concentrations of alcohol for rats, assessing the possible individual differences in consumption in relation to events involving frustrative nonreward. Situations involving reward loss (e.g., cSNC, consummatory extinction, instrumental extinction) induce a negative emotion, frustration, which encourages a selective increase in the preference for, and consumption of substances with addictive potential (e.g., alcohol, anxiolytic drugs; Manzo, Donaire et al., 2015; Manzo et al., 2014).

Third, this package of experiments sought to evaluate the effects of orexin-1 antagonism on voluntary oral consumption of high concentrations of alcohol. As mentioned above, orexin-1 receptors have been implicated in the rewarding properties of alcohol, and therefore have potential therapeutic value for the treatment of AUDs (Anderson et al., 2014; Hervieu et al., 2001; Jupp et al., 2011; Moorman et al., 2009; Moorman et al., 2017).

Further rationale for each individual manipulation is provided in the introduction to the relevant experiment.

**Experiment 1 – Preference testing with ascending and descending alcohol concentrations**

The purpose of Experiment 1 was to assess a wide array of alcohol concentrations in Wistar rats using a 2-h, 2-bottle, free-choice preference test. Two cohorts of animals were run at different time points. One cohort received access to all concentrations in an ascending manner starting with 1% alcohol and increasing to 66% alcohol. The other cohort received access to all concentrations in an abrupt, descending manner starting with 66% alcohol and ending in 1%
alcohol. Regardless of the presentation order (ascending vs. descending) rats consumed more alcohol in terms of g/kg intake for higher alcohol concentrations.

**Method**

**Subjects.** Subjects in the *ascending* group were 12 female Wistar rats and subjects in the *descending* group were 8 female Wistar rats (*Rattus norvegicus*), all experimentally naïve, bred from parents purchased from Envigo Laboratories (Indianapolis, IN). Their mean (±SEM) ad lib weight was 286.4 g (8.0 g).

All animals in the present experimental series were maintained under standard animal colony conditions with an approved Institutional Animal Care and Use Committee protocol. The colony room was maintained on a 12:12 h light:dark cycle with lights on at 07:00 h. Access to the colony room was restricted during the 12 h of darkness. Temperature was kept between 18-22 °C, with humidity levels ranging between 50% and 60%.

The following breeding protocol was followed in all experiments described here. Rat pups were housed with their mother for the first 21 days of life. On postnatal day (PND) 21, all animals were group housed based on litter in clear polycarbonate boxes, 45.4 x 24 x 20 cm, lined with 0.31-cm corn cob bedding, a single 5 x 5 cm cotton nestlet, and a red plastic enrichment hut to provide exercise and cover. On PND 31 all animals were single housed on wire-bottom cages that included the same red enrichment retreat. All animals had ad libitum access to filtered water via a metal lick sipper tube in the home cage. Animals were fed Purina lab rat chow on an ad libitum basis until PND 90. Except where otherwise notes, at PND 90 subjects were food deprived to a level between 81-84% of their ad libitum body weight.

**Apparatus.** Two-bottle free-choice preference testing took place in the animal’s wire-bottom home cages, on a transport rack in a room separate from the colony. Two 473-ml, wide-
mouth bottles with #8.5 rubber stoppers equipped with 10.2-cm curved ball bearing metal sippers (Ancare, Bellmore, NY) were secured to the home cage during the 2-h preference test through the attached feeder and the arm of the feeder. Plastic tape was applied to the bottom of each bottle to specify the contents and thus minimize potential experimenter error. All bottles were weighed prior to and following each session to assess the amount of fluid consumed (Ohaus Valor: Model 7000, Fisher Scientific, Waltham, MA).

**Procedure.** Rats in the *ascending series* (*N* = 12) were loaded in their home cages onto a transport rack and moved from their colony into a separate testing room where the 2-h, 2-bottle preference test began. The preference test involved simultaneously presenting two bottles, one containing a gradually increasing concentrations of alcohol mixed with varying quantities of deionized water and the other containing only deionized water. Rats received access to a specified concentration for two consecutive days before the concentration was gradually increased in accordance with the following progression: 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 26, 30, 34, 38, 42, 50, 58, 66% alcohol. Data collection ended once the 66% alcohol concentration was reached because rats were nearing toxic levels of consumption. Wiberg et al. (1970) asserted that the LD50 (lethal dose at which 50% of adult Wistar rats approximately 100 days old will die) is 10.0-11.2 g/kg of oral consumption. As shown below, given oral consumption levels of the 66% alcohol solution, rats were at this level of toxicity (see Figure 2, bottom). Bottle presentation order was counterbalanced daily using a standard double alternation procedure to control for spatial biases. Rats were left unmonitored in a brightly lit room during the preference test. Following the end of each daily session, animals were returned to the colony room and their home cages were placed back on their home rack. Animals were fed at least 15 min after their daily sessions, during the light cycle, to maintain 81-84% food deprivation levels.
Dependent variables of interest included: consumption of alcohol and water, and a preference ratio. The preference ratio was calculated by dividing the total amount of alcohol consumed within the 2-h testing window by the total amount of alcohol plus water consumed on that day. Preference ratios higher than 0.5 indicated a preference for alcohol over water, whereas preference ratios lower than 0.5 indicated a preference for deionized water.

Rats in the descending preference test ($N = 8$) received the same preference test described above, but starting at 66% alcohol then a gradually decreasing the concentration to 1% alcohol following the same sequence described above, but in reversed order. The dependent variables of interest were the same as described above for the ascending group.

**Results**

All data in this dissertation were analyzed with IBM SPSS Statistics Version 25. Further analyses were conducted on significant interactions using pairwise LSD tests derived from the main analyses. An alpha value was set at the 0.05 level for all analyses of significant effects.

Separate two-way repeated measures analyses of variance (ANOVA) with concentration (1-66%) and bottle (alcohol vs. water) as within-subject factors were conducted on the ascending and descending data due to data collection with these cohorts occurring at different time points.

Data for the ascending preference group are shown in Figure 1 (top). A Bottle (Alcohol, Water) by Concentration (1-66%) repeated measures ANOVA revealed a significant two-way interaction, $F(21, 231) = 3.67, p < 0.001, \eta^2 = 0.25$, as well as significant main effects of both bottle, $F(1, 11) = 111.10, p < 0.001, \eta^2 = 0.91$, and concentration, $F(21, 231) = 11.02, p < 0.001, \eta^2 = 0.50$. Group comparisons based on the overall analysis using pairwise LSD tests indicated that rats consumed more alcohol than water for the following concentrations: 6, 12, 14, 16, 18, 22, 26, 30, 34, 38, 42, 58, and 66%, $ps < 0.03$. Experience with increasing concentrations of
alcohol may lead to the development of tolerance to the toxic effects of alcohol (Carnicella et al., 2011). The descending series controls for tolerance since alcohol-naïve animals are exposed to the high concentrations from the outset of testing.

Data for the descending preference group are shown in Figure 1 (bottom). A Bottle (Alcohol, Water) by Concentration (1-66%) repeated measures ANOVA revealed a significant two-way interaction, $F(21, 147) = 1.86, p < 0.02$, $\eta^2 = 0.21$, as well as significant main effects of both bottle, $F(1, 7) = 44.80, p < 0.001$, $\eta^2 = 0.87$, and concentration, $F(21, 147) = 2.68, p < 0.001$, $\eta^2 = 0.28$. Group comparisons based on the overall analysis using pairwise LSD tests indicated that rats consumed more alcohol than water for the following concentrations: 6, 14, 16, 18, 20, 22, 26, 30, 34, 38, 42, 50, 58, and 66%, $ps < 0.03$.

Data for the preference ratio are shown in Figure 2, top. Each cohort was run separately, therefore they were not directly compared. Despite being run at different time points, the preference ratios were generally similar. A one-sample $t$-test was used to test the preference ratio for each concentration against 0.5 (indifference level) thus assessing the significance of alcohol preference over water. In the ascending group, the preference ratios were statistically significant for: 6, 12, 14, 18, 22, 26, 30, 34, 38, 43, and 58% alcohol, $t(7) > 2.58$, $ps < 0.04$, Cohen’s $d$s > 0.66. The preference ratio for 66% alcohol reached marginal nonsignificance, $t(7) = 2.16, p = 0.055$. A separate analysis conducted on the descending group revealed preference ratios statistically significant for: 6, 10, 14, 16, 18, 20, 22, 26, 30, 34, 38, 42, 50, 58, and 66% alcohol, $t(11) > 2.81$, $ps < 0.02$, Cohen’s $d$s > 1.32.
Figure 1. Consumption of various concentrations of alcohol (range: 1-66%) and water conducted in both an ascending and descending fashion showing that rats voluntarily consume more alcohol than water for a variety of concentrations.
**Figure 2.** Preference ratio for alcohol concentrations (range: 1-66%) relative to water conducted in both an *ascending* and *descending* fashion showing that rats accept and prefer a variety of concentrations above 6% alcohol (top). Alcohol consumption relative to body weight (g/kg) for all concentrations (bottom). The abscissa is shown in log units only for clarity of visualization of alcohol intake at lower concentrations.

Regardless of the presentation (ascending vs. descending), rats did not demonstrate a reduction in alcohol consumption. Alcohol intake by body weight (g/kg) is shown in Figure 2 (bottom). Values were calculated based on the amount of solution consumed for each
concentration. Calculations of alcohol per gram of solution were based on the density of 190 Proof, ACS-USP Grade 95% ethyl alcohol being 0.816 g/ml (Pharmco Aaper, Shelbyville, KY). Intake in g/kg was similar across groups with the exception of 50 and 66% alcohol differing between the ascending and descending groups.

Summary of Results

These results suggest that presentation order (ascending vs. descending) does not affect the voluntary, oral consumption of alcohol. Thus, the argument for tolerance to alcohol contributing to the current consumption levels can be discarded. In fact, a trend for more consumption of concentrations greater than 6% alcohol is observed in these data. This finding is inconsistent with previous findings from Manzo et al. (2012) where the drinking behavior and preference decreased once concentrations exceeded 6%. Experiment 1 established that rodents would voluntarily consume a wide array of alcohol concentrations. Experiment 2 was designed to assess pharmacologically high levels of BAC as a result of selected alcohol concentrations.

Experiment 2 – Blood alcohol levels

The primary objective of Experiment 2 was to assess whether a 30-min, 2-bottle free-choice preference test is sufficient to promote voluntary oral alcohol consumption resulting in pharmacologically relevant BAC levels. Previous data from our lab have shown voluntary oral consumption of high quantities of alcohol (i.e., greater than 2.0 g/kg) in 2-h-long, free-choice tests (see Figure 1). Animals in our lab exhibit higher alcohol intake in shorter periods of time compared to those reported in other studies (e.g., Carnicella et al., 2011; Vengeliene et al., 2014). These studies reported similar levels to those recorded in this Dissertation, but in 24-h-long tests. Unpublished pilot data from our lab using 8% alcohol suggest that the largest amount of drinking occurs during the first 30 min of access (see Figure 3). A shorter preference test was used in this
experiment to maximize detection of BAC, as blood samples taken after the regular 2-h long tests could be degraded after a long period of low consumption and alcohol loss through respiration and excretion. To better quantify the physiological effects of alcohol consumption in rats, an alcohol assay can be run on biological samples (e.g., blood serum, blood plasma, saliva, urine).

![Substance consumption graph]

*Figure 3.* Consumption of 8% alcohol and water during a 2-h preference testing pilot conducted inside of conditioning boxes equipped with a lickometer showing that rats consume more alcohol during the first 30 min of testing.

Previous research has measured BACs through blood serum and blood plasma for a variety of alcohol concentrations (Gilpin et al., 2009; Livy, Parnel, & West, 2003). Positive correlations were found between the BAC and the concentration of alcohol being tested with higher concentrations yielding higher BAC levels in rats regardless of the method of alcohol administration (e.g., oral or systemic). The rationale behind using colorimetric alcohol analyses in this experiment stemmed from McCarter et al. (2017) and Viudez-Martinez et al. (2018), who
used an alcohol assay kit (ab65343, Abcam, Boston, MA). Both studies evaluated BAC following low-concentration alcohol administration (10–13.5%).

**Method**

**Subjects.** Subjects were 10 Wistar rats, 5 male and 5 female, all experimentally naïve, bred from parents purchased from Charles River Laboratories (males from Raleigh, NC, and females from Sherbrooke, Canada). Breeders came from different facilities to ensure that they would not be closely related. Mean (±SEM) ad lib weights were 442.8 g (26.2 g) for males and 285.0 g (7.1 g) for females. All animals were maintained and deprived as described in Experiment 1.

**Apparatus.** The same two-bottle preference test was used as described in Experiment 1 with the exception of the duration being changed from 2-h to 30-min. A GDEALER DS1 Scale (GDEALER, Seattle, WA) was used to weigh the bottles before and after each preference test.

**Procedure.** Rats (N = 10) were moved in their home cages from the colony to a separate testing room. The preference test was as described in Experiment 1, except that it lasted a minimum of 30 min, see below. This reduction in the duration of the test was based on unpublished data showing that licking for alcohol during a two-hour test occurs predominantly during the initial 30 min. In addition, we estimated that the duration of the session plus a 15-min postsession interval, was optimal time to quantify blood alcohol concentration. Bottle location in the cage was changed according to a double alternation pattern to minimize spatial bias. Rats were left unmonitored in a brightly lit room during the preference test. Alcohol concentration was counterbalanced across animals and sessions. Each animal received access to a concentration for two consecutive sessions. The actual mean session length was 46.7 min, with a range between 30 and 75 min. The reason for this variability was delays related to the blood
sampling procedure. However, for any given animal, session length was relatively constant across days and, therefore, this variation affected equally the consumption of all alcohol concentrations. The mean individual range for session length (difference between the shortest and longest session, in min, for each rat) was 3.7 min. At the end of the second session of access to any given concentration, the bottles were removed and the animals were left undisturbed in the cage for 15 min. At the end of this waiting period, rats were individually carried into a separate room where a small sample of blood was collected from the lateral tail vein. Then, animals were returned to the colony room. Additional food to maintain the target deprivation weight was delivered in the home cage at least 30 min after their daily session and during the light cycle. The dependent variables of interest included consumption of alcohol and water (ml/kg), a preference ratio (alcohol/total consumption, calculated on ml of fluid consumed), and BAC (nMol/µL). Behavioral testing occurred during the light portion of the cycle, between 11:00 and 12:00 h, 7 days/week. Other aspects of the procedure were as described in Experiment 1.

**Alcohol preparation.** Alcohol solutions were prepared vol/vol by diluting 190 Proof 95% ethyl alcohol (Pharmco-Aaper, Shelbyville, KY) into deionized water and manually shaking (see Table 1). The same procedure was used to prepare 66% alcohol in all the experiments in this dissertation.

**Blood sampling.** On blood collection sessions, individual rats in their home cages were carried into a separate room one at a time and briefly restrained in a Decapicone sleeve (Braintree Scientific, Braintree, MA) 15 min following the end of the preference test. Based on a pilot experiment, it was estimated that introducing this 15-min postsession interval was the ideal time to quantify BAC in plasma. Once restrained, the tail of the animal was warmed with a heat lamp bulb prior to blood collection from the lateral tail vein via a 23-G needle. Whole blood
samples of approximately 0.2 ml were collected in standard 1.5-ml microcentrifuge tubes, put on ice for 15 min, taken off ice for 30 min, and then centrifuged at 4 °C for 10 min at 2000 x g. The supernatant was collected and immediately stored at -80 °C for up to 14 days. Serum was assayed using an Ethanol assay kit (ab65343, Abcam, Cambridge, MA). Serum samples were diluted in the ethanol assay buffer and standards were prepared according to the manufacturer’s protocol. Samples were plated in duplicate in a 96-well plate and incubated at room temperature in the dark for 60 min. Optical density was read at 570 nm (BMG LabTech FLUOstar Omega, Cary, NC).

Table 1

<table>
<thead>
<tr>
<th>Concentration (vol/vol)</th>
<th>Ethyl Alcohol (ml)</th>
<th>Deionized Water (ml)</th>
<th>Alcohol per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0.00</td>
<td>1500.00</td>
<td>0.00</td>
</tr>
<tr>
<td>2%</td>
<td>31.58</td>
<td>1468.42</td>
<td>0.02</td>
</tr>
<tr>
<td>10%</td>
<td>157.90</td>
<td>1342.10</td>
<td>0.17</td>
</tr>
<tr>
<td>66%</td>
<td>1042.11</td>
<td>457.89</td>
<td>2.28</td>
</tr>
</tbody>
</table>

Note. All solutions were diluted from 190 Proof 95% ethyl alcohol (Pharmco-Aaper, Shelbyville, KY). Alcohol per milliliter is the same as alcohol per gram of solution and indicates how much ethyl alcohol is in 1 ml of solution.

Results

The consumption, preference, and BAC results are presented in Table 2. To account for variation in session length (range: 15–75 min), consumption was further broken down into
amount consumed (ml) by animal’s daily weight (kg) by length of session (min). These data are represented in the g/kg/min column of Table 2.

The BAC values were subjected to a one-way, repeated-measures ANOVA with alcohol concentration (0, 2, 10, and 66%) as the within-subjects factor. The results revealed a significant effect of concentration, $F(3, 27) = 11.20, p < 0.001, \eta^2 = 0.46$. Pairwise LSD tests showed that 10% and 66% alcohol yielded significantly higher BAC levels than 0% or 2% alcohol, $p < 0.006$, but the two sets of concentrations (0 and 2% vs. 10 and 66%) were not significantly different from each other, $p > 0.25$.

Absolute consumption (ml/kg/min) was assessed with an alcohol concentration (0, 2, 10, and 66%) by Bottle (A, W) two-way, repeated-measure ANOVA. The results revealed a significant interaction, $F(3, 27) = 3.89, p < 0.03, \eta^2 = 0.30$, as well as a significant main effect of Bottle, $F(1, 9) = 6.07, p < 0.04, \eta^2 = 0.40$ but the effect across concentrations was nonsignificant, $F(3, 27) = 1.86, p > 0.16, \eta^2 = 0.17$. Pairwise LSD tests indicated that the foundation of the interaction was a significant preference for 10% and 66% alcohol over water, $p <0.03$, but not for 0 and 2% alcohol, $p > 0.50$. Similarly, a one-way, repeated measures ANOVA on the preference ratio revealed a nonsignificant difference between concentrations $F(3, 27) = 2.16, p > 0.11$.

The mean alcohol intake in g/kg/min during the preference test for each concentration was assessed with a one-way, repeated-measure ANOVA. The results revealed a significant difference between the concentrations, $F(2, 18) = 76.21, p < 0.001, \eta^2 = 0.46$. Pairwise LSD tests showed that each of the three alcohol concentrations (2, 10, 66%) were significantly different from the other two, $p < 0.001$. 
Table 2

*Consumption, preference, and BAC values for Experiment 2*

<table>
<thead>
<tr>
<th>Consumption (ml/kg/min)</th>
<th>Weight (g)</th>
<th>Alcohol</th>
<th>Water</th>
<th>Preference</th>
<th>g/kg/min</th>
<th>BAC (nMol/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>M: 374 (24)</td>
<td>0.26 (0.03)</td>
<td>0.23 (0.03)</td>
<td>0.53 (0.05)</td>
<td>108.76 (6.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: 238 (6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>M: 365 (24)</td>
<td>0.27 (0.04)</td>
<td>0.27 (0.03)</td>
<td>0.50 (0.03)</td>
<td>0.01 (0.00)</td>
<td>108.64 (8.6)</td>
</tr>
<tr>
<td></td>
<td>F: 237 (7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td>M: 365 (24)</td>
<td>0.37 (0.06)</td>
<td>0.22 (0.02)</td>
<td>0.61 (0.03)</td>
<td>0.04 (0.01)</td>
<td>263.36 (36.3)</td>
</tr>
<tr>
<td></td>
<td>F: 241 (6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66%</td>
<td>M: 362 (22)</td>
<td>0.34 (0.04)</td>
<td>0.23 (0.02)</td>
<td>0.58 (0.03)</td>
<td>0.77 (0.09)</td>
<td>339.86 (62.5)</td>
</tr>
<tr>
<td></td>
<td>F: 238 (5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note.* Mean (±SEM) for all above measures. Values were obtained from the second day of preference testing for each alcohol concentration. The conditions were presented in a within-subject design. M represents males, while F represents females.

**Summary of Results**

Experiment 2 demonstrated that voluntary, oral consumption via a free-choice preference test situation was capable of yielding pharmacologically relevant BAC levels in outbred Wistar rats. Alcohol consumption was initiated without the use of special methods. Naïve Wistar rats consumed 2, 10, and 66% alcohol from the very first exposure to the solution. Furthermore, significantly higher BAC levels were observed for the 10% and 66% alcohol conditions,
suggesting that it is possible to use a high-concentration alcohol solution in an animal model of alcohol consumption.

**Experiment 3 – High-concentration alcohol consumption under conditions of continuous access to food**

Experiment 1 showed that food deprived Wistar rats voluntarily consumed 66% alcohol. The primary objective of Experiment 3 was to assess the consumption of, and preference for high concentration, 66% alcohol under conditions of continuous access to rodent chow. In this experiment, rats were never subjected to conditions of food deprivation, and instead, had food freely available 24/7 in a metal hopper hanging inside of their home cages. During typical preference testing experiments, food is not available during the experimental session, but in this experiment, food and water were always available.

**Method**

**Subjects.** Subjects were 12 Wistar rats, 6 male and 6 female, all experimentally naïve, bred from parents purchased from Charles River Laboratories (males from Raleigh, NC, and females from Sherbrooke, Canada). Mean (±SEM) ad lib weights were 489.7 g (16.9 g) for males and 299.7 g (4.6 g) for females. All animals were maintained as described in Experiment 1 but were never subjected to food deprivation. Weights were assessed daily for 10 days prior to beginning the experiment, and daily during the 10 days of the experiment (20 days total) to measure potential changes in body weight as alcohol consumption started during preference testing.

**Apparatus.** The same 2-h, 2-bottle preference test was used as described in Experiment 1. Rats had access to only one concentration, 66% alcohol, and deionized water. A metal food
hopper was mounted inside of the home cages to ensure continuous access to food 24/7 during the experiment.

**Procedure.** Subjects began training once they reached the target deprivation weight. All rats ($N = 12$) in their home cages were placed on a transport rack and transported from the colony into a separate testing room to begin the preference test. Since food was continuously available, supplemental postsession feeding was not necessary. All other procedural aspects were as described in Experiment 1.

**Results**

The results from Experiment 3 are presented in Figure 4 for consumption (top) and preference (bottom), and Figure 5 for body weight. It is clear through visual inspection that preference for 66% alcohol disappeared with access to continuously available rodent chow. This drop in preference suggests the role of food deprivation as a contributing factor for developing a preference for high concentration alcohol. Despite a reduction in preference for 66% alcohol, two outcomes in this experiment are consistent with prior results. First, these animals displayed no signs of aversion to the 66% alcohol solutions, they just consumed more water than in previous experiments. In all sessions, animals consumed 66% alcohol, and select animals did maintain a preference ratio above 0.5. Second, the levels of absolute alcohol consumption observed in this experiment were similar to previous experiments from this lab.

A Bottle ($A$, $W$) by Session (1-10) ANOVA revealed significant interaction, $F(9, 81) = 2.67, p < 0.01, \eta^2 = 0.23$, and session effects, $F(9, 81) = 14.83, p < 0.001, \eta^2 = 0.62$, but a nonsignificant difference between the consumption of alcohol and water, $F < 1$. Pairwise LSD tests indicated that the consumption of alcohol was not significantly different from water in any of the 10 sessions, $ps > 0.09$. Preference ratios were analyzed using one-sample $t$-Tests against
the point of indifference (0.5). These analyses revealed nonsignificant differences from 0.5 for all sessions, $ps > 0.11$. Taken together, these results suggest no evidence for either preference or rejection of 66% alcohol relative to water.

Analyses were conducted on the rats’ daily weights. As expected, and consistent with visual inspection, the weights increased each day, and male rats weighed more than female rats. No indication of body weight being affected by preference testing or alcohol consumption was found. Fitting the weights to a straight line yielded coefficients of determination greater than 0.92 for male and female rats (see Figure 5).

Figure 4. Consumption of alcohol (A) and water (W) by animal’s body weight during the preference testing sessions (1-10; top). Preference ratio for 66% alcohol (bottom).
Summary of Results

Experiment 3 demonstrated that providing rats with continuous access to food eliminated the preference for 66% alcohol over water. It is important to note, however, that these animals did not reject the alcohol solution, nor did they refuse to consume it. Water consumption increased relative to previous experiments, yielding a reduction in preference for alcohol.

Experiment 4 – Rewarding properties of 66% alcohol in an operant licking paradigm

The primary objective of Experiment 4 was to assess the rewarding properties of a high concentration, 66% alcohol solution. Operant behavior is “instrumental” in that it yields some consequence and is not simply reflexive (Skinner, 1963). Operant conditioning paradigms are often used to explore the rewarding value of primary reinforcers such as food and water. Organisms are trained to engage in a response that yields some consequence (e.g., make one
lever press to receive one food pellet) and behavior is measured over time. Consequences with high reward value usually yield higher rates of responding than consequences with low reward value. During acquisition, responses that are met with reinforcement tend to be strengthened. However, during extinction reinforcement is withheld and responding usually decreases. If responding decreases once the reinforcer no longer follows a response, then it can be assumed that the behavior was maintained by access to the reinforcer.

Data from Experiments 1-3 demonstrated that rats will voluntarily consume 66% alcohol. In Experiment 4, as an initial test of the rewarding properties of 66% alcohol, rats were exposed to a self-administration protocol involving one 5-min free access pre-exposure session followed by ten 10-trial acquisition sessions, and ten 10-trial extinction sessions. In these sessions, one lick on an empty tube (Sipper 1) would give animals access to a second tube containing 66% alcohol (Sipper 2).

Method

**Subjects.** Subjects were 12 experimentally naïve Wistar rats, 6 males and 6 females. Animals were maintained and deprived as described in Experiment 1.

**Apparatus.** Self-administration testing took place in eight conditioning boxes (MED Associates, St. Albans, VT) constructed out of aluminum and Plexiglas (29.4-cm long, 24.7-cm wide, and 28.9-cm high) with floors made of steel rods (0.5 cm in diameter, 1.2-cm apart). A tray filled with 0.3-cm corn cob bedding was placed below the floor to collect feces and urine. Feces were removed at the end of each daily session and bedding was replaced as needed. Each box was equipped with two sipper tubes, but only two of them were used in this experiment. Sipper tubes were automatically inserted through two holes in the feeder wall (1-cm wide, 2-cm high, and 4 cm from the floor, 1 cm in diameter) and spaced 16.5 cm apart. A house light located in
the upper part of a wall opposite to the sipper tubes was used to produce diffuse light. Each conditioning box was enclosed in a sound-attenuating chamber equipped with a fan for ventilation and a speaker to deliver masking white noise, which collectively produced noise at an intensity of 80.1 dB. A computer equipped with MED-PC IV located in an adjacent room automatically controlled the presentation and retraction of the sipper tubes, and recorded contacts with the sipper tubes through a circuit connecting the steel rod floor and the sipper tubes.

Two 100-ml, glass bottles with a 3.81-cm opening equipped with #6.5 rubber stoppers with 8.89-cm long straight ball bearing metal sippers attached (Ancare, Bellmore, NY) were secured to each conditioning box during the self-administration test. Bottles were labeled on the side with plastic tape to indicate sipper number and contents, and thus minimize experimenter error.

**Procedure.** Subjects began training once they reached the target deprivation weight. All rats \((N = 12)\) in their home cages were placed on a transport rack and transported from the colony into a separate testing room to begin the self-administration test. Rats were randomly assigned to one of the eight conditioning boxes and placed into the same box throughout the experiment. Rats received free access to 66% alcohol (vol/vol) during a single 5-min preexposure session where Sipper 2 was presented alone. Self-administration training began the following day. Acquisition of self-administration behavior was conducted through ten 10-trial sessions using an FR1 schedule (i.e., all responses were reinforced). Rats received access to Sipper 1, an empty bottle, until one response had been detected or a maximum of 10 s had elapsed, whichever occurred first (a Pavlovian/instrumental procedure). Then, Sipper 1 was retracted, and Sipper 2 was presented. A response on Sipper 2 was followed by 5 s of access to the sipper. In the absence of a response, Sipper 2 remained available for a maximum of 10 s. Sipper 2 contained
66% alcohol during acquisition. A variable ITI (mean: 90 s; range: 60-120 s) followed the retraction of Sipper 2. Extinction training started one day after the tenth acquisition session, and lasted 10 daily sessions. Extinction sessions were as acquisition sessions, except that Sipper 2 contained deionized water. The relative position of Sippers 1 and 2 (right or left) were counterbalanced across rats.

Thus, training involved a mixture of Pavlovian/instrumental contingencies. Rats were able to decrease their wait time to access Sipper 2 by making a response (licking) to Sipper 1 (empty bottle). Subjects were fed at least 30 min after the end of the daily session to maintain the target body weight. The dependent measures were the latency to make first contact with Sipper 1 (measured in 0.01-s units) and the licks made on Sipper 2. Latency is an anticipatory response, whereas lick frequency is a consummatory response.

**Results**

Figure 6 shows the results for the latency to respond to Sipper 1 (top) and licks per trial on Sipper 2 (bottom). Here, an apparent reduction in latency occurred during the acquisition phase, that dissipates in extinction. Licks per trial to the 66% alcohol solution were also observed to increase during acquisition, whereas once extinction was implemented and the solution changed to water, the licks per trial visibly decreased. An ANOVA on the latency to respond to Sipper 1 during acquisition revealed a significant reduction in latency from session 1 to session 10, $F(9, 99) = 6.03, p < 0.001, \eta^2 = 0.35$. There was also a significant increase in latency during extinction (sessions 11-20), $F(9, 90) = 2.81, p < 0.007, \eta^2 = 0.20$. Licking behavior for Sipper 2 (66% alcohol in acquisition and water during extinction) demonstrated an significant increasing trend during the acquisition phase and a significant decreasing trend during the extinction phase, $Fs(9, 90) > 2.32, ps < 0.02, \eta^2 = 0.17$. These data suggest that 66% alcohol may possess
Rewarding properties as Wistar rats exhibit significantly increasing latencies, and significantly decreasing licks during extinction (water for Sipper 2).

Figure 6. Top: Latency to respond (seconds) to Sipper 1 (empty tube). Bottom: licks per trial made to Sipper 2 (66% alcohol during acquisition, water during extinction).

Summary of Results

Experiment 4 demonstrated that naïve rats were able to learn the operant licking self-administration task and lick at an empty sipper (Sipper 1) to gain access to a 66% alcohol
solution (Sipper 2). We observed a rapid acquisition of the operant licking behavior that was slowly extinguished when the 66% alcohol solution was replaced with water. The animals’ performance in the extinction phase suggests some reward value of the 66% solution because their licking behavior was successfully attenuated following the omission of the 66% alcohol.

**Experiment 5 – Progressive ratio performance and the reward value of alcohol solutions: Within-subject design**

The primary objective of Experiment 5 was to use a progressive ratio (PR) schedule to determine whether rats would develop persistence in the alcohol self-administration situation when the response requirement was increased in steps. PR schedules assess the point at which a rat would no longer engage in some number of responses to gain access the specified reward concentration (breakpoint). This provided a behavioral measure of the rewarding value of different concentrations of alcohol.

Czachowski and Samson (1999) trained rats to self-administer 10% alcohol on a FR4 schedule before exposing them to daily sessions of a PR schedule progressing from 4, 8, 12, 16, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, etc. The breakpoint was defined as failing to complete the required number of responses within 20 min. In a different PR test, Gilpin and Koob (2010) first trained Wistar rats to self-administer 10% alcohol on a FR1 schedule then exposed them to a single session of a PR schedule progressing as: 1, 1, 2, 2, 3, 3, 4, 4, 5, 5, 7, 7, 9, 9, 11, 11, 13, 13, 15, 15, 18, 18, 21, 21, 24, 24, etc. until the breakpoint was reached. This was defined as a rat failing to respond within a 15-min period during the single session.

A low breakpoint suggests that the alcohol concentration had little rewarding value, whereas a high breakpoint suggests that the specified concentration functioned as a high-value reward due to the animal exerting more response effort to access the concentration. Thus, a
motivational component also exists within the rats’ responding for alcohol. Additional test would be needed to determine whether the PR results are caused by motor impairment or facilitation caused by alcohol consumption. The breakpoints for 2, 10, and 66% alcohol were determined for each animal using a within-subject design.

**Method**

**Subjects and apparatus.** A total of 8 experimentally naïve Wistar rats (4 males and 4 females) served as subjects in Experiment 5. Animals were maintained and deprived as described in Experiment 1. The same conditioning boxes described in Experiment 4 were used in Experiment 5.

**Procedure.** Once rats reached their target deprivation weight, they began training in the self-administration paradigm on a FR1 schedule where every response yielded a reward. After three daily sessions of training under FR1 conditions a PR schedule was introduced for one of the alcohol concentrations. The number of responses required increased in a constant stepwise fashion (step=4): 1, 5, 9, 13, 17, 21, 25, etc., and the breakpoint for that concentration was defined as the point at which an individual rat stopped responding for alcohol for 10-min. The order in which rats received each alcohol concentration under PR conditions was counterbalanced across rats. Individual rats ended PR training for each daily session at different times depending on their individual performance. They were left in the chambers, in the dark, until all animals finished their respective sessions. At the end of the originally planned experiment, three additional sessions were added, in which all rats received access to water in Sipper 2. This addition provided a baseline responding. These sessions were run consecutively.
Results

Figure 7 shows the results for the PR breakpoint (top) and licks per trial on Sipper 2 (bottom). The PR breakpoint measure appears visually consistent for the 2, 10, and 66% alcohol concentrations, and the lowest for water. The mean lick frequency measure appears visually consistent for the 2 and 10% alcohol and water solutions. The mean lick frequency is the lowest for the 66% alcohol concentration. A within-subject ANOVA on the PR breakpoint for responding Sipper 1 revealed a significant effect of concentration, $F(3, 21) = 3.32, p < 0.04, \eta^2 = 0.32$. Pairwise LSD tests showed that 0% differed significantly from each of the other concentrations (2, 10, and 66% alcohol), $ps < 0.04$. A second analysis between the 2, 10, and 66% alcohol concentrations revealed a nonsignificant effect of concentration, $F < 1$.

A within-subject ANOVA on the licks per trial revealed no significant differences in licking for any of the concentrations $F(3, 21) = 2.74, p > 0.69, \eta^2 = 0.28$. A second analysis between the 2, 10, and 66% alcohol concentrations revealed a nonsignificant effect of concentration, $F(2, 14) = 3.20, p > 0.07, \eta^2 = 0.31$. Further analyses between 0% and 2% revealed a nonsignificant effect of concentration, $F < 1$, as well as a nonsignificant effect between 0% and 10%, $F(1, 7) = 5.26, p > 0.06, \eta^2 = 0.43$, and nonsignificant effects between 0% and 66%, $F(1, 7) = 2.51, p > 0.15, \eta^2 = 0.26$. Although some of the differences are borderline and effect sizes are considerable, none of them achieved significance by the standard used in these experiments.

Summary of Results

Interestingly, the breakpoints for 2, 10, and 66% alcohol were similar, and they all differed from 0%. This suggests that rats were willing to work equally for 2, 10, and 66% alcohol, but expended significantly less response effort when presented with water. Further,
when presented with the solutions, the mean lick frequency for all of the concentrations, including 0%, were not significantly different from each other. These data bring into question the rewarding value of each concentration, and present another question: What other factors contribute to their willingness to lick these solutions?

*Figure 7.* Top: PR breakpoint for 2, 10, 66% alcohol, and water. Bottom: Licks per trial for each concentration.
The use of a within-subject design could have enhanced the similarities among solutions according to several mechanisms. For example, hedonic responses in rodents are typically characterized by a direct correlation between increased licking for palatable (appetitive) solutions and decreased licking for unpalatable (aversive) solutions (Dwyer, 2012). If the hedonic value of all four solutions is similar, then it is reasonable to expect a similar number of licks for each of the solutions. However, the results of Experiment 1 suggest these concentrations are differentially preferred against water in a direct comparison. Perhaps the within-subject procedure obscured palatability differences among alcohol concentrations. In addition, since each rat tasted all the alcohol concentrations, this may have stimulated generalization of orosensory stimuli, thus supporting similarity in behavior among concentrations. Alcohol has also been shown to activate the insular cortex and other brain regions that modulate appetite, feeding, and drinking behaviors (Brasser et al., 2012). Similar levels of licking behavior for each of the concentrations could reflect that these concentrations were not discriminable for rats. However, this account seems unlikely since the results of Experiment 1 showed that rats are able to discriminate alcohol solutions of a variety of concentrations. If the within-subject design played a role in these results, then further testing using a between-subject design with naïve animals should produce different results. Experiment 6 was designed to provide an answer to this question.

**Experiment 6 – Progressive ratio performance and the reward value of alcohol solutions: Between-subject design**

The rationale behind Experiment 6 comes from the idea that perhaps being exposed to each alcohol concentration (2, 10, and 66%) in a counterbalanced order matched the breakpoints across alcohol concentrations via stimulus, hedonic, or appetite generalization. Accordingly,
Experiment 6 used a between-subject design to minimize generalization across alcohol concentrations. Based on the results of Experiments 2 and 5, the 2% concentration was dropped since it did not differ from water; only 0, 10, and 66% alcohol concentrations were used.

**Method**

**Subjects and apparatus.** A total of 24 experimentally naïve Wistar rats (12 males and 12 females) served as subjects. Animals were randomly assigned to one of three groups ($n = 8$): 0%, 10%, or 66% alcohol. Animals were maintained and deprived as described in Experiment 1. The same conditioning boxes described in Experiment 4 were used in Experiment 6. The same PR paradigm was used as described in Experiment 5, except that each animal was randomly assigned to only one of the three selected concentrations throughout the experiment.

**Results**

Figure 8 shows the results in terms of the mean breakpoint on Sipper 1 (top) across all sessions. The lowest breakpoint and mean lick frequency were observed for water, while the highest for both measures was 10% alcohol.

A between-subject ANOVA on the breakpoints for 0, 10, and 66% alcohol revealed a significant interaction between group and concentration, $F(2, 215) = 18.24, p < 0.001, \eta^2 = 0.15$. Pairwise LSD tests showed that 10% alcohol differed significantly from both 0 and 66% alcohol, $p < 0.001$, but that 0 and 66% did not differ significantly from each other, $p > 0.08$.

A between-subject ANOVA on lick frequency for 0, 10, and 66% alcohol revealed a significant interaction between group and concentration, $F(2, 215) = 30.91, p < 0.001, \eta^2 = 0.23$. Pairwise LSD tests showed that all concentrations of alcohol, 0, 10, and 66%, differed significantly from one another in terms of mean lick frequency, $p < 0.002$. 
Figure 8. Top: PR breakpoint for 2, 10, 66% alcohol, and water. Bottom: Licks per trial for each concentration.

These results also differ with respect to those obtained from Experiment 5. Here, the selected concentrations were differentiated, with 10% alcohol yielding the highest breakpoint and lick frequency, followed by 66% alcohol, and 0% alcohol (water). However, in Experiment 5 these measures for 10% and 66% alcohol were equivalent. These findings suggest that the within-subject nature of Experiment 5 and the presentation order of each concentration might have affected licking behavior. This could provide a potential explanation for the observed
differences in breakpoint and lick frequency when evaluated using a between-subject design. Experiment 8 also employed a between-subject design to evaluate PR responding for alcohol in one phase, though using only the 66% concentration.

**Summary of Results**

Though it was posited that rats would expend the most response effort for the high concentration, 66% alcohol, these data revealed that the highest breakpoint and lick frequency were allocated to 10% alcohol. As expected, water had the lowest breakpoint and lowest lick frequency. Though 66% alcohol did not have the highest breakpoint, these data suggest that rats were still willing to expend a considerable amount of response effort to obtain access to the solution. Taken together, these results suggest that high concentrations of alcohol, while still acceptable, may have less rewarding value than lower concentrations of alcohol.

These results also differ with respect to those obtained from Experiment 5. Here, the selected concentrations were differentiated, with 10% alcohol yielding the highest breakpoint and lick frequency, followed by 66% alcohol, and 0% alcohol (water). However, in Experiment 5 these measures for 10% and 66% alcohol were equivalent. These findings suggest that the within-subject nature of Experiment 5 and the presentation order of each concentration might have affected licking behavior. This could provide a potential explanation for the observed differences in breakpoint and lick frequency when evaluated using a between-subject design. Experiment 8 also employed a between-subject design to evaluate PR responding for alcohol in one phase, though using only the 66% concentration.
Experiment 7 – Individual differences in the rewarding properties of 66% alcohol vs. water

Experiment 7 was designed as an extension of Experiment 4. First, rats were exposed to the same self-administration protocol used in Experiment 4 except that the number of trials per session was increased from 10, to 20 and the length of access to Sippers 1 and 2 were increased. These changes were implemented based on the results of Experiment 4. It was expected that increasing the number of trials per session and the length of access to both sippers would allow animals to gain additional experience with the paradigm during the acquisition phase, and thus, experience the effects of the extinction phase more saliently. Two additional phases were added to Experiment 7 such that Phase 1 involved self-administration, Phase 2 involved a test of locomotor activity in the open field, and Phase 3 involved a reward downshift from 32% to 2% sucrose. I decided to add these phases based on the emergence of individual differences in behavior in animals with access to alcohol during the acquisition sessions, Phase 1.

Phase 2 was designed to measure the effects of presession alcohol (or water) exposure on locomotor activity in the open field. Rodents find open spaces threatening and the open field test detects that in terms of activity (Denenberg, 1969). Of particular interest was the time spent in the center vs. the periphery of the well-lit open field arena as this provides an index of the anxiolytic effects of 66% alcohol (Acevedo, Nizhnikov, Molina, & Pautassi, 2014).

Phase 3 was designed as an additional assessment of these animal’s response to an anxiety-inducing situation. Animals that responded differentially for alcohol were compared in their response to a 32-to-2% sucrose downshift. Such a downshift is similar to the downshift alcohol groups experienced in Phase 1 when they transitioned from acquisition with 66% alcohol to alcohol omission in extinction sessions. Cuenya et al. (2015) reported that training from one
task can impact observed performance in a separate, subsequent task. This phenomenon is referred to as a transfer effect. A series of studies by Glueck, Torres, and Papini (2018) explored transfer effects between anticipatory and consummatory tasks involving reward loss. Seven experiments were conducted, each involving at least two successive phases. Task sequence was evaluated for evidence of transfer effects. In one experiment, transfer from cSNC produced an increase in resistance to extinction in an autoshaping task. In other experiments, prior training in autoshaping, one-way avoidance, and taste conditioning either enhance the cSNC effect or had an impact on performance. This evidence suggests a potential role of transfer within the current experiment as animals move from Phase 1’s downshift into Phase 2’s locomotor activity test, and Phase 3’s self-administration test.

A final consideration in the planning of these additional phases was to model them after those proposed in Experiment 8. Having the same phases in a different order in Experiments 7 and 8 would provide insight on any potential effects of task order, though these experiments will not be directly compared statistically. As evidenced by Glueck et al. (2018), transfer effects involving cSNC are not always consistent, so that it is difficult to predict the specific effect that the order of task presentation could have in the current experiment.

Method

Subjects and apparatus. Subjects were 36 experimentally naïve Wistar rats, 18 males and 18 females. Initially, rats were to be evenly split between groups A and W; however, at the end of the first replication of Phase 1, individual differences in response latency became clear. Thus, rats were randomly assigned to Groups A ($n = 24$) and W ($n = 12$). In addition, rats in Group A were segregated into alcohol fast responders (AFR, $n = 6$) and alcohol slow responders (ASR, $n = 18$) as a function of the latency recorded during the final five sessions of acquisition.
Rats with latencies to respond to Sipper 1 (empty) lower than 7.5 s were classified as AFRs and those with latencies higher than 7.5 s were classified as ASRs. Animals were maintained and deprived as described in Experiment 1. Self-administration testing took place in the same conditioning boxes described in Experiment 4. Before this experiment began, each box was upgraded to be equipped with an additional sipper tube (totaling three per box), though only the two sippers in the lateral positions were used in this experiment.

**Procedure.** Once rats reached their target deprivation weight they were matched for weight and randomly assigned to one of two groups based on substance: A (n = 24), W (n = 12) with sex divided equally between groups. Subjects began training in the three phases of this experiment once they reached the target deprivation weight. For Phase 1, the same self-administration procedure was used as described in Experiment 4 with the following changes: the two lateral sippers were used, the number of trials per session was increased from 10 to 20 to allow more exposure to the task, the maximum presentation time of Sipper 1 was increased from 10 s to 15 s, and the amount of access to Sipper 2 was increased from 5 s to 10 s. The two experimental groups were further subcategorized into ASR (n = 18), AFR (n = 6), and W (n = 12) based on their performance during the acquisition phase of Phase 1. It became evident based on a visual inspection of the data during the first replication that there were large individual differences in the latency to respond to Sipper 1 (empty bottle).

In Phase 2, all animals received three 5-min free-access sessions with alcohol or water (depending on their group assignment) in the same modular chambers used for the self-administration task. On the third day of Phase 2, a 15-min postsession interval was implemented, and rats were placed into a well-lit open field arena for a 20-min session. Dependent measures included distance traveled (cm) as a function of the open field area: center vs. periphery.
In Phase 3, all animals were run through a reward downshift task where they received 10 daily sessions of 5-min free access to 32% sucrose followed by 5 daily sessions of 5-min free access to 2% sucrose. Lick frequency was the dependent variable.

**Results**

**Self-administration.** Figure 9 shows the results for the latency to respond to Sipper 1 (top) and licks per trial on Sipper 2 (bottom). Groups ASR and W exhibited similar response latencies to Sipper 1 during both acquisition and extinction, while the Group AFR exhibited a decreased latency throughout acquisition which stabilized during extinction at a somewhat higher level than that exhibited in the final acquisition sessions. The bottom panel of Figure 9 shows that animals in Group W tended to lick more than rats receiving 66% alcohol in Groups ASR and AFR. A Group (ASR, AFR, W) by Session (1-10) ANOVA on the latency to respond to Sipper 1 during acquisition revealed a significant interaction between Group and Acquisition Session, \(F(18, 297) = 3.16, p < 0.001, \eta^2 = 0.16\), as well as a significant reduction in latency from session 1 to session 10, \(F(9, 297) = 3.88, p < 0.001, \eta^2 = 0.11\), as well as a significant difference between groups, \(F(1, 33) = 19.26, p < 0.001, \eta^2 = 0.54\). Pairwise LSD tests on the main analysis suggest that the Group AFR was significantly different from the Groups ASR and W, \(ps < 0.001\), though Groups ASR and W did not differ significantly, \(p > 0.67\). A Group (ASR, AFR, W) by Extinction Session (11-20) ANOVA revealed a nonsignificant interaction between Group and Extinction Session, as well as a nonsignificant effects Session, \(F < 1\), however, the Group effect was significant, \(F(1, 32) = 14.05, p < 0.001, \eta^2 = 0.47\). Pairwise LSD tests revealed that Group AFR differed significantly from Groups ASR and W, \(ps < 0.001\), though Groups ASR and W did not differ from one another, \(p > 0.97\).
A Group (ASR, AFR, W) by Session (1-10) ANOVA on the lick frequency to Sipper 2 (containing alcohol or water) during acquisition revealed a significant interaction between Group and Session, $F(18, 297) = 48.21, p < 0.03, \eta^2 = 0.10$, as well as a significant effect of session, $F(9, 297) = 2.06, p < 0.04, \eta^2 = 0.06$, but not group, $F(1, 33) = 1.84, p > 0.17, \eta^2 = 0.10$. A Group (ASR, AFR, W) by Session (11-20) ANOVA on the lick frequency to Sipper 2 containing only water during extinction revealed a significant interaction between group and session, $F(18, 297) = 1.75, p < 0.03, \eta^2 = 0.10$, but nonsignificant effects of session and group, $F_s < 1.40$.

Figure 9. Top: Latency to respond (seconds) to Sipper 1 (empty tube). Bottom: licks per trial made to Sipper 2 (66% alcohol or water during acquisition, water during extinction).
**Alcohol and Open field.** Figure 10 shows the results for the licks per 5-min free access session to the substance originally assigned to each group (alcohol or water: top), and the center vs. periphery data in the open field (bottom). Rats in Group W exhibit more free access licks to water than rats receiving free access to 66% alcohol in Groups ASR and AFR. In the OF portion, all groups demonstrated more locomotor activity in the periphery of the OF arena than the center.

*Figure 10. Top: Licks per 5-min free access session to 66% alcohol or water. Bottom: Open field distance traveled in center vs. periphery. C: center. P: periphery.*
A Group (ASR, AFR, W) by Session (21-23) ANOVA on 66% alcohol or water consumption during the three sessions of free access revealed a nonsignificant interaction between group and session, $F(4, 66) = 1.80, p > 0.14, \eta^2 = 0.09$, as well as a nonsignificant effect of group, $F < 1$, but a significant effect of session, $F(2, 66) = 6.45, p < 0.03, \eta^2 = 0.16$. A second analysis comparing Groups ASR and AFR revealed a nonsignificant interaction between group and session, $F(2, 44) = 1.53, p > 0.22, \eta^2 = 0.06$, as well as nonsignificant effects of group, $F < 1$, and session, $F(2, 44) = 2.96, p > 0.06, \eta^2 = 0.12$. Next, an analysis was conducted between Groups ASR and W, revealing a nonsignificant interaction between group and session, $F(2, 56) = 2.23, p > 0.12, \eta^2 = 0.07$, as well as a nonsignificant effect of group, $F < 1$, but a significant effect of session, $F(2, 56) = 6.08, p < 0.04, \eta^2 = 0.18$. Pairwise LSD tests suggested a significant difference in free access licking between sessions 21 and 23, $p < 0.006$, but not between sessions 21 and 22, or 22 and 23, $ps > 0.05$. Finally, an analysis between Groups AFR and W revealed a nonsignificant interaction between group and session, $F(2, 32) = 1.29, p > 0.29, \eta^2 = 0.07$, as well as a nonsignificant effect of group, $F < 1$, but a significant effect of session, $F(2, 32) = 5.30, p < 0.01, \eta^2 = 0.25$. Pairwise LSD tests suggested a significant difference in free access licking between sessions 21 and 23, $p < 0.04$, but not between sessions 21 and 22, or 22 and 23, $ps > 0.06$.

A Group (ASR, AFR, W) by Area (Center, Periphery) ANOVA revealed a nonsignificant interaction between group and area, as well as a nonsignificant effect of group, $Fs < 1$, but a significantly higher distance traveled in the periphery than in the center, $F(1, 17) = 41.66, p < 0.001, \eta^2 = 0.10$. A second analysis was conducted between Groups ASR and AFR, revealing a nonsignificant interaction between group and area, as well as a nonsignificant effect of group, $Fs < 1$, but a significantly higher distance traveled in the periphery than in the center area, $F(1, 13)$.
was 18.99, a third analysis conducted between Groups ASR and W revealed a nonsignificant interaction between group and area, $F(1, 16) = 1.29, p > 0.27, \eta^2 = 0.07$, as well as a nonsignificant effect of group, $F(1, 16) = 1.27, p > 0.27, \eta^2 = 0.07$, but a significantly higher distance traveled in the periphery than in the center area, $F(1, 16) = 56.56, p < 0.001, \eta^2 = 0.78$. Finally, a comparison between Groups AFR and W indicated a nonsignificant interaction between group and area, $F < 1$, as well as a nonsignificant effect of group, $F(1, 5) = 1.13, p > 0.33, \eta^2 = 0.18$, but a significantly higher distance traveled in the periphery than in the center area, $F(1, 5) = 16.08, p < 0.001, \eta^2 = 0.76$.

**Reward downshift.** Figure 11 shows the results for the licks per 5-min free access session to sucrose. While licks to 32% sucrose in the preshift phase remain consistent between groups, a difference emerges during the postshift phase. Animals in Group AFR showed a faster recovery from downshift and engaged in more licks to the 2% sucrose solution than the other two groups. A Group (ASR, AFR, W) by Preshift Session (1-10) ANOVA on sucrose licks revealed a significant interaction, $F(18, 297) = 2.27, p < 0.003, \eta^2 = 0.12$, as well as a significant effect of session, $F(9, 297) = 5.67, p < 0.001, \eta^2 = 0.14$, but not of group, $F(1, 33) = 1.43, p > 0.25, \eta^2 = 0.08$. Pairwise LSD tests on the main interaction revealed that animals in Group W licked significantly less than animals in Group AFR on sessions 1 and 9, $ps < 0.03$. A Group (ASR, AFR, W) by Postshift Session (11-15) ANOVA on sucrose licks revealed a nonsignificant interaction between group and session, $F < 1$, but a significant effect of session, $F(4, 132) = 18.99, p < 0.001, \eta^2 = 0.37$, but not group, $F(1, 33) = 2.88, p < 0.08, \eta^2 = 0.15$. To further explore the larger suppression of consummatory behavior exhibited by AFR animals relative to ASR animals, an additional Group (ASR, AFR) by Preshift Session (1-10) ANOVA on sucrose licks was conducted. The results revealed a significant interaction between group and session, $F(9,)
198) = 3.08, $p < 0.003$, $\eta^2 = 0.12$, as well as a significant effect of session, $F(9, 198) = 5.02, p < 0.001$, $\eta^2 = 0.19$, but not group, $F < 1$. It should be considered that the interaction effect observed here may only be due to the crossing over of these functions as opposed to an orderly group effect across sessions. Upon visual inspection of Figure 11, it is clear that the animals in Group AFR recover more quickly in the postshift phase, and Groups ASR and W cross over on session 13, or the third downshifted session. A Group (ASR, AFR) by Postshift Session (11-15) ANOVA on sucrose licks revealed a nonsignificant interaction between group and session, $F < 1$, but significant effect of session, $F(4, 88) = 9.78, p < 0.001$, $\eta^2 = 0.31$, and, importantly, a significant group difference, $F(1, 22) = 5.63, p < 0.03$, $\eta^2 = 0.20$. This suggests that rats with reduced latencies to Sipper 1 signaling 66% alcohol during Phase 1 also exhibit a less pronounced effect after a 32-to-2% sucrose downshift.

![Graph](image-url)  

*Figure 11.* Licks per 5-min reward downshift session. 32% sucrose in sessions 1-10 and 2% sucrose in sessions 11-15.
Summary of Results

The data from Experiment 7 suggest that some rats (AFR) are motivated to respond for access to a 66% alcohol solution than others (ASR), and that this behavioral response can be extinguished when access to alcohol is replaced by access to water. In Phase 2 when animals were exposed to alcohol or water prior to their open field task, all three groups behaved similarly. However, AFRs traveled less distance than W animals, thus showing an anxiolytic effect of 66% alcohol intake. This anxiolytic effect was not observed in ASRs. In Phase 3 when animals were exposed to reward downshift, AFR rats showed less consummatory suppression than ASR rats. AFR animals, which exhibited learning in the operant licking task, also demonstrated faster recovery on sessions 11-12 relative to ASR animals. These AFR animals exhibited a decreased latency to respond to Sipper 1 signaling 66% alcohol in Phase 1 relative to both the ASR and W animals. Further, the AFR animals’ latency remained lower than the other groups’ during the extinction phase when they were working for access to water only.

Experiment 8 – Individual differences in reward downshift and alcohol consumption

The primary objective of Experiment 8 was to assess the potential connection between frustration induced by reward downshift and the voluntary oral consumption of 66% alcohol using an individual-differences approach. In a typical cSNC experiment animals are first trained to expect a high value 32% sucrose reward for ten sessions before their expectations are violated during five sessions with access to a low value 4% sucrose reward. Their consummatory behavior is compared to a control group that always receives 4% sucrose. On the first downshifted session (Session 11), 32-to-4% sucrose animals typically exhibit a marked decrease in consumption compared to their preshift performance and to the control group. This cSNC
effect induces frustration (Papini et al., 2015), though the effect is transient and the difference in consumption typically dissipates by Session 15 with consumption of the 4% solution rising to control levels (Flaherty, 1996).

Papini, Galatzer-Levy, and Papini (2014) statistically categorized three types of recovery profiles in rats following a 32-to-4% sucrose reward downshift. Within their sample of rats ($N = 262$), 83% of the animals recovered control levels of sucrose consumption by the end of the downshift period (recovery profile) while 11% were unable to recover to their preshift levels of consumption (no recovery profile). A no contrast profile was also observed in 6% of the animals and was characterized by no change in the level of sucrose consumption between the preshift and postshift phases.

Ortega et al. (2014) explored the effects of selective breeding on recovery from cSNC in Long-Evans rats. After being exposed to a 32-to-4% sucrose reward downshift, three groups of rats were bred for five generations. Two groups involved males and females classified as high-recovery or low-recovery animals based on their consummatory performance during the initial two downshift sessions. A third group of animals was bred irrespectively of their consummatory performance on these sessions (random-breeding controls). When all five generations were compared, researchers found that the cSNC effect was reduced in high-recovery rats, but not in low-recovery rats, which did not differ from random breeding controls. Pellegrini, Wood, Daniel, and Papini (2005) evaluated the role of opioid receptors in a reward downshift situation using a procedure similar to that followed in the present experiment. In one study, they exposed rats to a 32-to-4% sucrose downshift and separated them based on their speed of recovery from the downshift into fast-recovery or slow-recovery subgroups. Both fast- and slow-recovery subgroups were injected with the nonselective opioid antagonist naloxone or vehicle (saline) and
exposed to one session of locomotor activity in a narrow, dark compartment. Naloxone was shown to affect the locomotor activity of the slow-recovery subgroup more than that of the fast-recovery subgroup, which suggested that opioid receptors are connected to the speed of recovery from a frustrating event. These results provide the rationale for the present experiment. The aim was to uncover the potential connection between the style of coping with frustration induced by reward downshift and the motivation to consume a high-concentration 66% alcohol solution.

As in the study by Pellegrini et al. (2005), the present experiment will be silent as to whether the connection, if found, can be attributed to genetic or epigenetic effects related to early infancy. These factors are known to affect alcohol consumption. Lines of alcohol-preferring and alcohol-nonpreferring rats have been developed through selective breeding from Wistar stocks (Lumeng, Hawkins, & Li, 1977). Alcohol-preferring rats (P rats) typically consume 5 g/kg/day of alcohol (Bell et al., 2006). Differences in voluntary alcohol consumption have been observed in different strains of rats as well as in rats obtained from different suppliers (Gauvin, Moore, & Holloway, 1993; Goepfrich et al., 2013; Momeni, Segerstrom, & Roman, 2015; Palm, Roman, & Nylander, 2011).

Rockman and Gibson (1992) explored the effects of environmental rearing conditions on the voluntary oral consumption of alcohol in Sprague-Dawley rats. For the first 60 days animals were raised in enriched, isolated, or quasienriched (smaller space than normal enriched) conditions before being presented with gradually increasing concentrations of alcohol from 3-9% in a free-choice test with water and alcohol freely available. The results revealed significant increases in alcohol consumption for quasienriched animals, but no significant differences between enriched and isolated animals suggesting that isolation may not affect alcohol consumption. Conversely, Deehan, Cain, and Kiefer (2007) reported a relationship between
rearing conditions and alcohol consumption in outbred rats. Long-Evans rats were reared in one of three environments: impoverished (isolated in a wire-bottom cage), social (pair housed in a polycarbonate cage), or enriched (in a large metal cage with bedding, 14 novel objects, and 10 cage-mates) until PND 111. Once all animals reached PND 111 they were trained in a sucrose-fading procedure to lever press for 10% alcohol. The results revealed that impoverished rats had higher alcohol response rates than social and enriched rats. In a free-choice test where both 10% alcohol and water were freely available, only impoverished rats preferred alcohol to water.

Ramirez, Esperon, and Peris (2008) tested the effects of providing an environmental enrichment device (chew toy) to adult Sprague-Dawley rats on voluntary consumption of concurrently available alcohol gel. When comparing alcohol consumption across treatments, researchers found no significant differences suggesting that access to enrichment during consumption sessions does not affect voluntary consumption of alcohol gel.

Maternal separation is another factor that has been tested in rodents in relation to alcohol consumption. Lundberg, Abelson, Nylander, and Roman (2017) assigned newborn rat pups to groups receiving either 15 min or 360 min of maternal separation once per day during PND 1 through 21. Pups were removed from their mothers during the light period, placed into new polycarbonate boxes, and moved into an adjacent room during separation. At 12 weeks of age, females were selected to receive 20% alcohol and water 3 days/week in an intermittent access, free-choice preference protocol. After 14 weeks, a third bottle containing 5% alcohol was introduced into the preference test. Testing on the 3-bottle paradigm lasted 6 weeks. No significant differences in alcohol intake were found between the rats that received 15 min or 360 min of maternal separation as pups.
In addition to maternal separation, other stressors have been studied in relation to alcohol consumption. Previous studies have shown that rats selectively increase their voluntary oral consumption of alcohol following exposure to surprising nonreward (Manzo, Donaire et al., 2015; Manzo et al., 2014). This increase in alcohol consumption is posited to reduce frustration (ESM effect) induced by reward loss (Torres & Papini, 2016). In the present study, rats that were more sensitive to frustrative nonreward (slow recovery group) were hypothesized to engage in a higher rate of responding for alcohol relative to rats that are less sensitive to frustrative nonreward (fast recovery group). Experiencing a more intense state of loss-induced negative emotion (frustration) was expected to motivate the slow recovery animals to voluntarily consume more alcohol to experience the anxiolytic properties of alcohol.

**Method**

**Subjects and apparatus.** A total of 40 experimentally naïve Wistar rats (20 males and 20 females) served as subjects in Experiment 8. Animals were maintained and deprived as described in Experiment 1. The 32% sucrose solution was prepared on a weight by weight basis by diluting 320 g of pure cane sugar into 680 g of deionized water. The 2% sucrose solution was prepared by diluting 20 g of pure cane sugar into 980 g of deionized water. The same conditioning boxes described in Experiment 2 were used in Experiment 8.

**Procedure.** Once rats reached their target deprivation weight, they began behavioral testing in three successive phases.

In Phase 1, animals were tested in the reward downshift situation. All rats \((N = 40)\) were transported in their home cages into a separate testing room to begin the reward downshift training. Rats received access to 32% sucrose for 10 daily 5-min preshift sessions before being downshifted to receive access to 2% sucrose for 5 daily 5-min postshift sessions. Lick frequency
was automatically recorded via attached lickometers in the conditioning boxes. During Phase 1 sucrose was accessible via the middle sipper position through a single 8-oz plastic bottle with a #10 rubber stopper equipped with an 8.9-cm straight ball bearing. The lateral sippers were retracted with no bottles present.

After the reward downshift (Phase 1) was completed, rats were separated into two groups according to their rate of recovery from frustration: fast-recovery and slow-recovery. Animals were segregated according to their lick frequency on Sessions 11 and 12. Fast-recovery animals were those showing greater lick frequency on Session 12 than 11. Slow-recovery animals were those showing either equal or lower lick frequency on Session 12 than 11. For all animals, the lick frequency on Session 11 was less than 90% of that shown on Session 10 (i.e., all animals exhibited consummatory suppression upon experiencing reward downshift).

In Phase 2, the fast- and slow-recovery subgroups were exposed to the PR 66% alcohol self-administration procedure described in Experiment 5. Two lateral sipper tubes were used in this experiment as Sipper 1 and Sipper 2, and the middle sipper position, which contained sucrose in Phase 1, remained retracted with no bottle present. The dependent measures were the breakpoint responding on Sipper 1 and licks per trial on Sipper 2.

In Phase 3, animals were exposed to a single 20-min session in a well-lit open field arena. The dependent measure was the distance traveled (cm) in the center vs. the periphery of the arena. Due to equipment malfunction, only 23 (out of 40) animals were subjected to Phase 3.

Results

Reward downshift. Figure 12 shows the data from Phase 1, reward downshift. During the preshift phase, both the Fast and Slow recovery groups are undifferentiated, while in the postshift phase, the Fast recovery group appears to recover sucrose licking somewhat faster than
the Slow recovery group. A Speed (Fast, Slow) by Session (11-12) ANOVA revealed a significant interaction between speed and session, $F(1, 38) = 65.66, p < 0.001, \eta^2 = 0.63$, as well as a marginally nonsignificant effect of speed, $F(1, 38) = 3.78, p > 0.06, \eta^2 = 0.09$, and a nonsignificant effect of session, $F(1, 38) = 1.09, p < 0.31, \eta^2 = 0.03$. Follow-up comparisons from the main analysis using Pairwise LSD tests revealed that the source of the significant interaction between speed and session occurred on Session 12 of downshift, $p < 0.001$.

Figure 12. Sucrose licks during Phase 1: Reward downshift. 32% sucrose during sessions 1-10 and 2% sucrose during sessions 11-15.

**PR self-administration.** Figure 13 shows the data from Phase 2, PR self-administration. The breakpoint measure for the Fast recovery group appears lower than the Slow recovery group, while the mean lick frequency measures appear visually similar. A between-subject ANOVA on the PR breakpoint (Sipper 1) comparing Fast and Slow recovery groups revealed a significant difference between the groups, $F(1, 343) = 6.76, p < 0.01, \eta^2 = 0.02$. However, a between-subject
ANOVA on licks per trial to 66% alcohol (Sipper 2) found a nonsignificant difference between Fast and Slow recovery groups, $F < 1$.

![Figure 1. Top: PR breakpoint for Sipper 1 (empty bottle) to access Sipper 2 (66% alcohol). Bottom: licks per trial to Sipper 2 (66% alcohol).](image)

**Open field.** Figure 14 shows the data from Phase 3, open field testing. There is a clear difference in distance traveled between the center and periphery of the arena, with the periphery measure being higher for both groups. A Position (Center, Periphery) by Speed (Fast, Slow)
ANOVA on the open field distance traveled measure revealed no significant interaction between position and speed or speed, *F* < 1, but a significant effect of position, *F*(1, 21) = 329.82, *p* < 0.001, *η*² = 0.94. An additional analysis was conducted on fast- and slow-recovery groups focusing on the locomotor activity in the periphery of the arena. The results revealed no significant difference between the fast and slow recovery groups in the periphery of the arena, *F*(1, 22) = 1.48, *p* > 0.23, *η*² = 0.06.

*Figure 14.* Distance traveled (cm) by center (C) vs. periphery (P) in animals that had recovered from reward downshift in Phase 1 either Fast or Slow.

**Summary of Results**

Taken together, these results suggest that individual differences in coping with frustration had an effect on the willingness to work for access to 66% alcohol solution, as evidenced by the significant differences in breakpoint between the fast- and slow-recovery groups. Though there were significant differences in breakpoint, there were no detectable differences in how much
each of the groups licked the 66% alcohol solution. Further, no differences were observed in the open field task, other than as expected, both groups spent more time in the periphery of the open field arena than in the center. Animals that exhibit a fast recovery from contrast demonstrate behavioral resilience, while animals that exhibit a slow recovery from contrast are more vulnerable to reward downshift (Papini et al., 2014). Slow-recovery animals respond differently to reward uncertainty induced by reward downshift, and their performance during the downshift from a large, high value sucrose solution to a small, low value sucrose solution is markedly different from fast-recovery animals. Additionally, when provided with postsession access to anxiolytic substances such as alcohol, rodent strains with greater emotional reactivity have been shown to selectively increase their consumption and preference for anxiolytic substances (Manzo et al., 2014). With this in mind, animals that do not cope well with a downshift event and take longer to recover should be more likely to selectively increase their consumption of, and preference for alcohol, whereas animals that exhibit this resilience and recover quickly from the downshift should be less likely to selectively increase their substance consumption. These assumptions led to the development of Experiment 9.

**Experiment 9 – Individual differences in emotional self-medication with 66% alcohol**

The primary objective of Experiment 9 was to assess the potential for a 66% alcohol solution as a means of self-medicating against the negative emotion of frustration, induced by reward downshift. Experimental tests of the ESM hypothesis have used consummatory and instrumental tasks to induce negative emotion (Manzo et al., 2014; Manzo, Donaire et al., 2015). Standard protocols involve first, exposing animals to a task that triggers an aversive emotional state (i.e., a frustrative nonreward task) and second, providing the opportunity to voluntarily
consume an anxiolytic substance. Manzo et al. (2014) used Roman low-avoidance and high-avoidance rats to assess the voluntary, oral consumption of, and preference for alcohol, an anxiolytic, following an incentive loss situation (appetitive extinction). In one such test, consummatory extinction was used as the induction task. First, rats were exposed to 22% sucrose during the preshift phase, and downshifted to 0% sucrose (water) in extinction. Following each daily session of the induction task, rats were given a 2-h, 2-bottle preference test in their home cages. The experimental 22-to-0% group of each rat strain received access to 2% alcohol and tap water in the preference test, while the control group of each rat strain received access to only tap water in the preference test. The results revealed that the postsession consumption of 2% alcohol increased selectively after extinction was implemented in the induction task for the Roman low-avoidance rats, but not the Roman high-avoidance rats. Evidentiary support for the emotionality and coping methods of these two strains suggests that high-avoidance rats demonstrate proactive coping methods, while low-avoidance rats demonstrate more reactive coping methods, including more freezing and self-grooming behaviors (Piras, Corda, and Giorgi, 2006). The coping methods exhibited by low-avoidance rats allow them to be characterized as showing more emotionality than their high-avoidance counterparts. Considering the emotional states of the low-avoidance rats, the selective increase in postsession 2% alcohol consumption may be interpreted as an attempt to alleviate the salient, negative emotional state induced by the loss of incentive (extinction of 22% sucrose) in the induction task. The selective increase, in other words, can be described as the ESM effect.

Manzo, Donaire, et al. (2015) further tested the ESM hypothesis by using cSNC as the induction task. First, rats were trained to receive 32% sucrose during preshift and were downshifted to receive 4% sucrose, relative to an unshifted control group that always received
4% sucrose. In the daily preference test, rats were provided a choice between either chlordiazepoxide (CDP) or alcohol, and tap water. One bottle contained either 1 mg/kg of CDP or 2% alcohol, and the other water, or both contained water for the control groups. The data showed that preference for the anxiolytic substances (CDP and alcohol) increased following the 32-to-4% sucrose downshift, however, preference was not affected for water controls or unshifted 4-4% sucrose controls.

While these studies provide evidence of ESM, they use 2% alcohol, a low concentration that has failed to yield similar results in our lab or even support preference over water (see Experiment 1). The current experiment was designed to test the ESM effect using 66% alcohol. Consistent with the other experiments in this dissertation, 32% and 2% sucrose were used. First, rats were exposed to an induction task with the express purpose of generating a negative emotional state. Each day rats were given 5-min of free access to a 32% or 2% sucrose solution depending on their group assignment. Immediately following the daily induction task (cSNC session), rats were moved into a separate room for a 30-min preference test. Access to a bottle containing 66% alcohol and a bottle containing water were provided daily throughout the preshift phase to allow rodents to gain experience with the post effects of alcohol prior to the key sessions following the downshift from 32-to-2% sucrose.

Method

Subjects and apparatus. A total of 23 experimentally naïve female Wistar rats served as subjects in Experiment 9 in the 32-2% group (n = 12) and 2-2% unshifted control group (n = 11). Animals were maintained and deprived as described in Experiment 1. The 32% and 2% sucrose solutions were prepared as described in Experiment 8. The same conditioning boxes described in Experiment 2 were used in Experiment 9.
Procedure. Once rats reached their target deprivation weight, they began behavioral testing. All rats ($N = 23$) in their home cages were transported into a separate testing room to begin the cSNC task. The procedure was that described in Experiment 8, except for the addition of an unshifted control group. Rats received access to 32% or 2% sucrose for 10 daily 5-min preshift sessions depending on their group. On sessions 11-15, all rats received 2% sucrose. Lick frequency was automatically recorded via attached lickometers in the conditioning boxes as described in Experiment 8. Immediately after each session in the cSNC task, rats were moved into a separate room to start the same 30-min preference test described in Experiment 2. Consumption and preference measures were obtained as described in Experiment 2.

Results

Induction task. Figure 15 shows the data from the cSNC induction task. The unshifted 2-2 control group exhibited lower sucrose licks to the 2% solution during preshift than the 32-2 Fast and 32-2 Slow groups did to their 32% sucrose solution. A Group (32-2 Fast, 32-2 Slow, 2-2) by Preshift Session (1-10) ANOVA revealed a nonsignificant interaction between group and session, $F(18, 180) = 1.23, p > 0.24, \eta^2 = 0.11$, but significant effects of both group, $F(1, 20) = 26.83, p < 0.01, \eta^2 = 0.73$, and session, $F(9, 180) = 20.05, p < 0.001, \eta^2 = 0.50$. Pairwise LSD tests revealed that the unshifted 2-2 control group licked significantly less than the 32-2 Fast and 32-2 Slow recovery groups on sessions 1-2, and 5-9, $ps < 0.04$. A second analysis between the 32-2 Fast and 2-2 control groups revealed a nonsignificant interaction between group and session, $F(9, 117) = 1.74, p > 0.08, \eta^2 = 0.11$, but significant effects of both group, $F(1, 13) = 26.87, p < 0.01, \eta^2 = 0.69$, and session, $F(9, 117) = 13.68, p < 0.001, \eta^2 = 0.51$. A further analysis between the 32-2 Slow and 2-2 control groups revealed a nonsignificant interaction between group and session, $F < 1$, but significant effects of both group, $F(1, 19) = 24.19, p <
0.01, η² = 0.72, and session, F(9, 171) = 15.75, p < 0.001, η² = 0.45. An analysis between the 32-2 Fast and 32-2 Slow recovery groups revealed a nonsignificant interaction between group and preshift session as well as a nonsignificant effect of group, Fs < 1, but a significant effect of preshift session, F(9, 90) = 10.20, p < 0.001, η² = 0.51.

In the postshift phase when all groups received access to 2% sucrose the 32-2 Slow recovery group licked less than the 32-2 Fast and 2-2 groups. A Group (32-2 Fast, 32-2 Slow, 2-2) by Postshift Session (11-15) ANOVA revealed a significant interaction between group and session, F(8, 80) = 3.63, p < 0.001, η² = 0.27, as well as significant effects of both group, F(1, 20) = 31.79, p < 0.01, η² = 0.76, and session, F(4, 80) = 10.11, p < 0.001, η² = 0.34. Pairwise LSD tests on the main interaction suggest that the unshifted 2-2 control licked significantly more than the 32-2 Fast and 32-2 Slow recovery groups on sessions 11, 12, and 14, ps < 0.02. A second analysis between the 32-2 Fast and 2-2 control groups revealed a significant interaction between group and postshift session, F(4, 52) = 4.91, p < 0.002, η² = 0.27, as well as significant effects of both group, F(1, 13) = 17.59, p < 0.001, η² = 0.58, and session, F(4, 52) = 4.60, p < 0.003, η² = 0.26. An analysis between the 32-2 Slow and 2-2 control groups revealed a nonsignificant interaction between group and postshift session, F(4, 68) = 2.15, p > 0.08, η² = 0.11, but significant effects of both group, F(1, 17) = 71.84, p < 0.001, η² = 0.81, and session, F(4, 68) = 8.99, p < 0.001, η² = 0.35. Furthermore, a comparison between the 32-2 Fast and 32-2 Slow recovery groups revealed a significant interaction between group and postshift session, F(4, 40) = 4.76, p < 0.003, η² = 0.32, as well as significant effects of session, F(4, 40) = 14.14, p < 0.001, η² = 0.59, but not group, F(1, 10) = 2.72, p > 0.13, η² = 0.21.
Figure 15. Sucrose licks per 5-min session during the cSNC task. Preshift (sessions 1-10) and postshift (sessions 11-15).

**Preference test.** To explore the effects of individual differences on the recovery from contrast, animals that received the downshift were segregated into fast or slow recovery subgroups based on the same criteria used in Experiment 8. Due to animal performance on Sessions 11 and 12, the classification into subgroups yielded unequal group sizes. Further analyses were conducted on the fast recovery subgroup \((n = 4)\) and the slow recovery subgroup \((n = 8)\) to explore individual differences. Figure 15 shows similar levels of alcohol and water intake by animal’s daily weight for each of the three groups: 32-2 Fast (top left), 32-2 Slow (top right), 2-2 (bottom left), as well as the preference ratio (bottom right). A Group (32-2 Fast, 32-2 Slow, 2-2) by Preshift Session (1-10) ANOVA on the alcohol intake by animal’s daily weight revealed a nonsignificant interaction between group and preshift session, \(F < 1\), as well as nonsignificant effects of group, \(F < 1\), and preshift session, \(F(9, 180) = 1.18, p > 0.30, \eta^2 = 0.05\). Correspondingly, a Group (32-2 Fast, 32-2 Slow, 2-2) by Postshift Session (11-15) ANOVA on
the alcohol intake by animal’s daily weight revealed a nonsignificant interaction between group and postshift session, $F < 1$, and nonsignificant effects of group and postshift session, $Fs < 1$.

A Group (32-2 Fast, 32-2 Slow, 2-2) by Preshift Session (1-10) ANOVA on the water intake by animal’s daily weight revealed a nonsignificant interaction between group and preshift session, $F(9, 180) = 1.08, p > 0.37, \eta^2 = 0.09$, as well as nonsignificant effects of group and preshift session, $Fs < 1$. While a Group (Fast, Slow, 2-2) by Postshift Session (11-15) ANOVA on water intake by animal’s daily weight revealed a nonsignificant interaction between group and postshift session, $F < 1$, as well as nonsignificant effects of group, $F < 1$, and postshift session, $F(4, 80) = 1.30, p > 0.27, \eta^2 = 0.06$. A Group (Fast, Slow) by Postshift Session (11-15) ANOVA on water intake by animal’s daily weight revealed a nonsignificant interaction between group and postshift session, $F < 1$, as well as nonsignificant effects of group, $F < 1$, and postshift session, $F(4, 40) = 1.06, p > 0.38, \eta^2 = 0.09$.

A Group (32-2 Fast, 32-2 Slow, 2-2) by Preshift Session (1-10) ANOVA on the preference ratio for 66% alcohol revealed a nonsignificant interaction between group and preshift session, $F < 1$, as well as nonsignificant effects of group and preshift session, $Fs < 1$.

Correspondingly, a Group (32-2 Fast, 32-2 Slow, 2-2) by Postshift Session (11-15) ANOVA on the alcohol intake revealed a nonsignificant interaction between group and postshift session, $F < 1$, as well as nonsignificant effects of group and postshift session, $Fs < 1$. A separate Group (32-2 Fast, 32-2 Slow) by Preshift Session (1-10) ANOVA on the preference ratio for 66% alcohol revealed a nonsignificant interaction between group and preshift session, $F(9, 90) = 1.14, p > 0.37, \eta^2 = 0.10$, as well as nonsignificant effects of group and preshift session, $Fs < 1$. 
Figure 16. Consumption of alcohol and water (ml/kg) during preference testing sessions (1-15) for each group: 32-2 Fast (top left), 32-3 Slow (top right), 2-2 (bottom left). A indicates alcohol. W indicates water. Preference ratio for 66% alcohol (bottom right).

Preshift phase includes sessions 1-10. Postshift phase includes sessions 11-15.

Correspondingly, a Group (32-2 Fast, 32-2 Slow) by Postshift Session (11-15) ANOVA on the preference ratio revealed a nonsignificant interaction between group and postshift session, $F < 1$, as well as nonsignificant effects of group and postshift session, $Fs < 1$.

Summary of Results

The results of Experiment 9 fell short of establishing the ESM effect using 66% alcohol. The reward downshift effect observed in the induction task was clear. Moreover, there were significant individual differences in recovery, with Group 32-2 Slow recovering more slowly
than Group 32-2 Fast. Neither of the two downshifted groups reached the level exhibited by animals in Group 2-2, even by Session 15. The unshifted 2-2 controls consistently remained above that of the two downshifted groups. The preference ratio for 66% alcohol consistently remained above 0.5 (indifference) throughout the entire experiment, with the exception of the 32-2 Fast recovery group dipping below 0.5 on Session 1. Additionally, the preference ratio remained stable, neither increasing nor decreasing for any of the groups once reward downshift was implemented in the induction task. These findings are interesting, given the marked preference for 66% alcohol and the prior experience with the posteffects of the solution provided through the 10 preshift sessions. Though these rats consume and prefer the 66% alcohol solution, they do not exhibit any significant increase in the consumption of or preference for this concentration when experiencing a negative emotional state. Previous research has demonstrated the ESM effect using 2% alcohol (Manzo et al., 2014, 2015), but Experiment 9 did not replicate these findings using a high alcohol concentration. A possible explanation of the lack of significant ESM is a ceiling effect, wherein these rats consumed so much 66% alcohol (measured in ml) during the preshift phase that they were unable to increase their consumption and preference in the postshift phase. However, extensive unpublished research from our lab with a variety of alcohol concentrations have consistently failed to replicate the ESM effect that is routinely found in other labs. The reasons for the discrepancy have not yet been determined.

**Experiment 10 – Effects of orexin-1 antagonism on alcohol consumption**

The primary objective of Experiment 10 was to test the role of orexin-1 receptors on the consumption of alcohol solutions of high concentration. If a selective antagonist was
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administered via intraperitoneal (i.p.) injection, would it affect responding for 66% alcohol in a self-administration situation?

The orexin system resides primarily in the lateral hypothalamic (LH) region and has a widespread network of neural projection into other brain areas (Sharf, Sarhan, and DiLeone, 2010). Two G-protein coupled orexin neuropeptide receptors, orexin-1 and orexin-2, have been identified within the LH, and the system as a whole contributes to various processes such as sleep/wakefulness, feeding, reward, and addiction (Hervieu et al., 2001). Figure 17 depicts some brain areas known to receive projections of orexin neurons from the LH. Certain brain areas such as the ventral tegmental area (VTA), the amygdala, and the nucleus accumbens (NAc) are posited with a role in excitatory dopaminergic transmission (Plaza-Zabala, Maldonado, and Berrendero, 2012).

Pharmacological antagonism of the orexin-1 and orexin-2 receptors has been shown to decrease responding for drug rewards such as alcohol, nicotine, and opioids (Plaza-Zabala, Maldonado, and Berrendero, 2012). Due to the widespread projection of the orexin neurons from the LH, there is potential for other receptors, notably dopaminergic receptors, to also be affected (Hervieu et al., 2001). Volkow et al., (2011) demonstrated increased dopaminergic activity in the VTA and NAc following exposure to drugs of abuse. The projection of orexin neuropeptides expressed from the LH into these two brain areas further suggests a role of the orexin system in reward processing, and a potential relationship between orexin expression and dopaminergic expression (Baimel et al., 2015).
Figure 17. Projection of orexin neurons into other brain areas illustrated on a sagittal view of the rat brain. Modified from Plaza-Zabala, Maldonado, and Berrendero (2012). Grey dots contained within the circle represent orexin neurons from the lateral hypothalamus (LH). Grey lines indicate projection pathways of orexin neurons from the LH to various brain areas. *: Indicates a potential reward/motivation role. BNST: bed nucleus of the stria terminalis. NAc: nucleus accumbens. PFC: prefrontal cortex. VTA: ventral tegmental area.

Antagonists of the orexin system reduce responding for drugs of abuse as well as natural rewards, suggesting a role in motivation. Putula and Kukkonen (2012) characterized the orexin-1 and orexin-2 systems and the affinity for binding to the drug antagonist SB-334867. The antagonist SB-334867 was determined to have equal affinity for both orexin-1 and orexin-2 receptor sites localized within the LH.
To further characterize the role of antagonizing this system, Wiskerke, Moorman, and Aston-Jones (in press) trained rats in a cue-driven motivational task (stop-signal reaction time). In this task, rats were presented with “Go” and “Stop” trials and rewarded with small, edible rewards for correctly responding to the “Go” and “Stop” signals. Once rats were reliably performing in the stop-signal reaction time task, 10 or 30 mg/kg i.p. injections of the antagonist SB-334867 were administered prior to the task. They found that SB-334867 reduced overall task motivation to work for the small, edible reward.

Previous research has explored the relationship between the orexin/hypocretin-1 receptor and responding for alcohol in the operant self-administration situation (Anderson et al., 2014; Moorman et al., 2017). In studies involving 10-20% alcohol self-administration training, rats were given intraperitoneal injections of the selective orexin-1 receptor antagonist SB-334867, 30 min prior to testing. As a result of antagonizing this receptor, responding for alcohol decreased. However, less is known about the role orexin plays in the reward value of alcohol. To test the relationship between the orexin/hypocretin receptor and reward, SB-334867 will be administered prior to sessions of alcohol self-administration on a PR schedule. A decrease in the PR breakpoint after treatment with the orexin-1 antagonist would suggest a role of this receptor in the reward value of alcohol.

Method

Subjects and apparatus. A total of 16 experimentally naïve Wistar rats (8 males and 8 females) served as subjects for Experiment 10. Animals were maintained and deprived as in Experiment 1. The same conditioning boxes described in Experiment 2 were used in Experiment 10.
**Drug preparation.** The orexin-1 antagonist SB-334867 and its vehicle, dimethyl sulfoxide (DMSO) in saline were obtained from Sigma-Aldrich (St. Louis, MO). All components were weighed with an Acculab-AL64 scale (Fisher Scientific, Waltham, MA). The drug solutions were mixed by manually diluting the drug into a vehicle solution of 5% DMSO/95% sterile saline over low heat on a hotplate until the solutions were clear of flakes.

**Procedure.** Once animals reach the target deprivation weight, they were transported in their home cages from the colony into a separate testing room where they began training in the PR self-administration paradigm described in Experiment 6. After three consecutive sessions of PR self-administration all rats received 0 (DMSO/Saline vehicle), 1, 5, and 10 mg/kg i.p. injections of SB-334867, 30 min prior to testing, to examine whether antagonizing the orexin-1 receptor mediated the reinforcing properties of high-concentration alcohol in a dose-dependent fashion. Doses were counterbalanced across subjects such that four rats received the same dose per injection day. Other procedural details were as described in Experiment 7.

**Results**

Figure 18, top, shows the data from the 66% alcohol PR breakpoint under each dose of SB-334867 as well as the average of the three prior sessions to the injection day. Similar breakpoints are observed for all doses, though a suppression in breakpoint can be seen for all but the vehicle dose. A within-subject ANOVA on the breakpoint under the influence of four selected doses of SB-334867 was conducted. The results revealed a significant effect of drug dose, $F(3, 45) = 4.47$, $p < 0.09$, $\eta^2 = 0.23$. Further pairwise LSD tests indicated, that vehicle animals differed significantly from animals receiving 1 or 5 mg/kg, $ps < 0.03$, but not 10 mg/kg, $p > 0.16$. However, for each SB-334867 dose, the breakpoint on the injection session was significantly lower than the average breakpoint of the previous three sessions, $Fs (1, 120) > 5.60$, ...
$p < 0.02$, $\eta^2 > 0.04$. Whereas the breakpoint for the DMSO/Saline vehicle injection session was not significantly different from the average breakpoint of the three previous sessions, $F < 1$. An additional analysis on the PR breakpoint for 66% alcohol focusing on the average of the three sessions prior to the drug injection sessions confirmed that no significant differences in breakpoint were present, $F(3, 45) = 1.18, p > 0.33$, $\eta^2 = 0.07$.

**Figure 18.** Data separated by “Pre” (average of three sessions prior to the injection day) and “Inj” (injection day). Top: PR breakpoint by dose of SB-334867. Bottom: Licks per trial to 66% alcohol by dose of SB-334867.
Figure 18, bottom, shows the licks per trial to 66% alcohol under each dose of SB-334867. Here, it is shown that the 1 mg/kg dose produced less licking to the solution relative to the average of the three prior sessions to the injection day, whereas licking was generally similar for the other doses. A within-subject ANOVA on the mean lick frequency of 66% alcohol under the influence of four selected doses of SB-334867 (Figure 18, bottom) revealed a significant effect of drug dose, $F(3, 45) = 4.59$, $p < 0.02$, $\eta^2 = 0.23$. Pairwise LSD tests indicated that animals receiving 1 mg/kg licked significantly less than any of the other groups, $ps < 0.02$. An additional analysis on the mean lick frequency of 66% alcohol for the average of the three sessions prior to the drug injections revealed no significant differences in licking behavior for any of the doses, $Fs < 1$.

**Summary of Results**

Taken together, these results suggest that the orexin antagonist SB-334867 does affect the PR breakpoint for 66% alcohol, but not in the expected fashion. Antagonizing this receptor reduced the anticipatory response for the solution, as evidenced by the differences in breakpoint between each dose against saline (most clearly for the 1 and 5 mg/kg doses) and between each dose and the mean breakpoint of the three previous sessions (the reduction in breakpoint was significant for 1, 5, and 10 mg/kg). These results are consistent with those reported by Anderson et al. (2014) ad Moorman et al. (2017). In both studies, presession administration of the antagonist SB-334867 led to a consistent decrease in breakpoint and operant licking for the 10 and 20% alcohol solutions and the highest dose of the antagonist (20 mg/kg) produced the most suppression of behavioral responding. Therefore, my results show that the anticipatory response for a high-concentration alcohol solution can be reduced by the presession administration of SB-334867. The behavioral results of the current experiment are also in line with findings from
Experiments 6 and 8 in this dissertation in that the breakpoint values and licks per trial measures are similar. This shows that the breakpoints obtained here replicated previous findings from this Dissertation.

However, consummatory responses were reduced after administration of 1 mg/kg but not after 5 or 10 mg/kg of SB-334867, suggesting that a high-concentration alcohol solution may be differentially affected by these doses. I could not find any articles describing the effects of SB-334867 on consumption of solutions greater than 20% alcohol. Additional implications of these results will be developed in the General Discussion.

**General Discussion**

Considering the prevalence of alcohol use and the growing rate of AUD diagnoses, preclinical models of alcohol consumption and intoxication are needed. Establishing an animal model of alcohol consumption that does not involve lengthy initiation procedures also has practical value as it promotes rapid advancement of knowledge. The current research aimed to develop methods for establishing pharmacologically relevant levels of alcohol intake without the use of initiation procedures and based on the voluntary, oral consumption of high-concentration alcohol as opposed to forced administration methods. Most manipulations were explored in male and female rats, although sex was not of direct concern. Furthermore, the proposed research aimed at connecting alcohol consumption to frustrative nonreward and exploring the potential therapeutic role of orexin-1 receptor antagonism in the control of high-concentration alcohol consumption. Thus, the present dissertation has significant basic and translational impact.

The 10 experiments reported here are summarized in Table 3 and for the purpose of this General Discussion, they will be organized in three sections. First, I will discuss the evidence from experiments testing for the rewarding effects of high alcohol concentrations in three
procedures: free-choice preference tests, operant self-administration, and progressive ratio schedules of alcohol access (Experiment 1-6). Second, I will look at individual differences in alcohol self-administration in relation to reward downshift (Experiments 7-9). Third, I will consider the attempt at controlling the consumption of 66% alcohol by antagonizing the orexin-1 receptor (Experiment 10).

Table 3

*Summary of results from Experiments 1 through 10*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Design</th>
<th>Results</th>
</tr>
</thead>
</table>
| 1          | • Preference testing (2-h, 2-bottle)  
• Ascending: 1 to 66% A  
• Descending: 66 to 1% A | • A preferred to W for concentrations between 6 and 66%  
• Ascending or descending exposure produced similar results |
| 2          | • Preference testing (30-min, 2-bottle)  
• BAC assay  
• W (0%), 2, 10, 66% within-subject design | • A preferred to W in all concentrations  
• Highest BAC for 66% |
| 3          | • Continuous food access  
• Preference testing (2-h, 2-bottle)  
• 66% A vs. W | • A and W were consumed at similar levels.  
• There was no evidence of rejection of the 66% A solution |
| 4          | • Operant self-administration (10 trials/session)  
• 66% A in acquisition  
• W in extinction | • Acquisition: latency decreased across sessions and licking increased  
• Extinction: latency increased across sessions and licking decreased |
| 5          | • PR operant self-administration  
• 2, 10, 66% A and W, within-subject design | • PR breakpoint similar for 2, 10, 66% A and all higher than W  
• Lick frequency lower for 66% A |
| 6          | • PR operant self-administration  
• 0, 10, 66% A, between-subject design | • PR breakpoint highest for 10% A  
• Lick frequency highest for 10% A |
| 7          | • Individual differences in reward value  
• *Phase 1*:  
• Operant self-administration (20 trials/session) | • *Phase 1*:  
• AFR rats showed lower latency in acquisition and extinction than ASR and W rats  
• Similar licking between ASR and AFR in acquisition and extinction |
### Rewarding effects

The rewarding effects of alcohol solutions were assessed in terms of three different procedures. First, free-choice consummatory tests provided an indication of the degree of preference for alcohol solutions over water. To the extent that alcohol solutions have rewarding value, they should be consumed above the level of water consumption. Preference tests with solutions of different taste are routinely used in experiments assessing the value of particular reward (Tordoff, Alarcon, & Lawler, 2008). Preference tests were used in Experiments 1-3.

Second, operant responding (licking) was used to assess the extent to which animals would be...
willing to invest time/effort to gain access to 66% alcohol. Such a procedure is the cornerstone of operant conditioning studies and an objective way to characterize the motivational nature of a reward. By definition (Skinner, 1938), a stimulus is considered an appetitive reinforcer if an organism is willing to respond to obtain it. Experiment 4 used this procedure. Third, a PR schedule, as implemented in Experiments 5-6, has also been used to assess the reward value of appetitive reinforcers (Swanson, Goldbach, & Laubach, 2019). The higher the reward value of a substance, the higher the ratio value an organism is willing to complete before ceasing responding.

Experiment 1 sought to establish the voluntary, oral consumption of and preference for higher-than-usual concentrations of alcohol using a standard 2-bottle free-choice preference test. Gradual and abrupt presentations of alcohol were used to create an ascending and descending series. Common animal models of alcohol administration in rodents involve low concentrations of alcohol (i.e., less than 20% alcohol) and it is often reported that rodents struggle to consume concentrations that exceed 20% (Gilpin et al., 2009; Becker, McClellan, & Reed, 2017). The general view expressed in the literature is that rats have difficulty consuming alcohol solutions voluntarily and orally (Richter & Campbell, 1940). For Wistar rats, the range of concentrations in which they show at least 50% preference over water lies between 2.0 and 6.8%, which are considered low concentration solutions of alcohol (Goodwin & Amit, 2000). Research with Roman high- and low-avoidance rats using a testing procedure similar to that used here in Experiment 1 indicated that concentrations higher than 6% (in high-avoidance rats) or 2% (in low-avoidance rats) are rejected relative to water (Manzo et al., 2012). Becker and Ron (2014, p. 205), in an introduction to a special issue on animal models of alcohol consumption, pointed out that:
“Over several decades, numerous experimental approaches have been employed in developing rodent models of excessive alcohol self-administration. However, until about a decade ago, one of the major obstacles in this work was that rodents typically do not self-administer alcohol in sufficient amounts to produce overt signs of intoxication. Further, when given the opportunity to voluntarily drink alcohol, even under circumstances when access is unlimited, rodents rarely will consume alcohol in a manner that results in significant elevation in blood alcohol levels (above legal limits).”

Li et al. (2001, p. 117S) made a similar point:

“Rats and mice, in the aggregate, do not like to drink aqueous solutions of ethanol in concentrations greater than 5% and do not attain blood alcohol concentrations perceived to be “intoxicating.”

Spanagel (2003, p. 509) pointed out that:

“At higher alcohol concentrations (>6%), however, at which the taste of the solution is usually aversive to rodents, large differences in alcohol preference exist between individuals and between strains.”

In this context, the results of Experiment 1 were unexpected in that significant levels of preference for concentrations higher than 6% alcohol were observed in Wistar rats. Due to the abrupt, descending series, I can rule out tolerance to the substance as an explanation of current findings because these rats encountered the 66% alcohol solution for the first time at the start of the descending series of tests. Having no prior exposure to alcohol, these animals exhibited preference from the start of testing. Furthermore, the preference functions for both the ascending and descending series were similar across concentrations. The fact that these series were run at
different time points with different cohorts of animals is also notable and suggests the replicability of these findings.

Experiment 2 showed that the consumption of solutions with a high alcohol concentration led to measurable levels of BAC. Consumption of 0 (water), 2, 10, and 66% alcohol was examined following a 30-min preference test between each concentration and water using a within-subject design. Both the absolute consumption and preference ratios were similar to those found in Experiment 1. As expected, plasma BACs were higher after consumption of 10% and 66% alcohol solutions relative to 0% (water) and 2% alcohol. These findings suggest the viability of low-concentration (10%) and high-concentration (66%) alcohol as a means of inducing pharmacologically relevant BAC levels in Wistar rats.

Except for Experiment 3, all the experiments involved an 81-84% food-deprivation level. By contrast, Experiment 3 involved a continuous presentation of rodent chow to never-food-deprived Wistar rats to evaluate the impact of food-deprivation on the development of preference for high concentration, 66% alcohol. Preference for this concentration abated after a few sessions. This suggests that food-deprivation levels between 81 and 84% can be used as a non-lengthy means of inducing voluntary, oral alcohol consumption. Importantly, there was no evidence that rats rejected the 66% solution. In fact, the amount of 66% alcohol consumption in Experiment 3 was comparable to that of other experiments presented in this Dissertation, although the amount of water consumption increased, thus diminishing the preference ratio for 66% alcohol. Moreover, Long-Evans rats, which also exhibit preference for 66% alcohol to Wistars, showed unchanged levels of preference for 66% alcohol to water during periods of free-food vs. restricted-food access (Thompson et al., 2019, under review).
Experiments 1-3 established that rodents would voluntarily consume 66% alcohol under conditions of free access. Experiment 4 was designed to assess the rewarding properties of 66% alcohol under mixed Pavlovian/instrumental conditions (Experiment 7 also used this procedure). The procedure involved an operant licking task that required rats to lick at an empty tube (Sipper 1) to gain access to a 66% alcohol (or water) solution from a separate tube (Sipper 2). Failing to lick on Sipper 1 would also produce Sipper 2 after a preset time (i.e., the Pavlovian component), but rats rarely failed to respond to Sipper 1 during acquisition. This mixed Pavlovian-instrumental procedure was applied over 10 acquisition sessions, and the operant licking response was extinguished over 10 extinction sessions where only water was presented in Sipper 2. In Experiment 4, a clear pattern of decreasing latency to respond to the empty tube (Sipper 1) to gain access to 66% alcohol (Sipper 2) was observed in acquisition, which increased in extinction. A corresponding increase in lick frequency to 66% alcohol was observed in acquisition which was diminished in extinction when only water was available in Sipper 2. By current standards, these results demonstrate that access to 66% alcohol reinforced operant licking responses. Schultz (2015, p. 855) suggests that “anything that makes an individual come back for more is a positive reinforcer and therefore a reward.”

Experiment 5 further explored the reward value of 0, 2, 10, and 66% alcohol by means of a within-subject PR schedule of alcohol self-administration. The reward value was quantified here via a breakpoint, that is, the point at which response effort was no longer worth expending to gain access to a solution. Interestingly, the breakpoints for 2, 10, and 66% alcohol were similar, and higher than 0%, yet there were no significant differences in licking to 66% alcohol from Sipper 2. As an extension of this experiment, Experiment 6 was designed to tease apart any effects derived from the use of a within-subject design in Experiment 5, by conducting the PR
self-administration with naïve animals in a between-subject design. Three groups received 0, 10, and 66% alcohol (the 2% concentration was not included). The results revealed that 10% and 66% alcohol yielded the highest breakpoints, and that rats licked more to 10% alcohol than to 0 or 66%.

Taken together, the results of these initial six experiments provided strong evidence that Wistar rats find high concentrations of alcohol rewarding. As mentioned above, similar results using preference tests were obtained with Long-Evans rats (Thompson et al., 2019, under review). However, the reasons for the discrepancy between these results and the difficulties reported in the alcohol literature to induce acceptance and drinking of alcohol solutions in rodents (see Introduction) remain to be determined.

**Individual differences**

Experiment 7 sought to extend the findings of Experiment 4 by lengthening access to the 66% alcohol solution via more trials per session and a 5-s increase in available licking time per trial. During the initial phase of Experiment 7, it became clear that the procedural changes implemented uncovered measurable individual differences in the group receiving access to 66% alcohol. No such individual differences were observed in Experiment 4 using fewer trials per session and shortened exposure to alcohol per trial. Thus, animals were segregated into “Alcohol Fast Responders” (AFR) and “Alcohol Slow Responders” (ASR) subgroups. The criterion to qualify for the AFR subgroup required rats to have a stable and low latency to respond to the empty tube (Sipper 1) during the final sessions of acquisition (a mean latency lower than 7 s). To explore the potential connection of these individual differences with other behavioral traits, two additional phases were added to Experiment 7. Phase 2 involved three 5-min free access sessions to alcohol or water depending on original group assignment, followed by a single 20-min lighted
open field session after a 15-min delay on the third free access session. The goal of Phase 2 was to evaluate the effects of alcohol (or water) consumption on locomotor activity in the open field arena. There was evidence that 66% alcohol intake had an anxiolytic effect (it increased locomotor activity), but only in AFR rats. Phase 3 involved a reward downshift from 32% to 2% sucrose and was designed to identify possible relationships between the response to 66% alcohol in Phase 1 and the rate of recovery from frustrative nonreward induced by the reward downshift. Interestingly, AFRs also exhibited a faster recovery from reward downshift relative to ASRs.

Experiment 8 further explored the effects of high-concentration alcohol by examining individual differences in coping with frustrative nonreward induced by reward downshift. Here, the phases involving alcohol self-administration and reward downshift were reversed relative to the previous experiment. Now the question was to determine whether the rate of recovery from reward downshift would be correlated to alcohol self-administration. Thus, Phase 1 involved a reward downshift from 32% to 2% sucrose after which animals were separated into a fast- or slow-recovery subgroups based on their consummatory behavior in the first and second downshift sessions. Phase 2 involved the PR self-administration of 66% alcohol. Phase 3 involved a single 20-min lighted open field measurement of locomotor activity. As expected (see Introduction to Experiment 8), there were significant differences in breakpoint for the fast- and slow-recovery groups. Interestingly, fast-recovery animals quit responding for alcohol in the PR task faster than slow-recovery animals. No differences between these two subgroups were detected in terms of locomotor activity in the center or periphery of the open field arena. Thus, Experiments 7 and 8 uncovered a link between 66% alcohol consumption and frustrative nonreward.
The results of Experiment 8 are consistent with the ESM hypothesis (Torres & Papini, 2016). Assuming that slow recovery from reward downshift reflects vulnerability to frustrative nonreward (Papini et al., 2014), then higher breakpoints in the PR task in these animals could relate to the anxiolytic properties of alcohol. Forced alcohol administration does reduce the effects of reward downshift (Becker & Flaherty, 1982; Kamenetzky et al., 2008) in a similar manner to conventional anxiolytics, such as benzodiazepines (Flaherty, 1990; Phelps et al., 2015). In addition, as mentioned above, rats increase their consumption of alcohol and benzodiazepine anxiolytic solutions during periods of reward downshift (Manzo, Donaire et al., 2015). The general idea is, therefore, that animals that are vulnerable to the negative emotions induced by reward downshift assign a high value to consuming substances that alleviate such emotional states.

Experiment 9 provided a test of the ESM hypothesis using 66% alcohol as a means of alleviating frustration induced during reward downshift in the cSNC task. A group of rats received a downshift from 32-to-2% sucrose, and their behavior was compared relative to an unshifted control group that always received 2% sucrose. Interestingly, the preference ratio for both groups remained both similar and steady throughout the preshift and postshift phases. The preference ratio remained consistently above 0.5, thus indicating a preference for 66% alcohol over water, but it did not peak in a transient manner consistent with the observed reduction in sucrose consumption seen during reward downshift. This was true whether animals showed fast or slow recovery from reward downshift.

These results yield a complex view of the relationship between alcohol consumption and frustrative nonreward. On the one hand, individual variation in both variables seems to be connected in ways consistent with the ESM hypothesis, as shown in Experiment 7-8. On the
other hand, when the two procedures are used in tandem to assess their potential interaction, preference for 66% alcohol was not affected by reward downshift, thus failing to provide support for the ESM hypothesis. In fact, ESM effects routinely observed in other labs with Wistar rats exposed to a variety of alcohol concentrations (2-32% alcohol) after a 32-to-4% sucrose downshift (Manzo et al., 2014; Manzo, Donaire, et al., 2015; Manzo, Gómez, et al., 2015; Donaire et al., 2018; unpublished results) have systematically failed to occur in our TCU lab under a variety of conditions (unpublished results). The sources for these discrepancies remain to be determined.

**Role of the orexin-1 receptor**

Demonstrating the rewarding properties of high alcohol concentrations in rats opens the door to a potential animal model for alcohol intoxication. It was clearly of significant translational value to demonstrate from the outset that such a model would have some of the same properties described in experiments using lower alcohol concentrations. I was interested in the possibility of assessing the effects of SB-334867 (a selective orexin-1 receptor antagonist) as a treatment for the control of alcohol consumption that had been tried with some success in other experiments (Anderson et al., 2014; Moorman et al., 2017).

The use of the orexin-1 antagonist SB-334867 has the potential to serve as a successful method of diverting a person from engaging in problematic or risky drinking behavior, though the context in which this drug is administered should be carefully considered. Based on the parameters of Experiment 10, the drug being administered closely before the presentation of alcohol could affect its efficacy as a treatment. Some doses may effectively prevent a person from approaching alcohol-related cues and even reduce alcohol intake when drinking has started. However, higher doses may be ineffective.
Clinical studies that evaluated human responses to alcohol usually fail to distinguish between the response to alcohol cues (analogous to the anticipatory responses during PR training) and the response to alcohol drinking (analogous to the consummatory response for alcohol). Martins et al. (in press) examined two alcohol response phenotypes using ERP: sensitive to low-doses of alcohol and blunted sensitivity to alcohol. Participants were presented with images of a single person drinking and images of multiple people drinking. The images were mixed between alcoholic beverages and non-alcoholic beverages and they would induce anticipatory responses related to alcohol consumption. The P3 ERP was measured as a marker of the motivational significance of each image for each participant. Researchers found that individuals with an alcohol sensitivity phenotype had larger P3 ERP amplitude than individuals with blunted alcohol sensitivity. Similarly, heavy drinkers exhibited stronger fMRI activation than light drinkers in medial front cortex, hippocampus, amygdala, anterior cingulate cortex, and dorsal striatum when stimulated with alcohol imagery (Dager et al., 2013). However, these studies did not assess alcohol drinking within the same study.

Other studies come closer to assessing consummatory responses to alcohol. For example, Cyders et al. (2014) looked at brain responses to images of negative, neutral, or positive moods while participants were stimulated with alcohol odors. Participants were also asked to self-report their levels of alcohol craving. Using fMRI, this study found that the relationship between ventromedial prefrontal cortex activation and alcohol craving was stronger when participants were viewing negative-mood images than with neutral or positive images. Consistent with these findings, a fMRI study by Karch et al. (2015) involving participants diagnosed with AUD found that presenting alcohol related imagery induced feelings of craving (an anticipatory response) and produced activation in the prefrontal cortex, amygdala, hypothalamus, and striatum.
Hatchard et al. (2017) examined the effects of alcohol consumption on response inhibition using a go/no-go task presented 30-min after access to alcohol. Participants were classified as either infrequent alcohol users or regular alcohol users. Researchers found no significant difference in response inhibition during the task, but a significant difference in brain activation in the hippocampus and cerebellum. While the studies cited above explored differences in brain activation related to the anticipatory or consummatory components separately, a BOLD fMRI study reported by Gundersen, Specht, Gruner, Ersland, and Hugdahl (2008) directly compared the expectation of an alcoholic beverage (anticipatory response) vs. the oral consumption of an alcoholic beverage (consummatory response). Participants were given access to alcohol and administered a breathalyzer test to assess for BAC. Intoxication was defined as participants obtaining a BAC level of at least 0.08%. Neural activation in the dorsal anterior cingulate cortex and the prefrontal cortex decreased in response to alcohol intoxication, but neuronal activation increased in response to conditions of alcohol expectancy. These findings are interesting as they suggest different mechanisms are responsible for the anticipatory and consummatory components of alcohol use.

Conclusions

Unlike the prevailing view suggesting that rodents find alcohol solutions aversive, this series of experiments demonstrated that these animals will voluntarily and orally consume solutions of alcohol in a wide variety of concentrations. The preference remains above the level of indifference (0.5) under conditions of food-deprivation and, although such preference is abated in nondeprived animals, there was no evidence of aversion to alcohol. The argument for tolerance to the solutions can be combatted by the data from the abrupt, descending series in Experiment 1 which demonstrate that even on day 1, naïve food-deprived Wistars will accept
66% alcohol. Similar acceptance of 66% alcohol in alcohol-naïve animals was also observed in Long-Evans rats (Thompson et al., 2019, under review). A rewarding value is suggested via experiments involving Pavlovian-instrumental self-administration conducted both under an FR1 schedule and a PR schedule of reinforcement, though it remains unclear why the breakpoints for low- and high-concentration alcohol solutions were similar. Each concentration tested (2%, 10%, 66%) has been previously shown to be discriminable in situations of 2-bottle, free-choice preference testing, thus obtaining undifferentiated breakpoint values for each solution is puzzling. In these experiments 4-8, rats quickly learned to emit licking responses to an empty tube (Sipper 1) in order to gain access to the alcohol solutions (Sipper 2). The role of the orexin-1 system in reward was examined, however, further research exploring other doses of the antagonist SB-334867 as well as selective orexin-1 antagonists are necessary to provide more insight into the role of this system, as well as potential interactions with dopaminergic systems.

One implication of the current findings is that high concentration, 66% alcohol is acceptable to rodents under a variety of conditions (i.e., free access, operant licking, food deprivation, non-deprivation) though the conditions may affect the amount of consumption and preference. The data presented in Experiment 4 suggest that individual differences can arise in the willingness to work for 66% alcohol, as well as the inherent rewarding value of the substance for each animal. With respect to Experiment 7, the experimental group (Alcohol) was further subdivided into the AFR and ASR subgroups based on their performance in the acquisition phase of the self-administration task. Rats with fast latencies to respond to Sipper 1 (empty) to gain access to Sipper 2 (66% alcohol) in group AFR exhibited similar patterns of licking to the 66% solution, though differences emerged in the reward downshift phase. When a downshift from 32% to 2% sucrose was implemented, AFR animals recovered from downshift significantly
faster than ASR animals. Though the finding that some animals (ASR) did not learn to decrease their latency to respond to Sipper 1 may seem discouraging, this brings to light an interesting parallel between rodents and humans. Considering the commonality of alcohol consumption in humans, reports show that 86.4% of adults over the age of 18 have consumed alcohol. However, of that number only 6.2% develop AUDs (NIAAA, 2018b). Taken together, it seems reasonable and perhaps encouraging that individual differences were observed in our rodent model as it suggests an explanation for why only a sample of rats (group AFR) exhibited faster latencies to respond to the empty sipper in order to gain access to the alcohol solution. This type of response is anticipatory in nature, demonstrating an expectation of 66% alcohol from Sipper 2 which follows a response to Sipper 1. Later, in the reward downshift phase, these AFR rats demonstrate the expectation of the 32% sucrose solution, and exhibit a more robust demonstration of frustrative nonreward evidenced by a substantial decrease in licking when presented with the 2% sucrose solution relative to the ASR and W animals. Further testing employing genotyping is necessary to better explore individual differences at a genetic level.
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VITA

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ABSTRACT

ASSESSING THE REWARD VALUE OF HIGH CONCENTRATIONS OF ALCOHOL IN RATS

By Joanna Brooke Thompson, MA, MS, BCBA, 2019
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The misuse of alcohol is a growing problem in the United States. The scientific study of alcohol use via animal models possesses relevance for translational and theoretical problems in the following domains (1) establishing a rodent model of high-concentration alcohol use to better understand the transition from regular drinking to addictive drinking behavior, (2) exploring the rewarding properties of high-concentration alcohol, (3) assessing the role of motivation on preference for alcohol over water via conditions of food-deprivation and continuous food availability, (4) assessing the pharmacological basis of alcohol consumption by antagonizing the orexin-1 receptor. Throughout testing these problems, we found that rats preferred alcohol to water in concentrations ranging from 6-66% (Experiment 1), and that pharmacologically relevant BAC levels were obtained after access to 10% and 66% alcohol compared to 2% alcohol and water (Experiment 2). Providing rats with continuous access to food eliminated preference for 66% alcohol, but did not cause rats to stop drinking the solution (Experiment 3). Rats learned an operant licking task involving an empty tube to gain access to a tube containing 66% alcohol (Experiments 4 and 5) and were successful at this task under progressive ratio (PR) conditions for 2%, 10%, 66% alcohol and water in a within-subject (Experiment 6) and for 10% and 66% alcohol and water in a between-subject (Experiment 7) design. Individual differences in coping
with frustration were observed in a reward downshift task conducted prior to tasks involving 66% alcohol (Experiment 8). A test of emotional self-medication with 66% alcohol revealed no significant differences between recovery from frustration groups (Experiment 9). Finally, antagonism of the orexin-1 receptor was conducted 30-min prior to PR self-administration of 66% alcohol. Rats exhibited the lowest breakpoint for the lowest, 1 mg/kg dose of the antagonist SB-334867 as well as the most suppression of licking behavior. These experiments demonstrate that individual differences can affect the reward value of alcohol solutions, and that pharmacologically relevant BAC levels can be obtained through oral, self-administration methods.