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Searching for new medications for the treatment of alcohol use disorder

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SEARCHING FOR NEW MEDICATIONS FOR THE TREATMENT OF ALCOHOL USE DISORDER

by

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A Thesis

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Abstract

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Alcohol use disorder (AUD) affects more than 15 million people in the United States. Current pharmacotherapeutic treatments for AUD are only modestly effective, necessitating the identification of new targets for medications development. In this study, the effects of the D4 receptor antagonist, L-745,870, and the CB1 negative allosteric modulator, PSNCBAM-1, were both tested for effects in ethanol conditioned place preference (CPP) and oral ethanol self-administration. Food-restricted adult male mice were trained in operant chambers to nose poke for delivery of rewards, trained on ascending concentrations of alcohol with descending concentrations of Ensure and water, until the mixture self-administered was 8% w/v ethanol in water. L-745,870 did not significantly attenuate ethanol self-administration or ethanol CPP. These results suggest that D4R antagonism does not alter the rewarding value of ethanol. PSNCBAM-1 dose-dependently attenuated oral ethanol self-administration, significantly reducing ethanol rewards at a dose of 30 mg/kg but not at 10 or 18 mg/kg. However, 18 and 30 mg/kg PSNCBAM-1 also significantly reduced self-administration of a palatable food reward. These results suggest PSNCBAM-1 produces a non-specific anhedonic effect that may preclude its use in AUD or other neuropsychiatric conditions.

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Chapter 1

Introduction

Alcohol - What It Is and What It Does

Alcohol, also known as ethanol, is the ingredient found in beverages such as beer, wine, spirits, and mixed drinks that causes intoxication [1]. The chemical structure can be seen below in Figure 1.

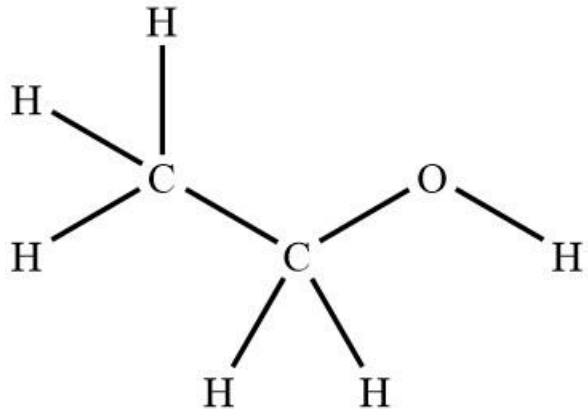

Figure 1. Structure of ethanol. The ingredient in beverages that causes intoxication.

Alcohol is formed when yeast ferments or breaks down the sugars in different foods, without the presence of oxygen [2]. The chemical formula for the fermentation of ethanol can be seen in Figure 2 below. For example, wine is made from the sugar in grapes; beer from the sugar in malted barley; cider from the sugar in apples; and vodka from the sugar in potatoes, beets, or other plants [3].

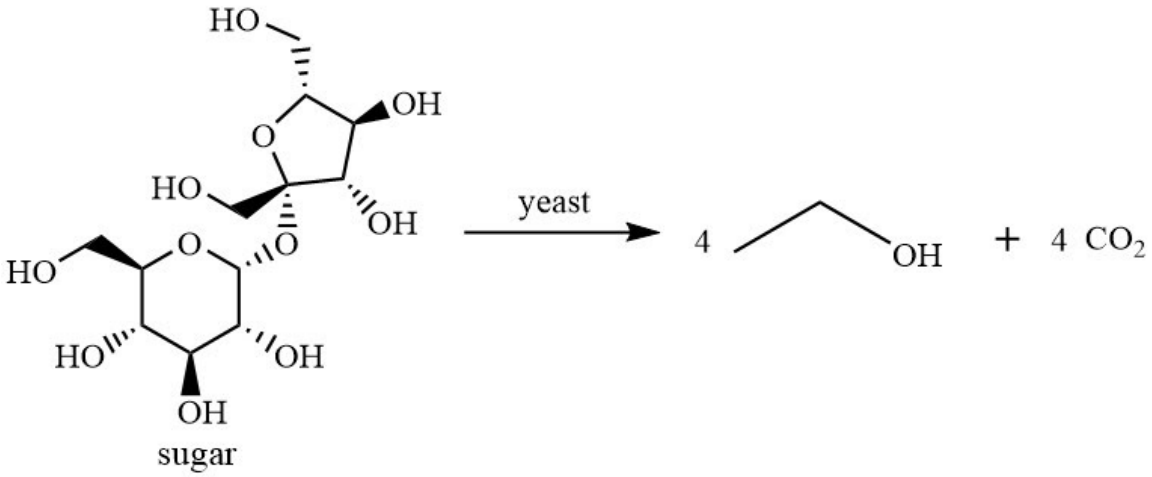

Figure 2. Chemical formula of the fermentation process of alcohol. The process involves yeast fermenting the sugar (in this case the disaccharide sucrose) in fruit and grains into ethanol and carbon dioxide.

The History of Alcohol

Drinking is a prevalent and deep-rooted feature of American life. Despite attempts by the government and other institutions to shape or even eliminate drinking, alcohol has been widely consumed throughout American history [4].

The first evidence of the preparation of alcohol appeared around 8000 BC, after humans started an agricultural society and established sedentary communities. The earliest evidence comes from chemical analysis of residues inside pottery jars found in Jiahu, North China. These clay vessels contained a fermented drink made with rice, honey, grapes, and hawthorn berries, dating back to 7000 to 6600 BC [5]. Proof that plants were being grown for the production of alcohol first emerged in the Fertile Crescent, a geographic area curving between the Mediterranean and Persian Gulf. Analysis of a yellow residue found in a jar at a Neolithic settlement in Haji Firuz Tepe (present-day Iran) dating back to 5400 to 5000 BC revealed the jar once held wine. In the city of Hierakonpolis, Egyptian ruins

contain evidence of the world's oldest brewery, dating back to 3400 BC [5]. The first proof that beer was being brewed in this area comes from residue found in a pottery vessel in the Zagros Mountains of Iran dating to 3100 to 2900 BC. It was established that, beer was considered the beverage for the working class and wine was considered the beverage of the elite, by the year 3100 BC. By the middle of the third millennium, 3000 to 2001 BC, it was clear that alcohol was more than just a sustenance to the people of the Fertile Crescent [5]. Wine production reached the Hellenic peninsula by 2000 BC and was a commonplace in Classical Greece by 1700 BC. The Romans were the next great drinking civilization to emerge in Europe. Rome was almost completely dry in its early years, but the economic benefits that vineyards offered to landowners helped alter the Romans' thoughts on alcohol. This led to the Romans adopting the drinking culture of the Greeks by the middle of the first century BC, 200 to 101 BC [5].

The Mesoamerican civilizations, between Mexico and Panama, were inventive in identifying potential sources of alcohol. Manufacturing of alcoholic beverages from cacti was widespread among hunter-gatherer tribes [5]. Maize, the principal cereal crop of the Americas, was fermented to make tesguino (maize beer) and balche, mead fermented with the bark of the balche tree. Although many of the native types of alcoholic drinks fell out of use after the Spanish conquest of the Incan and Aztec civilizations, pulque, the fermented sap of maguey (the agave plant), grew in popularity [5].

In 1584, Sir Walter Raleigh received letters from Queen Elizabeth for the foundation of an American colony. The first men returned within a few months of setting sail and described the new land with an abundance of vines [5]. Every tree seemed to be draped with vines and the native tribes were friendly, sober, and preferred water. Unlike

their neighbors in Central and South America, the North American natives did not drink alcohol [5].

In November 1620, under the leadership of John Carver, more than 100 Pilgrims and 36 sailors set out on a transatlantic voyage on the Mayflower, landing near Cape Cod. Winter was approaching and the Pilgrims settled where they had landed because resources were running scarce, especially the beer. Epidemics broke out among the Pilgrims and seamen, but the stock of beer was not offered to the sick for fear the supply would not be enough for the journey home [5]. There were only 53 Pilgrims still alive when spring arrived. The Mayflower returned to England in 1621 and the colonists continued to explore the new land. Over the next two decades, the colonists flourished and became self-sufficient in food and trade. In addition to the importation of liquor, home brewing of surplus food resulted in plenty of booze in the colonies [5].

During the 150 years before the American Revolution, the colonists of North America tended to regard heavy drinking as normal. The colonists brought with them from Europe a high regard for alcoholic beverages; people in all regions and of all classes drank heavily. Wine and sugar were consumed at breakfast; at 11:00 and 4:00 workers broke for their 'bitters'; cider and beer were drunk at lunch and toddies for supper and during the evening [4]. Alcohol was also a prominent feature of the colonists' social life. In addition, taxes on alcohol were an important source of revenue for the new colonial governments. In this society drunkenness was seen as a personal failing, as a sin against a natural order [4]. During the 150 years after the revolution, a quite different view of drinking took hold. Many people came to see alcohol as an addicting and even a poisonous drug. This view

found its institutional voice in the temperance societies of the 1800s and early 1900s. By 1916, 23 states had passed prohibitionist laws of various kinds [4].

The 18th Amendment of the United States Constitution was drafted by the Anti-Saloon League in 1917 and ratified by the states on January 16, 1919. The 18th Amendment brought about Prohibition by banning the manufacture and sale of alcoholic beverages. The amendment read: "After one year from the ratification of this article the manufacture, sale, or transportation of intoxicating liquors within, the importation thereof into, or the exportation thereof from the United States and all territory subject to the jurisdiction thereof for beverage purposes is hereby prohibited." [4]. Upon closer review of the wording, the amendment prohibits only the manufacture, sale, and transportation of alcohol, not the possession, consumption, or home production. The amendment gave people a year to dispose of their existing stocks. Many people were opposed to the enforcement of the new law and the government did not give a high priority to enforcing Prohibition [4]. The years during Prohibition were a time of widespread and blatant disregard of the law. Bootlegging, moonshining, and speakeasies all thrived during the period of Prohibition. Illegal marketers developed a strong black market in booze, especially with drinkers willing to pay three to four times the prewar prices for to get their hands on alcohol [4]. Illegal alcohol came from many sources, including skimming off the top of alcohol used for antifreeze in cars. The law was repealed by the 21st Amendment in December 1933, partially due to the economic collapse around 1929 [4].

In 1971, as part of the Alcohol, Drug Abuse, and Mental Health Administration, the National Institute on Alcohol Abuse and Alcoholism (NIAAA) was formed and is the main federal agency invested in and funding research on alcohol abuse and alcoholism [4].

The Sociocultural Relevance of Alcohol

The consumption of alcohol varies across gender, race, ethnicity, and economic standing. Men have statistically been reported across the world to drink more alcohol than women. People in developed countries drink more than those in developing countries [6]. Among racial and ethnic groups, Caucasians report the highest overall alcohol use among those aged 12 and older. American Indians and Alaskan Natives report the highest levels of binge drinking. People with a higher economic status drink alcohol more frequently and people with a lower economic status drink a larger quantity of alcohol [7].

Media exposure can influence social norms about the view of alcohol and drinking through advertising, product placements, and stories in a wide range of sources, including movies, television, social media, and other forms of entertainment. Although alcohol sales and marketing are highly regulated, people are exposed to a wide variety of alcohol and liquor advertisements [8]. Studies have shown that targeted alcohol marketing results in individuals developing positive beliefs about drinking and both create and expand environments where alcohol use is socially acceptable and encouraged. Increased use of social media for alcohol marketing has changed the communication methods for companies to advertise to adolescents and college-age youth. Social-networking sites such as Snapchat, Twitter, Instagram, and Facebook feature alcohol-related marketing and advertisements [8].

Drinking is a part of the culture and social life of many people in countries around the world. Drinking occurs in both casual and formal settings. From going to happy hour with colleges after work or having a drink with dinner to celebrating life milestones like weddings, funerals, and graduations. Going to happy hour with colleagues after work gives

a group of people that normally work in a professional setting the opportunity to relax and have an enjoyable time together. It is usually tradition for many loved ones to give toast to the newlyweds with a glass of champagne. Most social events people attend such as sporting events, concerts, and parties involve the consumption of alcohol as part of the culture or is seen as a way to have a more enjoyable time at the events. Many holidays in America also involve the consumption of alcohol as part of the celebration festivities. Alcohol plays a part in many of the aspects of people's lives around the world, weather we are conscious of it or not.

The Pharmacokinetics of Alcohol

Alcohol is classified as a sedative hypnotic drug; it acts to depress the central nervous system at high doses [2]. At lower doses, alcohol can act as a stimulant, inducing feelings of euphoria and loquaciousness [3, 9, 10]. Alcohol, an addictive drug, acts on the brain by increasing the effects of the neurotransmitter gamma aminobutyric acid (GABA), which suppresses activity in the nervous system and increases the levels of dopamine, in turn producing pleasurable effects.

Once swallowed, even one sip, alcohol is rapidly absorbed into the blood and moves to all parts of the body. A small amount of alcohol is immediately absorbed by the small blood vessels in the mouth and tongue. Up to 20% of alcohol that was initially consumed passes through the stomach into the blood stream [11]. The remaining 75% to 85% of the alcohol is absorbed through the small intestine into the blood stream. If the stomach is empty, the alcohol moves quickly down into the intestines. If there is food in the stomach, the alcohol stays in the stomach much longer so more is absorbed through the stomach [11]. An enzyme present in the stomach has time to break down some of the alcohol before most

of it moves down into the intestines. Effects of alcohol can be felt within five to ten minutes after consumption and usually peaks in the blood after 30 to 90 minutes [11].

Alcohol is quickly moved throughout the body via the blood stream to all the parts of the body. Alcohol stays circulating in the blood until the liver is able to break it down. The liver filters the blood and breaks down 80% to 90% of the alcohol to water, carbon dioxide and energy [11]. Alcohol is metabolized by several processes or pathways. The most common of these pathways involves two enzymes: alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). These enzymes help break apart the alcohol molecule, making it possible to eliminate it from the body. First, ADH metabolizes alcohol to acetaldehyde, a highly toxic substance and known carcinogen [12]. Then, in a second step, acetaldehyde is further metabolized down to another, less active byproduct called acetate, which then is broken down into water and carbon dioxide for easy elimination [12, 13].

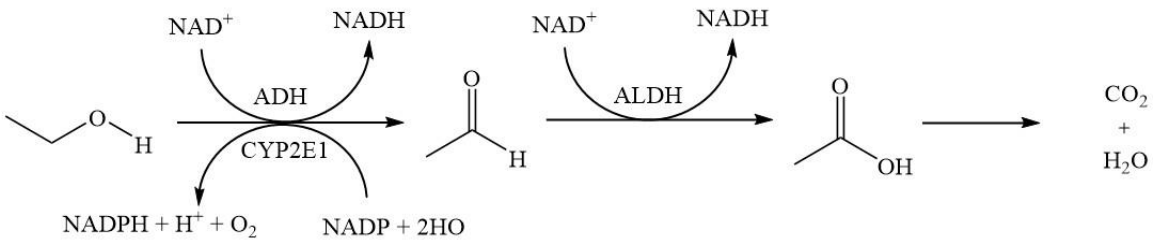

Figure 3. The chemical breakdown of alcohol. Most of the ethanol in the body is broken down in the liver by an enzyme called alcohol dehydrogenase (ADH), which transforms ethanol into a toxic compound called acetaldehyde (CH_3CHO), a known carcinogen. However, acetaldehyde is generally short-lived; it is quickly broken down to a less toxic compound called acetate (CH_3COO^-) by another enzyme called aldehyde dehydrogenase (ALDH). Acetate then is broken down to carbon dioxide and water, mainly in tissues other than the liver [14].

Due to the saturation of ALDH enzymes by even modest amounts of ethanol, the liver can only break down alcohol at an average rate of one standard drink per hour [11]. A reference scheme of what is considered a standard drink can be seen below in Figure 4.

Figure 4. Various standard drink sizes and their alcohol percentages. Each standard beverage is defined as containing 0.6 fluid ounces or 14 grams of pure alcohol. The percentage of pure alcohol is shown by volume. Figure taken from [15]. (National Institute on Alcohol Abuse and Alcoholism, "“What Is A Standard Drink?”," U.S. Department of Health and Human Services, 9 October 2019. [Online]. Available: www.niaaa.nih.gov/what-standard-drink. [Accessed 30 June 2020].)

The kidneys filter blood, balancing the amount of fluid in the body, and removing waste by excreting it into the urine. Alcohol makes the kidneys work harder and produce

more urine; up to 10% of alcohol leaves the body unchanged in the urine [11]. Alcohol causes changes in the function of the kidneys and makes them less able to filter the blood. Alcohol also affects the ability to regulate fluid and electrolytes in the body. When alcohol dehydrates the body, the drying effect can affect the normal function of cells and organs, including the kidneys. Alcohol can also disrupt hormones that affect normal kidney function [16].

A small amount of alcohol also evaporates from the fine blood vessels just under the skin. Some alcohol is evaporated from the blood through the lungs into the breath; up to 8% of alcohol is exhaled [11].

Blood Alcohol Concentration

The alcohol that is exhaled through the breath is the alcohol that a breathalyzer uses to measure blood alcohol concentration (BAC). When alcohol is consumed faster than the liver can break it down, BAC rises, and the feeling of drunkenness occurs. BAC does not correlate precisely with symptoms of drunkenness and different people can have different symptoms even after drinking the same amount of alcohol [1, 2, 17]. The BAC level and every individual's reaction to alcohol is influenced by: the ability of the liver to metabolize alcohol, which varies due to genetic differences in the liver enzymes that break down alcohol; the presence or absence of food in the stomach, since food dilutes the alcohol and dramatically slows its absorption into the bloodstream by preventing it from passing quickly into the small intestine; the concentration of alcohol in the beverage, highly concentrated beverages are more quickly absorbed; how quickly alcohol is consumed; body type, heavier and more muscular people have more fat and muscle to absorb the alcohol; age, sex, and ethnicity; and how frequently a person drinks alcohol [2, 10, 11, 17].

Alcohol Use Disorder and the Consequences

Alcohol Use Disorder (AUD), also known as alcoholism, is a medical diagnosis described as a chronic relapsing brain disease characterized by uncontrollable alcohol use, loss of control over alcohol intake, and having a negative emotional state when not using alcohol [18]. The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) defines AUD by: "a problematic pattern of alcohol use leading to clinically significant impairment or distress, as manifested by at least two of the criteria, occurring within a 12-month period. DSM-5 diagnoses AUD using the following criteria: alcohol is often taken in larger amounts or over a longer period than was intended; there is a persistent desire or unsuccessful efforts to cut down or control alcohol use; a great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects; craving, or a strong desire or urge to use alcohol; recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home; continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol; important social, occupational, or recreational activities are given up or reduced because of alcohol use; recurrent alcohol use in situations in which it is physically hazardous and alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol [19]. The DSM-5 defines the severity of AUD into three categories which include: mild, moderate, and severe. Mild is the presence of two to three of the criteria, moderate is the presence of four to five criteria and severe is the presence of six or more criteria [19].

AUD, like any addiction, is a mental illness and can interfere with a person's life, changing normal behavior and making it more difficult for a person to do daily activities such as go to work or school and have meaningful relationships with family and friends [20]. A person with AUD is at a greater risk of being diagnosed with other mental health disorders including depression, bipolar disorder, obsessive-compulsive disorder (OCD), anxiety, and insomnia [21]. A person that is abusing alcohol, on a continuous basis, is not only putting themselves in problematic situations in daily life, it could also lead to serious legal problems and possible jail time. Additionally, it could put other people in dangerous situations if the person under the influence of alcohol decides to get behind the wheel of a vehicle. The risk of death from car crashes, injuries, homicide, and suicide increase due to alcohol and its abuse [22].

Communication pathways in the brain are interrupted when a person is intoxicated, and it changes how the brain works and even looks [23]. A person under the influence of alcohol can have changes in their mood and behavior and it can affect cognition and motor function. Mental clarity is affected by alcohol use, which makes it difficult to make proper decisions and rational choices. Chronic heavy consumption of alcohol can also shrink the frontal lobes of the brain [24]. The frontal lobes of the brain control important cognitive skills and are tasked with emotional expression, problem solving, memory, language, judgment, and sexual behaviors [25]. How the brain makes memories can be affected by the consumption of alcohol; it is possible to have no memories of before, during or after being under the influence of alcohol. When a person is under the influence of alcohol his or her speech can become slurred and incoherent; this is one of the first noticeable signs that a person is intoxicated [24].

The World Health Organization (WHO) reported in 2014 that more than 200 diseases and injury-related health conditions could be contributed to alcohol and its abuse [26]. Long-term, heavy drinking can cause problems in all areas of the body. It increases a person's risk of having certain types of cancer, especially liver, but also cancer of the head and neck, throat, mouth, esophagus, colorectal, and in women, breast [23, 24]. Based on data from 2009, an estimated 3.5% of all cancer deaths, about 19,500 deaths, in the United States were alcohol related [27]. There is an increased risk of damage to the heart, leading to issues like cardiovascular disease, cardiomyopathy, arrhythmias, stroke, and high blood pressure. Drinking a large amount can also suppress immune function [23, 24]. A suppressed immune system can increase susceptibility to illness and disease; people that drink chronically are more likely to come down with serious lung infections such as pneumonia and tuberculosis. Heavy drinking can cause damage to the liver, preventing it from removing harmful substances from the body properly and causing steatosis, alcoholic hepatitis, fibrosis, and cirrhosis. Likewise, the pancreas can also be affected by excessive alcohol use, causing chronic pancreatitis, which is serious inflammation of the pancreas [23, 24]. Damage to the pancreas can also affect the body's sugar levels, which could be deadly for a person diagnosed with diabetes. This can cause low blood sugar because the organs are not functioning properly and on the other side it can also cause high blood sugar because the body is not producing enough insulin to use the sugar [28]. Those that drink heavily on a regular basis may feel fatigued or tired, which could be a sign of anemia, a condition where a person lacks enough red blood cells to supply sufficient oxygen to the tissues of the body. Stomach problems can also arise from drinking heavily, including bloating, gas, and ulcers; it can damage the intestines, which can lead to stomach pain and diarrhea [24].

Long periods of heavy drinking can cause infertility in women and men are likely to experience erectile dysfunction. The risk of having osteoporosis, thinning of the bones, increases when a person drinks alcohol. The body is not able to properly absorb all the vitamins, nutrients, and minerals that it needs. Damage can also occur to the central nervous system; this can cause tingling, numbness, or pain to the hands and feet. Muscle cramping, weakness, and muscle death can all be experienced by a person that drinks excessively [24].

Drinking alcohol during any stage in a pregnancy can harm the developing fetus. Mothers that drink alcohol during pregnancy can cause the developing fetus to have Fetal Alcohol Spectrum Disorders (FASDs), which can cause medical, behavioral, educational, and social problems [29]. Some of the problems that children diagnosed with FASDs can have include: abnormal facial features, small head size, shorter-than-average height, low body weight, poor coordination, hyperactive behavior, difficulty with attention and memory, learning disabilities and difficulty in school, speech and language delays, intellectual disability or low IQ, poor reasoning and judgement skills, sleep and sucking problems as a baby, vision or hearing problems, and problems with the heart, kidneys, or bones [29]. Fetal alcohol syndrome (FAS) is one of the most known and most serious types of FASD. Children diagnosed with FAS can have facial abnormalities, which include wide-set and narrow eyes, growth problems, and nervous system abnormalities. FASDs follow the child throughout their entire life because there is no cure. The only available treatments are medications that address FASD symptoms and interventions such as behavior and educational therapy [29].

Scale of AUD and Alcohol-Associated Morbidity and Mortality

In 2012, 3.3 million deaths (5.9% of all the world's deaths) were attributed to alcohol consumption. Those 3.3 million deaths included 7.6% of all deaths for men and 4.0% for women. In 2010, alcohol misuse was the fifth leading risk factor worldwide for premature death and disability [26]. This global risk factor increases to the first leading cause for those between the ages of 15 and 49 [30]. Approximately 25% of the total deaths worldwide for people between the ages of 20 to 39 are attributed to alcohol [31]. AUD is a common condition affecting an estimated 15 million people in the United States. In 2018, it was reported that 5.8%, or 14.4 million adults, ages 18 and older, in the United States had AUD. This statistic includes around 9.2 million men and 5.3 million women. It is unfortunate to also report that in 2018 an estimated 401,000 adolescents, ages 12 to 17, in the United States had AUD [32, 33]. It is estimated that 88,000 people are killed from alcohol-related causes. This is the third leading cause of preventable deaths in the United States, after tobacco, poor diet, and poor physical health [34, 35]. Alcohol-impaired driving fatalities accounted for 9,967 deaths in 2014, which accounted for 31% of the overall driving fatalities for that year [36]. Comparatively, in 2018, alcohol-impaired driving fatalities increased to 10,511, which accounted for 29% of traffic deaths for that year. Drunk driving costs the United States $199 billion annually, these costs include medical costs, property damage, emergency medical services, legal expenses, lost productivity, congestion costs, and insurance administration [37]. Alcohol misuse including binge drinking, underage drinking and drinking while pregnant, costs the United States $249 billion in 2010 [38].

Current Treatments for Alcohol Use Disorder

In the United States, of the 15 million people that suffer from AUD, less than 10% receive treatment [18]. Current treatments for AUD, including pharmacological treatments and medications, have low rates of success, indicating a clear need for new potential drugs. Current medications that are available and approved by the U.S. Food and Drug Administration (FDA) for those suffering from AUD include the following: disulfiram, naltrexone, and acamprosate.

Disulfiram, known by the brand name Antabuse, was one of the first medications approved for the treatment of alcohol dependence in 1948. The chemical structure of disulfiram can be seen below in Figure 5. This medication causes severe adverse reactions when a person drinks alcohol while on this treatment [39]. Physical reactions that disulfiram cause when a person drinks alcohol include nausea, vomiting, headaches and flushing of the face. This drug is thought to be a deterrent to drinking because a person that consumes alcohol while taking this medication will experience the physical adverse reactions [39]. Disulfiram acts as an active deterrent by triggering an accumulation of toxic acetaldehyde if alcohol is consumed. Disulfiram can have severe and even fatal effects and is normally prescribed only for those that are highly motivated to remain abstinent and have a strong support network. Compliance of those taking disulfiram are typically low, making supervised administration critical for its success [40].

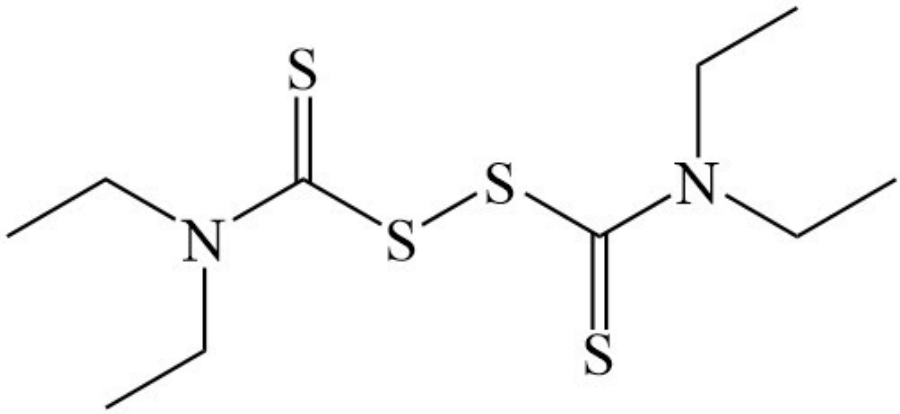

Figure 5. Structure of Disulfiram. One of the first medications approved for the treatment of AUD. It causes adverse reactions when a person consumes alcohol while taking this medication.

Naltrexone, known by the brand names Revia and Depade, is an opioid receptor antagonist; it works in the brain by blocking the receptors that give the feelings of euphoria from taking opioids. The chemical structure of naltrexone can be seen in Figure 6 below. For AUD, it similarly works by blocking the opioid receptors that encode the pleasurable feelings a person receives from drinking alcohol. Naltrexone helps some patients drink less or stop drinking altogether, and the desire to drink alcohol is decreased to a greater extent when taking naltrexone in combination with counseling, support, and lifestyle changes [41]. A meta-analysis of 64 clinical trials conducted between 1970 and 2009 showed that naltrexone was more effective in reducing cravings and the number of drinks per drinking day [40]. There is an injectable depot version of naltrexone, known by the brand name Vivitrol, that is comparable to the pill form, but is injected by a healthcare professional once a month [41]. The use of naltrexone for the treatment of AUD was approved by the FDA in 1994 [39].

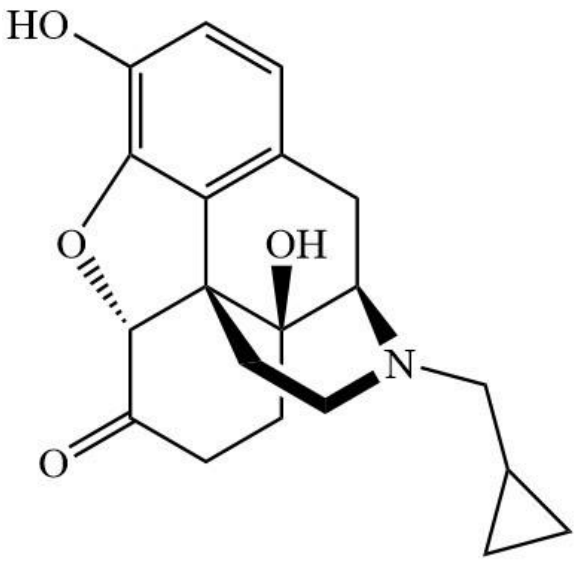

Figure 6. Structure of Naltrexone. Was first prescribed to treat opioid addiction. Helps with the treatment of addiction by blocking the receptor that give the feelings of euphoria from consuming alcohol or taking drugs.

Acamprosate, known by the brand name Campral, is prescribed to patients to help deal with the cravings of alcohol once the person has stopped drinking [42]. The chemical structure for acamprosate can be seen in Figure 7 below. A meta-analysis of 64 clinical trials conducted between 1970 and 2009 showed that acamprosate was more effective at promoting abstinence [40]. It was approved for the use of treatment for alcoholism in France and other countries throughout Europe for more than 20 years. However, it was not approved in the United States by the FDA until January of 2005 [39].

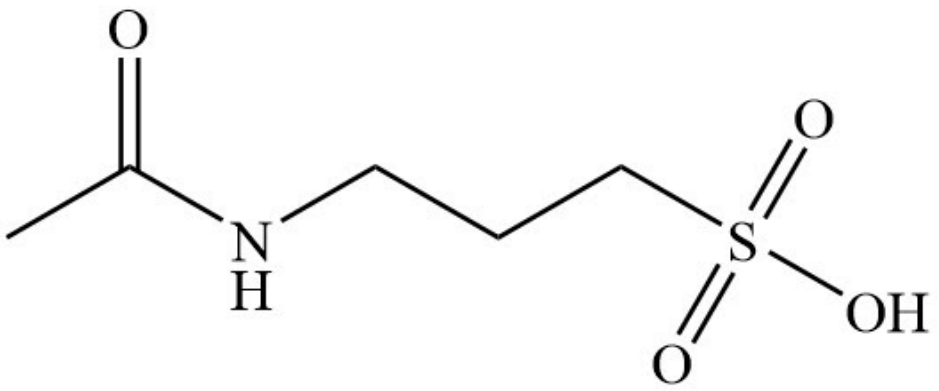

Figure 7. Structure of Acamprosate. One of the most recent drugs approved for the treatment of AUD. It helps patients deal with cravings after they have already stopped drinking.

There are several other non-medications available for the treatment of AUD that can be used in combination with drug treatments or in place of drug treatments. In-patient treatment centers or hospitals can help those trying to detox from alcohol and these facilities also allow people to go through withdrawal in a safe environment while being monitored by health care professionals. Psychological counseling, including talk therapy and cognitive behavioral therapy is another option for those suffering from AUD; many centers offer counseling for individuals and groups and therapy for couples and family. Alcoholics Anonymous (AA) is one of the most popular support groups that those that are addicted to alcohol use to help themselves and other become sober.

Gaps in Knowledge and Treatment for Alcohol Use Disorder

No new medications for the treatment of alcohol use disorder have been approved in the United States since acamprosate came on the market in 2004 [40]. Current treatments for AUD, including pharmacological treatments and medications, have low rates of success, indicating a clear need for new potential drugs. Non-pharmacological treatments

are generally the first and sometimes only method of treatment for people suffering from AUD. The drugs available for "treatment" of AUD only treat the side effects that result from quitting drinking or cause adverse effects if a person replaces in their drinking habits. None of the medications treat the actual addiction. The knowledge of the medical and scientific communities has gaps in how to treat addiction, including alcohol use disorder, in the most effective way. The first thing that needs to be known is where in the brain alcohol targets. The abuse liability of the addictive drug also needs to be determined. A drug needs to be found that completely and effectively targets the area in the brain that is attributed to AUD. A drug that is found to be successful could help people in the grips of their addiction to alcohol or for those that other treatments and therapies have not worked.

Research Goal

In this paper, two different drugs that each target separate receptors in the brain are tested to determine if either could be effective in the treatment in mouse models of AUD. The two drugs of interest are L-745,870 and PSNCBAM-1, both of which are described in more detail in subsequent chapters. The experimental methods and results of the experiments on both the drugs of interest are also described in detail in the following chapters. Our ultimate goal is to find new drugs that could help people in the grips of their addiction have a fighting chance.

Chapter 2

Methods and Materials

Animals

Adult, drug-naïve male C57BL/6 mice, obtained from Charles River Laboratories (Wilmington, MA), weighing 23 to 30 grams at the start of each experiment were used for all experiments. Upon arrival to the vivarium at Cooper Medical School of Rowan University (CMSRU), the mice were given free access to food and water during a one-week habituation period. The habituation period and housing happened in a room that was maintained at a constant temperature (21-23°C) and humidity (45-50%) on a 12-hour light/dark cycle. Mice were housed four per cage in polycarbonate cages with enrichment provided by paper Bio-Huts and/or nestlets. The mice were weighed and handled daily and monitored for general health and behavioral parameters, including potential signs of significant alcohol withdrawal. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (US National Academy of Sciences) and were approved by the Institutional Animal Care and Use Committee of Rowan University. The CMSRU animal facility is provisionally accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

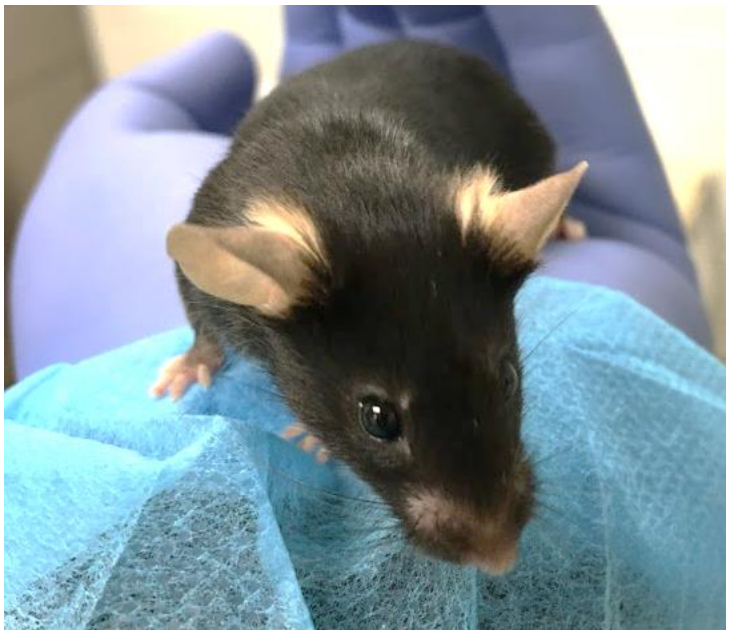

Figure 8. Picture of a C57BL/6 mouse that was used in our lab for an alcohol addiction study. The drug-naïve male mice were obtained from Charles River Laboratories.

Drugs

Two different drugs were tested in this study to determine if either could be effective in the treatment of alcohol use disorder, AUD, in mouse models. The two drugs of interest are L-745,870 and PSNCBAM-1, both of which are described in more detail in subsequent chapters. A vehicle was also used for each of the drugs, L-745,870 and PSNCBAM-1. The vehicle acts as a way for the solid drug to be dissolved and also acts as a delivery system for the drug to be introduced into the body. The vehicle for L-745,870 was physiological saline and for PSNCBAM-1 the vehicle was a mixture of 10% dimethyl sulfoxide (DMSO), 10% Tween 80, and 80% saline.

Initial Control Studies

Open field. Open field testing allowed experimenters to determine if locomotor function was disrupted due to the introduction of a drug. The results helped experimenters

determine if the drug was deemed fit to continue with additional studies. In this initial assessment, mice were placed in the open field chamber for an initial 20 minutes. This allotment of time allowed the mice to explore the chamber unhindered. After the initial 20 minutes, the mice were taken out and were each given an i.p. injection of the drug and dose being tested for that mouse for the specific experiment being performed. After the injection, the mice were placed in the open field chamber for an additional 40 minutes. The behavior and activity of the mice were recorded via a camera positioned over the open field chambers.

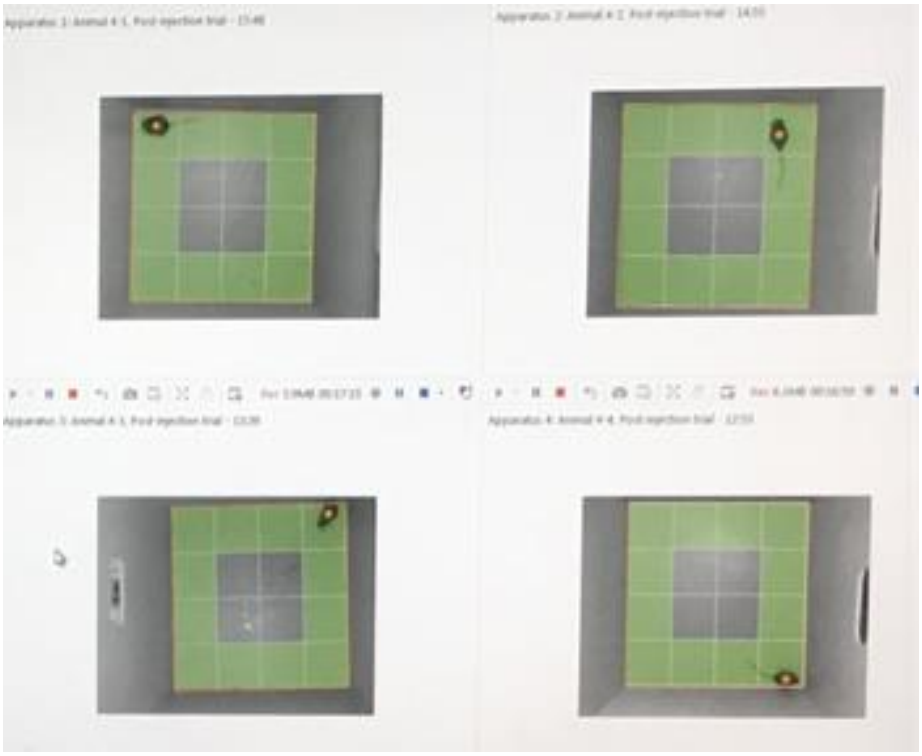

Figure 9. Open Field Layout. Each layout was observed by an individual camera. The chambers were divided into 16 squares, a four by four grid. The 16 squares were then grouped into the 12 outer squares of the perimeter and the 4 inner squares of the center. The camera tracked the mice with and orange dot and recorded the time comparatively of how much was spent in the perimeter squares verses the inner squares.

Rotarod. Rotarod testing allowed experimenters to determine if coordination function was disrupted due to the introduction of a drug. The results helped experimenters determine if the drug was deemed fit to continue with additional studies. In the initial assessment, mice were placed on the black rotating rod; the rod gradually increased in speed from 4 rpm to 40 rpm. The mice were tested for a limit of five minutes or until they fell off the rod–whichever happened first. Testing occurred for 20 minutes before the given injection and for 60 minutes after the given injection. Testing happened in ten-minute increments.

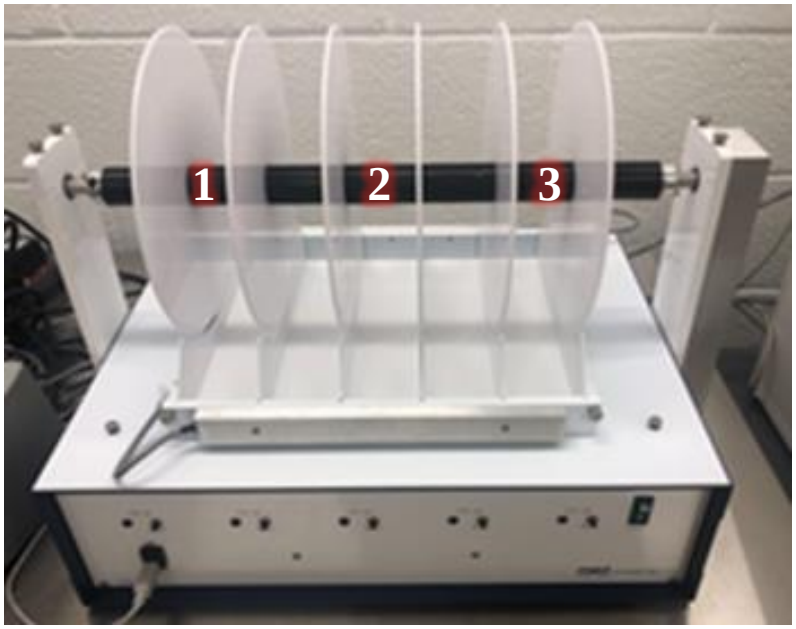

Figure 10. Rotarod Apparatus. The mice are placed apart from one another on the center black rod that goes through the apparatus. The black rod rotates at and increasing speed of 4 to 40 rpm. A sensor plate under the black rotating rod is able to sense when a mouse falls of the rod and records the time it took for the mouse to fall and at what speed the rod was rotating when the mouse fell. The mice are placed on the numbered slots indicated in the picture to allow for space in between the animals so there is no interruption with the other mice when one falls.

Conditioned Place Preference

Conditioned Place Preference (CPP), a preclinical behavioral analyzing model, has been widely used for the research of addiction and substances of abuse. CPP is a type of Pavlovian conditioning, which is utilized by different scientists to measure the motivational effects of addictive drugs [43]. The goal of this experiment was to test and analyze the effects of the dopamine D4 antagonist, L-745,870, and CB1 negative allosteric modulator, PSNCBAM-1, to determine if either could reduce ethanol induce preference behavior in adult male mice.

The conditioned place preference apparatus. The CPP apparatus is described as two adjacent chambers characterized by white- (left side) and black- (right-side) colored walls that are connected to each other through a small central gray-colored compartment. The end compartments had each have square stainless-steel grated floors and the central compartment has a smooth gray-colored floor. Access between the side and central compartments were controlled by guillotine-style doors. The center compartment allowed the mice to move freely between the two adjacent compartments when the gates of the side compartments were raised. The two end compartments were paired with either a drug or vehicle during experimental training. The center compartment was not paired with the drug or the vehicle; it was considered a neutral space within the entire compartment. A picture of the CPP apparatus can be seen in Figure 11 below.

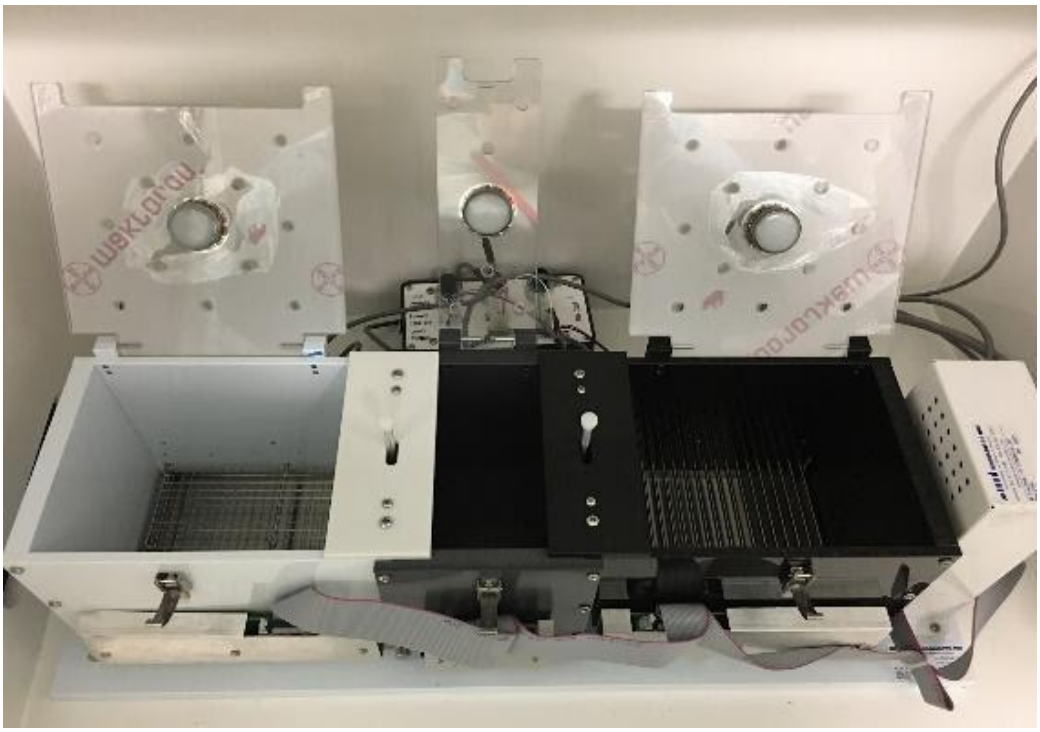

Figure 11. Conditioned Place Preference Chamber. In this picture you can see the white compartment located on the left-side and the black compartment located on the right-side. These two compartments during and experiment are either paired with the drug of interest or the vehicle being used with that drug. The neutral gray compartment can be seen in the middle. In this particular picture the gates that connect the three compartments are closed.

Initial preference testing for conditioned place preference. Before conditioning and training with any drugs of interest started, the initial preference, a predisposed liking, of the mice to either of the side chambers was tested to remove any bias from the experiment. An initial preference was measured by allowing the mice to explore the full apparatus unhindered for a session of 30 minutes. The ratio of time each mouse spent in an individual compartment was calculated, so a chart could be prepared of which mouse receives which drug paired side-compartment. Usually, in the experimental procedure, the drug-paired and vehicle-paired compartments were assigned at random regardless of initial

preference score. However, in a biased CPP study, the compartment which is least preferred is paired with the drug for that individual mouse.

Conditioning with ethanol. In this experiment place preference was explored at various dosages of ethanol, ranging from 1.2-2.0 g/kg. Mice were trained on alternating days with either ethanol or saline as the vehicle, with each mouse being paired to a particular compartment. During the training period, daily i.p. injections of ethanol were utilized to induce the effects of addiction. The 2.0g/kg dose of ethanol was the one that most optimized the addiction preference and was the dose used in subsequent experiments involving CPP.

Testing of pharmacotherapeutics for conditioned place preference. After establishing the effects of ethanol in CPP testing, the therapeutic potential of novel medications on ethanol abuse, including the dopamine D4 antagonist, L-745,870, and the CB1 negative allosteric modulator, PSNCBAM-1, were to be studied. In the L-745,870 study, the mice were subjected to a pre-injection of either 1.5 mg/kg L-745,870, 3.0 mg/kg L-745,870, or saline; and a post-injection of either 2.0 g/kg ethanol or saline. They were then placed in the CPP apparatus to determine the efficacy of L-745,870 as a potential anti-addiction drug. In the PSNCBAM-1 study, the mice were subjected to a pre-injection 10 mg/kg PSNCBAM-1, 30 mg/kg PSNCBAM-1, or the vehicle; and a post-injection of either 2.0 g/kg ethanol or saline. Again, they were then placed in the CPP apparatus to determine the efficacy of PSNCBAM-1 as a potential anti-addiction drug. In a second PSNCBAM-1 study, an intermediate dose of 18 mg/kg was tested in the same manner.

Self-Administration Operant Training

Operant conditioning is a method of learning that occurs through rewards and punishments for behavior that is performed. Through operant conditioning, an individual makes an association between a particular behavior and a consequence [44]. The purpose of performing trials of the two drugs of interest, L-745,870 and PSNCBAM-1, while the mice received vanilla Ensure was to determine if the drugs were decreasing the activity of the mice or made the mice lethargic. The goal of performing trials for the two drugs of interest, L-745,870 and PSNCBAM-1, while the mice received the ethanol solution was to determine if either of the drugs decreased the self-administration intake of ethanol.

Self-administration apparatus. Standard operant conditioning chambers that were utilized for the ethanol and food self-administration studies were housed in ventilated, sound-reducing boxes with fans. Each box was equipped with a reward receptacle in the middle of two nose poking response holes. A stimulus light was inside each response hole and a house light was present on the opposite side of the chamber. The ethanol or food was delivered through the reward receptacle via tubing attached to a syringe placed into a plunger pump located inside the box, but outside the chamber. A picture of the self-administration apparatus can be seen below in Figure 12.

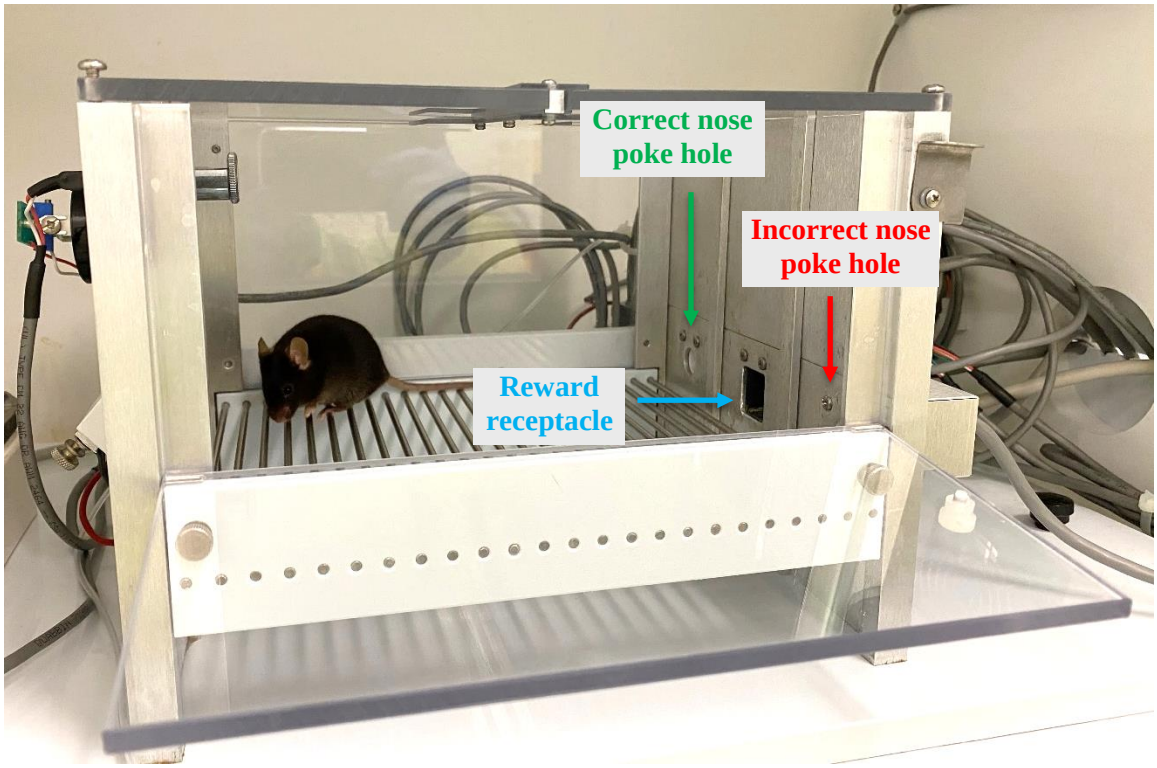

Figure 12. Self-Administration Operant Chamber. In this picture you can see the compartment that the mice are placed in during training and testing. Both nose poke holes, which are labeled in the picture, the correct hole being on the left side and the incorrect hole being on the right side. The reward receptacle is also labeled in the center of the picture, is where the mice consume the reward after correctly nose poking the proper amount of times. [1]

Training parameters for self-administration training. Self-administration training consisted of initial daily 60-minute sessions but was increased to daily 120-minute sessions during which mice were trained to nose poke the active hole, located on the left side, to obtain the reward either ethanol or diluted vanilla Ensure. The training was done on a fixed-ratio (FR) system, in which a fixed number of correct nose pokes were necessary to receive a programmed reward; training ratios started at an FR of one (FR1)—one correct nose poke on the programmed drug side results in an earned reward—and gradually

[1] Picture used with permission from Mohammad Atiqur Rahman.

increased to an FR4. Responses on the correct nose hole side (left side) by the mice resulted in a delivery of the reward over a three second period, presented with an associated light an tone; inactive nose pokes (right side) produced no programmed response but were recorded. In the FR4 training, a mouse had to nose poke four consecutive times at the correct hole to receive the reward; if the mouse nose poked correctly three times then incorrectly nose poked once, that mouse would have to start the count again.

Self-administration training. Food-restricted mice were trained to nose poke for liquid rewards, which were gradually changed across the training portion of the experiment. The mice were first taught to nose poke for a food reward of vanilla Ensure. The vanilla Ensure was watered down gradually until it was presented as a 50% Ensure: 50% water ratio. The mice were consistently nose poking for rewards at an FR4 before ethanol was introduced into the mixture. When the 50% Ensure: 50% water mixture had a stable response, the water was replaced in increments by ethanol. This was done until the ethanol was at a 10% w/v concentration in the mixture (i.e., a final 10% w/v mixture by volume; see Appendix A for details on ethanol dilutions used). The Ensure was replaced with water until the mixture was a 10% w/v mixture of ethanol in water, respectively. Upon training the mice for several months with the 10% w/v mixture, it was determined that the concentration of ethanol in the mixture was too high. The 10% w/v mixture was determined to be too high because the responses of the mice to nose poking for the alcohol mixture was decreasing over time instead of increasing. As a result of this, the concentration was lowered to an 8% w/v mixture of ethanol in water, respectively. The 8% w/v ethanol in water mixture was used in the operant chambers to the test of the drugs of interest, L-

745,870 and PSNCBAM-1 (i.e., a final 8% w/v mixture by volume; see Appendix A for details on ethanol dilutions used).

Testing of pharmacotherapeutics for self-administration. Before the testing of a drug of interest started, the eight mice that had the best nose poke response to the mixture being used were kept out of the sixteen trained mice. The testing sessions of the drug were each one day a week; the remaining days were used as training days. The testing sessions were performed in a Latin square design, wherein each of the mice received all drug or vehicle doses to be tested but the injections each mouse received varied each testing session; this was to account for the conditions not being the same during every testing session. At the end of all the testing sessions, all the mice had received each of the doses as well as the vehicle. The two different drugs of interest, L-745,870 and PSNCBAM-1, were tested during different sessions and with different sets of mice. During the L-745,870 testing session, the mice were injected with either 1.5 mg/kg L-745,870, 3.0 mg/kg L-745,870, or saline vehicle. During the first PSNCBAM-1 testing session, the mice were injected with either 10 mg/kg PSNCBAM-1, 30 mg/kg PSNCBAM-1, or the vehicle mixture, 10% Tween 80, 10% DMSO and 80% saline. In the second PSNCBAM-1 testing session, an intermediate dose of 18 mg/kg was tested in the same manner.

Statistical Analysis

All results are presented as means ± SEM (standard error of the mean). All data was analyzed in GraphPad Prism 8.3 (San Diego, CA, USA). During the one-way analysis of variance (ANOVA) tests, if a significant main effect was found, individual group comparisons were carried out using pre-planned Bonferroni t-tests. The one-way analysis of variance (ANOVA) is used to determine whether there are any statistically significant

differences between the averages of unrelated groups in an experiment. A Bonferroni test is a type of multiple comparison test used in statistical analysis that prevents data from incorrectly appearing to be statistically significant.

Chapter 3

Dopamine D4 Antagonist L-745,870 Does Not Affect Alcohol Reward or Self-Administration in Adult Male Mice

Abstract

The dopamine D4 receptor (D4R) is a target of interest in the development of medications for psychostimulant addiction but has been unexplored for AUDs. The dopamine D4 receptor has been of great interest in the study of several different types of addiction including cocaine. In this study, the effects of the D4R antagonist L-745,870 were investigated in rodent models of AUD using adult male mice. Initial control studies with L-745,870 indicated that the doses tested (1.5 and 3.0 mg/kg, i.p)—doses that alter cocaine-mediated behavior—did not significantly disrupt locomotor activity or rotarod coordination. It also did not significantly attenuate palatable food self-administration (diluted vanilla Ensure). L-745,870 did not significantly attenuate ethanol self-administration (8% w/v ethanol in water). Further testing determined that L-745,870 pretreatment during conditioned place preference training did not affect the rewarding value of 2.0 g/kg ethanol using a three-compartment chambered conditioned place preference apparatus. These results suggest that D4R antagonism does not alter the rewarding value of ethanol.

The Dopamine D4 Receptor: an AUD Target?

Dopamine primarily exerts its influence by interacting with and activating a family of G protein-coupled dopamine receptors [45]. It has long been thought that the dopamine systems in the brain were mainly involved in essential operations, such as the primary motivation for natural stimuli like food, water, and sex [46, 47]. Dopamine receptors are

classified into two groups based on their amino-acid sequence homology and modulation of adenylyl cyclase activity: the D1-like (D1 and D5) and D2-like (D2, D3, and D4) [48]. The dopamine receptor subtypes display different pharmacological profiles and expression levels in the brain, they are each involved in different roles in the development of substance use disorders as well as relapse [48, 49].

In alcohol addiction, the major neurotransmitters involved are dopamine, serotonin, GABA, glutamate, and acetylcholine. Dopamine is the major neurotransmitter that mediates reward behavior. Dopamine acts on the mesolimbic pathway, or reward pathway, including the amygdala that deals with emotions, nucleus accumbens that controls motor functions, the prefrontal cortex serving the purpose of attention and planning, and hippocampus which is involved in memory formation [50]. Acute or chronic alcohol exposure leads to an alteration in dopamine signaling. Acute alcohol consumption leads to the flooding of dopamine in ventral tegmental area and nucleus accumbens that leads to the overstimulation of neurons. Dopamine in the nucleus accumbens is critically involved in the development of alcohol addiction and promotes the changes in the body and brain as a result of the addiction [50].

The dopamine D4 receptor is expressed in the cortex, hypothalamus, hippocampus, and amygdala [51, 52]. A dopamine D4 antagonist works in the brain by blocking the receptor and inhibiting the normal function, this decreases the amount of dopamine able to bind to the receptor. The decrease in ability for dopamine to bind, in turn, decreases reward motivated behavior. The dopamine D4 receptor has been shown to change drug-seeking behaviors in animals [48].

The dopamine D4 receptor is of interest in the study of AUD due to its role in mediating the effects of dopamine—a neurotransmitter long implicated in the effects of alcohol use [53]. The dopamine D4 receptor has become of interest in the study of AUD and other neuropsychiatric disorders due to its unique highly genetic variant nature and the location of a variable number tandem repeat in its coding region [54]. The dopamine D4 receptor variable number tandem repeat has been hypothesized as a contributing risk factor of AUD, due to the wide allelic variability and high concentration of D4 receptors in the prefrontal cortex—a brain region known for its role in cognition, inhibition, and attention [55]. The D4 receptor has limited distribution within the brain suggesting it may have a unique role in drug abuse; however, few studies have evaluated the importance of the D4 receptor [45]. In addition to the animal models of reward and addiction, a number of clinical studies using human patient populations have suggested a link between D4 genetic variations and the occurrence of opiate or alcohol abuse. The D4 receptor genetic variations have also been associated with alcoholism [56]. Linkage studies performed by Hill et al. found evidence of a connection between the genetic variations near the D2 and D4 receptor and measures of physical dependence and early onset of alcoholism in families with histories of chronic alcoholism [57]. A study by Hutchison et al. revealed that subjects with genetic variations in the D4 receptor, displayed higher levels of alcohol craving following consumption of alcoholic beverages [58].

L-745,870

L-745,870 (Tocris Bioscience, Ellisville, Missouri, US) is a dopamine D4 receptor-selective antagonist (Figure 13) and is known to have excellent oral bioavailability and

brain penetration [59]. The IUPAC name for L-745,870 is 3-([4-(4-chlorophenyl)piperazin-1-yl]methyl)-1H-pyrrolo[2, 3-b]pyridine.

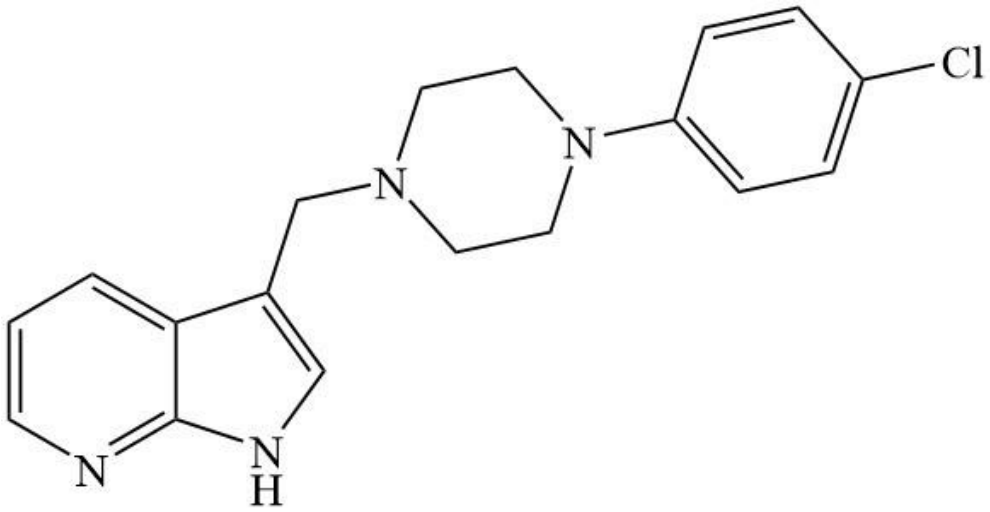

Figure 13. Structure of L-745,870. A D4 antagonist obtained from Tocris Bioscience in Ellisville, Missouri.

In 1997, just six years after the discovery of the dopamine D4 receptor, researchers from Merck published a series of manuscripts describing the discovery of a selective and CNS-penetrant dopamine D4 receptor antagonist, L-745,870, along with preclinical and clinical efficacy studies [59, 60, 61]. L-745,870 possessed sub-nanomolar binding at D4 (K_i = 0.43 nM), 5- to 20-fold higher than the typical antipsychotic haloperidol (K_i = 2.3 nM) or clozapine (K_i = 10 nM), and was >2000-fold selective versus D1–3,5 (D1 K_i > 10 μM, D2 K_i = 960 nM, D3 K_i = 2,310 nM, D5 K_i > 10 μM) [62].

L-745,870 has been tested in several studies. In one study, alcohol-dependent male rats were found to have diminished ability to execute several emotional-learning tasks during their abstinence period, but after giving levodopa, dopamine replacement agent, rats

showed rapid recovery [50]. In another study, L-745,870 had no beneficial effect on nicotine self-administration, however, it did reduce both cue- and nicotine-induced reinstatement of nicotine-seeking behavior in rats [63]. In a study, rats were trained to discriminate amphetamine from saline and L-745,870 partially blocked the discriminative stimulus effect [64].

L-745,870 was initially developed as a drug for the treatment of schizophrenia and its antipsychotic efficacy was tested in a phase IIa clinical trial in humans. The drug did not prove to be effective in the treatment of schizophrenia, but invaluable data regarding the safety, tolerability, and pharmacokinetics profile of the drug in humans was gathered during the development process [65].

L-745,870 was tested in a previous study in our lab and it attenuated cocaine CPP. We hypothesize, based on evidence from studies previously performed involving alcohol and other substances of addiction, that L-745,870 will reduce alcohol-taking and -seeking behavior in adult male mice. In order to test this hypothesis, we tested the effects of L-745,870 in ethanol CPP and self-administration studies. When these studies were initiated, L-745,870 had never before been evaluated for effects in models of alcohol addiction.

Results

Initial control studies. The effects of L-745,870 were tested in initial control studies to see if the drug decreased any locomotor activity (open field testing) or coordination function (rotarod testing) in mice, in order to determine if the drug is safe to use in further studies. The tests that were performed in the initial control studies included open field and rotarod.

For the open field study, mice were placed in the open field chamber for an initial 20 minutes. This allotment of time allowed the mice to explore the chamber unhindered. After the initial 20 minutes, the mice were taken out and were each given an i.p. injection of either 3.0 mg/kg L-745,870 or saline vehicle. After the injection, the mice were placed in the open field chamber for an additional 40 minutes. The behavior and activity of the mice were recorded via a camera positioned over the open field chambers. L-745,870 (3.0 mg/kg, i.p.) indicated that the doses tested did not significantly disrupt locomotor activity in the open field initial control study (Figure 14). An unpaired t-test of total post-injection locomotor activity revealed no significant effect of treatment ($t(20) = 0.073$, $p > 0.9$).

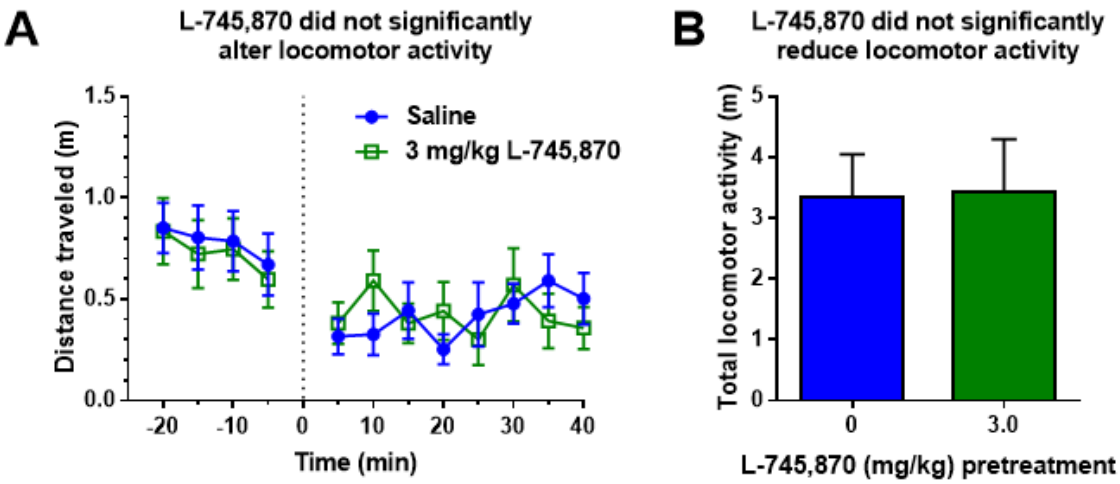

Figure 14. 3 mg/kg L-745,870 does not significantly affect locomotor activity in an open field test. Male mice were placed in an open field apparatus for 20 minutes and behavior was recorded. Then they were given i.p. injections of saline (n = 11) or 3 mg/kg L-745,870 (n = 11) and behavior was recorded for an additional 40 minutes. (A) 20 minutes after introduction into the open field, mice were injected with 3 mg/kg L-745,870 or vehicle and locomotor activity was recorded for an additional 40 minutes. Data are presented as means ± SEM of distance traveled in 5-minute bins. (B) Overall post-injection distance traveled was not significantly different across treatments. Data are presented as means ± SEM of total distance traveled in the 40 minutes following drug injection.

For the rotarod study, mice were placed on the black rotating rod; the rod gradually increased in speed from 4 rpm to 40 rpm. The mice were tested for a limit of five minutes or until they fell off the rod–whichever happened first. Testing occurred for 20 minutes before an i.p. injection of either 3.0 mg/kg L-745,870 or saline vehicle and for 60 minutes after the injection. Testing happened in ten-minute increments. L-745,870 (3.0 mg/kg, i.p.) also did not significantly disrupt coordination function in the rotarod initial control study (Figure 15).

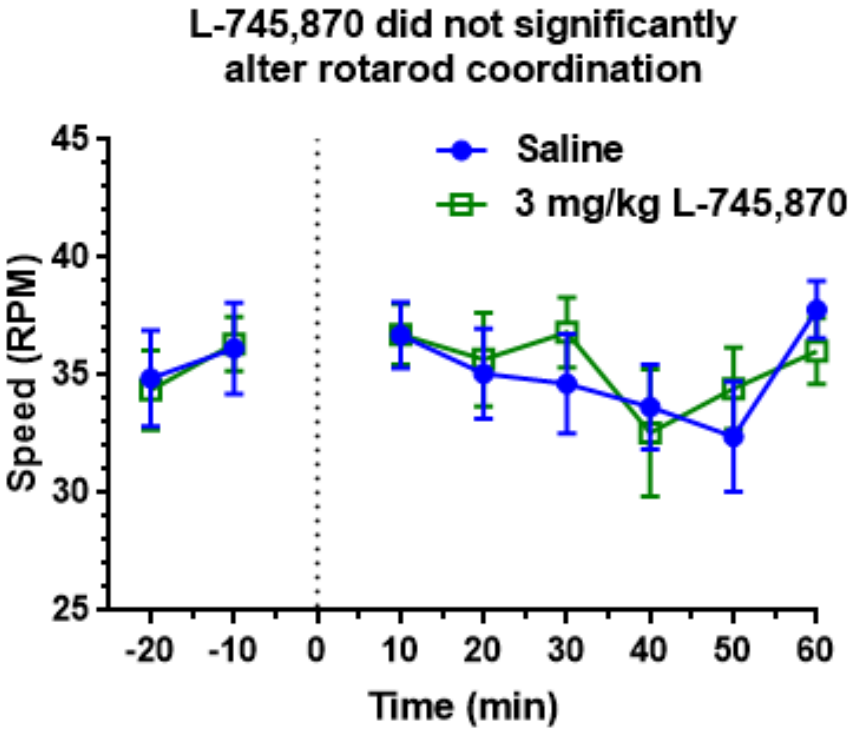

Figure 15. 3 mg/kg L-745,870 does not significantly affect coordination function in a rotarod test. Male mice were placed on a rotarod apparatus at 10-minute increments for a total of 20 minutes and the time and speed at which they fell off the apparatus was recorded. Then they were given i.p. injections of saline (n = 11) or 3 mg/kg L-745,870 (n = 11) and time and speed was recorded for an additional 60 minutes at 10-minute increments. Data are presented as means ± SEM of time spent at what speed the mice fell off in 10-minute bins.

Conditioned place preference. CPP training for 2.0 g/kg ethanol was modified to include a L-745,870 or saline vehicle pretreatment prior to standard side training. For the L-745,870 CPP studies, two different doses (i.p. injection), 1.5 mg/kg and 3.0 mg/kg and a saline vehicle were tested as a pretreatment to a 2.0 g/kg dose of ethanol or saline vehicle. Testing determined that pretreatment of L-745,870, at either dose, during conditioned place preference side-training did not affect the rewarding value of 2.0 g/kg ethanol (Figure 16). One-way ANOVA analysis of preference for the ethanol-paired compartment revealed no significant effect of L-745,870 treatment ($F(2,51) = 2.28$, $p = 0.11$).

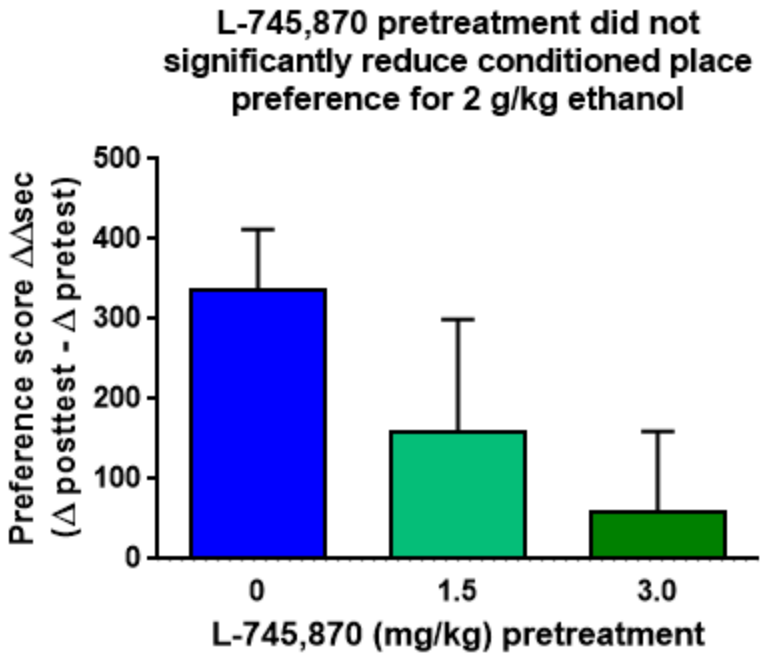

Figure 16. L-745,870 pretreatment does not significantly reduce CPP for 2.0 g/kg ethanol. CPP training for 2.0 g/kg ethanol was modified to include L-745,870 or vehicle pretreatment prior to standard side training. Neither dose, 1.5 mg/kg ($n = 13$) or 3.0 mg/kg ($n = 16$), L-745,870 pretreatment significantly attenuated ethanol conditioned place preference compared to vehicle ($n = 25$). All results are presented as means ± SEM.

Food and ethanol self-administration. One of the main goals of this study was to determine whether L-745,870 had any effect on reducing ethanol self-administration behavior. To determine this, two tests using the self-administration operant chamber were performed this includes food and ethanol self-administration.

For the ethanol self-administration, mice were trained to self-administer an 8% w/v ethanol in water solution. The mice were tested in a Latin square design using two different doses 1.5 mg/kg and 3.0 mg/kg of the L-745,870 along with saline vehicle. On test days, mice were given i.p. injections of L-745,870 or vehicle immediately prior to a 2-hour self-administration session. It was determined from the self-administration testing that L-745,870 did not significantly attenuate ethanol self-administration either dose that was tested (Figure 16). One-way repeated-measures ANOVA revealed no significant effect of L-745,870 treatment ($F(2,14) = 0.42$, $p > 0.66$).

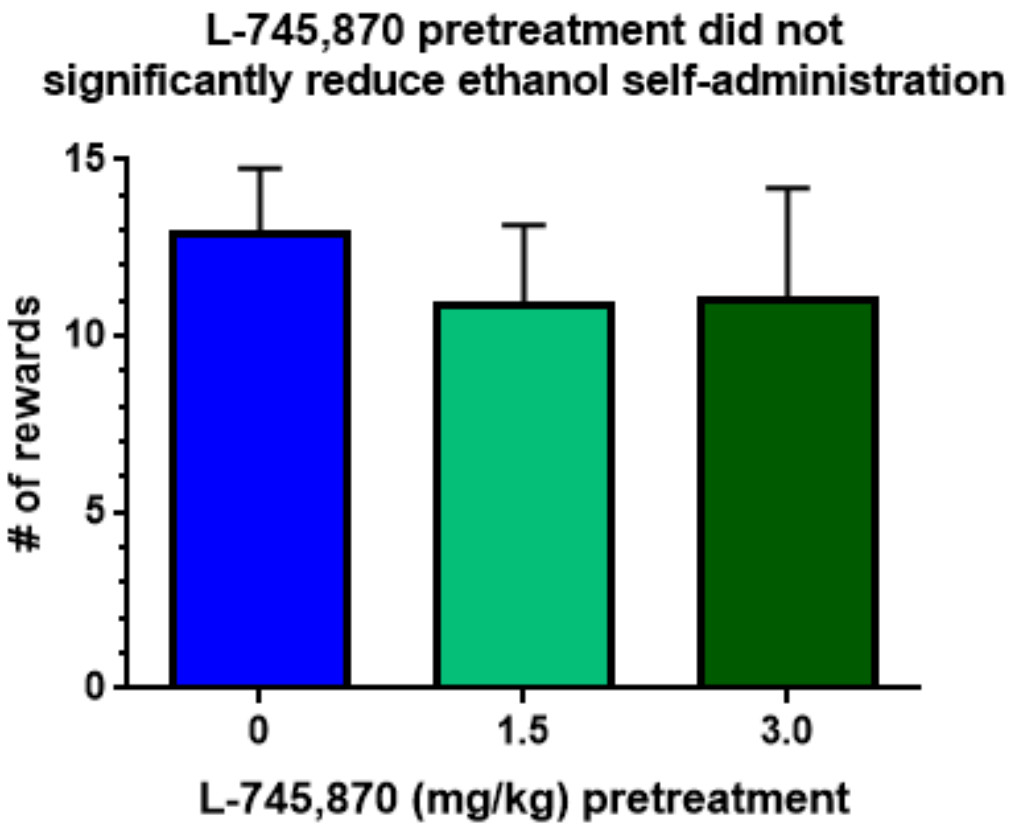

Figure 17. L-745,870 pretreatment does not significantly reduce oral ethanol self-administration. Self-administration training for oral 8% w/v ethanol in water was modified to include L-745,870 or vehicle pretreatment prior to standard side training. Neither dose, 1.5 mg/kg (n = 8) or 3.0 mg/kg (n = 8), L-745,870 pretreatment significantly attenuated oral ethanol self-administration. All results are presented as means ± SEM.

For the food self-administration, mice were trained to self-administer a mixture of 50% Ensure: 50% water. The mice were tested in a Latin square design using two different doses 1.5 mg/kg and 3.0 mg/kg of the L-745,870 along with saline vehicle (i.p. injection). On test days, mice were given injections (i.p.) of L-745,870 or vehicle immediately prior to a 2-hour self-administration session. It was determined from the self-administration testing that L-745,870 did not significantly attenuate palatable food self-administration at

either of the doses tested (Figure 17). One-way repeated-measures ANOVA revealed no significant effect of L-745,870 treatment ($F(2,14) = 0.37$, $p > 0.69$).

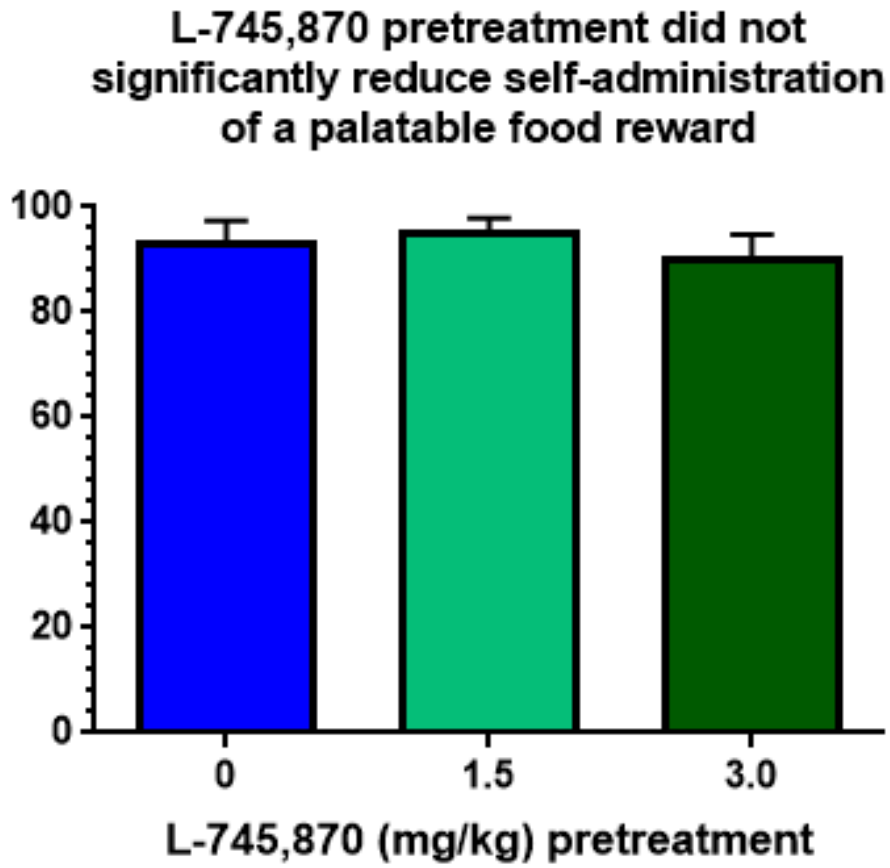

Figure 18. L-745,870 pretreatment does not significantly reduce palatable food self-administration. Self-administration training for the 50% Ensure:50% water mixture was modified to include L-745,870 or vehicle pretreatment prior to standard side training. Neither dose, 1.5 mg/kg ($n = 8$) or 3.0 mg/kg ($n = 8$), L-745,870 pretreatment significantly attenuated palatable food self-administration. All results are presented as means ± SEM.

Discussion

L-745,870 showed promise in initial studies as it did not affect locomotor activity in the open field tests or coordination function in the rotarod tests. L-745,870 (3.0 mg/kg,

i.p.) indicated that the doses tested did not significantly disrupt locomotor activity in the open field initial control study. L-745,870 (3.0 mg/kg, i.p.) also did not significantly disrupt coordination function in the rotarod initial control study.

L-745,870 did not perform as hypothesized in conditioned place preference and self-administration tests. During conditioned place preference side-training, L-745,870 did not affect the acquisition of place preference to 2.0 g/kg ethanol. These results suggest L-745,870 did not cause motivational changes toward alcohol and it did not eliminate or reduce ethanol induce preference behavior. L-745,870 has been tested, in previous studies in our laboratory and reported from others, to see if it has applicable value in other substance use disorders with different results of success reported. In a previous study in our laboratory L-745,870 did change cocaine induced behavior in mice. When comparing mice that received a pre-treatment of saline and posttreatment of cocaine versus mice that received a pre-treatment of L-745,870 (3 mg/kg) and posttreatment of cocaine our data indicated that L-745,870 mice have significantly less preference for the drug associated side.

L-745,870 also did not affect food self-administration with diluted vanilla Ensure or reduce ethanol self-administration in mice at the doses tested. These results suggest that L-745,870 does not alter the rewarding value of ethanol (or palatable food) and this is not an effective drug to be used for reducing ethanol self-administration behavior. Based on these results, L-745,870 is not likely to be successful in treatments for AUD.

L-745,870 was recently tested in another study (Kim *et al.*, 2020) by the laboratory of Dr. Bernard Le Foll at the Translational Addiction Research Laboratory, Centre for Addiction and Mental Health in Toronto, Canada. The study was done in a similar manner

to determine the effect of the dopamine D4 receptor antagonist, L-745,870, had on operant alcohol self-administration and reinstatement induced by either cues or stress. The selective D4 receptor antagonist L-745,870 attenuated alcohol self-administration and stress-induced reinstatement, without large effects on food self-administration in which response rates were high, suggesting that D4 receptor blockade influences motivational processes. For cue-induced reinstatement, analyses found no effects of D4 receptor modulation [66]. It is important to note that there were several experimental differences that could explain the difference between the results reported by Le Foll's lab and the ones reported here. Particularly the animals tested, and the maximum dose used for testing. In Kim *et al.*, Wistar rats were used while our study used C57/Bl6 mice. The report of Kim *et al.* also does not note the average weight of the rats at the time of the start of the study: the species of rodent and the size could have had an effect on drug in the body affect the results that were collected and observed [66]. The doses of L-745,870 that Le Foll's lab tested (0.5-10 mg/kg L-745,870) was a broader range of compared to the doses used in our study (1.5-3.0 mg/kg). They reported that the 10 mg/kg dose of L-745,870 did significantly reduce the number of active lever presses for alcohol compared to the other doses tested. They also reported that the 10 mg/kg dose of L-745,870 did significantly reduce the number of ethanol reinforcements when compared to the other doses tested [66]. Most significantly, when they compared the total alcohol intake of the rats during self-administration testing sessions, the 10 mg/kg dose of L-745,870 was significantly lower when compared to all other doses [66]. At doses of 0.3 to 3 mg/kg, L-745,870 was present at an estimated concentration of 4.2 to 52 ng/ml, which would be sufficient to produce in excess of 90% occupancy of dopamine D4 receptors. In contrast, at the highest dose of 3 mg/kg (the

highest dose used in our studies), only 25% of dopamine D2 receptors would be occupied [59]. The higher doses of L-745,870 (5 mg/kg and 10 mg/kg) that Le Foll's lab used in testing could have substantially inhibited dopamine D2 receptors. The positive effect seen by L-745,870 at the higher doses Le Foll's lab studied could have been a cause of the dopamine D2 receptor being partially occupied. The dopamine D2 receptor has been implicated in various psychiatric and neurological disorders related to addiction, stress, impulsivity, and other reward-related behaviors [67]. Dopamine release is regulated by dopamine D2 auto receptors and dopamine D2 receptor ligands are used to treat psychosis and addiction [68]. Another difference was the Fixed Ratio (FR), the number of times that the animal has to poke to receive alcohol reward, was different between the two studies: an FR3 for Kim *et al.* and an FR4 for our study [66]. This difference could have caused the motivation of the animals in each study to be different because one group had to work harder.

As with any scientific research study there are limitations taken into consideration results. Oral self-administration of alcohol can cause various levels of the alcohol in the blood. These levels can be affected by the animal's size and tolerance. It could also vary depending on if the animals drank any or all of the alcohol solution from the receptacle that was rewarded after successfully nose poking. This study only evaluated male mice and there are important sex-related differences in male and female mice in their behaviors and responses to drugs of abuse. In a biometrical study of locomotor activation and inhibition, it was shown by Dudek and co-workers that females were more sensitive to the locomotor activating effect of ethanol than were males [69]. Interestingly in the Dudek et al study, neither male nor female C57BL/6 mice evinced locomotor activation at 1.5 g/kg ethanol.

Middaugh and colleagues showed that in the C57BL/6 strain males evinced greater locomotor activation at 1.5 g/kg and greater locomotor inhibition at 2.5 g/kg (both IP) than did females [70]. Most studies of ethanol consumption in mice report higher consumption in females than in males [71]. In rats, females exhibited greater intake than males during the first 10 days, but after that time, total intake was not different between males and females [72]. In the Wistar rat, there are also sex differences in the rewarding versus aversive properties of ethanol that are dose dependent, with females displaying enhanced sensitivity to the rewarding effects of ethanol relative to males [73]. It is important to note though that there are some studies that do not see a sex difference in ethanol intake in adult rats [74, 75, 76].

These results that have been reported here would suggest more broadly that antagonism of dopamine D4 receptor signaling is unlikely to be a successful pharmacotherapeutic strategy for AUD. What needs to be understood is the reason behind why in our laboratory, did L-745,870 look promising for changing cocaine-mediated behavior but does not have potential to do the same for alcohol use disorder. For L-745,870 to be a promising drug of interest for another substance use disorder a method of how to determine if this could be a novel drug treatment needs to be developed to save time and resources. Considering the evidence that has shown promise with cocaine, a highly addictive substance, the success L-745,870 have on being a treatment for substance use disorder could depend on the abuse liability of the addictive drug. L-745,870 may only be an effective treatment for those drugs that are considered to have a different abuse liability or addiction potential than that of alcohol. It has always been thought that the dopamine D4 receptor plays a big role in substance use disorders, could the D4 receptor not actually

play as big of a role as previously thought or could there be another receptor that is also playing a role in the addiction.

In conclusion, in this study we examined the effects L-745,870, a D4 antagonist, had on mouse models of AUD. Several experiments we performed within this study to determine the potential for this drug to be a viable treatment for AUD. In initial control studies including open field and rotarod, PSNCBAM-1 did not significantly disrupt locomotor activity or coordination function. In the CPP experiment, L-745,870 pretreatment did not affect the rewarding value of 2.0 g/kg ethanol. In the ethanol self-administration experiments, L-745,870 did not significantly attenuate ethanol self-administration either dose that was tested. In the food self-administration experiments, L-745,870 did not significantly attenuate palatable food self-administration at either of the doses tested. Considering the results presented here it may not be completely known of how exactly addiction functions in the brain. With more research about substance use disorders including alcohol addiction and research about the dopamine D4 receptors role and involvement in addiction will come more knowledge and a better understanding of the best treatment.

Chapter 4

The Cannabinoid Type 1 Negative Allosteric Modulator PSNCBAM-1 has a General Anhedonic Effect in Mouse Models of Alcohol Addiction

Abstract

The cannabinoid receptor type 1 (CB1) is a target of interest for the development of medications for drug addiction, but CB1 antagonists/inverse agonists (e.g., rimonabant) have important side effects that limit their clinical utility, including anhedonia. Recent development of CB1 negative allosteric modulators (NAMs), including PSNCBAM-1, may provide an alternative mechanism of attenuating CB1 signaling with reduced side effects. PSNCBAM-1 has not yet been evaluated for effects in models of AUD. In this study, the effects of the CB1 NAM PSNCBAM-1 were investigated in rodent models of AUD using adult male mice. Initial control studies with PSNCBAM-1 indicated that the doses tested (10, 18 and 30 mg/kg, i.p) did not significantly disrupt locomotor activity. PSNCBAM-1 dose-dependently attenuated oral ethanol self-administration (8% w/v ethanol in water), significantly reducing ethanol rewards at a dose of 30 mg/kg but not at 10 or 18 mg/kg. PSNCBAM-1 also dose-dependently attenuated palatable food self-administration (diluted vanilla Ensure), significantly reducing food rewards at 18 and 30 mg/kg PSNCBAM-1. These results suggest PSNCBAM-1 produces a non-specific anhedonic effect that may preclude its use in AUD or other neuropsychiatric conditions.

The Cannabinoid Receptor Type 1 (CB1): an AUD Target?

At least two cannabinoid receptor subtypes are known to exist, CB1 and CB2, and there is also evidence of a CB3 receptor. These receptors belong to the G protein-coupled receptor (GPCR) superfamily. CB1 receptors are expressed in the peripheral nervous

system and the central nervous system, while CB2 receptors are expressed primarily in immune cells [77]. The CB1 receptor is involved in regulation of several physiological functions, such as food intake, energy balance, cardiovascular functions, reproductive functions, immune modulation, and cell apoptosis [78]. The cannabinoid receptor 1 is mainly expressed in the nervous system and regulates learning, memory processes, pain, and energy metabolism [79].

To date a number of endogenous cannabinoids and synthetic CB1 receptor ligands have been identified and many of these have therapeutic potential in a variety of disorders including obesity, nicotine and alcohol dependence, pain, multiple sclerosis, cancer, diarrhea, and cardiovascular diseases [77]. There is evidence to indicate that the CB1 receptor plays a key role in mediating the effects of alcohol [80]. The endocannabinoid system is probably involved in cognitive and motor responses to alcohol as well as behavioral effects, including tolerance to and dependence on alcohol; is also likely involved in the neural circuitry of the brain that controls the motivation for appetite stimuli and reinforcing effects of alcohol. A decreased CB1 receptor density and functionality was found in mice chronically exposed to alcohol and application of CB agonists was reported to stimulate alcohol intake [80]. CB1 receptors are downregulated, reduced response, by alcohol. The pharmacological data suggest that the endogenous CB system tonically increases the sensitivity of rodent and primate species to appetite reinforcers, possibly by modulating the mesolimbic activating system. There is substantial evidence that the endocannabinoid system is involved in the modulation of addictive behavior and in the mechanism of action of different drugs of abuse [80]. Recently, the cannabinoid receptor system has been explored as a possible target for treatment of substance use and addictive

disorders, including alcoholism, nicotine, and obesity. Drugs that block the CB1 receptors may represent medications with a novel mechanism of action in the treatment of addictive disorders [80].

Inhibition of CB1

Rimonabant was synthesized by Rinaldi-Carmona and is the first potent, selective, and orally active blocker of the cannabinoid receptor. Both *in vitro* and *in vivo* studies show that rimonabant antagonizes the behavioral and pharmacological effects induced by cannabinoid receptor agonists and reduces voluntary alcohol consumption in the animal model [80]. The drug has already been clinically studied for use in smoking cessation and is approved by Europe, the Middle East and Africa (EMEA) for its use in obesity treatment. There is also evidence from animal studies that it may be effective in alcohol treatment [80]. Rimonabant was shown to dose-dependently reduce alcohol intake in alcohol-preferring rodents. Pretreatment with rimonabant prevented an increase of alcohol consumption after treatment of a CB1 agonist. Recently, rimonabant was reported to reduce reward-related responses in rats [80]. Rimonabant has not been approved in the USA.

Many studies have been done on rimonabant for interest and concern about its safety and side effects. The most common side effect in studies of rimonabant leading participants to drop out were depression and anxiety [81]. In addition to depression and anxiety, there were more cases of irritability, insomnia, stress, and panic attacks [82]. Christensen et al. performed a meta-analysis of the four Rimonabant in Obesity Studies, further unveiling that patients receiving 20 mg rimonabant were 2.5- and 3.0-times more likely to discontinue treatment because of depression or depressive symptoms and because of anxiety, respectively. This meta-analysis also showed that, accordingly to the Hospital

Anxiety and Depression Scale, rimonabant was associated with significant increases in anxiety [83].

PSNCBAM-1

PSNCBAM-1 (Sigma Aldrich, US) is a CB1 negative allosteric modulator (NAM). The IUPAC name for PSNCBAM-1 is 1-(4-chlorophenyl)-3-(3-(6-(pyrrolidin-1-yl)pyridin-2-yl)phenyl)urea. The chemical structure of PSNCBAM-1 can be seen below in Figure 19. Negative allosteric modulators decrease protein function and reduce the effect a drug has on the receptor [84]. Negative allosteric modulators decrease the efficacy of the endogenous receptor agonist without inducing complete receptor inhibition caused by orthosteric inhibitors and therefore, maintain the native pattern of the receptor activation largely intact [85, 86, 87]. PSNCBAM-1 has not yet been evaluated for effects in models of alcohol addiction.

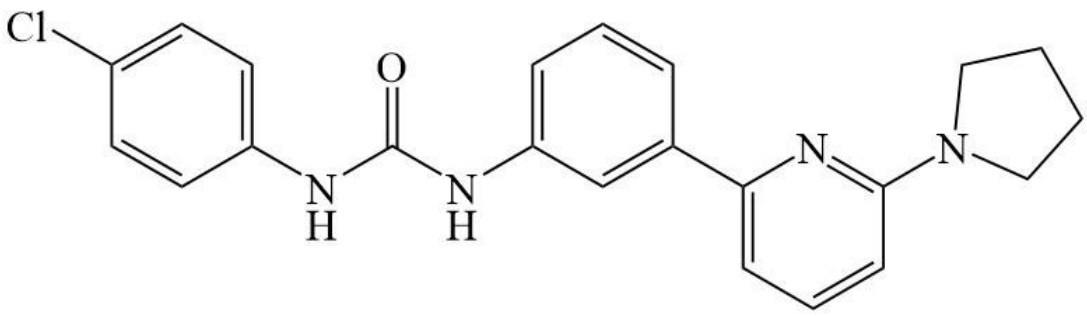

Figure 19. Structure of PSNCBAM-1. A cannabinoid type 1 negative allosteric modulator was obtained from Sigma Aldrich in the US.

We hypothesize that based on evidence from studies previously performed involving alcohol and other substances of addiction, that the CB1 NAM PSNCBAM-1 will

reduce alcohol seeking behavior in adult male mice. In order to test this hypothesis, we tested the effects of PSNCBAM-1 in ethanol CPP and self-administration studies. When these studies were initiated, PSNCBAM-1 had never before been evaluated for effects in models of alcohol addiction.

Results

Initial control studies. The effects of PSNCBAM-1 were tested in initial control studies to see if the drug decreased any locomotor activity in mice, in order to determine if the drug is safe to use in further studies. The tests that were performed in the initial control studies included open field.

For the open field study, mice were placed in the open field chamber for an initial 20 minutes. This allotment of time allowed the mice to explore the chamber unhindered. After the initial 20 minutes, the mice were taken out and were each given an i.p. injection of either 10 mg/kg PSNCBAM-1, 30 mg/kg PSNCBAM-1 or the vehicle mixture. After the injection, the mice were placed in the open field chamber for an additional 40 minutes. The behavior and activity of the mice were recorded via a camera positioned over the open field chambers. Initial control studies with 10 and 30 mg/kg PSNCBAM-1, i.p., indicated that the doses tested did not significantly disrupt locomotor activity in the open field test (Figure 19). One-way ANOVA analysis of total post-injection locomotor activity revealed no significant effect of treatment ($F(2,29) = 0.5594$, $p > 0.5$).

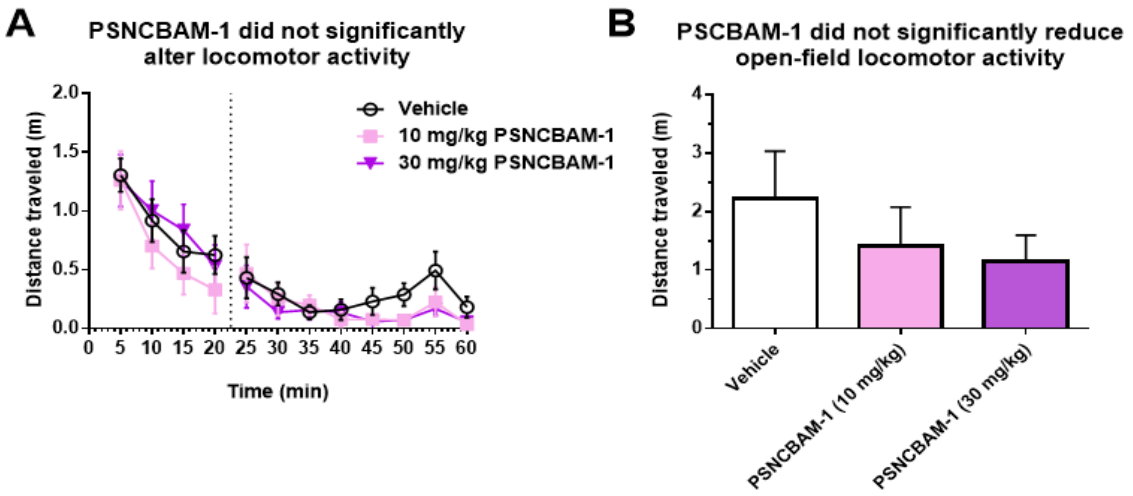

Figure 20. 10 mg/kg, 18 mg/kg or 30 mg/kg PSNCBAM-1 did not significantly affect locomotor activity in an open field test. Male mice were placed in an open field apparatus for 20 minutes and behavior was recorded. Then they were given i.p. injections of vehicle (n = 16), 10 mg/kg PSNCBAM-1 (n = 8), or 30 mg/kg PSNCBAM-1 (n = 8) and behavior was recorded for an additional 40 minutes. (A) 20 minutes after introduction into the open field, mice were injected with 10 or 30 mg/kg PSNCBAM-1 or vehicle and locomotor activity was recorded for an additional 40 minutes. (B) Overall post-injection distance traveled was not significantly different across treatments. Data are presented as means ± SEM of distance traveled in 5-minute bins.

Conditioned place preference. CPP training for 2.0 g/kg ethanol was modified to include a PSNCBAM-1 or vehicle mixture pretreatment prior to standard side training. For the PSNCBAM-1 CPP studies three different doses (i.p. injection), 10 mg/kg, 18 mg/kg, and 30 mg/kg and a vehicle mixture were tested as a pretreatment to a 2.0 g/kg dose of ethanol or saline vehicle. Testing determined that PSNCBAM-1 pretreatment during conditioned place preference training did not affect the rewarding value of 2.0 g/kg ethanol (Figure 20). One-way ANOVA analysis of preference for the ethanol-paired compartment revealed no significant effect of PSNCBAM-1 treatment ($F(2,26) = 0.3469$, $p > 0.7$).

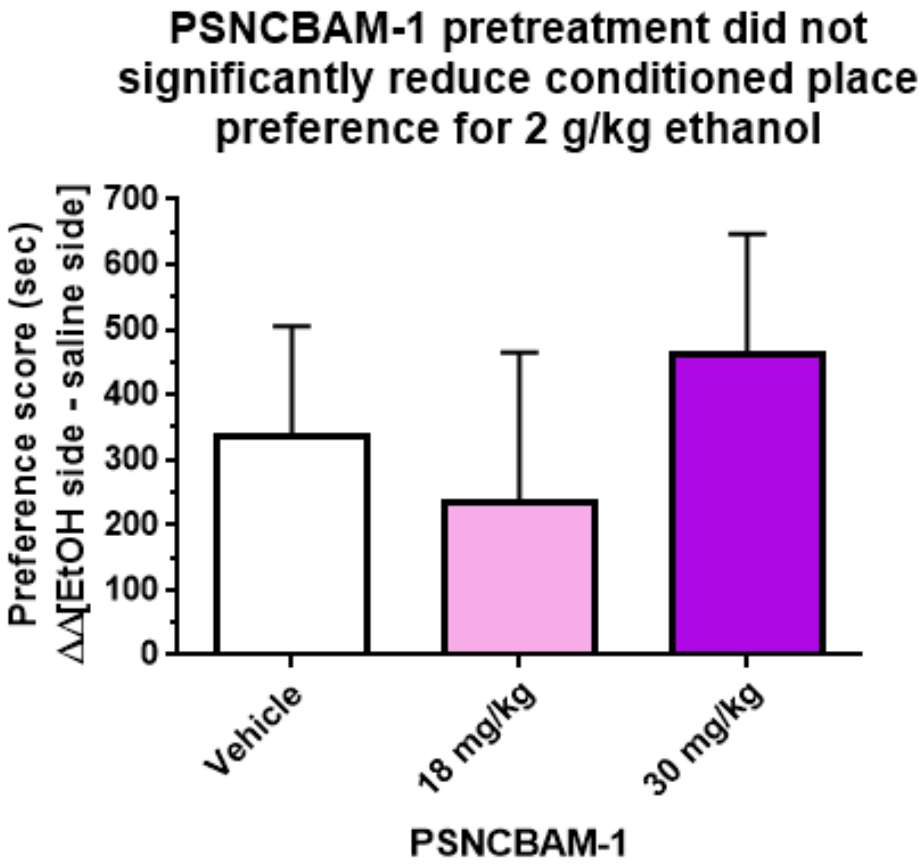

Figure 21. PSNCBAM-1 pretreatment does not significantly reduce CPP for 2.0 g/kg ethanol. CPP training for 2.0 g/kg ethanol was modified to include PSNCBAM-1 or vehicle pretreatment prior to standard side training. 18 mg/kg (n = 10) and 30 mg/kg (n = 10), PSNCBAM-1 pretreatment was not significantly different from vehicle pretreatment (n = 9) and did not attenuate acquisition of ethanol conditioned place preference. All results are presented as means ± SEM.

Food and ethanol self-administration. One of the main goals of this study was to determine whether PSNCBAM-1 has any effect on ethanol self-administration behavior. To determine this, two tests using the self-administration operant chamber were performed this includes food and ethanol self-administration.

For ethanol self-administration, mice were trained to self-administer an 8% w/v ethanol in water solution. The mice were tested in a Latin square design using two different

doses, 10mg/kg, and 30mg/kg, of PSNCBAM-1 and the vehicle (10% DMSO, 10% Tween 80, and 80% saline). In the second round of testing, an intermediate dose, 18 mg/kg, was tested. On test days, mice were given i.p. injections of PSNCBAM-1 or vehicle immediately prior to a 2-hour self-administration session. It was determined from the self-administration testing that PSNCBAM-1 dose-dependently attenuated ethanol self-administration reducing ethanol rewards at a dose of 30 mg/kg (Figure 21). One-way repeated-measures ANOVA revealed a significant effect of PSNCBAM-1 treatment ($F(3,31) = 8.410$, $p = 0.0007$). Pre-planned Bonferroni tests revealed a significant difference between vehicle treatment and 18 mg/kg ($t = 3.426$, $p < 0.05$) and 30 mg/kg PSNCBAM-1 ($t = 4.298$, $p < 0.01$).

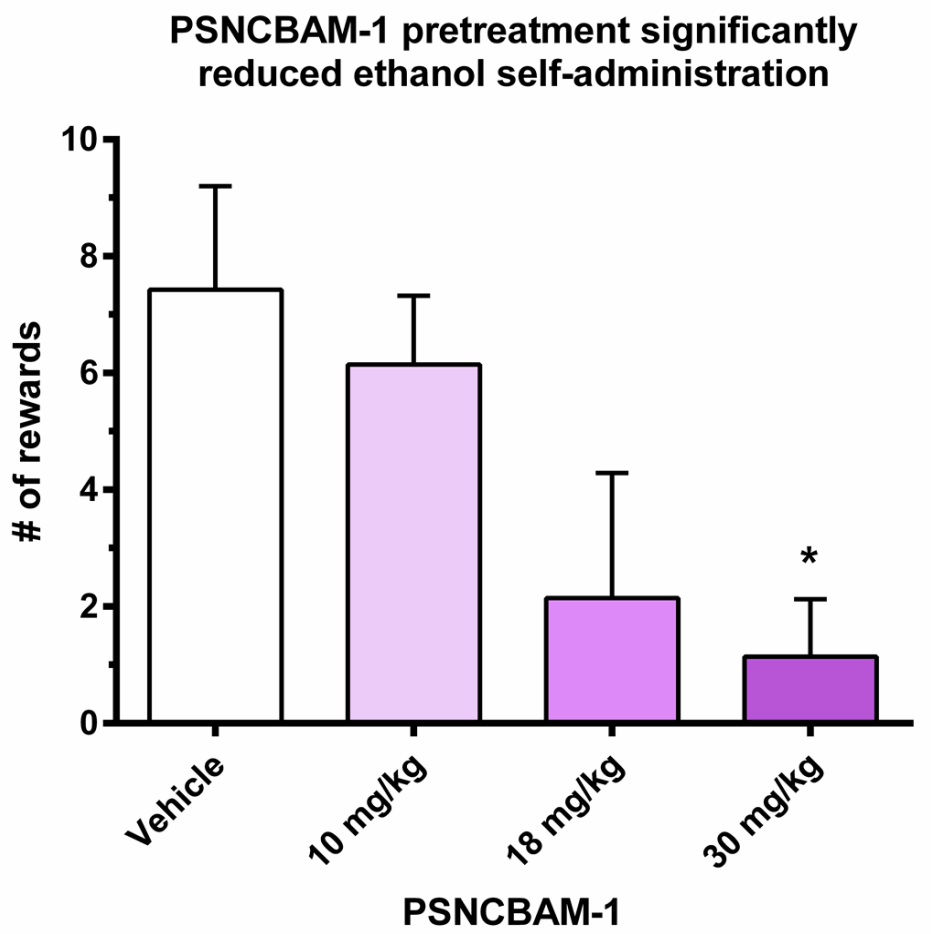

Figure 22. PSNCBAM-1 pretreatment does significantly reduce oral ethanol self-administration. Self-administration training for 8% w/v ethanol in water was modified to include PSNCBAM-1 or vehicle pretreatment prior to standard side training. 30 mg/kg (n = 8) PSNCBAM-1 pretreatment did significantly attenuate oral ethanol self-administration. 10 mg/kg (n = 8) and 18 mg/kg (n = 8), PSNCBAM-1 pretreatment did not significantly attenuate oral ethanol self-administration. All results are presented as means ± SEM; * $p < 0.05$ compared to vehicle pre-treatment.

For the food self-administration, mice were trained to self-administer a mixture of 50% Ensure: 50% water. The mice were tested in a Latin square design using two different doses, 10mg/kg, and 30mg/kg, of the PSNCBAM-1 and the vehicle. For this drug, the vehicle that was used was a mixture of 10% Dimethyl Sulfoxide, 10% Tween 80 and 80% saline. In the second round of testing, an intermediate dose, 18 mg/kg, was tested. On test

days, mice were given i.p. Injections of PSNCBAM-1 or vehicle immediately prior to a 2-hour self-administration session. It was also determined from the self-administration testing that PSNCBAM-1 dose-dependently attenuated palatable food self-administration significantly reducing food rewards at 18 mg/kg and 30 mg/kg (Figure 23). One-way repeated-measures ANOVA revealed a significant effect of PSNCBAM-1 treatment ($F(3,18) = 4.264$, $p = 0.0193$). Pre-planned Bonferroni tests revealed a significant difference between vehicle treatment and 30 mg/kg PSNCBAM-1 ($t = 3.016$, $p < 0.05$).

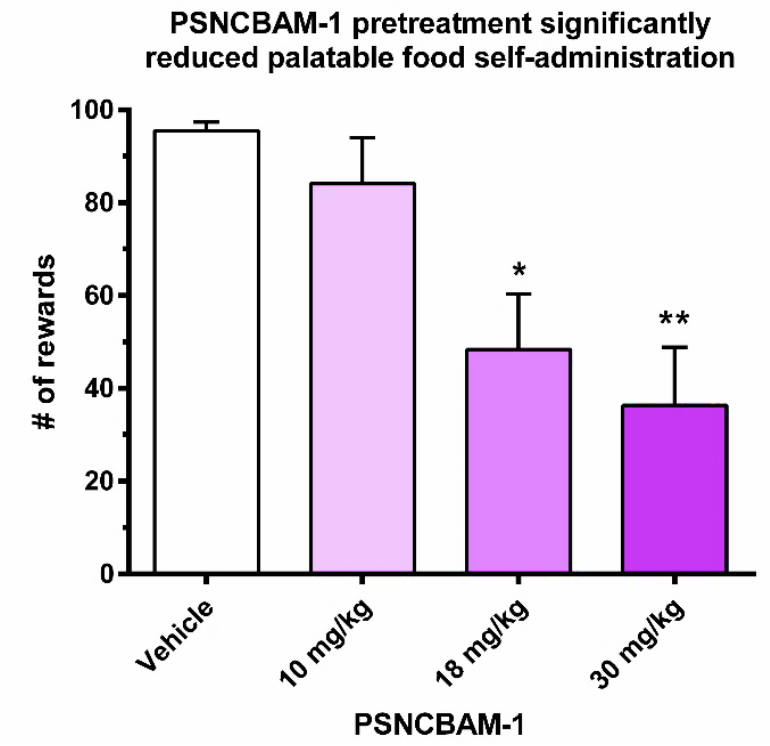

Figure 23. PSNCBAM-1 pretreatment does significantly reduce oral ethanol self-administration. Self-administration training for 50% Ensure:50% water was modified to include PSNCBAM-1 or vehicle pretreatment prior to standard side training. 18 mg/kg (*n* = 8) and 30 mg/kg (*n* = 8), PSNCBAM-1 pretreatment did significantly attenuate palatable food self-administration. 10 mg/kg PSNCBAM-1 (*n* = 8) pretreatment did not significantly attenuate oral ethanol self-administration. All results are presented as means ± SEM; * $p < 0.05$ compared to vehicle pre-treatment. ** $p < 0.01$ compared to vehicle pre-treatment.

Discussion

PSNCBAM-1 showed promise in initial studies as it did not affect locomotor activity in open field tests. PSNCBAM-1 did not perform as hoped in conditioned place preference and self-administration tests. PSNCBAM-1 indicated that the doses tested did not significantly disrupt locomotor activity in the open field initial control study.

During conditioned place preference side-training, PSNCBAM-1 did not affect the acquisition of place preference to 2.0 g/kg ethanol. These results suggest L-745,870 did not cause motivational changes toward alcohol and it did not eliminate or reduce ethanol induce preference behavior.

PSNCBAM-1 negatively affected both palatable food self-administration with diluted vanilla Ensure and ethanol self-administration with an 8% ethanol solution. It was concluded that PSNCBAM-1 had a non-specific anhedonic effect on both food and ethanol self-administration. Based on the results that have been gathered by this experiment, it was determined that this CB1 NAM, PSNCBAM-1, is not a suitable drug to be used for alcohol use disorder.

Most of the studies that have used PSNCBAM-1 are testing to determine its effects it has on obesity. There was a study done by Wong Kai In Lab out of Oxford, United Kingdom that study the hypophagic effect on rats that PSNCBAM-1 had specifically [77]. It was discovered from this study that in an acute rat feeding model, PSNCBAM-1 decreased food intake and body weight [77]. The conclusion made in this study can explain the results of PSNCBAM-1 having a non-specific anhedonic affect in the study we performed. This new knowledge could lead researchers to discover that CB1 receptor drugs

and specifically PSNCBAM-1 could be a novel drug in the treatment of obesity and diseases and conditions attributed to obesity.

An anhedonic effect means that there is a loss of the capacity to enjoy things, including the pleasurable effects of addictive drugs of and those of palatable food. PSNCBAM-1 has not been widely tested for studies involving addiction or other conditions. This may preclude its use in AUD or other neuropsychiatric conditions. What needs to be understood is the reason behind why, in our laboratory, did PSNCBAM-1 create an anhedonic effect for both food and ethanol. Considering the results reported here and in other studies PSNCBAM-1 could cause this non-specific anhedonic effect across the board, with no consideration as to what condition or disorder is being tested, the only way to positively know if this is true is to do more research with this drug in other areas of interest. Another thing that could be determined from more extensive studies of this drug and other CB1 NAMs is whether a general anhedonic effect is limited to this drug or if the effect extends to all CB1 NAM. The side effect PSNCBAM-1 has of reducing food intake needs to be compared and weighed against the possible success it may have on being a drug for the treatment of a substance use disorder or another medical condition. If the success of PSNCBAM-1 has on being a possible treatment for another does out way the side effect of reducing food intake, this medication would have to be taking with the ramifications of appetite suppression in mind and under clear direction and guidance from a medical professional. There are several medications approved for a variety of the treatments that have appetite loss and appetite suppression as listed side effects including but not limited to several high strength medications and many medications prescribed to treat attention deficient hyperactivity disorder (ADHD).

As with any scientific research study there are limitations taken into consideration results. Oral self-administration of alcohol can cause various levels of the alcohol in the blood. These levels can be affected by the animal's size and tolerance. It could also vary depending on if the animals drank any or all of the alcohol solution from the receptacle that was rewarded after successfully nose poking. This study only evaluated male mice and there are important sex-related differences in male and female mice in their behaviors and responses to drugs of abuse. In a biometrical study of locomotor activation and inhibition, it was shown by Dudek and co-workers that females were more sensitive to the locomotor activating effect of ethanol than were males [69]. Interestingly in the Dudek et al study, neither male nor female C57BL/6 mice evinced locomotor activation at 1.5 g/kg ethanol. Middaugh and colleagues showed that in the C57BL/6 strain males evinced greater locomotor activation at 1.5 g/kg and greater locomotor inhibition at 2.5 g/kg (both IP) than did females [70]. Most studies of ethanol consumption in mice report higher consumption in females than in males [71]. In rats, females exhibited greater intake than males during the first 10 days, but after that time, total intake was not different between males and females [72]. In the Wistar rat, there are also sex differences in the rewarding versus aversive properties of ethanol that are dose dependent, with females displaying enhanced sensitivity to the rewarding effects of ethanol relative to males [73]. It is important to note though that there are some studies that do not see a sex difference in ethanol intake in adult rats [74, 75, 76].

In conclusion, in this study we examined the effects of the CB1 NAM PSNCBAM-1 in mouse models of AUD. PSNCBAM-1 did not significantly disrupt locomotor activity. In the CPP experiment, PSNCBAM-1 pretreatment did not affect the rewarding value of

2.0 g/kg ethanol. In ethanol self-administration experiments, PSNCBAM-1 dose-dependently attenuated ethanol self-administration, reducing ethanol rewards at the 30 mg/kg dose. In the food self-administration experiments, PSNCBAM-1 dose-dependently attenuated palatable food self-administration, significantly reducing food rewards at the 18 mg/kg and 30 mg/kg doses. With more research about substance use disorders including alcohol addiction and research about the cannabinoid receptors role and involvement in addiction will come more knowledge and a better understanding of the best treatment.

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Appendix A

Calculations for Mixtures Used in Self-Administration

Mixture 1: 2.5% Ethanol (w/v) in 50% Ensure, diluted in water

EtOH density = 0.79 g/mL

2.5% EtOH (w/v), 50.0% Ensure, 47.5% H_2O

$\frac{2.5\%\ w/v}{0.79\ g/mL}$ = 3.16% v/v for 100% ethanol

Ethanol was purchased at 95% purity, therefore: $\frac{3.16\%\ v/v}{95\ \%}$ = 3.33% v/v (95%)

Ensure is individually packaged in 237 mL bottles. A 50% Ensure solution can have a maximum volume of 474 mL.

Therefore, to create a 474 mL solution of 2.5% Ethanol (w/v) in 50% Ensure, diluted in water:

$\frac{3.33\ mL}{100\ mL}$ x 474 mL = 15.78 mL EtOH

15.78 mL EtOH	237 mL	474 mL
237 mL Ensure (whole bottle)	+ 15.78 mL	- 252.78 mL
+ 221.22 mL H_2O	252.78 mL	221.22 mL
474 mL total		

Mixture 2: 5.0% Ethanol (w/v) in 50% Ensure, diluted in water

EtOH density = 0.79 g/mL

5.0% EtOH (w/v), 50.0% Ensure, 45% H_2O

$\frac{5.0\%\ w/v}{0.79\ g/mL}$ = 6.33% v/v for 100% ethanol

Ensure is individually packaged in 237 mL bottles. A 50% Ensure solution can have a maximum volume of 474 mL.

Therefore, to create a 474 mL solution of 5.0% Ethanol (w/v) in 50% Ensure, diluted in water:

$\frac{6.33\ mL}{100\ mL}$ x 474 mL = 30.0 mL EtOH

30.0 mL EtOH	237 mL	474 mL
237 mL Ensure (whole bottle)	+ 30.0 mL	- 267 mL
+ 207 mL H_2O	267 mL	207 mL
474 mL total		

Mixture 3: 7.5% Ethanol (w/v) in 50% Ensure, diluted in water

EtOH density = 0.79 g/mL

7.5% EtOH (w/v), 50.0% Ensure, 42.5% H_2O

$\frac{7.5\%\ w/v}{0.79\ g/mL}$ = 9.49% v/v for 100% ethanol

Ensure is individually packaged in 237 mL bottles. A 50% Ensure solution can have a maximum volume of 474 mL.

Therefore, to create a 474 mL solution of 7.5% Ethanol (w/v) in 50% Ensure, diluted in water:

$\frac{9.49\ mL}{100\ mL}$ x 474 mL = 44.9826 mL EtOH → 45.0 mL EtOH

45.0 mL EtOH	237 mL	474 mL
237 mL Ensure (whole bottle)	+ 45.0 mL	- 282 mL
+ 192 mL H_2O	282 mL	192 mL
474 mL total		

Mixture 4: 10.0% Ethanol (w/v) in 50% Ensure, diluted in water

EtOH density = 0.79 g/mL

10.0% EtOH (w/v), 50.0% Ensure, 40.0% H_2O

$\frac{10.0\%\ w/v}{0.79\ g/mL}$ = 12.66% v/v for 100% ethanol

Ensure is individually packaged in 237 mL bottles. A 50% Ensure solution can have a maximum volume of 474 mL.

Therefore, to create a 474 mL solution of 10% Ethanol (w/v) in 50% Ensure, diluted in water:

$\frac{12.66\ mL}{100\ mL}$ x 474 mL = 60.0 mL EtOH

60.0 mL EtOH	237 mL	474 mL
237 mL Ensure (whole bottle)	+ 60.0 mL	- 282 mL
+ 177 mL H_2O	282 mL	177 mL
474 mL total		

Mixture 5: 10.0% Ethanol (w/v) in 25% Ensure, diluted in water

EtOH density = 0.79 g/mL

10.0% EtOH (w/v), 25.0% Ensure, 65.0% H_2O

$\frac{10.0\%\ w/v}{0.79\ g/mL}$ = 12.66% v/v for 100% ethanol

A 25% Ensure solution in a total of 474 mL can have a maximum volume of 118.5 mL.

25.0% x 474 mL = 118.5 mL Ensure

Therefore, to create a 474 mL solution of 10% Ethanol (w/v) in 25% Ensure, diluted in water:

$\frac{12.66\ mL}{100\ mL}$ x 474 mL = 60.0 mL EtOH

60.0 mL EtOH	118.5 mL	474 mL
118.5 mL Ensure	+ 60.0 mL	- 178.5 mL
+ 295.5 mL H_2O	178.5 mL	295.5 mL
474 mL total		

Mixture 6: 10.0% Ethanol (w/v), diluted in water

EtOH density = 0.79 g/mL

10.0% EtOH (w/v), 90.0% H_2O

$\frac{10.0\%\ w/v}{0.79\ g/mL}$ = 12.66% v/v for 100% ethanol

Therefore, to create a 500 mL solution of 10% Ethanol (w/v), diluted in water:

$\frac{12.66\ mL}{100\ mL}$ x 500 mL = 63.3 mL EtOH

63.3 mL EtOH	500 mL
+ 436.7 mL H_2O	- 63.3 mL
474 mL total	436.7 mL

Mixture 7: 10.0% Ethanol (w/v) in 10% Ensure, diluted in water

EtOH density = 0.79 g/mL

10.0% EtOH (w/v), 10.0% Ensure, 80.0% H_2O

$\frac{10.0\%\ w/v}{0.79\ g/mL}$ = 12.66% v/v for 100% ethanol

A 10% Ensure solution in a total of 474 mL can have a maximum volume of 47.4 mL.

10.0% x 474 mL = 47.4 mL Ensure

Therefore, to create a 474 mL solution of 10% Ethanol (w/v) in 10% Ensure, diluted in water:

$\frac{12.66\ mL}{100\ mL}$ x 474 mL = 60.0 mL EtOH

60.0 mL EtOH
47.4 mL Ensure
+ 366.6 mL H_2O
474 mL total

47.4 mL
+ 60.0 mL
107.4 mL

474 mL
- 107.4 mL
366.6 mL

Mixture 8: 10.0% Ethanol (w/v) in 5% Ensure, diluted in water

EtOH density = 0.79 g/mL

10.0% EtOH (w/v), 5.0% Ensure, 85.0% H_2O

$\frac{10.0\%\ w/v}{0.79\ g/mL}$ = 12.66% v/v for 100% ethanol

A 5% Ensure solution in a total of 474 mL can have a maximum volume of 23.7 mL.

5.0% x 474 mL = 23.7 mL Ensure

Therefore, to create a 474 mL solution of 10% Ethanol (w/v) in 5% Ensure, diluted in water:

$\frac{12.66\ mL}{100\ mL}$ x 474 mL = 60.0 mL EtOH

60.0 mL EtOH	23.7 mL	474 mL
23.7 mL Ensure	+ 60.0 mL	- 83.7 mL
+ 390.3 mL H_2O	83.7 mL	390.3 mL
474 mL total		

Mixture 9: 8.0% Ethanol (w/v) in 10% Ensure, diluted in water

EtOH density = 0.79 g/mL

8.0% EtOH (w/v), 10.0% Ensure, 82.0% H_2O

$\frac{8.0\%\ w/v}{0.79\ g/mL}$ = 10.13% v/v for 100% ethanol

A 10% Ensure solution in a total of 500 mL can have a maximum volume of 50 mL.

10.0% x 500 mL = 50 mL Ensure

Therefore, to create a 474 mL solution of 10% Ethanol (w/v) in 5% Ensure, diluted in water:

$\frac{10.13\ mL}{100\ mL}$ x 500 mL = 50.65 mL EtOH → 50.0 mL EtOH

50.0 mL EtOH	50.0 mL	500 mL
50.0 mL Ensure	+ 50.0 mL	- 100 mL
+ 400.0 mL H_2O	100 mL	400 mL
500 mL total		

Mixture 10: 8.0% Ethanol (w/v), diluted in water

EtOH density = 0.79 g/mL

8.0% EtOH (w/v), 92.0% H_2O

$\frac{8.0\%\ w/v}{0.79\ g/mL}$ = 10.13% v/v for 100% ethanol

Therefore, to create a 500 mL solution of 10% Ethanol, diluted in water:

$\frac{10.13\ mL}{100\ mL}$ x 500 mL = 50.65 mL EtOH → 50.0 mL EtOH

50.0 mL EtOH	500 mL
+ 450 mL H_2O	- 50.0 mL
500 mL total	450 mL

Mixture 11: 8.0% Ethanol (w/v) in 5% Ensure, diluted in water

EtOH density = 0.79 g/mL

8.0% EtOH (w/v), 5.0% Ensure, 87.0% H_2O

$\frac{8.0\%\ w/v}{0.79\ g/mL}$ = 10.13% v/v for 100% ethanol

A 5% Ensure solution in a total of 500 mL can have a maximum volume of 25 mL.

5.0% x 500 mL = 25 mL Ensure

Therefore, to create a 474 mL solution of 10% Ethanol (w/v) in 5% Ensure, diluted in water:

$\frac{10.13\ mL}{100\ mL}$ x 500 mL = 50.65 mL EtOH → 50.0 mL EtOH

50.0 mL EtOH	50.0 mL	500 mL
25.0 mL Ensure	+ 25.0 mL	- 75.0 mL
+ 425 mL H_2O	75.0 mL	425 mL
500 mL total		

Appendix B

Calculations for Solutions

L-745,870

Solution 1 – 1.5 mg/kg

1.5 mg/kg L-745,870

1.5 mg/kg → 0.15 mg/mL

30 mL × 0.15 mg/mL = 4.5 mg L-745,870 → 0.0045g

In 30 mL of saline

Solution 2 – 3.0 mg/kg

3.0 mg/kg L-745,870

3.0 mg/kg → 0.3 mg/mL

30 mL × 0.3 mg/mL = 9.0 mg L-745,870 → 0.009g

In 30 mL of saline

PSNCBAM-1

Solution 1 – 10 mg/kg

10 mg/kg PSNCBAM-1

10 mg/kg → 1.0 mg/mL

50 mL × 1.0 mg/mL = 50 mg PSNCBAM-1 → 0.05g

In 50 mL of 10% Tween 80, 10% DMSO and 80% saline

Solution 2 – 30 mg/kg

30 mg/kg PSNCBAM-1

30 mg/kg → 3.0 mg/mL

50 mL × 3.0 mg/mL = 150 mg PSNCBAM-1 → 0.15g

In 50 mL of 10% Tween 80, 10% DMSO and 80% saline

Solution 3 – 18 mg/kg

18 mg/kg PSNCBAM-1

18 mg/kg → 1.8 mg/mL

50 mL × 1.8 mg/mL = 90 mg PSNCBAM-1 → 0.09g

In 50 mL of 10% Tween 80, 10% DMSO and 80% saline