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**EXPERIMENTAL PHARMACOTHERAPIES IN MODELS OF ALCOHOL
ADDICTION**

by

Mousumi Akter Sumi

A Thesis

Submitted to the
Department of Chemistry and Biochemistry
College of Science and Mathematics
In partial fulfillment of the requirement
For the degree of
Master of Science in Pharmaceutical Sciences
at
Rowan University
December 17, 2020

Thesis Advisor: Thomas M. Keck, Ph.D.

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Dedication

I dedicate this thesis to my parents Md. Osman Goni and Zarna Akter, and my best friend, Md. Shariful Islam.

Acknowledgments

In my entire education life, the best mentor, educator, or advisor is you, Dr. Keck, honorable thesis advisor. I want to give you thanks from my deepest heart for being my research advisor and being my guardian. Thank you for your mastermind direction, optimistic counseling, and ongoing backup since the conception of the present work to the submission of this thesis. I am highly obliged to the Department of Chemistry and Biochemistry and Rowan University for the fellowship and support to carry out the work successfully. I am grateful to Dr. Caputo and Dr. Subash for their support, making my journey easy. I would like to thank Dr. Grinias and Dr. Fischer for being a part of my thesis committee. Finally, I would like to thank all my well-wishers, family, friends, and special thanks to all collaborators and my research group for their cordial help.

Abstract

Mousumi Akter Sumi
EXPERIMENTAL PHARMACOTHERAPIES IN MODELS OF ALCOHOL
ADDICTION
2019-2020
Thomas M. Keck, Ph.D.
Master of Science in Pharmaceutical Sciences

The USA faces \$220 billion economic loss and eighty thousand deaths per year due to alcohol abuse while affecting more than 15 million people, making it the third largest life-style related cause of death. The U.S. FDA has approved four medications namely, disulfiram, acamprostate, oral naltrexone, and injectable long-acting naltrexone. These existing drugs are trashed with side effects, have a low success rate, indicating a demand for new potential drugs. We studied the effects of the CB1 negative allosteric modulator, PSNCBAM-1, and the dopamine D4 receptor antagonist, L-745,870 in mouse models of alcohol addiction. PSNCBAM-1 did not significantly reduce CPP for 2.0 g/kg ethanol or alter locomotor activity, but its dose-dependently attenuated oral ethanol self-administration at the dose of 30 mg/kg. 18 and 30 mg/kg PSNCBAM-1 significantly reduced self-administration of palatable food reward. These results suggest, PSNCBAM-1 produces a non-specific anhedonic effect that may preclude its use in AUD. L-745,870 did not significantly affect conditioned place preference for 2.0 g/kg ethanol, ethanol self-administration, locomotor activity in an open field, or co-ordination in the rotarod test. These results suggest that D4R antagonism does not alter the rewarding value of ethanol.

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Chapter 1

Introduction

What is Ethanol?

Ethanol (C_2H_5OH) is a type of alcohol used extensively in foods, beverages, pharmaceuticals, chemical syntheses, and more (Pohanka, 2016). It is an organic compound characterized by its colorless nature, volatility, flammability, and water-soluble nature, with a slight chemical odor (Levy, 2020). The chemical structure of ethanol is shown below in Figure 1.

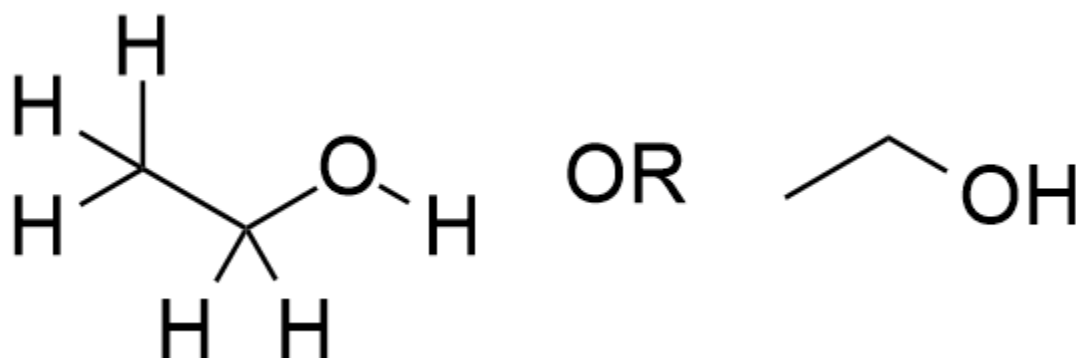


Figure 1. Structure of ethanol. The ingredient in beverages, food, pharmaceuticals, etc.

Preparation of Alcohol

Ethanol can be naturally manufactured using the fermentation protocol for certain carbohydrates, namely sugars, starches, or synthetically, using ethylene's chemical hydration. (Weathermon & Crabb, 1999). Ethanol production as alcoholic beverage using the fermentation process has been practiced for centuries. It is difficult to determine when humans first learned to produce alcohol, but one of the most widely known alcoholic

beverages, wine, is thought to have been produced in ancient Greece and China, in the same time period when iron was discovered (Pohanka, 2016). The chemical formula for the fermentation of ethanol can be seen that is presented in Figure 2 below.

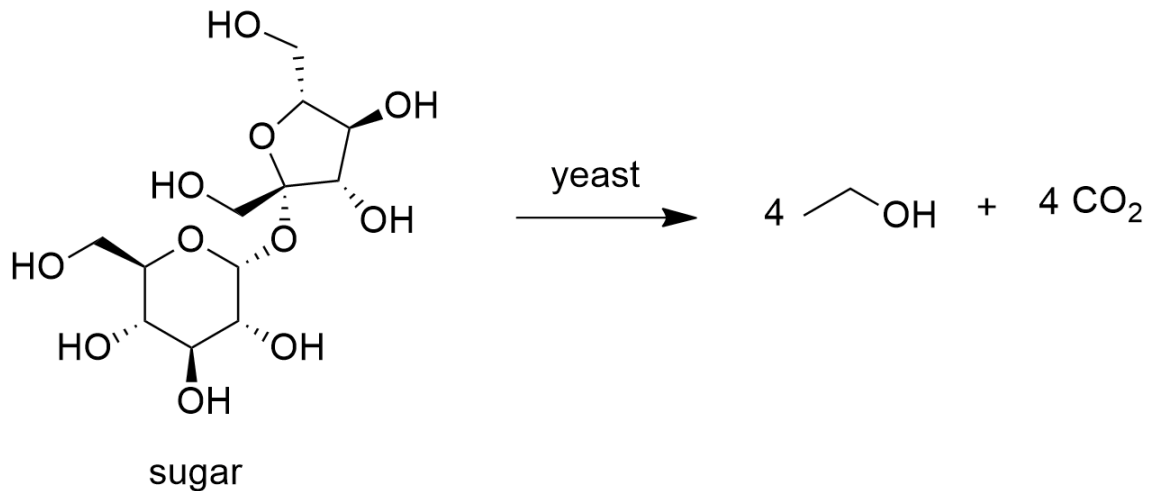


Figure 2. The fermentation process of alcohol in which yeast ferments the sugar (in this case the disaccharide sucrose) in fruit and grains into ethanol and carbon dioxide.

Why Alcohol Consumption

The most common reason for alcohol consumption is its mind-altering properties, including its ability to alter mental and emotional states via actively inducing euphoria, relaxation, disinhibition, and suppressing stress and anxiety. (Gilman, Ramchandani, Davis, Bjork, & Hommer, 2008).

Alcohol Pharmacology

Pharmacologically alcohol can be characterized as a γ -aminobutyric acid (GABA) and serotonin agonist that contains a number of neurotransmitter receptors located in the

brain, altering the nature of permeability of various ions namely, Cl^- , K^+ , Na^+ , or Ca^{2+} through the help of their respective channels, i.e., enhancing the passage of Cl^- ions through GABA-A channels, and suppressing the passage path of Na^+ and Ca^{2+} through N-methyl D-aspartate (NMDA) and non-NMDA channels (Albanese, 2012). These processes eventually cause upregulation of dopamine release in the ventral tegmental area of the midbrain and dopamine release in the nucleus accumbens that enhance pleasurable sensations and euphoria (Friedlander, Marder, Pisegna, & Yagiela, 2003; Gessa, Muntoni, Collu, Vargiu, & Mereu, 1985).

Ethanol is actively absorbed into the systemic circulation via the mucous membranes of the mouth, stomach, small intestine, and colon (Weathermon & Crabb, 1999). Once it is absorbed, alcohol is consequently transported to the liver via the portal vein. A portion of the administered alcohol is metabolized during its primary passage through the liver; the remainder of the administered alcohol exits from the liver, enters into the systemic circulation and is finally distributed throughout the entire body tissues (Weathermon & Crabb, 1999). The concomitant intake of various kinds of foods with alcohol generally results in a diminished area under the curve (AUC) on a blood alcohol concentration (BAC) curve, in addition to a lower peak concentration and an elevated time to reach the peak BAC (Sedman, Wilkinson, Sakmar, Weidler, & Wagner, 1976).

Essentially, an expanded threat for heavy drinkers and a relatively lower threat for lighter alcohol drinkers results in the 'J'-shaped alcohol-mortality curve (Klatsky, Friedman, Armstrong, & Kipp, 2003). Not to mention, an unbiased view of alcohol drinking and health should always consider its deleterious and helpful implications, amount of alcohol taken, choice of beverage, and last but not least, drinking patterns (Klatsky et

al., 2003). Having profound impact on mood and mental state, alcohol is one of the most widely known psychoactive substances that is produced by yeasts that digest the sugar in certain carb-rich foods, like grapes are used to make wine, or grains are used to produce beer (Steele & Josephs, 1990).

Negative Health Effects of Alcohol Consumption

The consequences of excessive alcohol use in terms of health risk is huge. The USA faces \$220 billion in economic losses and eighty thousand deaths per year due to alcohol abuse, which makes it the third largest life-style related cause of death. (Yeligar et al., 2016)

The excessive use of alcohol is closely related to an enhanced risk of various physical injuries and accidents. Even a single dose of excessive drinking can possibly bring about moderate to severe negative consequences. In a broad sense, alcoholism and chronic use of alcohol are linked to various medical, psychiatric, social, moral, and family problems. Children in families who are exposed to a first-degree relative's alcohol addiction are at risk. Children of parents with alcohol dependence have shown a higher degree of alcoholism than children who do not have parents with alcohol dependence. Nowadays, alcohol dependence related violence has become a serious social phenomenon where we observe more violent behavior in alcohol-dependent individuals (Heinz, Beck, Meyer-Lindenberg, Sterzer, & Heinz, 2011). It is extremely significant for social workers to bear in mind that alcohol and alcohol-related problems largely affect human health, safety, and well-being (Moss, 2013).

Physiological Systems Affected by Alcohol Abuse

Several physiological systems are potentially harmed by long-term, excessive alcohol use (Albanese, 2012). Firstly, the central nervous system (CNS) is mostly affected by many psychiatric problems. Alcohol abuse is known to cause various disorders, including peripheral neuropathy, reduced sleep latency, Wernicke-Korsakoff syndrome, blackouts, dementia, cerebellar degeneration, and so on (Fitzpatrick, Jackson, & Crowe, 2008; Hariharan, 2013; VITIELLO, 1997). Secondly, the gastrointestinal tract is affected by diseases like elevated gastric acid secretion, esophagitis, enteritis, gastritis, etc. (Bode & Bode, 1997). Alcohol abuse also lowers the esophageal sphincter tone, increases the absorption process of iron, slows down the absorption of some vitamins, increases toxicity to pancreatic cells, elevates the likelihood of various cancers, including gastric, esophageal, hepatocellular, pancreatic, and colorectal cancers (Albanese, 2012). Fatty liver and liver cirrhosis are also an outcome of the gastrointestinal tract being affected by excessive alcohol consumption (Diehl, 2002).

Thirdly, hematopoietic systems are also affected by alcohol abuse, which results in pancytopenia, increased mean corpuscular volume (MCV), and toxic granulocytosis (Heermans, 1998). Fourthly, alcohol abuse affects cardiovascular systems by increasing high-density lipoprotein (HDL), decreasing myocardial contractility and peripheral vasodilatation, decreasing blood pressure (BP) in small doses, though BP is enhanced when used for long-term in high doses (Zakhari, 1997). Additionally, cardiomyopathy and arrhythmias are also outcomes of excessive alcohol intake. Fifthly, genitourinary tract - modest doses potentially increase sex drive but reduce erectile capacity, sometimes testicular atrophy with shrinkage of the seminiferous tubules, responsible for amenorrhea,

and decreased ovarian size, infertility, and consequentially, spontaneous abortions (Havers, Majewski, Olbing, & Eickenberg, 1980). Lastly, other body systems affected by alcohol abuse are known to cause fetal alcohol syndrome, alcoholic myopathy, osteonecrosis with elevated fractures, avascular necrosis of the femoral heads, and reversible decreases in T3 and T4 hormones (Albanese, 2012).

History of Alcohol Use

The history of using alcohol dates back thousands of years. Even prehistoric people used alcohol for different purposes, but many of those were unknowingly done. Although procedures to produce alcohol and related beverages have transformed in some way over the years, very little has changed from the basic. Dating back from the prehistoric ages, the idea of taking alcohol to swing human mood and behavior is not very new at all (Albanese, 2012). The ninth chapter of the two thousand years old Biblical book of Genesis tells a story of Noah, where the most righteous man on earth became drunk. The first actual evidence of alcoholic drinks production dates back around 8000 B.C., after humans have started agriculture and started to establish sedentary communities (Khaderi, 2019). The oldest evidence of alcohol comes from chemical analysis of residues inside pottery jars found in Jiahu located at North China (McGovern et al., 2004). Dating back to 7000 to 6600 B.C., these clay pots help a fermented drink made from rice, honey, grapes, and hawthorn berries (Lee et al., 2009). Since then, different kinds of fermented grains, fruit extracts, and honey have been successfully used to manufacture alcohol for thousands of years.

Different kinds of beer were produced in ancient Egypt (A. H. Joffe, 1998). The reliable evidence for most was the fermentation of wheat or barley, triggered by malting,

efficient manipulation of germinated grains as a source of enzymes to transform carbohydrates into sugars, further intensified by elongated heating, and finally flavored by adding dates, fruits, and wines (Darby, 1977; Gastineau, Darby, & Turner, 1979). The Levantine Early Bronze Age, which was thought to be between 3500–2350 B.C., experienced the uprising of a small-scale urban civilization when the production of wines and oils became a key focal point for the new emerging economy (Esse, 1991; A. Joffe, 1993). Most of the manufacturing of wines, oils, and vessels in the Levant appears to have been incorporated for intrasocietal use, distribution and consumption. Different kinds of ceramic pots from the Mesopotamian civilization suggests that manufacturing and intake of alcoholic beverages enhanced extensively throughout the 4th and 3rd millennia (A. H. Joffe, 1998). The considerable rise in workshop-produced spouted jars and flasks in the Uruk-period certainly suggests increased use of beverages produced and stored in closed vessels (Delougaz, 1952; A. H. Joffe, 1998). The gradual evolution of alcohol use and consumption patterns in India can be roughly divided into four broad eras; it began with the Vedic era (ca. 1500–700 B.C.) (Sharma, Tripathi, & Pelto, 2010). From 700 B.C. to 1100 C.E., is the time of emergence and flourishing of Buddhism and Jainism, with some new anti-alcohol doctrines within the religions, and the post-Vedic cultures in the Hindu traditions and its scholarly writing and documents (Sharma et al., 2010). The writings of the two famous traditional medical practitioners, Charaka and Susruta, added a new era of thought, where they added arguments titled “moderate alcohol use” for particular health benefits (Sharma et al., 2010).

Alcohol as Medicine Through History

Throughout human history, the use of alcohol is established with numerous complications, including social norms, culture, rituals, religion, economics, traditions, medical beliefs, fun times, gatherings, and perhaps much more. The therapeutic use of alcohol with different medications appears to be as old as alcohol.

Rice wines and herbal remedies in Ancient China. Alcohol-based herbal medicines encompass a major portion of almost all Chinese works on herbal prescriptions and medicine, and on a general level, alcohol was thought to promote blood warming and blood revitalization (Nunn, 1996). Chinese medical literature also refers to some circumstances that necessitate alcohol quantities to be imbibed and discusses the adverse outcomes of its excessive use (Nunn, 1996).

Beer and wine in Pharaonic Egypt. Dating back to nearly 3400 B.C., the most ancient known brewery is thought to have been situated at Nekhen (also known as Hierakonpolis) in Egypt. Brewing was considered a very sophisticated art then, in Pharaonic Egypt, and beer was then also known as "necessity of life," was very popular among mass people. Osiris, known as the God of life and death, was the God of wine at the same time, which was largely imported for the people of upper classes. The intake of alcohol was extensive than and generally used for "pleasure, nutrition, medicine, religious purposes, gifts, and funereal purposes" (Hanson, 2013; Murray, 1999).

Wine and sophistication in Classical Greece. The wine was considered as an effective therapeutic agent for both body and the mind, for both men and women, was largely prescribed by the then physicians in classical Greece for ailments like bad breath, cancer, and wound healings, or to "loosen bowels" (Hanson, 2013; Links, PRO, Start, &

by Step; Villard, 1997). Even Hippocrates considered wine an efficient remedy for various disease conditions except for those involving "an overpowering heaviness of brain" (Hanson, 2013). According to their judgment, the wine had an active role in disease pathology and treatments (Links et al.; Villard, 1997).

Wine remedies and binging in the Roman Empire. The utilization of wine as a remedy in the ancient Roman Empire was mostly guided by Greek and Etruscan traditions (Faria, 2015). The Romans used to make a composition of wine and frankincense or myrrh to mask the muscle senses before any surgery, a practice thought to derive from the Talmudic medicine system (Links et al.). The widespread intake of wine for various purposes, mostly for sustenance and pleasure, mainly increased in the second century B.C. and, with the further extension of the great empire, the usage of wine spread very far and wide (Brownlee, 2002). Probably for the first time in history, binge drinking turned into a popular drink in leisure hours.

The water of immortality in the middle ages. During the Middle Ages, brewing was one of the major occupations for many people throughout entire Europe for monasteries and religious rituals (Links et al.). Among the brewed goods, beer was a vital source of sustenance: between 1000 and 1500 A.D., the average adult population in England is thought to have taken approximately 1 gallon of it per day (Barr, 1999; Links et al.). At that time, physicians and local practitioners continued to believe in the therapeutic values of alcohol, including those of new distilled concoctions, as for example, aqua vitae, also then known as "divine medicament" (Hanson, 2013). During the eighth and ninth century in Poland and Russia, an alcoholic drink, named vodka, made from fruit, herbs, spices, wormwood, acorn, birch, chicory, sorrel, dill, horseradish, mint, lemon, was highly

regarded for its estimated therapeutic values (Barr, 1999). One of the famous ancient physicians, Arnaldus de Villanova, took aqua vitae as a "cure for all ailments," which was effective against general fevers and other cold diseases and prophylaxis against the ever-present life-threatening plague disease (Berdan & Anawalt, 1997; Links et al.).

Pulque, mead, and maize-based alcohols in Mesoamerica. The widespread consumption of alcohol includes ancient societies in Mesoamerica. Evidence suggests that Mayans brewed mead and maize-based alcohol around 1000 B.C. in ancient Mesoamerica (Gately, 2008). Alcohol was extracted from cacti, fruits, and barks, as found in surviving artifacts and in many Spanish historians (Hanson, 2013). Therapeutic use of alcohol is believed to have been rampant in the age of pre-Columbian Americas, differing with the position in societies and cultures (Hanson, 2013).

A question of dosage. During the Renaissance and Enlightenment period, there was a deeply rooted belief in beer and wine's restorative and therapeutic potentials despite having ample anatomical understanding. In those years, alcohol was frequently prescribed in the London hospitals, but eventually, with a considerable rise in medical knowledge, growing disbelief was found among medical practitioners and general people about the role of alcohol in health promotion (Hanson, 2013). Although more knowledgeable and sophisticated medical personnel began to question the role of alcohol-based folk medicines, a child named David Hume was cured with drink therapies for his nervous problem. Hence, alcoholic drinks were still in the prescription for children in several illnesses (Hanson, 2013). During that period, tea imported from Asia started to achieve more traction as a new panacea, which was probably superseding alcohol as the new miracle of cure to all ailments (Hanson, 2013).

The gin crazes. Among the starting seeds of new transformation brought to England from Holland by King William, a brand new alcoholic drink, popularly known as distilled juniper water ("geneva" or "gin"), which was essentially an ideal tonic for the ailments of the stomach, gout, and gallstones, kidneys, liver, and heart (Berdan & Anawalt, 1997). According to the renowned Irish physician Dr. Robert Bentley Todd, a medical professor at King's College in London, it certainly helped natural disease healing processes in humans (Barr, 1999).

Mint julep, cocktails, and tonics in the United States. The mint julep, an amalgamation of mint and whiskey, and probably the ancestor of the modern known cocktail was first invented in the southern United States during the 18th century. It was given to the patients by some practitioners as a treatment for "all sorts of pathological conditions and ailments of the southern climate" (Berdan & Anawalt, 1997). As a tonic or a possible cure to all, mint julep was mostly a part of a wave of pseudo therapeutic drinks familiar as "cordials," "patent medicines," and "stomach elixirs," often used extensively to "treat women's constitutions." Other popular tonics of that time, such as, less cocktail than medicine and often sold as an alcohol-free drink, like Parker's Tonic, that contained 42% alcohol; Dr. Kaufmann's Sulphur Bitters, which contained 26% alcohol; Dr. Hoofland's German Bitters, that had 26% alcohol; Whiskol with 28% alcohol; Colden's Liquid Beef Tonic with 27% alcohol; and Lydia E. Pinkham's Vegetable Compound for "female complaints" (Berdan & Anawalt, 1997).

Prohibition and the winds of change. The extensive use of alcohol for medical purposes brought about division in the medical profession in the early days of the 20th century. In the absence of other remaining options, it was in some circumstances used as medication during the Spanish influenza epidemic in 1920 and as a treatment for pneumonia (Berdan & Anawalt, 1997). Nonetheless, an ever-growing awareness about the adverse effects of its abuse fueled the claim for a total and widespread ban of alcohol, leading to its prohibition in many countries, such as Russia (1916-1917), Norway (1919-1927), Finland (1919-1932), and the United States (1920-1933) (Links et al.). In the United States, alcohol with medicinal values reached a higher new level during the tumultuous prohibition years. US doctors were permitted for 100 prescriptions for "medicinal whisky" per three months span, which in total amounted to about 1.8 million gallons of alcohol in the year 1927 (Barr, 1999).

The Historical and Cultural Relevance of Alcohol

The craving for alcohol differs from men to men, and certainly, ethnicity, race, gender, etc. have essential roles to play here. Throughout the world, men drink more alcohol than women, and women in relatively more developed countries consume more alcohol than women in relatively less developed countries (Rehm et al., 2009). In contrast with men, more women are lifetime abstainers of alcohol, consume less, and are less prone to fall into drinking disorder and alcohol withdrawal symptoms (Erol & Karpyak, 2015). Misuse of alcohol is a burning health problem, especially among the youth, which often leads to severe consequences. Not to mention, identifying factors in the religiosity-alcohol relationship has vital implications for the intervention development process (Hai, 2019).

Alcohol and Ethnicity

Along with religious influence, the role of diversified cultural norms and social beliefs can't be ignored when predicting current drinking patterns and frequency of heavy drinking (Brooks-Russell, Simons-Morton, Haynie, Farhat, & Wang, 2014). Across all races and ethnicities, conservatism about drinking is observed more among the African-Americans and Latinos than the Whites (Caetano & Clark, 1999). Adolescents, passing more times with their friends than with their families, are more frequently involved in heavy drinking.

Alcohol and Sociocultural Influence

Studies have also pointed out the parents who are highly involved in heavy drinking increase the possibility of their children getting involved in regular alcohol intake (Caetano & Clark, 1999). Media have a huge role in regulating the rate of alcohol consumption within a society. TV advertisements, movies, TV series, various social media platforms, etc. bear a significant influence in setting alcohol usage patterns within a society. Every day, people view a considerable number of TV advertisements on alcohol and related beverages, despite the fact that alcohol marketing is greatly regulated by most countries (Grenard, Dent, & Stacy, 2013). Several socio-cultural influencers estimate the prevalence of alcohol consumption, including social discrimination and associated stigma. The huge role of discrimination and mental and physical stress in health-associated risk behaviors, including alcohol abuse, is very well rooted in liquor advertisements, especially in the US (Dawson, Grant, & Ruan, 2005; Sudhinaraset, Wigglesworth, & Takeuchi, 2016).

Government Regulation

After the tobacco and obesity problem, the overconsumption of alcohol is the third largest cause of death in the United States. Moreover, the death rate because of alcohol

abuse has nearly doubled in recent years, which includes premature deaths that are associated with alcohol abuse, such as motor vehicle accidents. Heavy drinking habit worsens the morbidity rate among the mass population. It can cause various chronic diseases, including hypertension, hepatitis, diabetes mellitus, etc. Heavy drinking habit is certainly a problem for those who usually administer different types of medicines daily. Drug metabolism and its therapeutic efficacy are greatly hampered by alcohol present in the stomach. Societal costs of over alcohol consumption have far-reaching consequence, including increased rate of severe alcohol injury, accidental deaths in roads and highways, income loss, wastage of the country's healthcare resources, and disruption of social and family life (Bouchery, Harwood, Sacks, Simon, & Brewer, 2011).

Since chronic alcohol consumption is the burning issue, the National Institutes of Alcohol Abuse and Alcoholism (NIAAA) has published a guideline for acceptable upper limits of alcohol consumption by adults. According to the NIAAA, men aged 21–65 should take a maximum of fourteen standard drinks per week and four standard drinks per day. The rules for women and older adults are a bit different. Adult women (age 21–65) and older adults (aged over 65) should not take more than seven standard drinks per week and three standard drinks per day.

All the standard drinks have the same percentage of alcohol, which is less harmful to human physiology. But, it is true that the percentage of alcohol in the standard drinks and guided upper limits differ from state to state (Suzanne & Kril, 2014). In the United States, a standard drink contains 14 g of alcohol. Among the standard drinks, 355 ml of beer, 237 ml malt liquor, 148 ml wine, 44 ml 80-proof spirits are mentionable which contain 5, 7, 12, and 40 % of alcohol, respectively (Kalinowski & Humphreys, 2016;

Miller, Heather, & Hall, 1991; Turner, 1990). In Australia and New Zealand, a standard drink is defined as 10 grams of ethanol and four drinks per day, and 14 drinks per week are the upper limit of drinking. In Japan, a standard drink is allowed to bear 19.75 grams of alcohol, whereas a standard drink contains 8 grams of alcohol in the United Kingdom. In the European Union countries, the alcohol content in a standard drink differs from country to country, ranging from 6 to 17 grams of alcohol in alcoholic drinks. Among most of the guidelines, it is recommended that pregnant and breastfeeding women should abstain from consuming alcohol (Bouchery et al., 2011).

Pharmacokinetics

Distribution. Ethanol is reasonably insoluble in fatty materials, yet it can surpass the mammals' biological membranes as water does. Essentially, ethanol evenly circulates into the blood via absorption from the gastrointestinal tract, then passes into all body tissues and other body fluids via a consistent ratio with their comparative water content. The active concentration of ethanol within body tissues heavily relies on the relative water content of the tissues. It swiftly reaches the equilibrium condition in relation to ethanol concentration in the body plasma (Frezza et al., 1990). But alcohol does not bind to any plasma proteins. Dose-response mechanisms for alcohol consumption for different individuals vary largely. The same amount of alcohol dose per unit of body weight can exhibit quite different blood-alcohol concentrations in different individuals, mostly because of variations in fat ratios to water in different bodies and low lipid: water partition coefficient of ethanol within the bodies (Cole-Harding & Wilson, 1987). Women's physiological condition generally shows relatively a smaller volume of distribution for alcohol than men, mainly because of their larger percentage of fat present in the body. Alcohol first-pass metabolism is generally

more significant in males than females, which also influences the higher concentration of alcohol in blood, particularly in females (Cole-Harding & Wilson, 1987; Frezza et al., 1990).

Absorption. Alcohol absorption is quicker in the duodenum and jejunum than in the stomach; therefore, the rate and prevalence of gastric emptying time is a vital determinant factor in terms of the rate of absorption of orally administered alcohol (Halsted, Robles, & Mezey, 1973). Alcohol can pass through the biological membrane barriers via the passive diffusion method down towards its concentration gradient. Hence, a higher concentration of alcohol results in a larger concentration gradient of alcohol and faster absorption. Alcohol is also known for its irritant properties, and higher concentrations of alcohol can result in superficial tissue erosions, hemorrhages, and insensitivity of the smooth muscle cells in the stomach. This incident is responsible for reducing alcohol absorption. Blood alcohol concentration is generally higher if ethanol is consumed as a single dose rather than several tiny doses, perhaps because the concentration gradient of alcohol will be greater in the previous case. Different alcoholic beverages containing the same amount and concentration of alcohol are expected to be continuously absorbed. That's why the blood alcohol concentration remains all the same after administering different types of alcoholic beverages with the same concentration of alcohol (Baraona et al., 2001; Kwo et al., 1998; Wilkinson, Sedman, Sakmar, Kay, & Wagner, 1977). Stomach filled with foods certainly delays the gastric emptying time, and that's why it decreases the absorption of alcohol and supports the concept that “Don't drink on an empty stomach” (Moxnes & Jensen, 2009).

Metabolism. The major enzyme systems that are involved in the oxidation process of ethanol, among them alcohol dehydrogenase and cytochrome P450-dependent ethanol-oxidizing system, are abundantly in the liver (Morgan & Levine, 1988). Most of the consumed ethanol in the body is metabolized in the liver by a specific enzyme named alcohol dehydrogenase, which converts ethanol into a toxic metabolite called acetaldehyde (CH_3CHO), a well-known carcinogenic agent. But acetaldehyde is normally a short-lived compound; it is consequently converted into a comparatively less toxic compound, named acetate (CH_3COO^-) through interference from an enzyme called aldehyde dehydrogenase. Acetate is then further converted into carbon dioxide and water, mainly in tissues except for the tissues in the liver (Alert, 2007).

Elimination. Alcohol elimination is a zero-order kinetic process that certifies that alcohol is eliminated from the body at a constant rate. Elimination of alcohol from the body occurs principally via enzymatic oxidation in the liver, with usually minor non-hepatic oxidation pathways and minor excretion of unchanged alcohol in the urine, breathe, and perspiration (Dubowski, 1985).

Introduction Alcohol-Use Disorders (AUDs)

The very term "alcohol-use disorders" basically consists of long time alcohol dependence and alcohol abuse or excessive use (Association, 2000). According to U.S. Department of Health and Human Services, AUD is a medical diagnostic state that can be characterized as a chronic relapsing brain disease described by uncontrollable alcohol use, loss of self-regulation over alcohol consumption has a negative and depressing mental state when not consuming alcohol. Alcohol overconsumption and its related toxicities are

responsible for nearly eighty-eight thousand deaths per year in the United states (K Witkiewitz, Litten, & Leggio, 2019). AUD is such a sort of psychiatric problem which affects approximately one-third of the US adult population at some point in their life span. In addition to health risks, AUD costs the United States nearly \$249 billion per year (K Witkiewitz et al., 2019). However, it is a matter of joy that recent advances in medical treatment patterns have helped patients manage AUD. Although various researches during the last one or two decades have enlarged the understanding of AUD on a broad level, more research is necessary at the same time to identify the etiological and treatment-related influencers of this disease (K Witkiewitz et al., 2019).

The important influencers under study include genetic, neurobiological, epigenetic, psychological, social, and environmental factors. It is most significant to implement this research-based knowledge in different clinical practice layers to ensure efficient diagnosis and treatment of AUD. As far as AUD and its consequences are concerned, these life-threatening disorders mimic and exacerbate a large variety of additional medical and psychiatric complexities and compress the lifespan of the addicted people (Schuckit, 2006). However, a big problem within this reality is, most people suffering from AUD are difficult to identify since they are likely to be involved in jobs, families, and daily life and generally present with symptoms including anxiety, malaise, sadness, insomnia, etc. (Organization, 1993).

History of Treatment for AUD

A physician from Pennsylvania named Benjamin Rush first talked about the possible treatment patterns of alcohol abuse during the edge of the 18th century (Rush, 1823). He studied different cases after cases and eventually came up with some possible

ways to manage AUD. He emphasized the practice of religion strictly, which might help people to realize guilt and shame. The physician also suggested giving much importance to personal passions and likings, while a person's diet would only include vegetarian foods (Rush, 1823). During the last two centuries, it is extensively preached by many social and voluntary assistance groups that over alcohol consumption is an ethical failure. We can also find traces of some permanent and temporary asylums to provide house treatments to alcohol-addicted patients during the same era. Though these asylums only ensured forced abstinence from alcohol consumption (Baumohl, 1990). An organization named “Alcoholics Anonymous” was found in the year 1935 with a view to restricting people from alcohol consumption through motivations and different processes (White & Kurtz, 2008). The idea of AUD first emerged in the 1940s; consequently, various treatment options were invented, some of which have been practiced for so long and exist even today (Jellinek, 1942). According to the World Health Organization’s Global Status Report on Alcohol and Health-2018, we have observed enforcement of several public health policy initiatives, including increased taxation on alcohol sales, restrictions on advertising of alcoholic drinks, and brief scale intervention programs, such as revitalization of social norms and rules that can help control the abundance of AUD.

The Broader Impact of AUD in Society

Till today, AUD is socially regarded as a personal lousy habit or fault rather than a disease. To some particular extent, this judgment is winning the public opinion as well as among the health care providers (Neuberger, Adams, MacMaster, Maidment, & Speed, 1998). But it is also to acknowledge that public perception has started to change among the mass population due to widespread education campaigns. Today, it is accepted that

alcoholism is a disease (Gitto, Vitale, Villa, & Andreone, 2014). Thus, AUD is largely regarded as a clinical circumstance related to a substantial amount of disability and loss of quality of life (Samokhvalov, Popova, Room, Ramonas, & Rehm, 2010). Not to mention, alcoholism is one of the main reasons for frequent road accidents and many other social violence episodes (Mathurin & Bataller, 2015).

Classification of AUD

Patients having AUD can be classified based on the craving pattern for alcohol (Sinha & O'Malley, 1999). Alcohol craving is vitally responsible in AUD that broadly impacts the pharmacological and physiological choice and the principal prognosticator of alcohol addiction (Addolorato, Abenavoli, Leggio, & Gasbarrini, 2005). AUD can be classified into the following categories: (a) reward craving (due to family history of alcoholism involving the loss of regulation of dopaminergic or receptors and representative personality characteristics); (b) relief craving (due to deregulation of GABAergic or glutamatergic receptors); (c) obsessive craving (involves deregulation of serotonergic receptors, typical personality traits consisting of the absence of alcohol inhibition, compulsive drinking pattern, loss of control on alcohol consumption, and alcohol-related impairment) (Addolorato, Leggio, Abenavoli, Gasbarrini, & Group, 2005). Although it has to be mentioned that "The Craving Typology Questionnaire" is not yet considered an authenticated diagnostic aid that can classify patients by their craving typology for alcohol (Martinotti et al., 2013).

Biological Mechanisms Underlying AUD: Focus on GABA Receptors

The biological pathways of AUD are yet incompletely understood. Alcohol intake has a powerful impact on brain functioning and behaviors (Bayard, McIntyre, Hill, & Woodside,

2004). Continued over alcohol intake can gradually build up a physical dependence on alcohol for a considerable amount of time. In that case, discontinuation or abruptly decreased alcohol intake stimulates AWS4 (alcohol withdrawal syndrome). Today, the pathways for how excessive alcohol intake guides physiological changes in the human brain functioning that generate alcohol dependence prevail quite dark. AUD is a long-term generation process, and thus it is characterized as a chronic disease. For instance, a relapse in alcohol intake might be voluntary and spontaneous. Various intrinsic stimuli inside the body, such as anxiety, mood swing, etc. can cause a relapse.

Some external factors like drinking culture in the family and society or even the bottles of alcoholic drinks can cause a relapse. Regardless of both of this view-point, the profound impact of alcohol on the human brain and nervous system cannot be overlooked by any mean, provided lot of neuropharmacological and psychological impacts of ethanol, including its sedative, anxiolytic, intoxicating, reinforcing, and addictive potentials (Hobbs, 1996; Paul, 2006).

GABA is one of the main inhibitory neurotransmitters located in the mammalian brain systems. A GABAergic neuron triggers an action potential, and then the presynaptic nerve terminus helps to release GABA into a synaptic cleft. GABA_ARs are a family of ligand-gated chloride anion route communicated throughout the entire central nervous system and consist of five subunits along with several isoforms, namely, $\alpha 1-6$, $\beta 1-3$, $\gamma 1-3$, δ , ϵ , θ , π , $\rho 1-3$ (Nayeem, Green, Martin, & Barnard, 1994) (Barnard et al., 1998; Macdonald & Olsen, 1994; Nayeem et al., 1994; Olsen & Sieghart, 2008, 2009).

GABA attaches to the GABA_ARs, altering their conformational systems and consequently unfolding the pore to permit the passage of chloride (Cl⁻) ion to move down

towards an electrochemical gradient. GABAARs induce fast and phasic inhibition in the postsynaptic membrane. The metabotropic GABA_BRs slow down the synaptic inhibition. GABAARs are known to induce several pharmacological impacts of alcohol in the brain. Evidence shows that GABAARs are the main target of ethanol in the CNS (Becker, Veatch, & Diaz-Granados, 1998; Boehm II et al., 2004; Koob, 2004; Olsen & Spigelman, 2012; Weiner, Zhang, & Carlen, 1994). Several studies have demonstrated that alcohol consumption for a shorter duration of time upregulates the GABAARs inhibitory effects. Yet, many more factors decide whether GABAARs would respond to alcohol exposure for a short duration or not (Mihic & Harris, 1997). Alcohol can function as a depressant by upregulating inhibitory neurotransmission, and by downregulating excitatory neurotransmission, or via a combination of the both (Lithari et al., 2012; Valenzuela, 1997).

In general, alcohol intake can affect mental attention, alter memory functions, reduce executive decision-making capability, change the mood, and cause drowsiness. GABAARs are known to mediate sedation, anxiolysis, inconsistency in motor coordination, and withdrawal symptoms, including hyperexcitability, anxiety, insomnia, and random seizures (Buck & Finn, 2001; Buck & Reynolds, 1996; Davies, 2003; Grobin, Matthews, Devaud, & Morrow, 1998; Hanchar, Dodson, Olsen, Otis, & Wallner, 2005; Kumar et al., 2009; Liang, Cagetti, Olsen, & Spigelman, 2004; Liang, Spigelman, & Olsen, 2009; Rudolph & Knoflach, 2011; Tobler, Kopp, Deboer, & Rudolph, 2001). Ethanol works on some specific sub-groups of GABAARs, and their subunit assembly is swiftly altered, which subsequently changes the functional characteristics of these GABAARs (Grobin et al., 1998; Kang, Spigelman, Sapp, & Olsen, 1996). Consequently, GABAAR-induced behaviors are altered after alcohol consumption (Cagetti, Liang, Spigelman, &

Olsen, 2003; Liang et al., 2004; Liang et al., 2009). Evidently, it can be stated that GABAARs have a pivotal role in response to ethanol, thereby modulating the altered balance between inhibition and excitation and contributing largely to the withdrawal syndrome.

Types of Treatment

As alcohol abuse is a long-existed problem on this planet, there was always an attempt within many human societies to resolve this issue. Woods, leaves, and barks from various trees were used to contain the habit of over alcohol consumption among mass people. Several treatment approaches have been attempted so far to treat people having AUD. Proper and sustained treatment of alcohol use disorders mainly depends on its proper and early diagnosis and understanding of the fact that there exists a wide spectrum of drinking disorders.

Pharmacologic Therapies Approved by the U.S. Food and Drug Administration

So far, the U.S. Food and Drug Administration (FDA) has approved four medications for the treatment of alcohol use disorders. The medications are disulfiram, acamprostate, oral naltrexone, and injectable long-acting naltrexone.

Disulfiram. Disulfiram (Antabuse™) is the first medication for AUD approved by the FDA in 1948 (Liang & Olsen, 2014). It is an anti-craving drug that blocks acetaldehyde's transformation into acetate by the aldehyde dehydrogenase enzyme. However, disulfiram causes some side effects like headache, nausea, flushing, vomiting, flushing, etc. when taken simultaneously with alcohol consumption. That's why there is a black box warning for disulfiram intake, which says it should not be prescribed to patients

who have administered alcohol during the last 12 hours. This drug's dose range is 125 to 500 mg/d, although the higher dose range is possible at high dosing intervals. Recent reports have demonstrated that this drug has only a moderate short-term reduction capacity in alcohol use (Jørgensen, Pedersen, & Tønnesen, 2011). The chemical structure of Disulfiram is shown below.

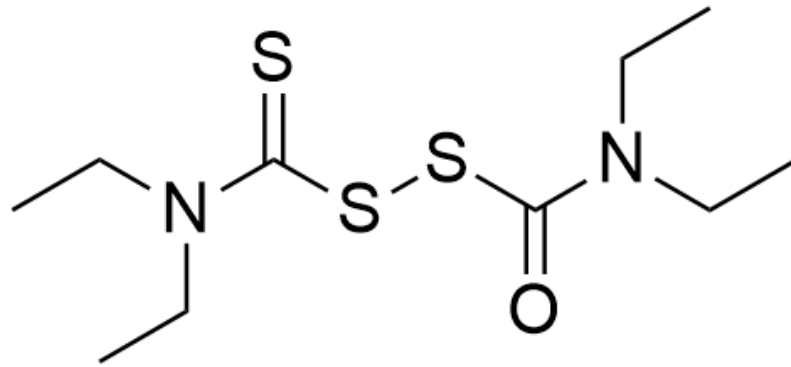


Figure 3. Structure of Disulfiram. One of the first FDA approved medications for the treatment of AUD.

Acamprosate. Acamprosate (Campral™) is given to patients to help them manage alcohol cravings when a person has given up drinking (Buechler, 2020). The FDA approved it in 2004, although it has been used in Europe since the 1980s. The exact mechanism of action acamprosate in reducing alcohol craving is still unclear, but it contains significant structural homogeneity with GABA receptor and thought to modulate the glutamate action at the N-methyl-D-aspartate (NMDA) receptor in the human brain. It has been extensively studied in different doses to assess its real efficacy at a certain dose. Acamprosate has a dose range of 1332 to 3000 mg/d, usually prescribed as 666 mg three

times daily. It is noteworthy that acamprosate has no black box warning. It also has no specification based on gender (Kranzler & Gage, 2008). Acamprosate helps to increase the duration of abstinence from alcohol. The possibility of getting back to drinking after taking this drug is very low. It is relatively more effective than any other drug used in the management of AUD (Garbutt, West, Carey, Lohr, & Crews, 1999; Katie Witkiewitz, Saville, & Hamreus, 2012). The structure of Acamprosate is given below in Figure 4.

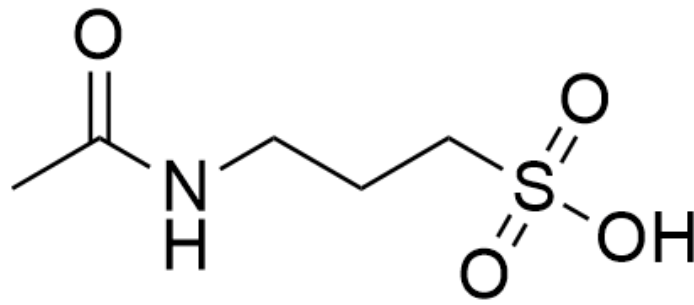


Figure 4. Structure of Acamprosate. One of the most recent drugs approved for the treatment of AUD. It helps patients with withdrawal symptoms.

Oral naltrexone. FDA approved oral naltrexone in 1994 as an anti-craving agent for the management of alcohol dependence (Liang & Olsen, 2014). Naltrexone actively antagonizes μ -opioid receptor (OPRM1) and most possibly work via suppressing the brain reward systems, which use the neurotransmitter dopamine to communicate. The anti-craving action of naltrexone is most profound in patients with specific genetic polymorphisms in them. A black box warning for oral naltrexone says not to use this in patients diagnosed with acute hepatitis or hepatic failure. Caution also should be followed by patients with severe liver or renal insufficiencies, yet no dose adjustment is

recommended so far. Patients already using naltrexone should not use opioids at the same time. Patients are often suggested to carry a card to notify medicine suppliers that they are administering an opioid receptor blocking agent. The structure of Naltrexone is shown below in Figure 5.

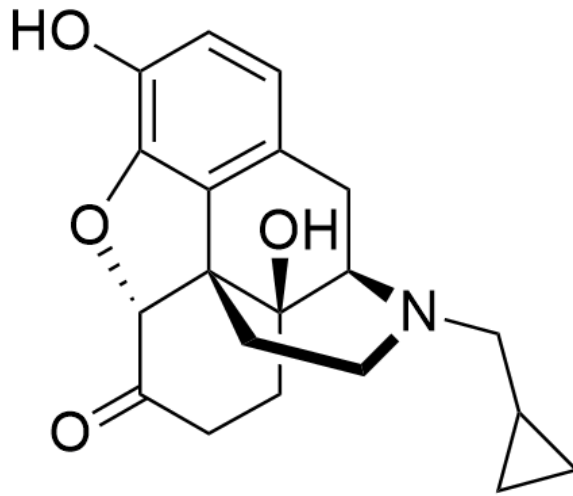


Figure 5. Structure of Naltrexone. It was first prescribed for the treatment of opioid addiction.

Other pharmacologic therapies. The existing FDA-approved drugs for AUD are trashed with several side effects and less effective in many cases, as they do not fully cure it. Thus investigation and search for new drugs are on the rise (M Edwards, A Kenna, M Swift, & Leggio, 2011). Topiramate, baclofen, ondansetron, sertraline, nalmefene, and aripiprazole are currently under investigation. Topiramate is thought to exert its mechanistic effect as a GABA receptor agonist and glutamate receptor antagonist. Side effects of this investigated medication include anorexia, paresthesia, and taste perversion. Baclofen is a GABA-B receptor agonist, according to the published data currently under

investigation to assess its efficacy (Johnson, 2005). Sertraline is a selective serotonin reuptake inhibitor used to treat anxiety, sleep disorder, depression, and other psychiatric disorders. It is now under assessment for its potential efficacy in managing AUD.

Why New Treatments are Needed?

One of the common side effects of one of the FDA-approved drugs, oral naltrexone (vs. placebo), includes somnolence, nausea, vomiting, reduced appetite, abdominal pain, insomnia, and dizziness (Roesner et al., 2010). This particular drug blocks the therapeutic efficacy of opioid analgesics and can precipitate the incidence of opioid withdrawal in a patient who is already physically dependent on opioids. Additionally, long-acting naltrexone can bring about the same adverse outcomes as oral naltrexone and injection-site reactions (Garbutt et al., 2005). Furthermore, naltrexone is quite less effective for maintaining abstinence from alcohol in most patients with alcohol use disorder (Carmen, Angeles, Ana, & María, 2004; Srisurapanont & Jarusuraisin, 2005). Naltrexone is also well known to induce fatigue and anxiety; in addition, it impairs the patient's thinking process or reactions (Losekam, Kluge, Nittel, Kircher, & Konrad, 2013; Sonne & Brady, 2000; Sullivan & Nunes, 2005). Another drug for alcohol use disorder, acamprosate, has the most commonly observed side effects in the clinical trials, including headaches, diarrhea, flatulence, nausea, etc. (Kiefer & Wiedemann, 2004; Mann, 1996). It is also known that disulfiram is trashed with peripheral neuropathy's side effects (Filosto et al., 2008).

It is notable that current treatment patterns for AUD, including pharmacological treatments and available medications, certainly have lower success rates, suggesting a clear necessity for new potential drugs. Till today, most often, non-pharmacological therapies are still the first and sometimes only available treatment method for the people suffering

from AUD. The drugs currently available for treating AUD only deal with the side effects of quitting the drinking habit. The currently available medications try to suppress or manage the adverse effects that originate from alcohol use disorders. But it is a matter of great concern that none of the medications treat the actual addiction due to alcohol use. The medical and scientific communities' existing knowledge and expertise have certain gaps in how to treat addiction in a sustainable matter, including alcohol use disorder. One of the first things that need to be known is where in the brain or in which receptor alcohol targets. The abuse-related liability of addictive drugs is also needed to be ascertained. It is now a demand for an effective drug that can be found that can utterly and effectively target the specific area in the brain or specific receptor protein attributed to the alcohol use disorder. A sustainable drug that is found to be successful could hugely help people manage their addiction to alcohol or for those where other treatment options and therapeutic approaches have not worked.

Research Goal

In this thesis, two different drugs, each of which target separate receptors in the brain, were tested to determine whether either of these drugs could be effective in the treatment of alcohol use disorder in mice models. The two interest drugs are L-745,870 and PSNCBAM-1, both of which are described in more detail in the subsequent chapters. The experimental methods and results for each drug are also described in detail in the following chapters of this work. Additionally, I describe the optimization of a two-bottle choice procedure for future use as a model of AUD. Our goal is to find a new drug that could help people in their addiction grips to have a fighting chance against alcohol addiction.

Chapter 2

Materials and Methods

Animals

For all experiments, we used drug naïve male C57BL/6 mice purchased from Charles River Laboratories (Wilmington, MA). Animals were grouped-housed in the temperature- (21-23°C) and humidity-controlled (45-50%) vivarium at Cooper Medical School of Rowan University under a 12 h light/dark cycle (lights on at 0700, off at 1900). Housing enrichment is provided by polycarbonate cages with ad libitum food and water. Animals received enrichment provided by paper Bio-Huts and/or nestles. Testing animals arrive in vivarium approximately 28 days of age, weighing 23-30 grams, and need to be familiarized about 7/10 days before testing. During the habituation period, the mice were given free access to foods and water. The picture of C57BL/6 mice is shown below in figure 6.



Figure 6. Picture of a C57BL/6 drug naïve mouse obtained from Charles River Laboratories.

Drugs

PSNCBAM-1 (a negative allosteric modulator of the cannabinoid CB1 receptor) and L-745,870 (a dopamine D4 receptor) were used in this study to determine if either could be work in the treatment of alcohol use disorder. The vehicle used for PSNCBAM-1 was a mixture of 10% dimethyl sulfoxide (DMSO), 10% Tween 80, and 80% saline. DMSO is commonly used to dissolve drugs (Brayton, 1986). The vehicle for L-745.870 was physiological saline. PSNCBAM-1 and L-745.870 were purchased from Tocris Bioscience (Ellisville, Missouri).

Open-Field Locomotor Test

In this experiment, three groups of 20 Male C57/B16 mice were used. PSNCBAM-1 (0, 10, or 30 mg/kg) was administrated by intraperitoneal (i.p.) injection. The PSNCBAM-1 vehicle was 10% dimethyl sulfoxide (DMSO), 10% tween 80, and 80% physiological saline.

Apparatus. Open-field locomotor test apparatus was a 40 × 40 × 35 cm Plexiglas® open-field, and a camera mounted overhead recorded and tracked locomotion of animals that was connected to the Any-maze behavioral analysis software of a computer. Two regions of this field are the center region by 20*20 cm and the rest of the outer regions of the apparatus.



Figure 7. Picture of Open Field apparatus. Camera mounted overhead that records the locomotion of animals

Procedure. To measure animal behaviors, the open field is one of the oldest and most widely used platforms. This experiment is easy and quick to determine different types of behavioral information (Seibenhener & Wooten, 2015). An open field test was done to determine whether locomotor activity was disrupted due to the injection of a drug in mice. At the beginning of these experiments, mice were placed in an open field chamber for 20 minutes to explore the chamber unchecked. The mice were then taken out, and intraperitoneal injection of either 3.0 mg/kg L-745,870 or saline were given during the L-745,870 study, and either 10 mg/kg PSNCBAM-1, 30 mg/kg PSNCBAM-1 or the vehicle mixture were given during PSNCBAM-1 study. After that, the mice were placed in the open field compartment for 40 minutes. There is a camera on top of the open field chamber that records the movements of mice. After every use, the chamber was cleaned by 70% isopropyl alcohol to make the apparatus ready for the next mice.

Statistical analysis. The total distance, time in the center zone, and time in outer zones were collected for further behavioral analysis. The total distance traveled by animals was statically analyzed using GraphPad Prism 6.0 statistical analysis software.

Rotarod

The rotarod performance test is a behavioral analysis to test motor coordination and balance in rodents, especially to test the effects of different drugs and substances. Additionally, testing rotarod coordination gives the ability to characterize the motor phenotype of rodents. This study aimed to determine if dopamine D4 antagonist L-745,870 disrupted the mice model's coordination function. Evaluating the period, the mice could maintain their coordination in an increasing rotarod speed. In the initial study, mice were placed on a black round rotating rod that gradually increased its speed from 4 RPM to 40 RPM. Before injecting, there were two 10 minutes segments, where 6 minutes of run and 4 minutes resting period and then again 60 minutes of post-injection experiments with the same procedure as six of ten minutes segments with six minutes run and 4 minutes resting period. In the first part of the experiment, different concentrations of ethanol (1.2 g/kg, 1.6g/kg, 2.0 g/kg) EtOH were injected.

Rotarod apparatus. The animals were placed apart on a horizontal black rod that rotates about its long axis. Initial rotation was set at 4 RPM and increased to 40 RPM. and the mice had to walk forward to remain upright and not fall from the rod (Deacon, 2013). The mice were either injected with EtOH or L-745,870 to determine whether the dosages of each altered their coordination activity. A white sensor plate under the rotating rod that senses the time when mice fall from the rod. This rotarod machine was connected to Med

PC that indicated the falling time of mice in second and RPM speed in the falling time. We recorded falling time and speed manually.

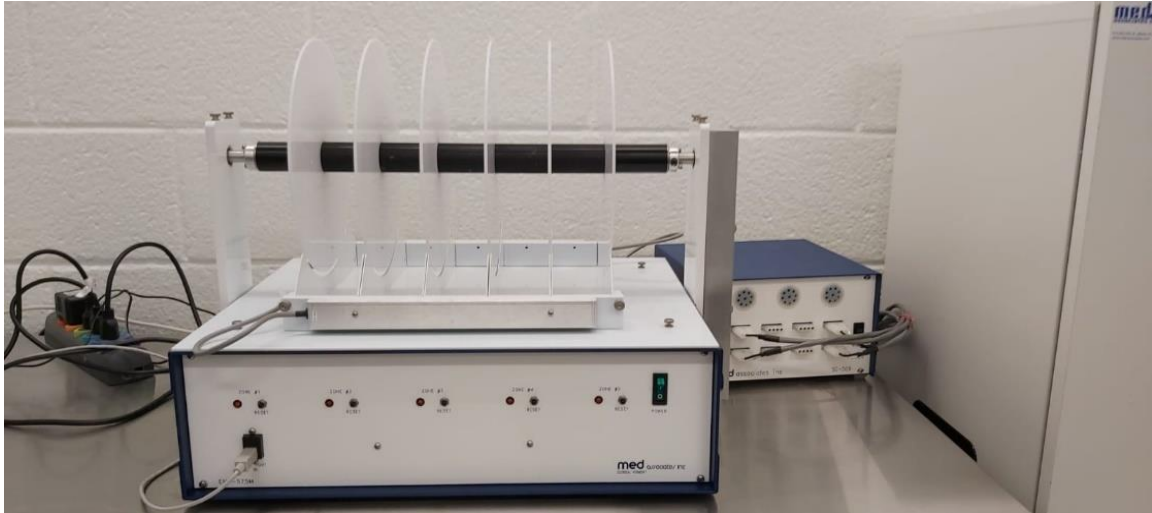


Figure 8. Rotarod Apparatus. The center black rod rotates about its long axis. A sensor white plate under the rotating rod.

Self-Administration Operant Training

Self-administration is the process of a subject (usually animal) administering a certain pharmacological substance to themselves. Self-administration is a kind of operant conditioning where the reward is usually a drug. Alcohol operant self-administration training is a critical tool for studying the neural circuits both in alcohol-seeking and consummatory behaviors for studying the neural basis of underlying alcohol use disorders. Ethanol self-administration using operant conditioning procedures has been firmly established in several species, including monkeys, rats, and mice (Lopez & Becker, 2014). It was tried to observe whether the two drugs, L-745,870 and PSNCBAM-1, reduced ethanol's self-administration.

Operant chamber. Standard operant conditioning chambers were well-ventilated and sound-attenuated with fans. Every box contained a reward vessel in the center and two nose poking holes in two sides of the vessel. A house light inside the chamber and a stimulus light were also available in each response hole. Tube connected with syringes was put into a plunger pump inside the box that was used to supply either ethanol or food. An image of the self-administration apparatus was demonstrated below.

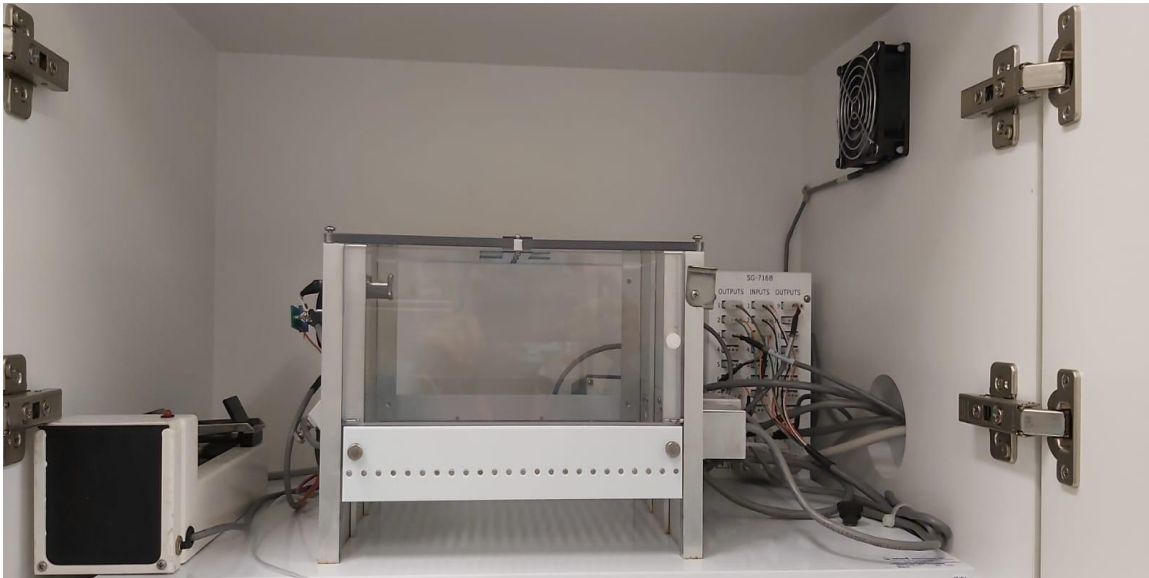


Figure 9. Self-Administration Operant Chamber. There are two nose poke holes inside the chamber, the correct hole being on the left side and incorrect hole being on the right side and a reward receptacle in the middle of right and left hole.

Self-administration training. This training was primarily for 60 minutes daily, but the training session increased to 120 minutes daily when mice could nose poke into the active hole to attain the reward of either diluted vanilla Ensure or ethanol. A fixed-ratio (FR) system was used for training where a fixed number of correct nose pokes were required to get a reward. Training ratios started at FR1- for a reward to earn, one correct

nose poke on the programmed drug side is required. The training ratio was gradually risen to FR4- four consecutive nose pokes at the correct hole was required to get the reward. Three correct and consecutive nose pokes and then one incorrect nose poke would require starting from the beginning again. According to the training, mice that were restricted for food, nose poked for rewards and for a food reward of Ensure, mice were couched to nose poke. Dilution of Ensure into a 50% Ensure: 50% water ratio was done gradually by adding water. Then ethanol replaced the water when the Ensure: water ratio showed a stable response. When the ethanol concentration came down to 10% w/v, this process was stopped. Water was added in place of the Ensure to make a mixture of 10% w/v ethanol in water. The 10% w/v mixture was too high as the number of nose pokes for the alcohol mixture reduced instead of increasing and hence the mixture was converted to 8% w/v ethanol in water. The 8% w/v ethanol in water was used in the operant chambers for the two drugs, L-745,870 and PSNCBAM-1.

Testing of pharmacotherapeutics for self-administration. We used eight mice which showed best nose poke response for the mixture out of sixteen trained mice. One day of the week was the testing day with drug and rest of the days were used as training days. Latin square design was used to arrange the testing session. The two drugs, L-745,870 and PSNCBAM-1 were tested at different sessions and with different sets of mice each time. In case of L-745,870, the mice were injected with either 1.5 and 3.0 mg/kg L-745,870, or saline vehicle, respectively. At the time of PSNCBAM-1 testing session, the mice were injected with either 10 and 30 mg/kg PSNCBAM-1, or the vehicle (10% Tween-80, 10% DMSO and 80% saline solution). In the second testing session of PSNCBAM-1, an intermediate dose of 18 mg/kg was also given in the same way.

Statistical analysis. GraphPad Prism, version 8.3 (San Diego, CA, USA) was used to analyze the data. All the results are demonstrated as means \pm SEM (standard error of the mean). Pre-planned Bonferroni t-test was used to conduct individual group comparisons, in case of a significant effect using one-way analysis of variance (ANOVA). Bonferroni t-test is a multiple level comparison tool used extensively in statistical analysis that does not allow data to incorrectly appear as statistically significant. Presence of any statistically significant difference between the averages of the unrelated groups were ascertained using ANOVA as well.

Conditioned Place Preference

The conditioned place preference (CPP) is a standard preclinical behavioral analyzing model which has been widely used for the research of abuse and addictions for drugs, food, sex, etc. CPP, has four phases-acquisition, expression, extinction, and reinstatement (Wu, Yang, & Wang, 2016). The goal of the study was to test the effects of the CB1 negative allosteric modulator, PSNCBAM-1 and dopamine D4 antagonist to determine if it could reduce ethanol self-administration behavior in adult male mice.

For this PSNCBAM-1 and L-745,870 related studies, we used modular CPP chambers from Stealing for using with Any-Maze software. These chambers included two rectangular shapes compartments, described by the white and black wall which are connected through one small central compartment which has gray color wall. There is no specific feature of this center compartment and two doors between two adjacent compartments connected with this middle compartment that allows animals to move freely. The compartments are either circular grid or square grid flooring with the similar marked wall. Two adjacent chambers characterized by the white and black surroundings are

connected through a small central gray compartment. The two end compartments are either paired with a drug or vehicle during experimental training. The center compartment is not paired with either the drug or the vehicle, it is considered neutral space within the entire compartment. The center compartment allows the mice to move freely between the two adjacent compartments when the gates to those compartments are raised.

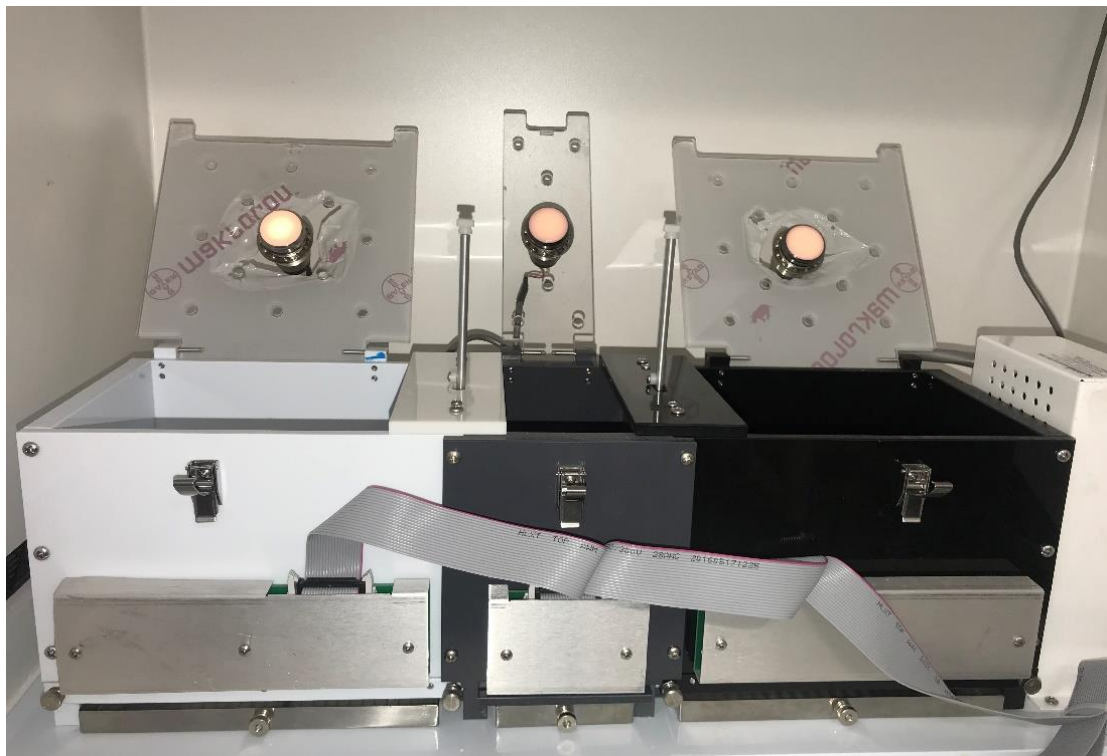


Figure 10. CPP Chamber. The white compartment is located on the left side and black compartment located on right side. The middle gray compartment on the picture is the neutral compartment.

Initial preference test. Initial preference test is common for most of the place preference studies. During the initial preference test, animals can access both chambers to

show pre-existing preference for any of the two chambers before administration or train with any drug. The initial preference was measured by allowing the animal to explore in both compartment for 30 minutes. The amount of time animals spends in the compartment is recorded by the software. Before conditioning, we calculated the ratio of time animal spent in an individual chamber. After completion of conditioning, the initial preference value is used to assess the rewarding effects of drugs and sometime, this value is used to fix which chamber can be related to drug or vehicle. In unbiased experimental procedure the drug-paired and vehicle paired compartment were assigned randomly but in a biased procedure, least preferred compartment is paired with the drug of interest.

Drug conditioning. The standard procedure for place conditioning with drugs is to pair one distinct chamber with a drug injection for one session, and pair a second chamber with vehicle in a separate session. Conditioning can be defined as training or acquisition. During training, animals are repeatedly and alternately exposed to either the investigative drug while confined to one compartment or vehicle to the other compartment. When the drug side and the vehicle side were determined for the individual, the mouse was placed to drug paired chamber after injecting with drug and the alternative session, the same mouse was placed to vehicle paired compartment after injecting with vehicle. Animals were trained off 10 days of acquisition period to develop place preference. Proper training was required to start testing the animals. Everyday before start the experiments, animals health and behavior parameter was observed and noted down. Only healthy animals with proper percent of weight gain or lose, no injury, no infection, no hyper activity animals were injected with drug or vehicle in this experiments.

CPP expression. In this stage, animals were undergo testing to get place preference score. There is no injection in this CPP expression stage. Mice were allowed to access any of the compartment in the apparatus.

CPP extinction. After CPP training and expression, the conditioned mice were repetadely exposed to all CPP apparatus freely in absence of drug or vehicle. This extinction placed 3 days, for this reason, animales may losse of place preference.

CPP reinstatement. It can be indused by re-exposure to the drug which is known as drug primed reinstatement. The forced swim and the foot-shock are the two important and widely used testing methods for stress indused reinstatement. In our study, we used forced swim technique to induce stress. A 30cm hight and 20cm diameter and build of transparents plexiglas inescapable cylindrical tank was used where mice were placed. The water temperature was 25-28C and the water level was 5-20 cm. One mouse was placed inthis cylinder for force swim for 6 minutes and then was dried and placed in CPPchember for 30 minutes. The anti-stress, anti-addiction, anti-anxiety effect of drugs was tested on this stressed mice.

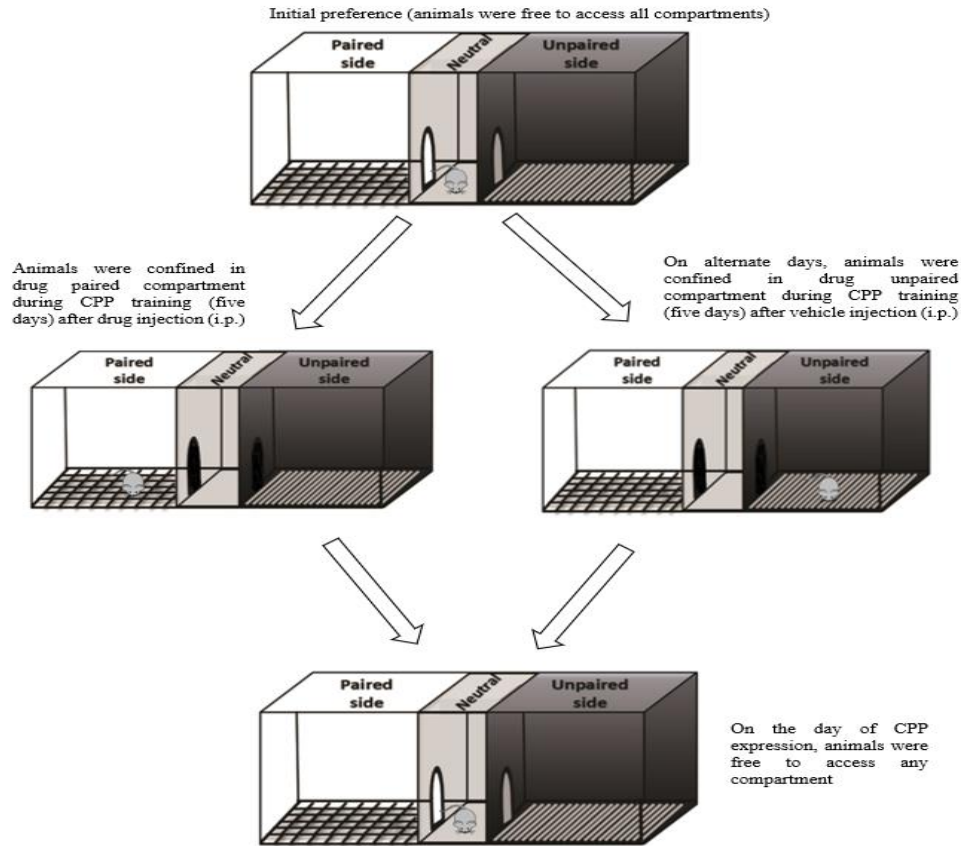


Figure 11. General schematic presentation of CPP procedure adapted from Fernandes, M.F.A. and S. Fulton, 2016 (Fernandes & Fulton, 2016).

In this CPP study, place preference is explored at various dosages of ethanol (1.2g/kg, 1.6g/kg, 2.0 g/kg). Mice were trained on alternating days with either ethanol or saline vehicle, with each being paired to a compartment. 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.

After establishing the effects of ethanol in CPP testing, the therapeutic potential of novel medications on ethanol abuse, including the CB1 negative allosteric modulator, PSNCBAM-1, and dopamine D4 antagonist L-745,870 were studied. In the PSNCBAM-1 study, the mice were subjected to pre-injection 10 mg/kg PSNCBAM-1, 30 mg/kg

PSNCBAM-1, or the vehicle and post-injection of either 2.0 g/kg ethanol or saline and 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. 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. 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.

Chapter 3

The CB1 Negative Allosteric Modulator PSNCBAM-1 has a General Anhedonic Effect in Mouse Models of Alcohol Addiction

Abstract

Previous research has determined that substance use disorders could be treated by attenuating the signaling of the cannabinoid receptor type 1 (CB1). However, the clinically used CB1 antagonist/inverse agonist rimonabant produced profound adverse effects, including anhedonia and suicidality. Current CB1 drug development focuses more on CB1 negative allosteric modulators (NAMs) such as, PSNCBAM-1. These NAMs are thought to be quite promising in the sense that different pathway of action might be provided for restricting CB1 signaling along with diminished adverse effects. PSNCBAM-1 has never before been assessed in AUD models. This study evaluated the effects of CB1 NAM PSNCBAM-1 (10, 18, and 30 mg/kg, i.p.) in adult male mice. PSNCBAM-1 did not significantly interfere with the locomotor activity. Oral ethanol self-administration was suppressed by PSNCBAM-1 in a dose-dependent manner, where ethanol rewards were significantly decreased at 30 mg/kg dose, but not at the 10 or 18 mg/kg doses. Self-administration of palatable food (diluted vanilla Ensure) was also reduced by 18 and 30 mg/kg PSNCBAM-1. Taken together, these results indicate that PSNCBAM-1 induced a non-specific anhedonic effect. This may hinder its possible use in AUD treatment.

The Cannabinoid Receptor Type 1 (CB1) as a Target for AUD Treatment

Treatment of various complexities like pain, obesity, addiction, inflammation, etc. are focused on cannabinoid signaling, making it an important medicinal compound (Ye, Cao, Wang, & Zhou, 2019). The discovery of Δ^9 -tetrahydrocannabinol is a landmark incident in the pharmacological research of cannabinoids, principally known for its effect

on the brain via cannabinoid receptors(Horswill et al., 2007). The unique endocannabinoid system in the vertebral CNS and PNS mainly includes the endogenous cannabinoids, its receptors and the enzymes which are responsible for destroying and synthesizing endocannabinoids (Mackie, 2008).

Although some evidence exists in favor of the existence of a cannabinoid “CB3” receptor, the two major subtypes are CB1 and CB2. CB1 and CB2 belong to the superfamily of G-protein-coupled receptors (Horswill et al., 2007). Subtype CB1 is primarily expressed in the brain, and found in peripheral tissues, including testis and adipose tissue. In contrast, the CB2 subtype is unique to the immune cells for immune response (Horswill et al., 2007). The cannabinoid receptor subtypes, not being homogenously scattered in the brain, remain densest in territories where cannabinoids exert effects on cognition, short-term memory preservation, motor function and movement (Pertwee, 1997). Therefore, many other psychoactive outcomes of cannabinoids are mediated by these receptors. In addition, CB2 receptor is known for its more specific and targeted distribution, residing in brain neurons other than its existence in few immune cells (Mackie, 2008). Beside its presence in the peripheral tissues and immune cells, CB2 receptors are also found to be present in nearly all hematopoietic cells including natural killer cells, lymphocytes, neutrophils, macrophages, etc. (Kumawat & Kaur, 2019).

Sometimes they are found in kidneys and pancreas as well. Natural psychotropic reflex in the brain is disrupted in the brain when CB2 receptors are absent. Not only this, but many other pathophysiological circumstances are also vastly regulated by CB2 receptors. For instance, progression and management of a number of diseases, including atherosclerosis, cardiovascular abnormalities, diabetes, cancer, etc. are conducted by CB2

receptors (Kumawat & Kaur, 2019). As CB2 is a highly clinically studied receptor, evidences suggest that CB2 receptor agonists are capable of effectively suppressing neuropathic pain and inflammation (Kumawat & Kaur, 2019). Since both receptors are known to associate with inhibitory G proteins and consequently fall into similar pharmacological effects, hence, some important events such as, partial agonism, inverse agonism or functional selectivity have determinant role in the cellular response of certain cannabinoid receptor ligands (Mackie, 2008).

In recent times, allosteric binding site, that is quite specific from the orthosteric site, has emerged as an effective alternative mechanism for the management of G-protein coupled receptors (GPCRs) (German et al., 2014). Essentially, allosteric modulators can be characterized as ligands which tie up to these sites to modify the receptor signaling characteristics of the orthosteric ligand, and altering the functional efficacy, potency, and ligand affinity. Allosteric modulators can be chosen over traditional orthosteric drugs, firstly because of the fact that subtype selectivity is relatively greater for the allosteric modulators (German et al., 2014). This higher subtype selectivity is related to the higher sequence diversity at extracellular allosteric binding sites, in comparison with the traditional orthosteric domains for specific GPCR subtypes. Secondly, we have greater selectivity of tissues for allosteric modulators, exerting effects only where endogenous ligands are present. The third and final reason behind this is, saturable effect of the allosteric modulators, as they are dependent on endogenous ligands for signaling (German et al., 2014). Recently, a number of allosteric ligands that are specific for CB1 receptor have been reported (German et al., 2014). Compound PSNCBAM-1 and Org27569, -

27759, -29647 are extensively studied among them, though there remains complexity regarding the pharmacological index of these allosteric modulators (German et al., 2014).

There are two types of allosteric modulators; one is positive allosteric modulators or PAMs, another is negative allosteric modulators or NAMs (Bertini et al., 2017). This classification is based on the type of modulation in accordance with the affinity and efficacy of the orthosteric agonists. A number of compounds are already established as CB1 receptor allosteric modulators, for example, NAM Org27569 (an indole derivative), NAM PSNCBAM-1 (a urea derivative), PAMs RTI-37133, and ZCZ01134 (Bertini et al., 2017). During the year of 2007, high throughput screening of a small library led to discovery of PSNCBAM-1. The pharmacological index of this compound is quite like Org27569. In an in vivo study of acute feeding model, PSNCBAM-1 demonstrated reduced food consumption and body weight in an in-vivo acute feeding model (Bertini et al., 2017).

One of the similar pharmacological attributes of PSNCBAM-1 with Org27569 is both of the allosteric modulators enhance [³H]CP55,490 binding levels, which is positive allosteric modulation, but inhibit agonist-mediated responses in functional assays, which is negative allosteric modulation (Nguyen et al., 2018). The positive allosteric modulator-antagonists (PAM-antagonists) systematically enhance the agonist affinity for the receptor but at the same time reduce the co-bound agonist functional efficacy (Nguyen et al., 2018).

Some other reasons for which researchers prefer to target allosteric binding sites include spatiotemporal control, specificity of mechanistic pathways, selectivity of subtypes, and more importantly a ceiling effect is attainable which helps to manage the risk of overdosing (Dopart, Immadi, Lu, & Kendall, 2020). The characteristic property of PSNCBAM-1 demonstrated that it promotes an active CB1 conformation, where it actively

assists the binding of CB1 agonist CP55,940, at the same time suppressing the inverse agonist SR141716A binding. However, it was observed that PSNCBAM-1 showed noncompetitive, inhibitory outcomes in GTP γ S and cAMP assays (Dopart et al., 2020).

Inhibition of the Cannabinoid Receptor Type 1 (CB1)

Rimonabant (SR141716) is amongst one of the prominent CB1 receptor antagonists or inverse agonists which was formerly accepted in Europe for the management of obesity (Nguyen et al., 2018). In addition to this, significant clinical benefits were observed via using CB1 receptor antagonists in abrogating drug seeking tendency in animals and ceasing the craving for smoking (Nguyen et al., 2018). Based on these outcomes, it can be rationalized that CB1 antagonism may pave the way for the treatment of drug addiction. Rinaldi-Carmona first synthesized Rimonabant and is the very first selective, potent, and orally active antagonist of the cannabinoid receptor (Soyka et al., 2008). It is evident from in vitro and in vivo assays that rimonabant demonstrates antagonistic property in behavioral and pharmacological aspects mediated through cannabinoid receptor agonists and as a result reduces voluntary alcohol intake in animal studies (Soyka et al., 2008).

Along with Europe, the drug has already been approved in The Africa and Middle East for the management of obesity (Soyka et al., 2008). As far as the role of rimonabant in the treatment of addiction is concerned, it dose-dependently decreased alcohol consumption in the alcohol-preferring rodents (Soyka et al., 2008). It is observed in clinical study, rimonabant pretreatment abrogated the up rise of alcohol intake after CB1 agonist treatment. Of late, it was found that rimonabant significantly decreased reward-related responses in animal studies. But rimonabant has not attained approval in the US till date

(Soyka et al., 2008). Safety profiling and side effects evaluation is very important for any prospective drug like compound. So far, quite a few studies have been conducted on the safety and side effects of rimonabant where depression and anxiety are found to be the most common side effects (Després, Golay, & Sjöström, 2005). In some other studies there were presence of cases like visible irritability, stress, insomnia, and sudden panic attacks (Buechler, 2020). A meta-analysis was run by Christensen *et al.* on four of the Rimonabant in obesity Studies, which revealed that patients who took 20 mg dose of rimonabant, have shown to cease the treatment 2.5 to 3.0 times more as symptoms of anxiety and depression persisted (Buechler, 2020).

Due to presence of abundantly devastating side effects, rimonabant was immediately withdrawn from the market. Thus, allosteric modulators are in need of an alternative that can target the CB1 receptor signaling pathway for therapeutic outcome. That's where PSNCBAM-1 came into prominence which is one of the earliest CB1 receptor allosteric modulators (Nguyen et al., 2018). 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 is given below in figure 12.

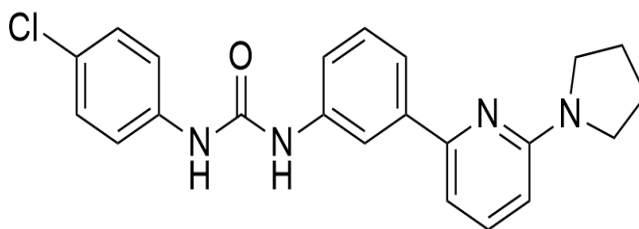


Figure 12. Structure of PSNCBAM-1, the cannabinoid type 1 negative allosteric modulator was obtained from Sigma Aldrich in the US.

Results

Open-field locomotor test. Ascertaining the safety profile of any drug is very important before commencement of further clinical studies. Initial control studies were conducted for PSNCBAM-1 to observe whether the drug reduced any locomotor function in mice. The initial control studies were performed in the open field method. To allow the mice to experience the open field chamber unhindered, experimental mice were put into the open field chamber for a period of initial 20 minutes. Following initial 20 minutes, each mouse received an i.p. injection of either 10 or 30 mg/kg PSNCBAM-1, or the vehicle mixture and then put into the open field chamber for another 40 min period. The mice behavior being recorded using a camera, both doses of 10 and 30 mg/kg PSNCBAM-1, i.p. did not significantly suppress the locomotor activity (Figure 13).

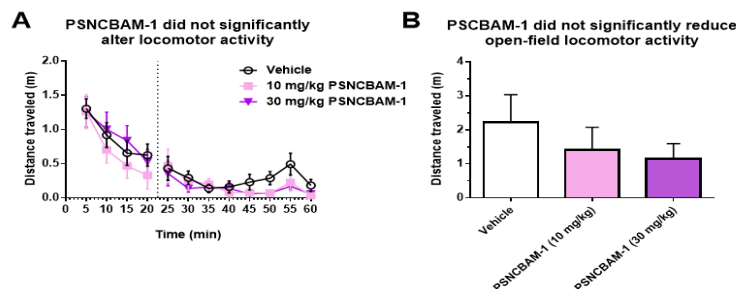


Figure 13. Neither of the doses of 10, 18 or 30 mg/kg PSNCBAM-1 significantly impacted locomotor activity in the open field method test. Male mice were put in the open field chamber for initial 20 minutes, behavior was recorded and then they were treated with i.p. injections of vehicle (n = 16), 10 mg/kg PSNCBAM-1 (n = 8), or 30 mg/kg PSNCBAM-1 (n = 8), finally, behavior was recorded for additional 40 minutes. (A) 20 minutes after introduction into the open field chamber, mice were injected 10 or 30 mg/kg PSNCBAM-1 or vehicle alone and locomotor activity was assessed for additional 40 minutes. (B) Overall post-injection distance traveled by mice was not found significantly different throughout the experiment. Data is demonstrated as means \pm SEM of distance traveled by mice in 5-minute bins.

Conditioned place preference. Prior to standard side training, conditioned place preference (CPP) training was modified for 2.0 g/kg ethanol to provide a PSNCBAM-1 or vehicle mixture pretreatment. As a pretreatment to 2.0 g/kg ethanol or saline vehicle dose, varieties of doses like 10, 18, and 30 mg/kg and a vehicle mixture as i.p. injections were assessed, where the PSNCBAM-1 pretreatment did not affect the rewarding value of ethanol (Figure 14). Statistical one-way ANOVA analysis did not show any significant effect of PSNCBAM-1 pretreatment ($F(2,26) = 0.3469, p > 0.7$).

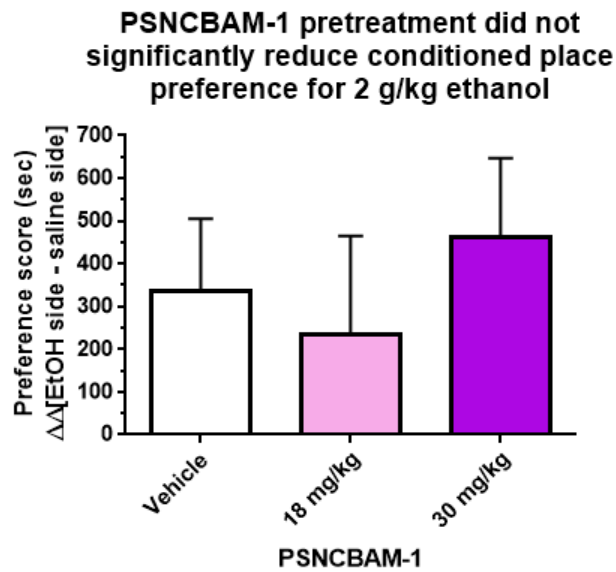


Figure 14. CPP training was modified to include PSNCBAM-1 or vehicle pretreatment prior to 2.0 g/kg ethanol. PSNCBAM-1 did not significantly decrease place preference for 2.0 g/kg ethanol. Vehicle pretreatment ($n = 9$) was not significantly different from 18 mg/kg ($n = 10$) or 30 mg/kg ($n = 10$) PSNCBAM-1 pretreatment and did not affect acquisition of ethanol conditioned place preference. All results are presented as means \pm SEM.

Ethanol self-administration. To satisfy one of the main objectives of this study, the effects of PSNCBAM-1 on ethanol self-administration were measured. Mice were trained to self-administer 8% w/v ethanol in water 10, 18, and 30 mg/kg PSNCBAM-1 and vehicle (10% DMSO, 10% Tween 80, and 80% saline) were tested using a Latin square design. Mice were injected PSNCBAM-1 or vehicle i.p. immediately prior to a two-hour ethanol self-administration session. PSNCBAM-1 exhibited dose-dependent attenuation of ethanol self-administration via decreasing ethanol rewards at 30 mg/kg dose (Figure 15).

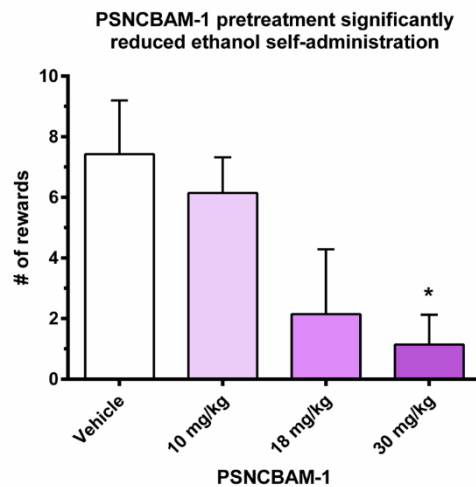


Figure 15. Pretreatment with PSNCBAM-1 significantly decreased oral ethanol self-administration. 8% w/v ethanol in water was modified to provide PSNCBAM-1 or vehicle pretreatment in the self-administration training just before the standard side training. Pretreatment with 30 mg/kg (n = 8) PSNCBAM-1 significantly attenuated oral ethanol self-administration, but 10 and 18 mg/kg (n = 8) doses of PSNCBAM-1 did not. All results are presented as means ± SEM; * p < 0.05 in comparison with vehicle pre-treatment.

Palatable food self-administration. Mice were trained to self-administer a mixture of 50% Ensure: 50% water for the food self-administration and a Latin square design was used to test two doses, 10 and 30 mg/kg of PSNCBAM-1 and vehicle (10% DMSO, 10% Tween 80 and 80% saline). An intermediate dose of 18 mg/kg PSNCBAM-1 was given in the second round of testing. On test days, i.p. injections of PSNCBAM-1 or vehicle were given just before a 2-hour self-administration session. The test revealed that palatable food self-administration was dose-dependently attenuated by PSNCBAM-1 by decreasing food rewards at 18 and 30 mg/kg doses. Statistical one-way ANOVA analysis showed a significant effect of PSNCBAM-1 treatment ($F(3,18) = 4.264, p = 0.0193$), where the pre-planned Bonferroni tests showed a significant difference between vehicle and 30 mg/kg PSNCBAM-1 treatment ($t = 3.016, p < 0.05$).

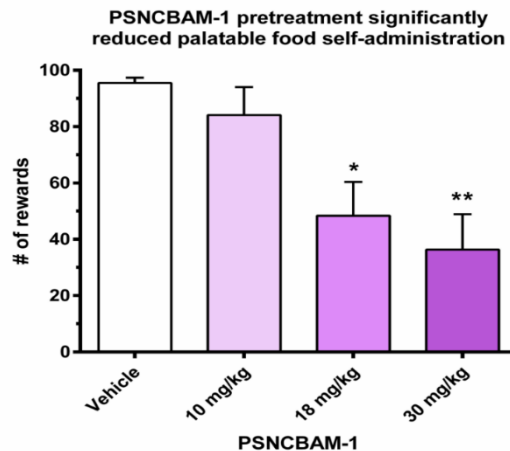


Figure 16. Pretreatment with PSNCBAM-1 significantly decreased palatable food self-administration. 50% Ensure:50% water was modified for self-administration training to provide PSNCBAM-1 or vehicle pretreatment just before standard side training. Doses of 18 and 30 mg/kg ($n = 8$) PSNCBAM-1 significantly attenuated palatable food self-administration, but the dose of 10 mg/kg did not. All results are presented as means \pm SEM; * $p < 0.05$ in comparison with vehicle pre-treatment. ** $p < 0.01$ in comparison with vehicle pre-treatment.

Discussion

In open field tests, PSNCBAM-1 did not significantly affect locomotor activity. This was conducted to determine PSNCBAM-1 doses tested did not produce general behavioral disruption or did not significantly disrupt locomotor activity in the open field initial study. PSNCBAM-1 pretreatment did not significantly influence the acquisition of place preference to 2.0 g/kg ethanol, suggesting that it did not cause motivational changes for alcohol as it did not suppress the preference behavior induced by ethanol. PSNCBAM-1 also dose-dependently reduced both palatable food and ethanol self-administration. These results suggest that PSNCBAM-1 produces a non-specific anhedonic effect that inhibits both food and ethanol self-administration. Prior to these experiments, most studies involved in investigating the drug PSNCBAM-1 have attempted mainly to determine its effect on obesity-related disorders (Dopart et al., 2020). A study investigating the hypophagic effects of PSNCBAM-1 in rats concluded that, in an acute rat feeding model, PSNCBAM-1 significantly reduced food intake as well as body weight (Horswill et al., 2007). Another study done by Wong Kai in a lab out of Oxford University, studied the specific hypophagic effect of PSNCBAM-1 on rats. (Horswill et al., 2007). One study reported that PSNCBAM-1 has abolished the reinstatement of extinguished cocaine-seeking behavior in rats (Nguyen et al., 2017).

Though the effects of PSNCBAM-1 on obesity are well documented, studies on addiction are not well mentioned yet, precluding its potential use in AUD or other neuropsychiatric disorders. It is a demand of analytical understanding that why in our laboratory tests PSNCBAM-1 exerted an anhedonic effect both for food and ethanol. With respect to our results and other reports, PSNCBAM-1 could exert this non-specific

anhedonic effect in most conditions, excluding the issue of what condition or disorder is being assessed here. To justify whether this is true or not, more research work needs to be conducted on this drug in other areas as well. The side effect of reduced food intake of the drug PSNCBAM-1 can be contrasted against the potential success of being a drug for the treatment of a substance use disorder. If PSNCBAM-1 is found successful as a potential treatment option for another disease or disorder, it needs to be ensured that both patient and medical professional are quite aware of the consequence of appetite suppression exerted by this drug. Yet there are a high number of medications with the side effect of appetite loss and not limited to high strength medications and attention deficient hyperactivity disorders (ADHD) (Bristow et al., 1997). There are several important limitations of this study. The sex of experimental animal can influence the test results; we only used C57BL/6 male mice and conducted all of our tests only in the light cycle. A study performed by Dudek and co-workers stated that female mice have greater sensitivity to the locomotor-activating action of ethanol than males (Dudek, Phillips, & Hahn, 1991). Other studies indicate that female mice have higher consumption rate of ethanol than males (Schramm-Sapyta et al., 2014). Though some reports have denied the effect of sex among adult rats on ethanol consumption. (van Haaren & Anderson, 1994). Additionally, oral self-administration of alcohol can result in different blood alcohol concentrations based on each animal's size, tolerance, and alcohol consumption from the receptacle that was given after successful nose poking.

Conclusion

In this study, the effect of PSNCBAM-1 in AUD using mice model is assessed. It was found that locomotor activity was not significantly suppressed by PSNCBAM-1.

PSNCBAM-1 pretreatment did not significantly influence the rewarding value of 2.0 g/kg ethanol in the condition place preference experiment. Ethanol self-administration was dose dependently attenuated by PSNCBAM-1 in ethanol self-administration experiment, decreasing ethanol rewards at only 30 mg/kg dose. Palatable food self-administration was dose-dependently attenuated by PSNCBAM-1 in the food self-administration experiments, decreasing food rewards at 18 and 30 mg/kg doses. More research in this arena, particularly on alcohol addiction, cannabinoid receptors, and their role and mechanism in addiction will provide deeper insight in discovering better treatment strategy for AUD.

Chapter 4

The Dopamine D4 Antagonist L-745,870 Does Not Affect Locomotor Activity in Adult Male Mice Model of Alcohol Addiction

Abstract

The dopamine D4 receptor (D4R) receptor has been the center of profound interest in the study of several addictions, i.e., cocaine addiction and a popular target in the development of drugs for psychostimulant addiction but has not been tested for AUDs till today. During this study, the effects of L-745,870, a D4R antagonist were assessed for AUD in rodent models, using adult male mice. L-745,870 was investigated for initial control studies with 1.5 and 3.0 mg/kg, i.p. doses which was found to exert no significant disruption on locomotor activity. Tests were conducted with diluted vanilla Ensure and ethanol, where it was found that palatable food and ethanol self-administration (8% w/v ethanol in water) was not significantly attenuated as well. Conditioned place preference training was conducted using a three-compartment chambered conditioned place preference apparatus with L-745,870 pretreatment that did not hamper the rewarding value of 2.0 g/kg ethanol. Taken together, all these results signify that D4R antagonism does not significantly alter the rewarding value of ethanol.

The Dopamine D4 Receptor: an AUD Target?

Considerable amount of interest has been given to the dopaminergic system because of its pivotal role in regulating the central nervous system (CNS), motor functions, cognition, reward and endocrine action (Oak, Oldenhof, & Van Tol, 2000). Dopamine receptors are categorized within the G protein-coupled receptors super-family and is involved in mediating diseases like Parkinson's and addiction. These receptors are the

focus when it comes to studying antipsychotic medications in treating schizophrenia. Five distinct dopamine receptor subtypes are found in mammalian species in molecular cloning studies (Oak et al., 2000).

Depending on their amino-acid sequence homology and adenylyl cyclase activity modulation, dopamine receptors are broadly divided into two groups: D1-like (D1 and D5) and D2-like (D2, D3, and D4) (Siegel, Miller, & Jemal, 2020). The capacity of dopamine binding at the time of evolution of biogenic amine receptors have helped to lay down the origin of these subfamilies (Di Ciano, Grandy, & Le Foll, 2014). Specific downstream signaling pathways are activated when the D1- and D2-like subfamilies are attached with different G proteins, such as DRD1 and DRD5 are present in postsynaptic sites of dopamine-receptive neurons (Di Ciano et al., 2014). Reward and movement regulation pathways in the brain are highly regulated by dopamine and it is produced in the ventral tegmental area and in nerve cell bodies via the reward pathway (Juárez Olguín, Calderon Guzman, Hernandez Garcia, & Barragán Mejía, 2016). Dopamine D4 receptor has structural and pharmacological similarity with dopamine D2 receptor which gave it considerable level of attention in the management of psychiatric disorders (Patel et al., 1997).

L-745,870

The IUPAC name of L-745,870 is 3-([4-(4-chlorophenyl) piperazin-1-yl] methyl)-1H-pyrrolo [2, 3-b] pyridine. L-745,870 (Tocris Bioscience, Ellisville, Missouri, US) is evaluated in this study which is a high-affinity, selective dopamine D4 receptor antagonist via using rodent behavioral models to predict its antipsychotic nature and probable side-effects in humans (Bristow et al., 1997). Six years after the discovery of dopamine D4

receptor, the selective and CNS-penetrant dopamine D4 antagonist, L-745,870, was discovered by researchers from Merck. Preclinical and clinical efficacy studies on L-745,870 were published in 1997 (Patel et al., 1997).

Different studies have been conducted using this particular drug, L-745,870. A group of researchers reported that rats were trained to discriminate amphetamine from saline and L-745,870 partially abolished the discriminative stimulus effect. (Marona-Lewicka & Nichols, 2011). Another study demonstrated that alcohol-dependent male rats have reduced ability to full-fill a number of emotional-learning tasks during their abstinence period, but after applying the dopamine replacement agent, levodopa rats recovered rapidly.(Chouhan et al., 2020). L-745,870 was found to have no beneficial effect on nicotine self-administration according to another report, however, it did suppress both cue- and nicotine-induced reinstatement of nicotine-seeking behavior in rats. (Yan et al., 2012). Additionally, although there are a number of evidences which signify that a selective D4 receptor antagonist might be a fruitful antipsychotic agent with a lower tendency to facilitate extrapyramidal side-effects, L-745,870 was found ineffective as an antipsychotic agent in humans. (Bristow et al., 1997). L-745,870 has also been studied as a possible treatment option for schizophrenia with a view to ensuring an aim that it would not show the extrapyramidal side effects that are often seen when using classical antipsychotic agents. (Zhang et al., 2000).

L-745,870 was formerly studied in our lab and was found to attenuate cocaine CPP. Based on previously study derived outcome on alcohol and other substance addiction, it can be hypothesized that L-745,870 will decrease alcohol seeking behavior and overall alcohol consumption in adult male mice. With a view to justifying this hypothesis, we

assessed L745,870 effects in ethanol CPP and self-administration studies and this drug was never assessed in alcohol addiction models.

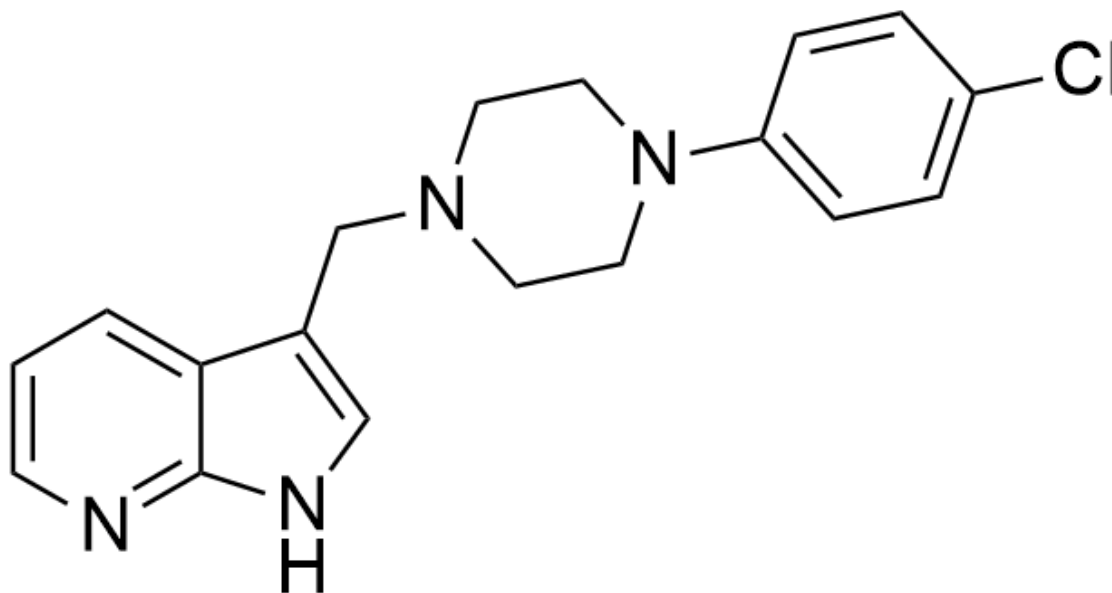


Figure 17. The Chemical Structure of L-745,870, a dopamine D4 receptor antagonist.

Results

Initial control studies. Initial control studies were conducted to ascertain the safety parameters for L-745,870 to observe any considerable change in locomotor activity using open field-testing method and coordination function using rotarod testing method in mice. To allow the mice to experience the open field chamber unhindered, experimental mice were put into the open field chamber for a period of initial 20 minutes. Following initial 20 minutes, each mouse received an i.p. injection of either 3.0 mg/kg L-745,870 or saline vehicle. Mice were taken into the open field chamber for the next 40 minutes to record the behaviors of the mice using a camera attached with the open field chamber. 3.0 mg/kg, i.p

dose of L-745,870 suggested that, mice locomotor activity was not significantly disrupted during the open field initial control study. No mentionable effect of the treatment was found after an unpaired t-test of total post-injection locomotor activity ($t(20) = 0.073, p > 0.9$).

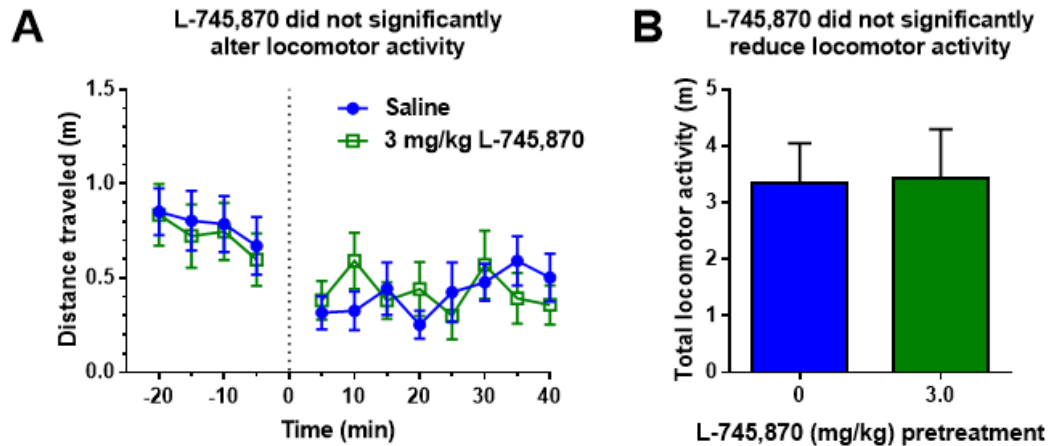


Figure 18. Mice locomotor activity was not significantly affected with 3 mg/kg dose of L-745,870 in the open field test using male mice. Open field apparatus was used to assess behavioral aspects for initial 20 minutes. Mice were treated with i.p. injections of saline or 3 mg/kg L-745,870 ($n = 11$) followed by recording of mice behavior for next 40 minutes. (A) after remaining for 20 minutes into the open field, mice were given 3 mg/kg L-745,870 or vehicle dose, followed by recording of locomotor activity for the next 40 minutes. All data are presented as means \pm SEM of distance traveled by mice in 5-minute bins. (B) Post-injection distance traveled by mice does not vary much across the treatment. All data are presented as means \pm SEM of total distance traveled by mice in the 40 minutes.

To conduct rotarod study, mice were put onto the black rotating rod where the rod speed went up from 4 rpm to 40 rpm. Limited five minutes was allocated to conduct this test or until the mice fell from the rod—whichever first occurred. Testing was carried out for 20 minutes prior to an i.p. injection of either 3.0 mg/kg L-745,870 or saline vehicle solution and for 60 minutes after introducing injection. Testing was conducted in ten-

minute increments. Coordination function of mice in the rotarod study was not significantly disrupted in the initial control study using 3.0 mg/kg, i.p. dose of L-745,870.

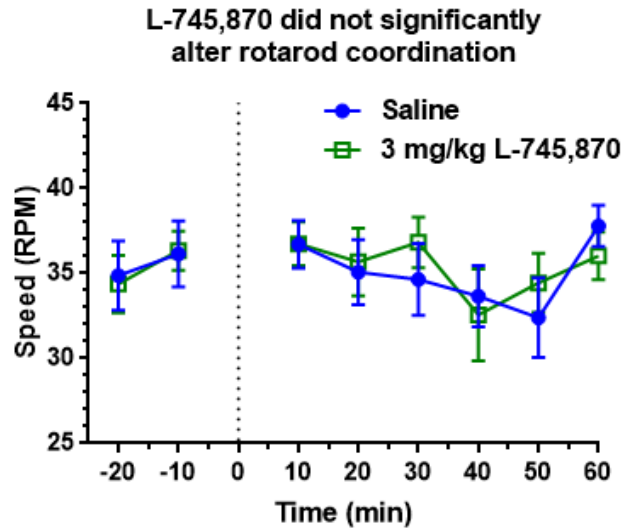


Figure 19. Coordination function is not significantly influenced after providing 3 mg/kg doses of L-745,870 in a rotarod test using male mice. At 10-minute increments, mice were put onto a rotarod apparatus for 20 minutes in total and the speed and time at which mice fell down from the apparatus was carefully recorded. Then i.p. injections were introduced for injecting saline vehicle or 3 mg/kg L-745,870 ($n = 11$) and time and speed recording was continued for rest of the 60 minutes at 10-minute increments. All data are presented as means \pm SEM of time spent and at what speed the mice fell down from the rotarod apparatus in 10-minute bins.

Conditioned place preference. Prior to standard side training, CPP was modified for 2.0 g/kg ethanol to provide a L-745,870 or saline vehicle pretreatment. After that, two different doses, 1.5 and 3.0 mg/kg along with a saline vehicle dose of i.p. injections were given as a pretreatment prior to giving a 2.0 g/kg dose of ethanol or saline vehicle solution. Our test result demonstrated that rewarding value of 2.0 g/kg ethanol is not significantly affected (Figure 20). Statistical one-way ANOVA analysis for the ethanol-paired

compartment of conditioned place preference demonstrated no significant effect of L-745,870 treatment ($F(2,51) = 2.28, p = 0.11$).

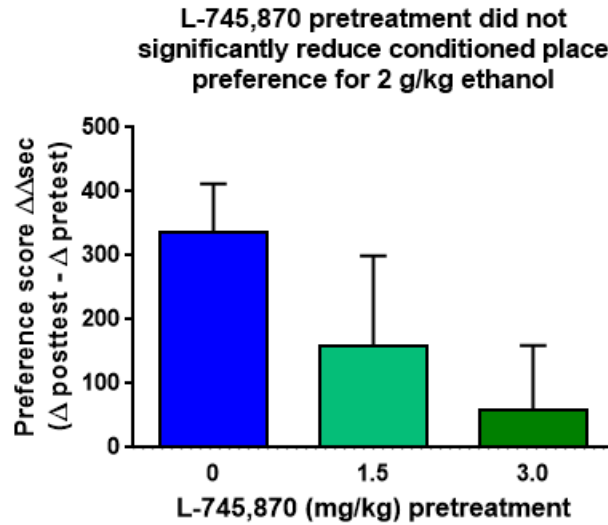


Figure 20. L-745,870 pretreatment did not significantly decrease the CPP for the dose of 2.0 g/kg ethanol which was modified prior to standard side training to include L-745,870 or vehicle pretreatment. Neither of the doses, 1.5 mg/kg ($n = 13$) or 3.0 mg/kg ($n = 16$) L-745,870 significantly suppressed ethanol CPP when it is compared to vehicle ($n = 25$). All results are presented as means \pm SEM.

Food and ethanol self-administration. To satisfy one of the main objectives of this study, which is determining ethanol self-administration behavior of L-745,870, two tests were performed using the self-administration operant chamber that included food and ethanol self-administration. In case of ethanol self-administration, the trained mice self-administered 8% w/v ethanol in water and then tested with two doses of 1.5 and 3.0 mg/kg of L-745,870 along with saline vehicle in a Latin square design. Mice were injected L-745,870 or vehicle i.p. prior to two-hour self-administration session on each test day. L-745,870 did not exhibit dose-dependent attenuation of ethanol self-administration at either

dose that were tested. Statistical one-way ANOVA analysis demonstrated no significant effect of L-745,870 treatment ($F(2,14) = 0.42, p > 0.66$).

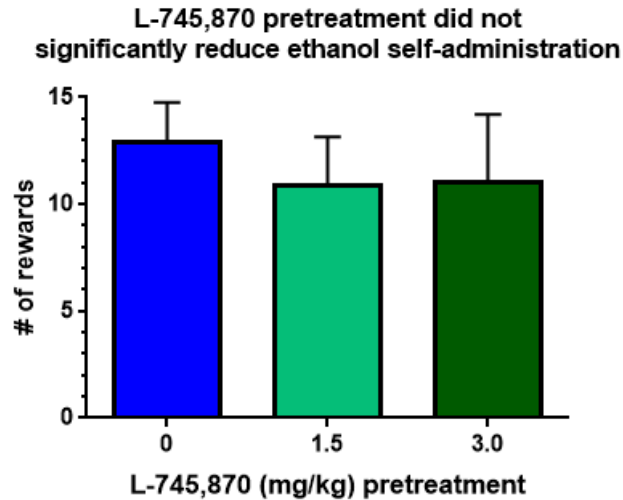


Figure 21. Pretreatment with L-745,870 does not significantly decrease oral ethanol self-administration. 8% w/v ethanol in water was modified to provide L-745,870 or vehicle pretreatment in the self-administration training just before the standard side training. Pretreatment with neither of the doses, 1.5 or 3.0 mg/kg ($n = 8$) L-745,870 significantly attenuated oral ethanol self-administration. All results are presented as means \pm SEM.

Mice were trained to self-administer a mixture of 50% Ensure: 50% water for the food self-administration and a Latin square design was used to test two doses via i.p. injection, 1.5 mg/kg and 3.0 mg/kg of the L-745,870 and along with the saline vehicle solution. On test days, i.p. injections of L-745,870 or vehicle were given just before a 2-hour self-administration session. The test revealed that palatable food self-administration was not significantly attenuated by L-745,870 at either of the doses tested (Figure 22). Statistical one-way ANOVA analysis showed no significant effect of L-745,870 treatment ($F(2,14) = 0.37, p > 0.69$).

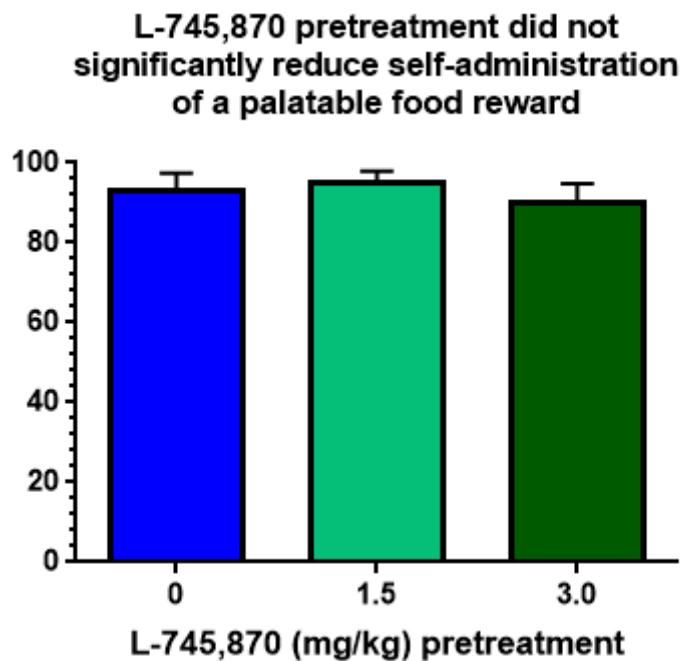


Figure 22. Pretreatment with L-745,870 does not significantly decrease palatable food self-administration. 50% Ensure:50% water was modified for self-administration training to provide L-745,870 or vehicle pretreatment just before standard side training. Neither of the doses, 1.5 or 3.0 mg/kg (n = 8), L-745,870 pretreatment significantly attenuated palatable food self-administration. All results are presented as means \pm SEM.

Discussion

L-745,870 was found promising in initial studies since the locomotor activity was not greatly affected in the open field tests or coordination function in the rotarod tests. L-745,870 with 3.0 mg/kg i.p. dose did not significantly hamper the locomotor activity in the open field initial control study and with the same dose it also did not significantly hamper the coordination function in the rotarod initial control study.

According to our current study, L-745,870 did not work as we expected in conditioned place preference and self-administration tests. L-745,870 did not significantly

influence the acquisition of place preference with 2.0 g/kg dose of ethanol at the time of conditioned place preference side-training, suggesting that L745,870 could not mediate motivational changes towards alcohol or abolish ethanol induced preference behavior. L-745,870 has been previously assessed in our laboratory settings were changed cocaine mediated behavior in mice. Comparing our experimental outcome for pretreatment of L-745,870 (3 mg/kg) and post-treatment of cocaine with the previously studied experimental outcome of pretreatment of saline with post-treatment of cocaine, L-745,870 was found to have less preference than cocaine. L-745,870 did not affect both palatable food and ethanol self-administration which helped us to conclude that L-745,870 does not influence the rewarding value of palatable food or ethanol, hence effectivity of this drug is questioned to manage ethanol self-administration behavior or AUD.

Till date, L-745,870 has been assessed in various studies where it is extensively searched for cure against various diseases. Among them, some studies are focused on alcohol self-administration study. Dr. Bernard Le Foll's lab recently worked on this drug where they tested the effect of this drug on operant alcohol self-administration and reinstatement (Siegel et al., 2020). The study added that dopamine D4 antagonists decrease ethanol consumption and stress-induced reinstatement at highest dose. The doses of L-745,870 that Le Foll's lab tested (0.5-10 mg/kg L-745,870) were at a broader range compared to the doses used in our study (1.5-3.0 mg/kg).

No scientific study is free of shortcomings. We only used male mice in our tests, though it is established that there are significant variations in mice behavior and response to drugs that is closely linked to sex. Dudek and co-workers demonstrated in one of their biochemical evaluation of locomotor activity that female mice were relatively more

sensitive to the locomotor activating effect of ethanol than male mice (Dudek et al., 1991). It is evident from most scientific studies that ethanol intake is higher in female mice than in male mice (Schramm-Sapyta et al., 2014). Though some reports have denied the effect of sex among adult rats on ethanol consumption. To become a potentially promising drug for other substance use disorder, new methods are demanded to be developed to save time and resources. Considering the promising effect of cocaine, the effectivity of L-745,870 for becoming a treatment option would depend on the abuse liability of the addictive drug. It was always under profound focus that the dopamine D4 receptors have a pivotal role in various substance use disorders. But it needs to be further investigated that whether this receptor has that much role as it is thought to have. Additionally, search needs to be conducted to investigate, if there are other receptors like the dopamine D4 that might have a bigger role in the management of addictions.

Conclusion

We performed several experiments for L-745,870, a D4 antagonist, to assess its effectivity in the management of AUD. Initial control study consisting of open field and rotarod testing, L-745,870 did not significantly hamper the locomotor or coordination function. Pretreatment with L-745,870 demonstrated that it would not affect the rewarding value ethanol with a dose of 2.0 g/kg in the CPP experiment. L-745,870 also did not significantly attenuate the ethanol and palatable food self-administration behavior and at any of the doses tested in the ethanol and palatable food self-administration experiments, respectively. The analysis that we presented here regarding our experimental results may not provide the proper and broader insight about the exact mechanistic function of addiction in the brain. With much more cutting-edge research on other substance use

disorders including AUD will put us many more steps ahead, as far as research on substance abuse is concerned. Focus also needs to be drawn into the dopamine D4 receptor and its mechanistic involvement in addiction and addictive behaviors, which may bring more knowledge and a wider understanding about the treatment of AUD and other disorders related to substance abuse.

Chapter 5

Two-Bottle Choice Preference Test

Concerns about alcohol use disorder (AUD) have risen along with the increased incidence of patients with diagnosable AUDs. Currently, more than 15 million people in the United States have a diagnosable AUD annually and nearly one-third of the US adult population will have a diagnosable AUD at some point in their life. This disorder persists in humans because of chronic dependence on alcohol (Association, 2000). The U.S. Department of Health and Human Services characterizes AUD as a medical diagnosis characterized as a chronic relapsing brain disease with uncontrollable alcohol use, and loss of self-regulation over alcohol consumption. Alcohol abuse facilitates approximately 88,000 deaths per year in the United States (K Witkiewitz et al., 2019) and costs nearly \$249 billion to the US each year (K Witkiewitz et al., 2019).

Non-pharmacological treatment options are a preferred treatment method for AUD patients. The U.S. Food and Drug Administration (FDA) has approved four medications to treat AUDs, disulfiram, acamprosate, oral naltrexone, and injectable long-acting naltrexone, that can be paired with non-pharmacological treatments. These current treatments have low success rates, indicating a need for new potential drugs. In order to identify new candidate medications for AUD, we have developed and used a number of different behavioral pharmacology techniques. In this experiment, we sought to optimize the two-bottle choice behavioral paradigm as an experimental protocol to evaluate the effects of different medications on the preference of mice to consume ethanol over water.

Background

The two-bottle choice alcohol preference test is a well-known and widely used experimental technique used in AUD research. In this experimental model, animals are provided with two bottles, one with a diluted ethanol solution and another with water or other control liquid. The effect of morphine in lower doses on alcohol and sucrose self-selection was assessed by Ronald R Ulm's lab (Stromberg, Meister, Volpicelli, & Ulm, 1997). It was observed that, when rats were provided to choose between sucrose sweetened ethanol and plain water, significant preference was observed for the sweetened ethanol. However, when rats were given to choose between sweetened ethanol and sweetened water, they showed preference for sweetened water, suggesting that the morphine treatment in lower doses enhanced the sweetened alcohol consumption that was induced by the reinforcing capacity of sucrose, but not ethanol (Stromberg et al., 1997). The lab of Rosana Camarini demonstrated using a two-bottle choice model that, ethanol pre-exposure may enhance ethanol intake in both adolescent and adult mice (Carrara-Nascimento, Hoffmann, Contó, Marcourakis, & Camarini, 2017). In another study, rats being experimented in three-day long operant self-administration of 10% sucrose + 10% ethanol where sucrose was found to be completely faded out (Carrillo et al., 2008). The ethanol self-administration was established by ethanol intake, pre- and post-procedure 2-bottle choice preference tests, and extinction trials. It was observed from this study that, mean ethanol intake was 2.2 times greater on day 2 when compared with day 1 on the 10% sucrose + 10% ethanol solution (Carrillo et al., 2008). When the sucrose faded out, the daily consumption of 10% ethanol over 5 days was observed stable where ethanol preference was nearly 3-times greater.

We sought to establish this experimental protocol as an effective tool in order to assess alcohol preference behavior in our lab, with a view to being able to test animal liquid consumption preferences, addiction-like behaviors, anhedonia, and depression-like responses. We monitored consumption of liquids from each bottle over 24 hours for several months to establish how mice preferred to intake various ethanol- and sucrose-containing aqueous solutions. Progress on this experiment was halted due to the outbreak COVID-19 pandemic and while we were unable to complete our experiment, though we were able to evaluate several experimental design parameters.

Methods

Animals. In the first portion of the experiment, we used eight adult C57 male mice weighing 23-31 g/mice. Upon arrival to the laboratory, the mice were given free access to food and water during one-week habituation period in a room that was maintained at a constant temperature of 21-23°C and humidity of 45-50% on a 12h light-dark cycle. Mice were singly housed to better monitor liquid consumption and handled daily throughout the experiment.

Solution components. In this study, we used ethanol, sucrose, and regular tap water to assess the consumption preference behaviors of mice. 95% ethanol (VWR) was diluted as noted in each experimental condition. Sucrose (Fischer Science) was weighed and added to solutions as noted in each experimental condition.

General Procedure

At the beginning of the two-bottle choice experiment, all mice were single-housed and provided with two separate plastic conical graduated bottles with rubber stoppers

containing a metal dispenser; experimental liquid was released via pressure provided by mice licking the dispenser. Each bottle contained 10-15 ml of solutions that varied with changing experimental conditions. Each bottle was replenished daily, and consumption of each liquid was assessed every day between 10 am to 12 pm. The position of each bottle was stable in some treatment conditions and in other conditions was changed at regular intervals to evaluate or eliminate any potential positional preference. Experimental data was collected by gross eye observation and weighing the bottles, recording the liquid volume and mass consumed from each bottle. Procedure used here is adapted from Carrillo, J., et al., 2008, Holgate, J.Y., et al. 2017 and Grim, T.W., et al., 2018 (Carrillo et al., 2008; Grim et al., 2018; Holgate, Garcia, Chatterjee, & Bartlett, 2017)

Two-Bottle Choice Experimental Conditions

In this experiment, we attempted to identify important variables that might affect the preference behavior of mice between alcohol and water. We used 8 cages of mice in total and recorded daily alcohol and water consumption over several different conditions.

Condition 1. We started our experiment with 2% ethanol (w/v) in one tube and regular tap water in the other tube. Our plan was to gradually increase the concentration of ethanol to 8%. But we observed that mice drank almost no liquid from either tube. In fact, they were losing weight because of dehydration. From our observations, the mice were not used to this type of bottle which might be the reason they avoided drinking any liquid. Based on this observation, we decided to change our experimental plan and gave them a sucrose solution to make them drink from the bottles.

Condition 2. Then we started giving the mice only 10% (w/v) sucrose in water in both tubes for more than one month. Bottles were filled daily with up to 10 ml of solution and consumption data were collected. The cages of mice were placed in a regular water rack to give full access to water to keep the mice hydrated. After two hours of regular water access time, the mice were placed in a separate rack with no water access other than the two bottles which were filled with the sucrose solution. In Condition 2, we witnessed the mice developed a consumption preference for one tube over the other based on its location. In Condition 3, we swapped the tubes and determined whether the preference for one side persisted.

Condition 3. After substantial sucrose habituation, we replaced one tube with a 2% ethanol while the other tube remained 10% sucrose for one week. The ethanol tube was placed on the side that held the drinking preference seen in Conditions 2 and 3.

Condition 4. Following the mixed results in Conditions 1-4, we altered our training procedure using a new set of mice who had never experienced the standard housing conditions that included one large water bottle. Over two weeks, the new set of mice received 2% EtOH and 10% sucrose together in one tube and water in the other tube. The position of the tubes was interchanged every other day to reduce any potential positional preference. The mice were placed into the rack where there was no water access for two hours other than the tubes in the cage. The data were collected daily and recorded into an excel sheet. This group of mice demonstrated a promising drinking pattern by preferring to drink EtOH and sucrose solution than water.

Results

Condition 1. In the first portion of the experiment, mice did not show any significant EtOH preference. We started our experiment with 2% ethanol on one tube and regular tap water on the other tube. We found almost no drinking of any liquid from the tube, and the mice were losing their weight because of dehydration.

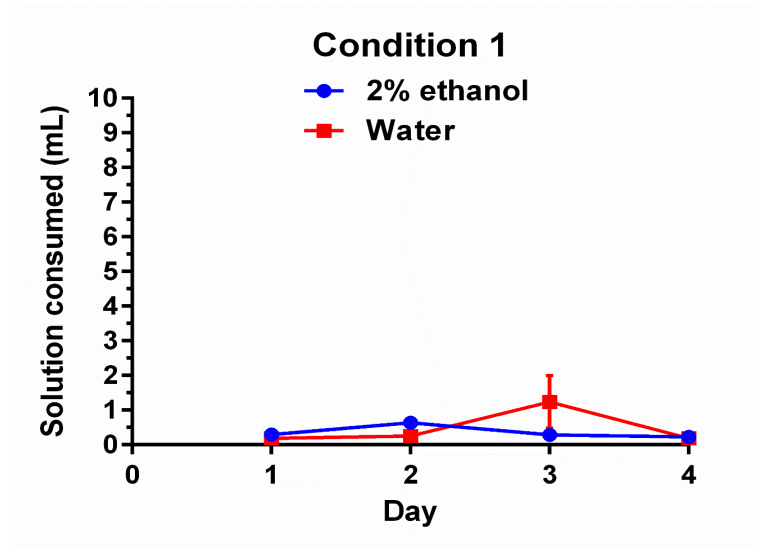


Figure 23. Eight C57 male mice were given 2% w/v ethanol in one tube and water in another tube. The graph showed that water consumption is relatively higher than 2% w/v EtOH. The mice do not have preference for 2% EtOH over water.

Condition 2. Then we changed our working protocol where we gave them 10% sucrose water on both tubes to make them comfortable with drinking from those specialized tubes for the next four weeks. During this time, they started to drink sucrose water, but the amount was not significant.

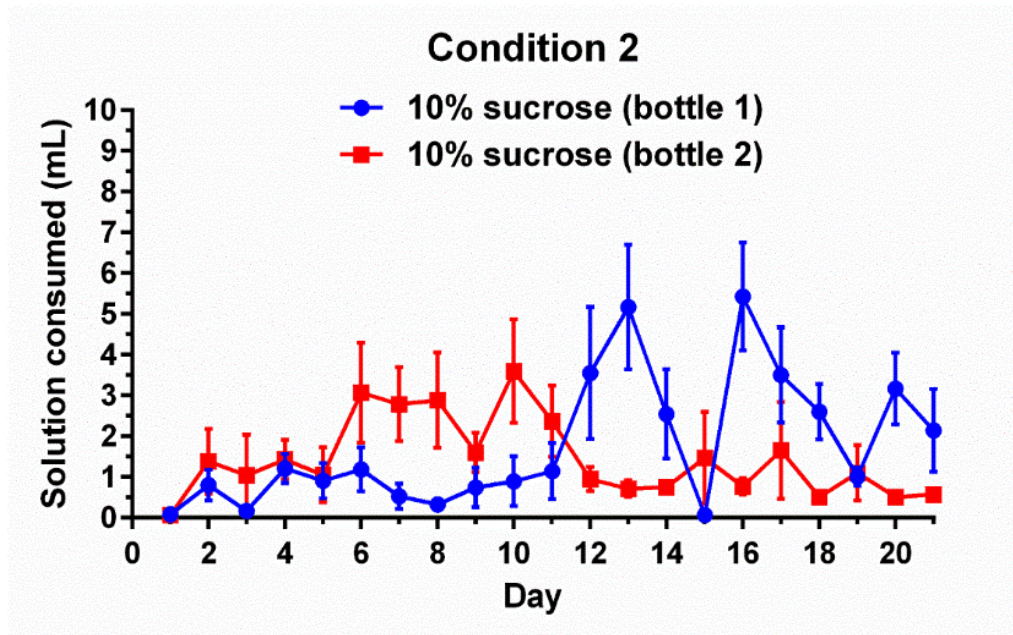


Figure 24. Eight C57 male mice were given 10% w/v sucrose solution in both bottles for one month. The mice were preferred drinking solution two over solution one for first two weeks and next two week they preferred to drink solution one over solution two.

Condition 3. After that, we started giving them ethanol on one tube and sucrose in the other via following the same experimental procedure. The ratio of sucrose and ethanol was 10% w/v sucrose with 2% w/v ethanol. Mice preferred to drink 10% sucrose over ethanol.

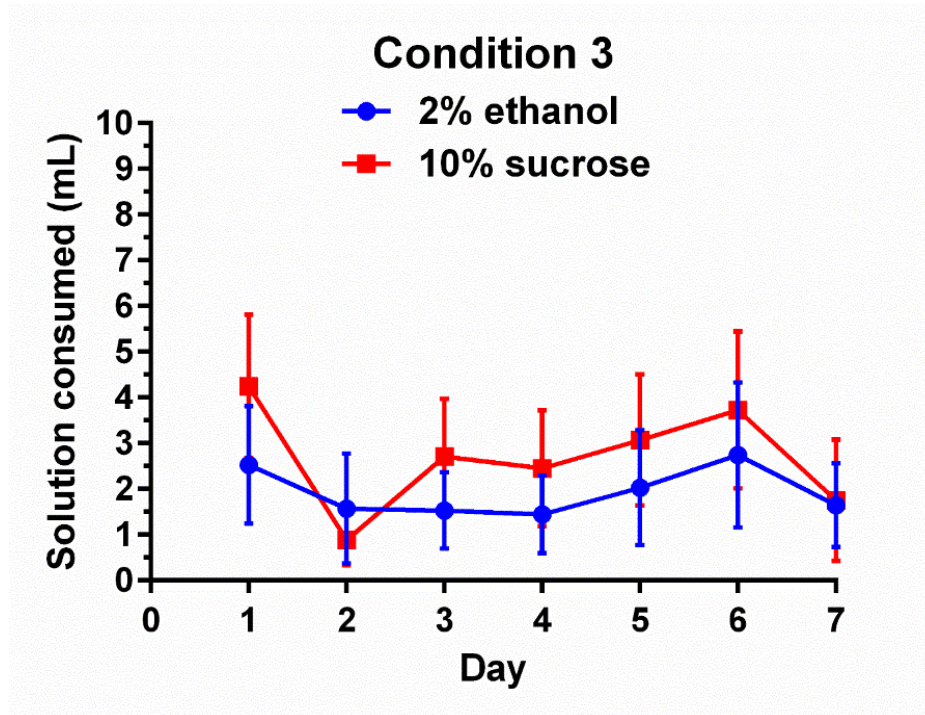


Figure 25. Same group of mice were given 2% w/v ethanol in one tube and 10% w/v sucrose in other tube. Consumption of 10% sucrose is higher than the 2% EtOH. There is no preference for EtOH over sucrose.

Condition 4. In the second part of our experiment, we got some promising data from the mice where they demonstrated a preference for EtOH and sucrose mixture solution over water. Mice were given 2% EtOH+10%Sucrose solution together in a tube and water in another tube for two weeks. Tubes were interchanged every other day. After 14 days of experiment, we found that mice were drank from solution 1 over solution 2. Mice liked to drink sweetened ethanol over water.

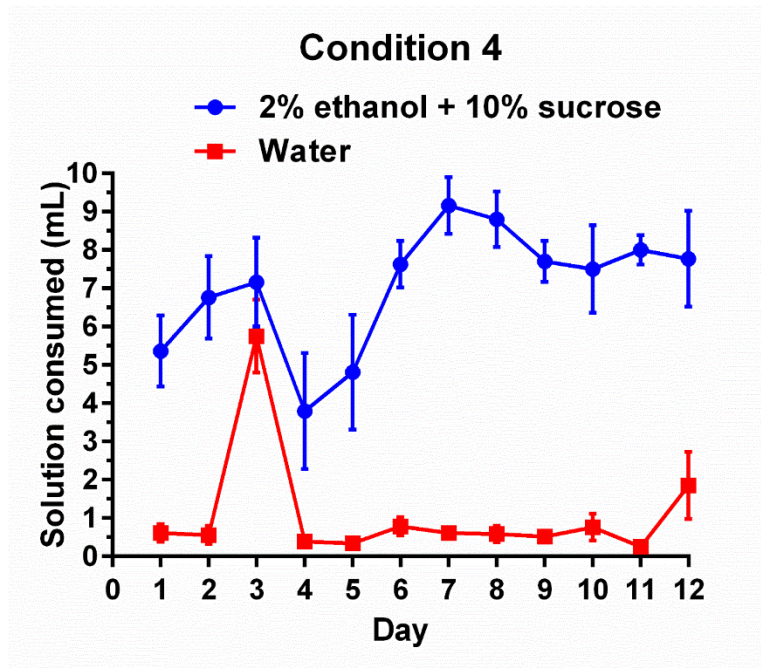


Figure 26. A group of eight C57 male mice were given 2% ethanol + 10% sucrose solution in one tube and water in other tube. Consumption of 2% ethanol + 10% sucrose solution from one tube was significantly higher than consumption of water from another tube. Mice showed clear preference for EtOH+ 10% sucrose solution over water.

Discussion

In the first portion of the experiment, we used eight adult C57 male mice. We fixed the initial housing conditions for mice which included a large water bottle on one top side of the cage for liquid consumption. Here, we observed that the mice were not interested or previously learned to drink from the specific bottle we used to give them solutions. Hence, they started to lose their body weight because of dehydration. To fix this issue, we provided them with 10% w/v sucrose solution over one month and thankfully they started drinking sucrose solution at that point.

In the second portion of our experiment, we used the same group of mice and observed no significant effect or preference for 2% w/v EtOH over water, rather they preferred 10% sucrose solution. Then we increased the concentration of EtOH and decreased the concentration of sucrose, but still there was no significant preference for EtOH. Then we decided to start with a new set of mice who had never experienced the standard housing conditions with one regular water bottle. The new set of mice received 2% EtOH and 10% sucrose together in one tube and water in the other tube where they showed clear preference for 2% EtOH and 10% sucrose mixture solution over water.

Two bottle choice preference tests have been used for different types of experiments so far, with different research interests. A study conducted by Ronald R. Ulm demonstrated that sweetened ethanol is preferable for rats than water (Stromberg et al., 1997). Selena E. Bartlett's lab has conducted a study that stated, social and environmental experiences in alcohol and sucrose consumption are critical for generating preventative actions and treatment options for AUDs and obesity (Holgate et al., 2017).

Further investigation process of our experiment was cut short due to outbreak of the COVID-19 pandemic though we had planned to test whether PSNCBAM-1 (CB1 negative allosteric modulator) or L-745,870 (dopamine D4 receptor antagonist) altered patterns of ethanol consumption in the 2-bottle choice procedure.

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