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Behavioral Effects of Novel Treatments for Pain and Alcohol Use Disorder

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BEHAVIORAL EFFECTS OF NOVEL TREATMENTS FOR PAIN AND ALCOHOL USE DISORDER

by

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Eric, there is not a day that goes by that I do not think about you. I often feel your presence in lab and wish you were doing this master's program with me because we would have made a great team. I know you would have really enjoyed this type of research. You were so passionate about pharmaceuticals and were destined for great things in this industry. I hope I am making you proud with my research efforts. Everything I have done and continue to do in the lab, I dedicate to you.

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Abstract

Christa M. Donegan
BEHAVIORAL EFFECTS OF NOVEL TREATMENTS FOR PAIN AND ALCOHOL USE DISORDER
2020-2021
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Master of Science in Pharmaceutical Sciences

Opioid use disorder (OUD) and alcohol use disorder (AUD) are pressing public health problems in the United States that require new pharmacotherapies to be explored. Current FDA-approved treatment options for these two disorders are only moderately effective. Thus, there is a demand for the identification of new targets for drug development. Previous research initiatives have shown that when morphine is co-administered with the novel imidazodiazepine, MP-III-024, synergistic effects in models of analgesia and antinociception are produced. Our research efforts were concerned with understanding if morphine in combination with MP-III-024 produced synergistic effects in measures of undesirable pharmacological responses: opioid side effects. The results of our operant self-administration tests demonstrated that MP-III-024 does not enhance morphine induced disruptions; and in models of locomotor function, our 1.0:0.94 morphine:MP-III-024 ratio demonstrated a statistically significant subadditive (anti-synergistic) effect. With these findings, we now know that morphine and MP-III-024 are not *universally* synergistic. Concerning AUD, the dopamine D4 receptor (D_4R) full antagonist, CAB-01-019, was studied through palatable food self-administration testing. Our results showed that CAB-01-019 did not significantly reduce behavioral responses at any of the three tested doses (10, 17.8, and 30 mg/kg). These palatable food self-administration tests with CAB-01-019 will serve as a critical control for future alcohol tests.

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Chapter 1

The Impact of Opioids and Alcohol on Society and the Need for New Treatment Methods for Substance Use Disorders without Addiction Risks

1.1. Part 1 – Opioids

1.1.1. Definitions

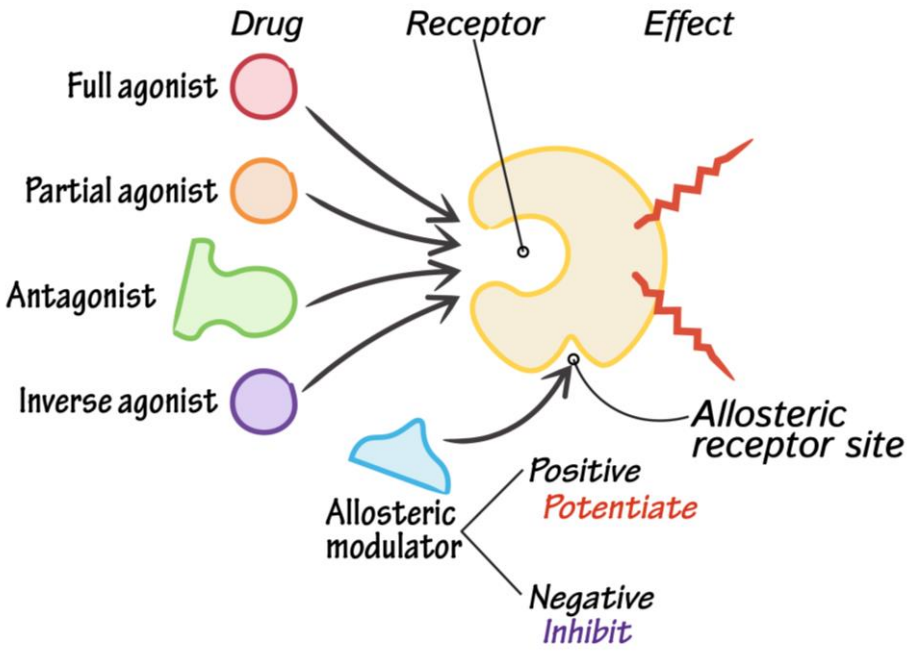
1.1.1.1. Drug Interactions.

- Additive effect: occurs when the effects of a drug combination equal the sum of the expected effects of the two drugs alone (e.g., 1 +1 = 2)[1]
- Subadditive (anti-synergistic) effect: occurs when the effects of a drug combination are less than the expected sum (e.g., 1 + 1 < 2)[2]
- Superadditive (synergistic) effect: occurs when the effects of a drug combination are greater than the expected sum (e.g., 1 + 1 > 2)[1]
- Homergic effect: refers to two drugs having the same maximal effect[3]
- Heteroergic effect: refers to one drug having an effect and the other drug lacking the effect[3]
- Agonist: a substance that binds to receptors in the brain and produces a physiological response; some agonists imitate neurotransmitters associated with pain and pleasure (e.g., nicotine and heroin)[4]
- Antagonist: a substance that binds to receptors in the brain and interferes with or blocks certain chemical reactions (e.g., naloxone and naltrexone)[4]

- Allosteric modulator: a substance that binds to a receptor at a site distinct from the active (orthosteric) site; causes a conformational change in the receptor, thus altering the endogenous ligand's affinity (probability the ligand will bind to the receptor) and efficacy (ligand's ability to activate the receptor)[5,6]
  - Positive allosteric modulator (PAM): increases affinity and/or efficacy[5]
  - Negative allosteric modulator (NAM): decreases affinity and/or efficacy[5]

Figure 1

Drug Interactions: Agonists, Antagonists, and Allosteric Modulators



Note. The active (orthosteric) site of a receptor is where agonists or antagonists bind in order to produce an effect. Contrastingly, the allosteric site of a receptor is where allosteric modulators bind to exert their effect; agonists and antagonists are unable to bind to the

allosteric site. There are two main types of allosteric modulators: positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs). PAMs help to potentiate the effect of the receptor and increase the affinity and/or efficacy of agonists or antagonists, whereas NAMs inhibit the effect of the receptor and decrease the affinity and/or efficacy of agonists and antagonists.[7]

1.1.1.2. Responses to Pain.

- Analgesia: the process of relieving or reducing pain, oftentimes through the use of pharmacotherapy with an analgesic (e.g., Tylenol® and Advil®)[8]
- Hyperalgesia: an increased sensitivity to pain; opposite of *antihyperalgesia*[9]
- Antinociception: the inhibition of nociception, which is the neural processes used to detect painful or noxious stimuli[10,11]

1.1.2. History of Opioids

There is evidence to suggest that opioid-based analgesics have been used for thousands of years.[12] In Mesopotamia (what is now present-day Iraq and sections of Iran, Turkey, Syria, and Kuwait), the opium poppy plant, also known as *Papaver somniferum*, was cultivated for its active ingredients as far back as 3000 BCE.[12] More recently, throughout the 1500s to 1800s, laudanum, an alcoholic solution containing opium and several other ingredients, was used as a popular painkiller and cough suppressant in European medicine.[12] Nevertheless, modern opioid pharmacology was not truly born until the discovery of morphine in 1806 by Friedrich Sertürner, a German pharmacist.[12] Sertürner was the first scientist to successfully isolate morphine crystals from the opium

poppy plant.[13] Along with being the first alkaloid to be extracted from opium, morphine was also the first alkaloid to be isolated from any plant.[13] Therefore, Sertürner is often recognized as a pivotal pioneer of alkaloid chemistry.[13] However, it was not until the invention of the hypodermic needle and syringe by Scottish physician, Alexander Wood, and French surgeon, Charles Gabriel Pravaz, in 1853, that the administration of morphine became clinically widespread.[12,14]

In the 1860s, the use of opioids for the purpose of treating pain started to become prevalent during the United States' Civil War.[15] When injured, soldiers were administered morphine as a means of analgesia.[15] In the years following wartime, many men began developing dependencies and addictions to this drug, thus foreshadowing the emergence of a public health crisis that would occur years later.[15]

In 1898, heroin was introduced to the public by the German pharmaceutical firm, the Bayer Company, after investigating a chemical modification to morphine that made it more palatable as a cough suppressant.[15,16] The Bayer Company named their new, what they thought to be "wonder" drug, heroin, and advertised it as being less habit-forming than morphine.[15,16] Not long after hitting the market, physicians and pharmacists began noticing that many patients taking heroin were becoming very dependent on the drug and larger doses were required to produce therapeutic effects after repeated administration.[16] Eventually, due to its highly addictive nature, heroin was removed from commercial sale and a total ban was placed on its production.[17]

During the 1910s and 1920s, the United States began enforcing stringent regulations on drugs classified as opioids and narcotics.[15] These types of drugs now required formal prescriptions to be written by a licensed physician in order for patients to

receive them; opioids and narcotics were no longer available over the counter.[15] In the 1970s, further restrictions on opioids and narcotics were passed with the issuance of the Controlled Substances Act, which divides drugs into different groupings based on their abuse liability and imposes regulations contingent on the class.[15]

Moreover, in 1995, Purdue Pharma introduced the drug, OxyContin®, a version of the already available opioid, oxycodone.[15] Scientists at Purdue Pharma claimed OxyContin® to be a gentler and less addictive analgesic when compared to other opioid pills.[15] Therefore, over the next couple of decades, doctors would readily prescribe OxyContin®, along with other opioids as an easy means of treating pain.[15] This over prescription of exceedingly addictive drugs would eventually fuel what we now know to be the Opioid Epidemic. As of October 2017, the United States has officially declared a public health emergency in response to the over prescription and misuse and abuse surrounding opioids.[15]

1.1.3. Classification of Opioid Drugs

Clinical opioids can be classified into three groups:

- Naturally occurring: alkaloids that are directly extracted from the opium poppy plant[18]
- Semi-synthetic: opioids produced by scientists in a laboratory from natural opiates; for example, chemical manipulations to the natural plant alkaloid, morphine, have yielded semi-synthetic compounds, such as heroin[18]
- Synthetic: opioids that are entirely manmade in a laboratory[18]

Opioids can be classified into three groups based on their interaction with opioid receptors:

- Full agonists: tightly bind to opioid receptors to produce a maximal effect[19]
- Partial agonists: activate opioid receptors but to a lesser extent than full agonists[19]
- Antagonists: bind to opioid receptors to reverse or block the effects of opioids[20]

Table 1

Classifications of Opioids

Origin	**Function**	**Analgesic Effects**
Naturally occurring: • Morphine • Codeine • Thebaine • Papaverine	Full agonists: • Morphine • Codeine • Heroin • Oxycodone • Hydrocodone • Fentanyl • Methadone	Strong: • Morphine • Fentanyl • Methadone • Meperidine
Semi-synthetic: • Heroin • Oxycodone • Hydrocodone • Buprenorphine	Partial agonists: • Buprenorphine • Tramadol	Intermediate: • Buprenorphine
Synthetic: • Fentanyl • Methadone • Tramadol • Meperidine	Antagonists: • Naloxone • Naltrexone	Weak: • Codeine

Note. Opioids categorized by their origin, function, and analgesic potency (strength)[12,21]

1.1.4. Classification of Opioid Receptors

Opioids work by attaching to proteins, called opioid receptors, which are found throughout the central and peripheral nervous system at various levels of expression in different tissue types. Many opioid receptors are located on nerve cells in the brain, spinal cord, and gut, as well as a plethora of other areas of the body associated with regulating pain.[22] There are currently four known opioid receptors which include:

- μ-opioid receptors (MORs): found primarily in the periaqueductal grey region of the midbrain and the superficial dorsal horn of the spinal cord; responsible for opioid analgesia, as well as many opioid side effects (e.g., respiratory depression, constipation, tolerance, dependence, and abuse liability)[21,23]
- κ-opioid receptors (KORs): located in the limbic and other diencephalic areas, brain stem, and spinal cord; help mediate spinal analgesia[21]
- δ-opioid receptors (DORs): largely present in the brain; effects are not well known but also thought to have antinociceptive effects[21]
- Nociception/orphanin opioid receptors (NOPs): found predominantly in the brain (i.e., cortex, amygdala, bed nucleus of the stria terminalis, medial prefrontal cortex, ventral tegmental area, lateral hypothalamus, and nucleus accumbens) and many brainstem areas (i.e., locus coeruleus and raphe); modulate nociceptive sensitivity primarily associated with anxiety- and stress-like states[24]

When opioids attach to these opioid receptors, pain messages sent from the body through the spinal cord to the brain are blocked and an individual experiences a sense of alleviation.[22] In this study, the opioid receptor we were focused on was the μ-opioid receptor, since it is the key receptor for analgesia.

1.1.5. Common Uses of Opioids

1.1.5.1. Pain Management. Opioids are medications most commonly prescribed by physicians to treat acute or chronic pain, whether it be moderate or severe.[22] They are frequently utilized for chronic headaches and backaches, post-operative pain, pain associated with cancer, and injuries resulting from playing sports, falls, and auto accidents.[22]

1.1.5.2. Cough Suppression. When it comes to coughs, many of which are the result of infections of the upper and lower airways, asthma, chronic obstructive pulmonary disease (COPD), lung cancer or lung metastases, interstitial pulmonary processes (e.g., lymphatic tumor spread or pulmonary edema), gastroesophageal reflux, aspiration, or a side effect of certain drugs, opioids are understandably the only effective centrally acting anti-tussive drugs on the market.[25] This class of medication is believed to work by suppressing the brainstem cough center through μ- and κ-opioid receptor agonism.[25] Opioids are typically used as a first-line treatment option for severe coughs.[25] Three of the most common opioids prescribed to individuals with distressing coughs are codeine, dextromethorphan, and hydrocodone.[25] Nevertheless, each comes with its own set of side effects, which includes but is not limited to sedation, constipation, and nausea.[25]

1.1.5.3. Chronic Diarrhea. Through clinical experience, it has been found that opioids, particularly opioid agonists, are an effective treatment option for people experiencing chronic diarrhea.[26] Loperamide, also known as Imodium®, is a peripherally acting opioid that is often recommended as a first-line therapy due to the fact it does not cause harsh side effects, like sedation and addiction.[26] Other opioids, including diphenoxylate-atropine, more commonly known as Lomotil®, are also effective at treating

chronic diarrhea, but they have the potential of being habit forming and are associated with a variety of adverse effects, such as sedation, dizziness, and dry mouth.[26] Opioids other than loperamide should only be used for more refractory cases of chronic diarrhea.[26]

1.1.5.4. Anesthetics. According to the *Journal of Pain and Symptom Management*, opioids are widely used in the practice of anesthesia.[27] A number of opioids are used for preanesthetic medication, systemic and spinal analgesia, supplementation of general anesthetic agents, as well as primary anesthetics.[27] Opioids used as primary anesthetics are commonly utilized during major surgical operations involving patients with cardiovascular disease.[27] This form of anesthetic helps to prevent the occurrence of cardiac depression.[27] Examples include sufentanil, alfentanil, remifentanil, and fentanyl.[28]

1.1.6. Adverse Effects of Opioids

1.1.6.1. Respiratory Depression. Although opioids can effectively relieve pain and discomfort, they do pose some notable risks and can be extremely addictive.[22] Addiction is especially of concern for people taking opioids in order to manage chronic pain over an extended period of time.[22] These critical side effects limit their safety and utility. One of the most life-threatening side effects associated with opioids is respiratory depression.[29] When taken, opioids induce profound respiratory changes in the body (e.g., sleep apnea and hypoventilation), which can cause complete respiratory arrest, especially if a person overdoses.[29] Opioids bind to MORs in the brain and inhibit the body's respiratory circuits, as well as depress breathing by affecting important respiratory structures of the brainstem.[29] Suppression of these respiratory circuits and structures

induces respiratory depression by lowering the body's respiratory rate and chemosensitivity, the brain's ability to detect changes in carbon dioxide (CO_2) levels.[29,30]

1.1.6.2. Constipation. Opioid drugs are known to cause opioid-induced constipation (OIC).[31] Opioids inhibit gastric emptying and promote peristalsis in the gastrointestinal (GI) tract, thus causing increased absorption of fluid in the body.[31] As a result of lower levels of fluid in the intestines, stool becomes hardened, oftentimes leading to constipation.[31] Many patients who experience OIC report straining and are unable to fully empty the rectum during defecation.[31] Additionally, opioids have been found to impair the defecation reflex, which in turn leads to anal sphincter dysfunction and anal blockage.[31] Likewise, opioids cause a decrease in pancreatic juice and bile emptying, resulting in delayed digestion and contributing to the emergence of constipation.[31]

1.1.6.3. Sedation. According to the *Clinical Journal of Oncology Nursing*, opioid-induced sedation manifests in 20-60% of patients taking opioids.[32] Sedation is typically defined as depression of brain function due to a medication that results in sleepiness, drowsiness, fatigue, slowed brain activity, reduced wakefulness, and impaired performance.[32] Although sedation is a prevalent side effect of opioid analgesics, its exact mechanism remains elusive.[32] So far, scientists are aware that opioids bind to opioid receptors in the central nervous system (CNS) and inhibit the firing of certain neurons.[32] The impact of opioids at these receptors hinders the brain's arousal mechanism and decreases content processing, thus contributing to decreased wakefulness and slowed interpretation of the environment.[32]

1.1.6.4. Tolerance. In regard to drugs, tolerance is defined as a decrease in effect of a particular substance following prolonged administration, thus resulting in a reduction

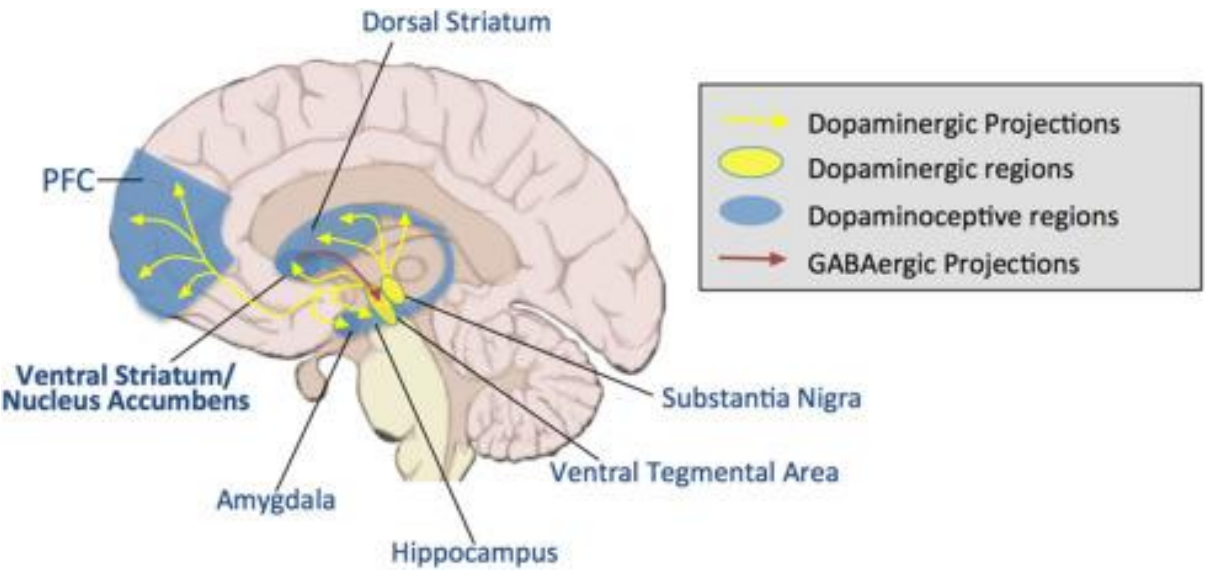
in potency.[33] This requires a person to need more of the drug in order to generate the same effect.[33] The development and extent of an individual's tolerance are correlated to the drug's interaction with opioid receptors, as well as its dose and frequency of administration.[33] There are a variety of mechanisms that impact opioid tolerance, such as upregulation of drug metabolism (i.e., metabolic tolerance), desensitization of receptor signaling, and downregulation of receptors.[33] Initiation of compensatory/opponent processes may also impact tolerance at a behavioral level.[33] Although analgesic tolerance can often be overcome by simply increasing the therapeutic dose, this is not always a safe and effective tactic because of other pharmacological effects, like constipation.[33] In addiction medicine, tolerance strongly influences dependence and abuse liability.[33]

1.1.6.5. Abuse Liability. Abuse liability refers to the tendency of a drug to be utilized for non-medical purposes (i.e., recreationally) as a result of the substance's underlying psychoactive effects, like euphoria and sedation.[34] Not only is abuse liability dependent on a drug's properties (e.g., neurochemical effects on the brain, formulation, and pharmacokinetics), but it is also contingent on the population being studied; this may incorporate age, vulnerability to addiction, and psychiatric and physical health morbidities.[34] Moreover, medications have the ability to cause cognitive effects through direct (e.g., crossing the blood-brain barrier) and indirect (e.g., peripheral mechanisms in the body) effects on the brain.[34] Over the years, scientists have found several brain mechanisms to be associated with abuse liability: (1) direct effects on the brain's reward pathway (prefrontal cortex and ventral striatum, also known as the nucleus accumbens), (2) indirect effects on stress-related neuronal pathways (amygdala), and (3) activation of the ventral tegmentum and amygdala via exposure to drug-related cues.[34] Each one of

these circuits are controlled by the neuromodulatory effects of dopamine, GABA, and glutamate, as well as the nicotinic, opioid, and cannabinoid systems of the CNS.[34]

Figure 2

Notable Structures and Pathways of the Brain



Note. One of the most important pathways when it comes to abuse liability is the mesolimbic dopaminergic pathway, the major reward circuit in the brain. During rewarding experiences, like drinking alcohol or using drugs, the dopamine system is stimulated, causing an abundance of dopamine to be released and pleasurable feelings to be felt throughout the body. This pathway contributes to the rewarding and reinforcing effects of opioid consumption, which in turn can lead to misuse, abuse, and addiction.[35,36]

1.1.7. Opioid Use Disorder (OUD)

Opioid use disorder (OUD) is defined as a "problematic pattern of opioid use that leads to serious impairment or distress."[37] The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), published in 2013, put together a list of 11 symptoms that help diagnose OUD.[37,38] Severity is based on the number of symptoms a person presents within a 12-month period of time—mild (2-3 symptoms), moderate (4-5 symptoms), or severe (6 or more symptoms).[37] The DSM-5's criteria for diagnosis of OUD is as follows:

Table 2

DSM-5's Criteria for Diagnosis of OUD

LOSS OF CONTROL	
1	Opioids are often taken in larger amounts or over a longer period than was intended.
2	There is a persistent desire or unsuccessful efforts to cut down or control opioid use.
3	A great deal of time is spent in activities necessary to obtain the opioid, use the opioid, or recover from its effects.
4	Craving, or a strong desire or urge to use opioids.
SOCIAL PROBLEMS	
5	Recurrent opioid use resulting in a failure to fulfill major role obligations at work, school, or home.
6	Continued opioid use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of opioids.
7	Important social, occupational, or recreational activities are given up or reduced because of opioid use.

RISKY USAGE	
8	Recurrent opioid use in situations in which it is physically hazardous.
9	Continued opioid use despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance.
PHARMACOLOGICAL PROBLEMS	
10	Tolerance, as defined by either of the following: a. A need for markedly increased amounts of opioids to achieve intoxication or desired effect. b. A markedly diminished effect with continued use of the same amount of an opioid. **Note:** This criterion is not considered to be met for those taking opioids solely under appropriate medical supervision.
11	Withdrawal, as manifested by either of the following: a. The characteristic opioid withdrawal syndrome. b. Opioids (or a closely related substance) are taken to relieve or avoid withdrawal symptoms. **Note:** This criterion is not considered to be met for those individuals taking opioids solely under appropriate medical supervision.

Note. **Source:** *Diagnostic and Statistical Manual of Mental Disorders: DSM-5.* (Fifth Edition). (2013). American Psychiatric Association.[39]

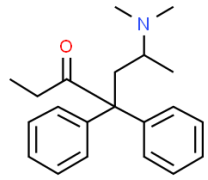
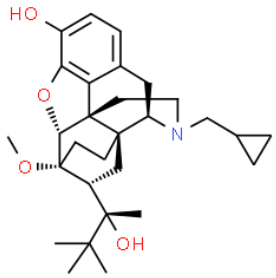
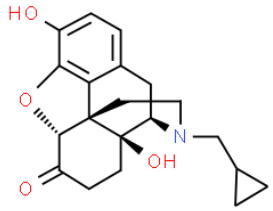
Along with the DSM-5's criteria for diagnosis of OUD, clinicians may require patients showing symptoms of problematic opioid usage to undergo drug testing. This may include the collection of matrices, the biological material used for analysis in a drug test.[40] Examples of matrices include blood, urine, oral fluid (spit/saliva), hair, nails, sweat, and

exhaled breath samples.[40] Currently, urine is the most commonly used biological specimen when it comes to drug and alcohol testing in a clinical setting.[40] Nevertheless, these types of drug tests are classified as presumptive tests and only provide preliminary evidence regarding the absence or presence of drugs or metabolites in a sample (often used for screening purposes).[40] Generally, presumptive testing utilizes immunoassay technology.[40] Immunoassays use antibodies formulated to bind to certain drugs, metabolites, or a class of compounds in a given sample.[40] If there are no drugs or drug byproducts present in the sample, then the antibodies will bind to a conjugate compound and produce a colored line in the test readout area.[40] Since presumptive tests may display less accurate results because of fast turnaround times and are susceptible to tampering, definitive testing can also be used to detect specific substances in samples.[40] Definitive testing typically incorporates gas or liquid chromatography in combination with mass spectrometry.[40] Chromatography is used to separate a specimen into its individual components, whereas mass spectrometry helps to identify those parts.[40] These definitive techniques often help to confirm and quantify presumptive positive and negative drug test results.[40]

1.1.7.1. Current FDA-Approved Treatments for OUD.

Table 3

Pharmacological Treatments for OUD

Name	**Function**	**Structure**
Methadone	Opioid full agonist	 [41]
Buprenorphine	Opioid partial agonist	 [42]
Naltrexone	Opioid antagonist	 [43]

Note. Name, function, and structure of current pharmacological treatments for OUD

1.1.8. Opioid Addiction Research in the Keck Animal Behavior Lab

1.1.8.1. Background. Opioid analgesics are crucial therapeutic techniques for the management of acute and chronic pain.[3] However, their side effects—respiratory depression, constipation, sedation, tolerance, and abuse liability—limit their safety and utility.[3] Therefore, in order to provide patients with safer analgesic options, it is imperative for researchers to identify new pharmacotherapeutic strategies to treat pain.[3]

Currently, scientists are aware that activation of the μ-opioid receptors (MORs) in the central and peripheral nociceptive pathways help to mediate opioid analgesia, as well as their side effects.[3] Likewise, antinociception can be attained through selective enhancement of gamma-aminobutyric acid (GABA) signaling at ionotropic GABA type A ($GABA_A$) receptors.[3] The $GABA_A$ receptor has six known α subunits, denoted as α1, α2, α3, α4, α5, and α6.[3] $GABA_A$'s α2 and α3 subunits (α2/α3$GABA_A$) are co-expressed with MORs in the dorsal horn spinal pathways, a neuronal pathway important to nociceptive transmission.[3] The dorsal horn functions as an intermediary processing center comprised of a complex network of excitatory and inhibitory interneurons, along with projection neurons that are in charge of transmitting processed somatosensory information from the spinal cord to the brain.[44] Recent work in our lab and with collaborators has determined that α2/α3$GABA_A$ can be selectively targeted with novel imidazodiazepine positive allosteric modulators (PAMs), like our drug of interest, MP-III-024, which produces antinociceptive effects with limited behavioral disruption.[3,45] $GABA_A$ PAMs facilitate the action of GABA by increasing the rate of channel opening, as well as enhancing receptor affinity for GABA.[3]

Recently, Mohammad Atiqur Rahman, one of my fellow graduate students at Rowan University, *et al.* showed that MP-III-024 co-administered with morphine produces synergistic antinociceptive and antihyperalgesic effects in rodent models.[46] He demonstrated this using two techniques: hot plate and von Frey testing. During hot plate testing, antinociception was assessed using a hot plate analgesia meter.[46] Before experimentation, animals were injected with either morphine, MP-III-024, or a drug mix, consisting of morphine and MP-III-024.[46] Certain behavioral changes, such as paw licking or fluttering and/or jumping from the hot plate surface, after placing the animal on the hot plate were recognized as a pain response.[46] In comparison, during von Frey testing, antihyperalgesic effects were studied following inflammation of the right hind paw evoked by zymosan A, a substance often used to induce experimental sterile inflammation.[46,47] The non-injected left hind paw was used as the control.[46] 24 hours after the zymosan A injection, mechanical sensitivity was assessed through the use of von Frey filaments of increasing stiffness following an injection of either morphine alone, MP-III-024 alone, or the morphine + MP-III-024 drug mix at varying ratios.[46] The mid plantar surface of each animal's right and left hind paws was poked with these thin, plastic filaments in order to determine the threshold that produces a hind paw withdrawal response.[46] A positive response was considered to be any type of paw withdrawal reflex after being poked by one of the filaments; mechanical sensitivity of this testing was defined as the minimum force necessary to elicit paw withdrawal behavior.[46]

Rahman *et al.* demonstrated that morphine was a potent analgesic in both the hot plate and von Frey testing procedures.[23] However, MP-III-024 only produced analgesia in the von Frey test.[23] On the hot plate assay, MP-III-024 did not show significant

analgesic effects at any of its administered doses (3.2, 10, and 32 mg/kg).[23] Contrastingly, on the von Frey assay, morphine and MP-III-024 displayed effectiveness simultaneously, with their dose effect curves being parallel ($p < 0.05$).[23] Moreover, when morphine was given in combination with MP-III-024, leftward shifts in the dose-response function for both assays were observed.[23] Therefore, Rahman *et al.* was able to deduce that the analgesic effect of each of the drug mixes was better than the effect produced by morphine alone, with respect to antinociception and antihyperalgesia.[23] Additionally, graphical analysis of each mixture indicated that morphine in combination with MP-III-024 produced superadditive, or synergistic, effects.[23] The synergistic effects of morphine + MP-III-024 suggest that this new drug combination may be used as an analgesic that requires a lower dosage in order to yield its desired effect.[23] Still, the safety profile of this combination needs to be studied, which brings us to my research.

1.1.8.2. Research Goal. Thanks to the groundbreaking research conducted by Rahman *et al.*, we now know that morphine in combination with MP-III-024 produces a superadditive, or synergistic, effect in models of pain. With this newfound information, two questions arise: (1) Does this mean that MP-III-024 is *universally* synergistic with morphine? (2) Does this drug mix have a synergistic side effect profile, as well? Since morphine + MP-III-024 had a synergistic analgesic property, we would predict that MP-III-024 enhances all of morphine's side effects, whether desirable or undesirable. In order to determine if MP-III-024 is universally synergistic with morphine, we have observed how varying doses (3.2, 10, and 32 mg/kg) and combination drug ratios (1.0:0.31, 1.0:0.94, and 1.0:2.8 morphine to MP-III-024, respectively) of morphine, MP-III-024, and morphine + MP-III-024 affect the behavior of mice trained to self-administer palatable food rewards.

The goal of this experiment is to find new drug types that can be paired with opioids to improve analgesia with fewer side effects and lower rates of dependence and addiction.

1.2. Part 2 – Alcohol

1.2.1. What is Alcohol?

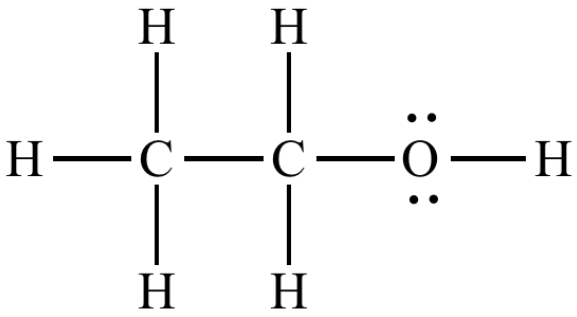
Alcohol, also known as ethanol or ethyl alcohol, is the main ingredient found in beer, wine, and spirits.[48] It is considered to be a sedative-hypnotic drug and is classified as a depressant, which means it depresses the central nervous system (CNS) at high doses and slows down vital bodily functions.[48,49] Usually, the amount of alcohol an individual consumes determines its effect on the body.[49] At lower doses, alcohol acts as a stimulant and causes feelings of euphoria and wellbeing, talkativeness, increased alertness, and so on.[49,50] Typically, the majority of people who drink alcohol are looking to obtain its stimulant effect, rather than its depressant effect.[49] However, if a person consumes more than his or her body can accommodate in a single session, alcohol's depressive effects, such as drowsiness, respiratory depression, slurred speech, unsteady movement, decreased reaction time, and distortion in judgment and rationality, start to ensue.[48,49]

Alcohol is formed when yeast ferments (i.e., breaks down without oxygen) the sugars in various foods, most notably fruits, vegetables, and grains.[48] For example, wine comes from the sugar found in grapes, beer is made from the sugar in malted barley (a kind of grain), cider originates from the sugar produced by apples, and vodka is the product of fermented sugar in potatoes, beets, and other plants.[48] Although there are many types of

alcohol out there (e.g., isopropyl, methyl, and ethyl), the only kind of alcohol that can be consumed by humans and therefore the one found in all our alcoholic beverages is ethyl alcohol, more commonly known as ethanol.[49]

Figure 3

Chemical Structure of Ethanol



[51]

1.2.2. History of Alcohol

For thousands of years, fermented grain, fruit juice, and honey have been utilized to produce alcohol.[49] There is evidence to suggest that fermented beverages existed in early Egyptian civilization, as well as China around 7000 BCE.[49] Similarly, an alcoholic beverage known as sura, which came from distilled rice, was consumed between 3000 and 2000 BCE.[49] As early as 2700 BCE, there is also record of the Babylonians worshipping a wine goddess; and in Greek literature, which dates back thousands of years, there is warnings of excessive drinking.[49] In Greece, one of the first noted alcoholic beverages was mead, a fermented drink produced from the fermentation of honey and water.[49]

Additionally, there is evidence to suggest that a number of Native American civilizations developed alcoholic beverages in pre-Columbian times.[49] A large sum of the fermented beverages produced in the Andes region of South America were made from corn, grapes, and apples, referred to as "chicha."[49] Likewise, in the 1500s, alcohol, often called spirits, was commonly used for medicinal practices.[49] At the start of the 1700s, the British parliament passed a law encouraging people to use grain in order to distill spirits.[49] As a result of this request, cheap spirits filled the British markets and reached peak sale mid-eighteenth century.[49] During this time, one of the most popular spirits among the British people was gin.[49] Due to its high consumption rate throughout this period, gin is often blamed for commencing widespread alcoholism in Britain.[49]

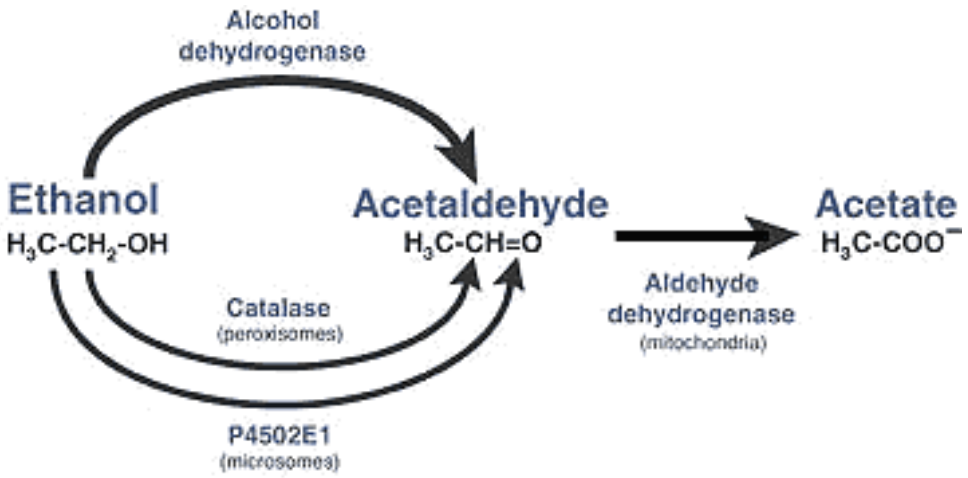
Attitudes towards alcohol began changing in the nineteenth century with the temperance movement promoting moderate use of alcohol in order to halt the spread of alcoholism.[49] This change in attitude towards fermented beverages ultimately became a driving factor in the push for total prohibition.[49] The prohibition movement took the United States by storm and in 1920, the United States' government passed a law prohibiting the manufacture, sale, import, and export of all alcoholic beverages.[49] This strict law forbidding the consumption of intoxicating liquors prompted the illegal alcohol trade and by 1933, the prohibition of alcohol was repealed.[49] More recently, in 1971, as part of the Alcohol, Drug Abuse, and Mental Health Administration (ADAMHA), the National Institute on Alcohol Abuse and Alcoholism (NIAAA) was founded to act as the primary federal agency overlooking alcohol abuse and alcoholism in the United States.[52] The NIAAA also funds many research efforts focused on combating conditions, like alcohol use disorder (AUD).[52]

1.2.3. The Pharmacokinetics of Alcohol

To date, alcohol is one of the most widely consumed legal drugs around the world.[53] In regard to its pharmacokinetics, alcohol is absorbed into the bloodstream through small blood vessels located in the walls of the stomach, as well as the small intestine.[49] After an individual consumes an alcoholic beverage and a substantial amount of oral absorption has taken place, alcohol begins to travel from the stomach to the brain, where it begins initiating its stimulatory then depressant effects, if the person continues drinking.[49] Along with being absorbed by the stomach and the small intestine, alcohol is also taken up by the liver, where it is metabolized and converted into acetaldehyde, a very toxic byproduct of alcohol, then acetate.[49,54] Eventually, acetate leaves the liver and is converted into CO_2 and H_2O by means of the Krebs cycle.[54] Nevertheless, the liver only has the ability to metabolize a certain amount of alcohol at a time, thus leaving the remainder to circulate throughout the body and cause adverse effects.[49] Moreover, when the amount of alcohol found in a person's blood surpasses a certain concentration, the respiratory system begins slowing down remarkably and adverse effects, such as coma and death, can result if oxygen is unable to reach the brain.[49] In most people, though, after a day or night of drinking, the excess alcohol left in the body that is not oxidatively metabolized by the liver is removed unchanged by means of excretion via the kidneys (urine), lungs (breath), or skin (sweat) and alcohol's side effects cease.[54]

Figure 4

Metabolism of Alcohol in the Liver



Note. In the first step, alcohol (i.e., ethanol) is metabolized by the enzyme, alcohol dehydrogenase (ADH), and is converted into a highly toxic substance and known carcinogen, acetaldehyde. Then, in the second step, acetaldehyde is metabolized by the enzyme, aldehyde dehydrogenase (ALDH), to a less active byproduct, acetate, which is eventually broken down into CO_2 and H_2O, allowing for easy excretion from the body. Other enzymes, including cytochrome P450 2E1 (CYP2E1) and catalase, are also involved in this process, helping to break down alcohol to acetaldehyde. Interestingly, CYP2E1 tends to only be active after an individual has consumed large quantities of alcohol.[55]

1.2.4. Short- and Long-Term Effects of Alcohol

Table 4

Side Effects of Alcohol Usage

Short-Term Effects:	**Long-Term Effects:**
• Slurred speech • Drowsiness • Nausea • Vomiting • Diarrhea • Headaches • Difficulty breathing • Distorted vision and hearing • Impaired judgment • Decreased perception and coordination • Blackouts (memory lapses) • Unconsciousness • Coma • Anemia	• Family problems/broken relationships • Loss of productivity in the workplace • Unintentional injuries: car crashes, falls, burns, drownings • Intentional injuries: firearm injuries, sexual assaults, domestic violence • Alcohol poisoning • High blood pressure, stroke, or other heart-related diseases • Liver disease • Cancer of the mouth and throat • Nerve damage • Permanent brain damage • Sexual dysfunction • Vitamin B_1 deficiency • Ulcers • Gastritis • Malnutrition [49]

1.2.5. Alcohol Use Disorder (AUD)

According to the National Institutes of Health (NIH), alcohol use disorder (AUD) is defined as a "chronic relapsing brain disease that causes a person to drink compulsively despite adverse consequences to daily life and overall health."[56] The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), published in 2013, put together a list of 11 symptoms that help diagnose AUD.[38] Severity is based on the number of symptoms a person presents within a 12-month period of time—mild (2-3 symptoms), moderate (4-5 symptoms), or severe (6 or more symptoms).[57] The DSM-5's criteria for diagnosis of AUD is as follows:

Table 5

DSM-5's Criteria for Diagnosis of AUD

LOSS OF CONTROL	
1	Alcohol is often taken in larger amounts or over a longer period than was intended.
2	There is a persistent desire or unsuccessful efforts to cut down or control alcohol use.
3	A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects.
4	Craving, or a strong desire or urge to use alcohol.
SOCIAL PROBLEMS	
5	Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home.
6	Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol.

7	Important social, occupational, or recreational activities are given up or reduced because of alcohol use.
RISKY USAGE	
8	Recurrent alcohol use in situations in which it is physically hazardous.
9	Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol.
PHARMACOLOGICAL PROBLEMS	
10	Tolerance, as defined by either of the following: a. A need for markedly increased amounts of alcohol to achieve intoxication or desired effect. b. A markedly diminished effect with continued use of the same amount of alcohol.
11	Withdrawal, as manifested by either of the following: a. The characteristic withdrawal syndrome for alcohol. b. Alcohol (or a closely related substance, such as a benzodiazepine) is taken to relieve or avoid withdrawal symptoms.

Note. **Source:** *Diagnostic and Statistical Manual of Mental Disorders: DSM-5.* (Fifth Edition). (2013). American Psychiatric Association.[39]

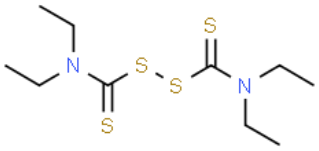
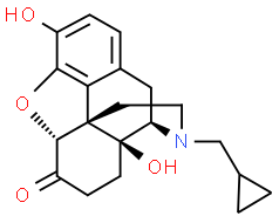
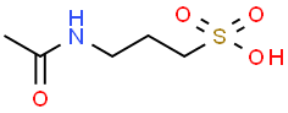
Similar to the preceding section discussing the various drug testing methods for people suffering from OUD, along with utilizing the DSM-5's checklist for diagnosing AUD, clinicians may require patients showing symptoms of concerning and problematic alcohol usage to undergo routine blood, urine, or other lab tests in order to assess the severity of their alcohol consumption. These tests may again require the use of presumptive (e.g., immunoassay) and/or definitive (e.g., chromatography/mass spectrometry) drug

testing techniques to determine the presence or absence of alcohol or its metabolites in a given sample, as well as the alcohol concentration within a sample.[40]

1.2.5.1. Current FDA-Approved Treatments for AUD.

Table 6

Pharmacological Treatments for AUD

Name	**Function**	**Structure**
Disulfiram	Acetaldehyde dehydrogenase antagonist	 [58]
Naltrexone	Opioid antagonist	 [43]
Acamprosate	Mechanism of action is unclear; believed to act as a GABA receptor agonist and glutamate receptor antagonist [59,60]	 [61]

Note. Name, function, and structure of current pharmacological treatments for AUD

Non-FDA approved medications for AUD include nalmefene (which is approved in Europe for AUD), gabapentin, topiramate, baclofen, and ondansetron.[62] The American Psychiatric Association (APA) guidelines recommend naltrexone and acamprosate be offered to patients as first-line treatment options for individuals suffering from moderate to severe AUD.[62] Second-line treatment options may incorporate the administration of disulfiram, topiramate, and gabapentin if patients are intolerant or have not responded well to naltrexone or acamprosate, or if they prefer one of these second-line drugs over the first-line options.[62] Likewise, naltrexone should not be administered to patients diagnosed with severe hepatic impairment, since it can cause hepatotoxicity, or concomitant opioid use.[62] Similarly, acamprosate should not be taken by people who have severe renal impairment, since this drug is unable to be metabolized by the liver and is excreted renally.[62] The APA has not yet acknowledged the use of baclofen or ondansetron for AUD.[62]

1.2.6. Alcohol Addiction Research in the Keck Animal Behavior Lab

1.2.6.1. Background. Along with drug addiction, alcoholism is a major health problem in the United States, as well as on a global level. Although there are treatment options currently on the market to help combat alcohol use disorder (AUD), the side effects associated with these medications are not always desirable and their success rates are often low due to poor compliance.[63] Therefore, non-pharmacological techniques, like behavioral treatments (e.g., alcohol counseling or talk therapy) or mutual-support groups (e.g., Alcoholics Anonymous, also known as AA), are typically the first and only treatment methods explored by individuals suffering from AUD.[52,57] Depending on the individual, these non-pharmacological approaches are not always successful and pose a risk of relapse

occurring without the implementation of drug intervention. With AUD being such a prominent health issue around the world, there needs to be pharmacological treatment options available to people diagnosed with AUD that are not only efficacious but have limited adverse effects, thus resulting in increased patient compliance and potentiating a promising, new cure.

One of the main hurdles that comes with creating a drug for individuals suffering from alcoholism is knowing where exactly in the brain alcohol targets. In the past, scientists believed that alcohol acted as a membrane disruptor that inflicted a generalized effect all over the brain.[64] However, scientists now know that there are actually specific structures in the brain that alcohol targets, the most notable being γ-aminobutyric acid (GABA) receptors, glutamate receptors, and the nucleus accumbens.[64] The nucleus accumbens is a pivotal component of the brain's mesolimbic pathway, the major dopaminergic pathway that gets stimulated during rewarding experiences, like using drugs or drinking alcohol.[65] Since alcohol has been found to have a profound effect on dopamine release in the brain's reward center, thus inducing its stimulating, pleasurable effects, it is plausible to hypothesize that medications that target dopamine receptors may be up-and-coming treatment options for people suffering from AUD.

When dopamine is released, it acts on receptor proteins termed the dopamine receptors. There are five dopamine receptors identified including dopamine D1 receptor (D_1R), dopamine D2 receptor (D_2R), dopamine D3 receptor (D_3R), dopamine D4 receptor (D_4R), and dopamine D5 receptor (D_5R). So far, there have been several lines of evidence indicating that pharmacologically targeting D_4R, may be advantageous when it comes to substance use disorders, such as AUD.[66] Preliminary data utilizing the full D_4R antagonist,

CAB-01-019, has shown promising results in rodent models of cocaine addiction.[66] Through behavioral analysis it was revealed that injections of CAB-01-019 prior to experimentation attenuated cocaine self-administration at all three of the drug's tested dosages (5, 15, and 30 mg/kg).[66] Cocaine is a highly addictive stimulant drug that, similar to alcohol, has been found to have a profound effect on the dopaminergic receptors of the brain.[67,68] In conjugation with these findings of CAB-01-019 dose-dependently decreasing cocaine intake, as well as the commonalities between cocaine and alcohol's effect on dopamine, it is assumed that CAB-01-019 will also attenuate drug-taking and -seeking behaviors in animal models of alcohol addiction and prove to be a promising new avenue for AUD medication development in the near future.[66]

1.2.6.2. Research Goal. In order to determine if CAB-01-019 is a good drug candidate for treating AUD, we must first explore how behaviorally disruptive it is. In the past, antagonism of the D_4R was found to disrupt processes involved with memory and cognition, therefore indicating that it may be important to maintain a level of D_4R activation through partial agonism rather than full antagonism.[66] A reliable method of testing behavioral disruption is through the use of operant responding by means of self-administration of palatable food rewards. Thus, the goal of this experiment is to study the effects that CAB-01-019 evokes during sessions of self-administration in mice trained to self-administer palatable food rewards. The results of this testing will be used as 1) a critical control for alcohol tests, separating alcohol-specific effects from non-specific behavioral or appetitive effects, and 2) a training precursor for ethanol self-administration. Future research plans include additional testing to determine if CAB-01-019 affects operant alcohol self-administration in mice.

Chapter 2

The Effects of Morphine and MP-III-024 Co-Administration on Food Self-Administration and Open Field Testing

2.1. Abstract

Each year, millions of people across the globe suffer from opioid use disorder (OUD). Opioid misuse, abuse, and addiction, along with overprescribing are responsible for fueling the Opioid Epidemic, a major health crisis in the United States. Thus, there is a dire need for new treatment techniques for pain management that are less addictive and less subject to misuse and abuse. In previous work, our lab determined that the novel imidazodiazepine, MP-III-024, when co-administered with the opioid analgesic, morphine (in 1.0:0.31, 1.0:0.94, or 1.0:2.8 ratios of morphine:MP-III-024), produced synergistic effects in models of analgesia and antinociception. In this study, the combination effects of morphine and MP-III-024 were analyzed in food self-administration and open field testing, tests representative of a subset of opioid side effects. Based on prior research studies, we hypothesized that morphine + MP-III-024 would produce synergistic effects in these behavioral tests. The results of our self-administration testing demonstrated that morphine co-administered with MP-III-024 had statistically indistinguishable effects compared to morphine alone; but, adding MP-III-024 to morphine did not make morphine more disruptive in regard to operant responding. In open field testing, however, our 1.0:0.94 morphine:MP-III-024 ratio attenuated morphine-induced hyperlocomotion and was found to be statistically less than morphine alone, a subadditive (anti-synergistic) effect. With these findings, we now know that morphine and MP-III-024 are not *universally*

synergistic. Therefore, this drug mix may be able to produce more potent analgesia with reduced risks of opioid-induced side effects, potentially increasing the safety of opioid analgesia treatments.

2.2. Introduction

Opioid misuse, abuse, and addiction are major health problems around the globe. In 2019, in the United States alone, approximately 50,000 individuals died from opioid overdoses.[69] Since opioids are so addictive, many people who do not take them as prescribed or use them recreationally often develop a chronic and relapsing illness known as opioid use disorder (OUD).[70] In simple terms, OUD is a physical and psychological dependence on opioids frequently characterized by symptoms of uncontrollable cravings and the inability to control usage.[70] Likewise, OUD increases the likelihood of disability, overdose, and in some cases, death.[70]

To better understand the purpose of this study, it is important to know some background information regarding opioids. Opioids are derived from the opium poppy plant, known scientifically as *Papaver somniferum*.[71,72] However, nowadays, many opioids are synthetically formulated in a lab by scientists.[72] These types of substances are highly potent and effective analgesics, commonly used to treat moderate to severe pain.[71,72] Nevertheless, opioids are highly addictive due to their euphoric effects and as a result of this are often abused.[72] To date, one of the most dangerous and addictive opioids is the illicit drug, heroin.[72] Common prescription opioids include codeine, fentanyl, hydrocodone (Vicodin®), morphine, oxycodone (OxyContin®, Percocet®), and oxymorphone.[72]

Opioids affect the body in many different ways. Although they are often beneficial when it comes to reducing pain triggered by surgery, trauma, disease, and other painful conditions, they come with a lot of risks.[70,73] Approximately 80% of the people who take opioids have experienced at least one adverse event, with the most common being gastrointestinal problems (e.g., constipation, nausea, and vomiting).[74] Important side effects include respiratory depression and hypoxia, which is the reason people die of overdoses, infections of the heart, lungs, and liver, and tolerance, dependence, and addiction.[72,75] These side effect risks tremendously increase when taking illegal, unregulated drugs, like heroin.[72] For example, new diagnoses of Hepatitis C, an infection that attacks the liver, are often linked to people who inject drugs using contaminated needles, syringes, or injection equipment.[75] According to the Centers for Disease Control and Prevention (CDC), opioids, predominantly synthetic opioids, are the main cause of drug overdose deaths in the United States.[76] Therefore, there is a dire need for new treatment techniques that are less addictive and subject to misuse and abuse.

In regard to their mechanism of action (MOA), opioids bind to and activate opioid receptors predominantly found in the central nervous system (CNS), which includes the brain and spinal cord, and the peripheral nervous system (PNS), as well as other organs in the body associated with pain and pleasure.[72,46] After attaching to these opioid receptors, opioids inhibit pain signals being conveyed from the brain to the rest of the body, also causing large quantities of dopamine to be released, which is the neurotransmitter that helps mediate pleasure in the brain.[72] Currently, there are four known opioid receptors designated as mu (μ), kappa (κ), delta (δ), and nociception/orphanin FQ.[46,77] Activation of μ-opioid receptors (MORs) is responsible for the prototypic opioid effects of analgesia,

reward, and withdrawal.[77] Additionally, MORs in the CNS are associated with respiratory depression, analgesia, euphoria, and miosis, and those in the PNS are linked to cough suppression and constipation.[77]

Scientists are aware that opioid's analgesic properties can also be attained through selective enhancement of γ-aminobutyric acid (GABA) signaling at ionotropic GABA type A ($GABA_A$) receptors.[3] The $GABA_A$ receptor has six known α subunits.[3] $GABA_A$'s α2 and α3 subunits ($\alpha2/\alpha3GABA_A$) have been found to be co-expressed with MORs in the dorsal horn spinal pathways, where they help to mediate the transmission of pain sensory signals throughout the body.[3] It has been found that $\alpha2/\alpha3GABA_A$ can be selectively targeted with novel imidazodiazepine positive allosteric modulators (PAMs), like our drug of interest, MP-III-024, which produces antinociceptive effects with limited behavioral disruption.[3] $GABA_A$ PAMs facilitate the action of GABA by increasing the rate of channel opening, as well as enhancing receptor affinity for GABA.[3]

There are currently three FDA-approved treatments for OUD, which include methadone, an opioid agonist, buprenorphine, an opioid partial agonist, and naltrexone, an opioid antagonist.[78] Opioid agonists work by binding and activating opioid receptors, the same receptors that are activated by the body's endogenous opioids, β-endorphin, met- and leu-enkephalins, and the dynorphins.[78,79] In contrast, opioid antagonists block opioid receptors instead of activating them, thus stopping opioids from producing any effect.[78] Methadone is a full μ agonist that helps block the euphoric effects of opioid drugs, as well as minimizes the symptoms caused by opioid withdrawal.[78] Buprenorphine is a partial agonist, which means that it binds to opioid receptors and blocks the effects of opioid drugs like methadone; however, it is considered to be a partial agonist because it has high affinity

for the μ-opioid receptor but low intrinsic activity.[78,80] Nevertheless, buprenorphine still helps to reduce cravings and withdrawal symptoms.[78] Naltrexone is an antagonist, meaning it has no intrinsic signaling effects on its own and prevents other opioids from binding and activating opioid receptors altogether.[78] As a result, if an individual takes opioids while on naltrexone, the opioids will not produce an effect; although, this drug should really only be prescribed to people who have completely detoxed from opioids in order to avoid precipitating withdrawal.[78,81]

Even though there are FDA-approved treatment options out there for people suffering from OUD, new treatment strategies that reduce opioid exposure need to be explored, since they may help decrease the likelihood of OUD development. One of the research objectives of the Keck Animal Behavior Lab is testing new drug combinations that may help to reduce the doses of clinically prescribed opioids. In this study, we tested the combination effects of morphine, a μ opioid agonist, and MP-III-024, a novel imidazodiazepine with PAM effects at α2- and α3-subunit containing $GABA_A$ receptors.[45,46] Our main goal is to find new candidate medications that can be co-administered with opioids to selectively enhance analgesia, reducing the risks of opioid-induced side effects, including opioid addiction.

Thanks to some groundbreaking research conducted by Mohammad Atiqur Rahman, a recent Rowan University graduate, *et al.*, we know that MP-III-024 co-administered with morphine produces synergistic antinociceptive and antihyperalgesic effects in mouse models of thermal and inflammatory pain.[46] However, with this newfound information, an important question arises: Is MP-III-024 *universally* synergistic with morphine and, therefore, will morphine/MP-III-024 co-administration produce a

synergistic side-effect profile as well? Our working hypothesis for these studies is that because morphine/MP-III-024 co-administration produced synergistic analgesic effects, we predict that MP-III-024 similarly enhances morphine-mediated side effects. In pharmacology, synergism (derived from the Greek word "synergos" which means "working together") is defined as "an interaction between two or more drugs that causes the total effect of the drugs to be greater than the sum of the individual effects of each drug."[1,82] Synergistic effects can be harmful or beneficial to one's health.[1]

According to the findings of several other preclinical research studies, it is likely that morphine and MP-III-014 will have a synergistic side effect profile. Biological data published by Gueye *et al.* (2002), Megarbane *et al.* (2005), and Nielsen and Taylor (2005) have demonstrated that these two drug classes have synergistic effects in regard to sedation and respiratory depression when administered concurrently and may contribute to the chance of fatal overdose.[83,84] There are serious risks associated with the concomitant use of opioids and benzodiazepines, especially since benzodiazepines are known to enhance the sedating and respiratory effects of other medications and substances, including full opioid agonists, which morphine acts as on the μ-opioid receptor.[84] Gueye *et al.* analyzed this potentially dangerous synergistic relationship in rodent models.[83] In Gueye *et al.*'s study, it was shown that while high doses of the opioid, buprenorphine (30 mg/kg, i.v.), and the benzodiazepine, midazolam (160 mg/kg, i.p.), alone caused mild but significant increases in partial pressure of arterial carbon dioxide ($PaCO_2$), when co-administered, these two drugs promoted rapid, substantial, and prolonged respiratory depression and hypoxia.[83] Similarly, in Megarbane *et al.*'s preclinical research study, rodents who received the opioid, buprenorphine (30 mg/kg, i.v.), co-administered with the

benzodiazepine, flunitrazepam (40 mg/kg, i.v.), displayed rapid and sustained respiratory depression.[83] However, that dose of buprenorphine alone had no significant effect on the animals' respiration rates.[83] Likewise, investigators, such as Nielsen and Taylor, have proposed potential mechanisms to explain the synergistic impact that comes with the simultaneous use of opioids and benzodiazepines. Nielsen and Taylor found that buprenorphine administered in combination with diazepam seemed to abolish the protective plateau, or ceiling effect (the optimal effect of a drug; once a therapeutic limit is achieved, increases in doses may cause side effects but no further beneficial effects), of buprenorphine, thus resulting in a higher risk of respiratory depression and death.[83.85]

Based on Rahman *et al.*'s results, as well as Fischer *et al.*'s research studies investigating MP-III-024, we hypothesized that the synergistic analgesic effects seen with morphine and MP-III-024 co-administration result from simultaneous enhancement of signaling by MORs and $\alpha 2GABA_A$ and $\alpha 3GABA_A$ receptors, co-expressed in key nociceptive pathways in the brain and spinal cord.[45,46] We also hypothesized that morphine and MP-III-024 co-administration will produce synergistic effects in measures of undesirable pharmacological responses: opioid side effects. This synergistic side effect profile is further anticipated based on the results from the three studies discussed above regarding the negative impacts (i.e., sedation and respiratory depression) associated with concurrent use of opioids and benzodiazepines. To test our hypothesis, we focused on two opioid-induced behavioral effects: opioid-induced disruption of food-maintained operant responding and opioid-induced hyperlocomotion in open field.

In regard to locomotor function, morphine is known to induce hyperactivity in mice. In fact, most drugs of abuse have been found to have a stimulatory effect on

locomotor activity in laboratory animals, especially after repeated exposure.[86] In relation to self-administration, if morphine produces dose-dependent increases in locomotor activity, then we expect to see a decrease in the number of earned food rewards for morphine administered alone, as well as the morphine/MP-III-024 combination therapy, especially if there is a synergistic side effect profile for this drug mix. It is assumed that with the employment of hyperlocomotion, the animals will be less focused on the task at hand, that being nose poking for palatable food rewards. As a result of this, we expect to see dose-dependent decreases in operant response rates for self-administration. When taking this into consideration, as well as our working hypotheses, we predict that morphine/MP-III-024 co-administration will synergistically enhance morphine-induced hyperlocomotion and morphine-induced disruption of palatable food self-administration. As previously mentioned, morphine is well-characterized to produce dose-dependent increases in locomotor and disrupt operant responding, thus causing a decrease in operant response rates and a corresponding decrease in food consumption (i.e., rewards), since the mice are more preoccupied with moving around than searching for food. If our hypotheses are correct, MP-III-024 given in combination with morphine, will enhance morphine-induced locomotion which in turn will enhance morphine-induced behavioral disruption, resulting in a leftward shift on the dose-response curves.

2.3. Materials and Methods

2.3.1. Drugs

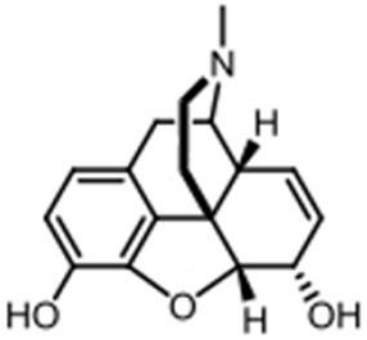
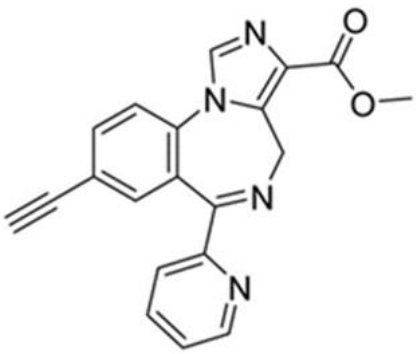
2.3.1.1. Morphine. Morphine, also known by its scientific name, (4R,4aR,7S,7aR,12bS)-3-methyl-2,4,4a,7,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7,9-diol, is a natural plant alkaloid and is recognized as the main alkaloid of opium.[87,88] Over the years, chemical manipulations to morphine's structure have yielded semi-synthetic opioids, for example heroin, and fully synthetic opioid compounds.[88] Morphine and other opioid agonists bind to and activate opioid receptors.[87] Morphine, its metabolites, and other opioid analgesics act as agonists at the μ-, κ-, and δ-opioid receptors.[88] Activation of these opioid receptors result in the inhibition of pain signals being sent from nociceptors, specialized peripheral sensory neurons which warn the brain and spinal cord, specifically the dorsal horn neurons, of damaging or potentially damaging stimuli to the body.[88,89] This in turn provides a sense of temporary relief if a person is experiencing pain.

2.3.1.2. MP-III-024. MP-III-024, also known by its scientific name, methyl 8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate, is an imidazodiazepine positive allosteric modulator (PAM) at α2GABA$_A$ and α3GABA$_A$ receptors in the CNS and PNS.[45] Other research studies have determined MP-III-024 to be α2GABA$_A$- and α3GABA$_A$-selective over α1GABA$_A$ and α5GABA$_A$ receptors.[45] α2GABA$_A$ and α3GABA$_A$ receptors are thought to mediate the antihyperalgesic effects of benzodiazepines.[45] α1GABA$_A$ receptors are associated with the negative side effects of benzodiazepines, such as sedation and dependence.[45] α5GABA$_A$ receptors are involved in

certain memory processes impacted by benzodiazepines.[45] MP-III-024 was chosen for this combination drug study because of its high subtype selectivity, as well as its time course of action, which aligns well with morphine.[3] More importantly, MP-III-024 has negligible affinity for opioid receptors.[3]

Table 7

Chemical Structure of Morphine and MP-III-024

Morphine	**MP-III-024**
 [90]	 [45]

2.3.2. Drug Dosing

2.3.2.1. Food Self-Administration Drug Dosing. The doses for morphine alone and morphine + MP-III-024, our combination therapy, were 3.2, 10, and 32 mg/kg, with the drug mixes being a 1.0:0.31, 1.0:0.94, and 1.0:2.8 ratio of morphine to MP-III-024, respectively. The drug dosing for MP-III-024 alone had an additional 100 mg/kg dose, since that set of testing was conducted by Fischer *et al.* in 2017. MP-III-024 was

synthesized at the Department of Chemistry and Biochemistry at the University of Wisconsin-Milwaukee. Morphine was purchased from Henry Schein, Inc. The standard vehicle for these experiments (including vehicle controls) was 0.5% methylcellulose dissolved in 0.9% NaCl (physiological saline). Doses were administered to mice via intraperitoneal (i.p.) injections at a volume of 10 mL/kg. Animal weights were determined on the morning of designated injection days.

Table 8

Food Self-Administration Drug Dosing for Morphine + MP-III-024

Ratio → 1.0:0.31		**Ratio → 1.0:0.94**		**Ratio → 1.0:2.8**	
Morphine	MP-III-024	Morphine	MP-III-024	Morphine	MP-III-024
3.2 mg/kg	0.992 mg/kg	3.2 mg/kg	3.008 mg/kg	3.2 mg/kg	8.96 mg/kg
10 mg/kg	3.1 mg/kg	10 mg/kg	9.4 mg/kg	10 mg/kg	28 mg/kg
32 mg/kg	9.92 mg/kg	32 mg/kg	30.08 mg/kg	32 mg/kg	89.6 mg/kg

Note. Food self-administration drug dosing for our combination therapy, morphine + MP-III-024, at the three tested ratios (1.0:0.31, 1.0:0.94, and 1.0:2.8). The ratios were derived using log-linear interpolation by linear regression based on Rahman *et al.*'s dose-response curve results for morphine and MP-III-024.[23]

2.3.2.2. Open Field Drug Dosing. Open field testing used cumulative dosing in which test subjects were repeatedly administered drug doses of increasing concentrations and then tested after each incremental dose. Locomotor function can be evaluated through this type of dosing, which is the side effect of interest during this part of experimentation.

The doses of morphine, MP-III-024, and morphine + MP-III-024 that were selected were 1.0, 2.2, 6.8, 8.0, and 14.0 mg/kg. Likewise, the ratios of morphine to MP-III-024 that were tested were 1.0:0.31, 1.0:0.94, and 1.0:2.8.

Table 9

Open Field Drug Dosing for Morphine + MP-III-024

Ratio → 1.0:0.31		**Ratio → 1.0:0.94**		**Ratio → 1.0:2.8**	
Morphine	MP-III-024	Morphine	MP-III-024	Morphine	MP-III-024
1.0 mg/kg	0.31 mg/kg	1.0 mg/kg	0.94 mg/kg	1.0 mg/kg	2.8 mg/kg
2.2 mg/kg	0.682 mg/kg	2.2 mg/kg	2.068 mg/kg	2.2 mg/kg	6.16 mg/kg
6.8 mg/kg	2.108 mg/kg	6.8 mg/kg	6.392 mg/kg	6.8 mg/kg	19.04 mg/kg
8.0 mg/kg	2.48 mg/kg	8.0 mg/kg	7.52 mg/kg	8.0 mg/kg	22.4 mg/kg
14.0 mg/kg	4.34 mg/kg	14.0 mg/kg	13.16 mg/kg	14.0 mg/kg	39.2 mg/kg

Note. Open field drug dosing for our combination therapy, morphine + MP-III-024, at the three tested ratios (1.0:0.31, 1.0:0.94, and 1.0:2.8). The ratios were derived using log-linear interpolation by linear regression based on Rahman *et al.*'s dose-response curve results for morphine and MP-III-024.[23]

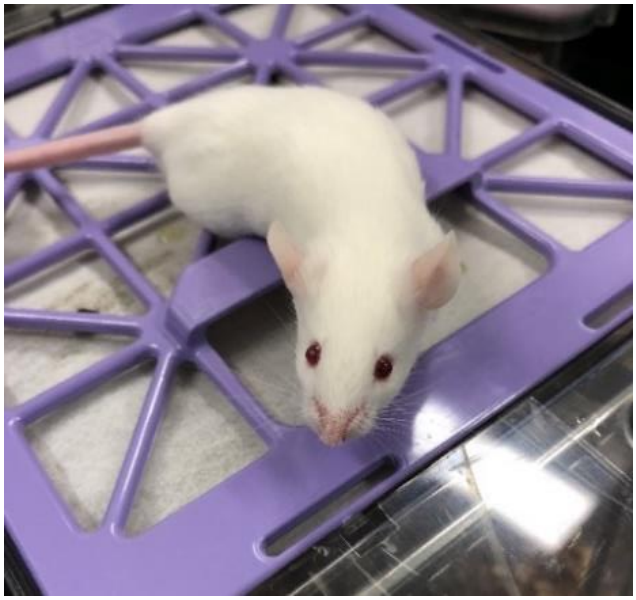
2.3.3. Animals

Drug-naïve adult male CD-1 mice obtained from Charles River Laboratories were used for these studies. Prior to this experiment, the animals were not exposed to any kind of behavioral or pharmacological manipulation, which could potentially skew the data and results of this study. The mice were albino and therefore had white fur and red eyes. They weighed anywhere between 30-45 grams; however, their weights often fluctuated due to

daily fasting. Upon arrival to the vivarium located at Cooper Medical School of Rowan University (CMSRU), the animals were grouped in fours and housed in standard plexiglass cages, equipped with food, water, bedding, nestlets, and enviropaks and allowed a two-week habituation period. Each group of animals was housed in a colony room, also known as a holding room, with a controlled environment (i.e., temperature, humidity, and light/dark cycle) when not undergoing testing. Throughout the study, the mice had access to food when not being fasted and continuous amounts of water. The animals utilized in this experiment were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of Rowan University and all testing followed the "Guide for the Care and Use of Laboratory Animals."[45]

Figure 5

CD-1 Mouse



Note. Picture of a CD-1 mouse, the strain used in these studies

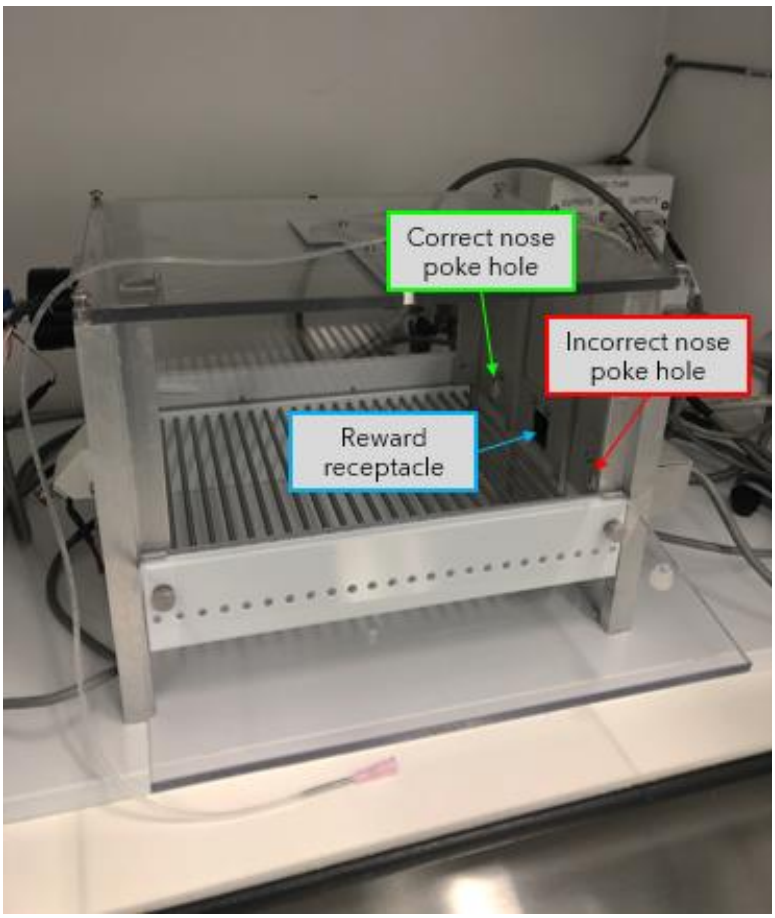
Mice are often utilized in behavioral testing for a number of reasons. Research regarding the physiology and treatment of pain often requires subjectively testing animals' reactions to drugs with abuse liability.[3] In order to test behavioral responses, an intact nervous system is necessary.[3] Since humans cannot be ethically used for this type of work, mice are a well-accepted model for studying experimental compounds and provide a handful of advantages over other animal models.[3] Firstly, mice's murine central nervous system (CNS) is comparable to humans, thus allowing the extrapolation of results.[3] Secondly, because of mice's small size, smaller amounts of drugs can be used for testing.[3] Lastly, the complete mouse genome is known, allowing for genetic study and manipulation.[3] CD-1 mice, specifically, are a very popular strain of mouse for these types of behavioral studies because they are well-characterized behaviorally and have robust behavioral responses to analgesics and drugs of abuse.[3] Additionally, CD-1 mice have a common outbred genetic background, allowing for potential genetic follow-up studies to be carried out in order to identify genetic variables affecting behavioral and cellular responses.[3]

2.3.4. Equipment

2.3.4.1. Operant Chambers. The apparatuses used for the first part of this behavioral study were operant chambers. Each operant chamber was equipped with a reward receptacle, also referred to as a liquid dipper, located between two nose poking response holes. The left nose poke hole was designated the correct hole and when poked, the animals received a reward, that being food, a mixture composed of 50% vanilla Ensure and 50% water, for this specific study. Contrastingly, the right nose poke hole was designated the incorrect hole and when poked, generated an incorrect response reading. The animals did not receive a reward when the right-side hole was poked. The mice were trained to nose poke using a fixed ratio—a fixed number of correct nose pokes required to obtain a programmed reward—or FR, system.[52] We started at an FR 1, meaning one reward per one nose poke, and increased to an FR 4, meaning one reward per four nose pokes. Additionally, each chamber consisted of a house light, ventilator fan, and a syringe pump that assisted with administering the Ensure/water rewards. The operant chambers were controlled by a PC running MED-PC (MED Associates).

Figure 6

Operant Chamber

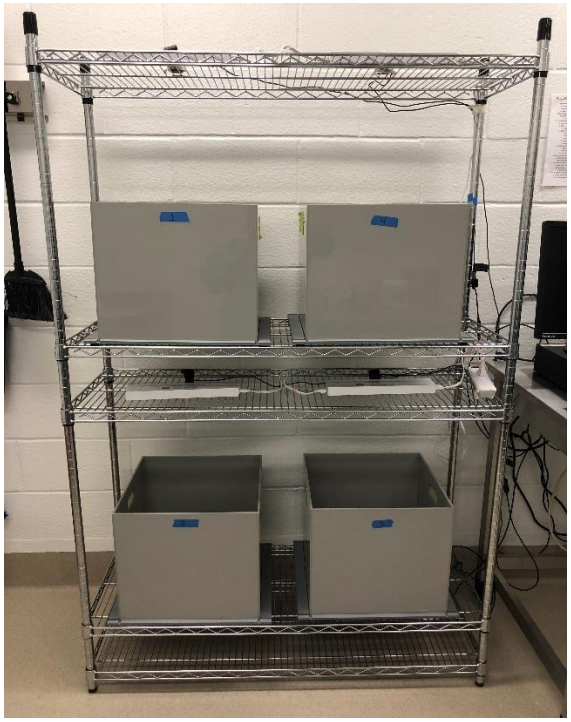


Note. Self-administration operant chamber from the Keck Animal Behavior Lab. The left hole was designated the correct nose poke hole, while the right hole was designated the incorrect nose poke hole. A reward receptacle was situated in between the two holes, which administered the mice their palatable food rewards after correctly responding.

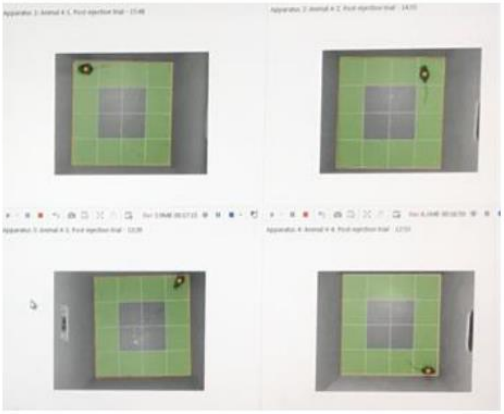
2.3.4.2. Open Field Chambers. The apparatuses used for the second part of this behavioral study were open field chambers. Each chamber was grey in color (i.e., opaque) and approximately 40 cm × 40 cm × 35 cm. The chambers consisted of high walls so that the mice were unable to escape from the apparatuses during testing. The walls fit into a slotted base that was detachable for easy cleaning. Each floor insert was also grey in color and did not consist of any gridlines, unlike some other open field chambers. Rather, the ANY-maze program that computed all the data from the testing sessions provided the gridlines for the chambers, as depicted in Figure 7 (B). A camera positioned over top of each chamber in combination with ANY-maze's tracking software tracked the individual mice's movement and location (center or perimeter) in the chambers throughout the duration of the experiment.

Figure 7

Open Field Chambers and ANY-Maze Layout



(A)



(B)

Note. **(A)** Open field chambers from the Keck Animal Behavior Lab. **(B)** Open field layout using the ANY-maze program. Each open field chamber was divided into 16 squares. The outer 12 squares represented the chamber's perimeter, as highlighted in green in the above picture, while the inner four squares represented the chamber's central region. A camera, as well as ANY-maze's tracking software tracked the mice's movement in the chambers in order to compare how much time was spent in the perimeter squares versus the inner squares after drug administration.[52]

2.3.5. Experimental Procedures

2.3.5.1. Food Self-Administration Procedure. Eight CD-1 mice were trained to nose poke for diluted Ensure for approximately 1.5 months. Each testing session was 120 minutes, or two hours, and was carried out seven days a week. Once the mice reached a consistent reward response at an FR 4, the animals were injected on designated injection days with either morphine or morphine + MP-III-024 and placed in the operant chambers. The data and results for MP-III-024 were already preestablished by Fischer *et al*. and thus the animals were not reinjected with MP-III-024 alone. The drug dose each animal received was determined using a Latin square design, so that every mouse received each drug or the vehicle exactly one time; Latin square designs are also useful when it comes to controlling potential variation between test subjects and their assigned drug doses caused by nuisance factors dependent on the day. Once in the chambers, the MED-PC program analyzed the animals' disruption in food self-administration caused by the drugs. This analysis included important information, such as number of rewards received and response rates. A "rest" day was placed in between each injection day, which consisted of regular self-administration testing (i.e., training). Furthermore, the animals were allowed to eat for two hours after daily testing sessions, then fasted overnight for ~20 hours for the following, next day experiment, since food deprivation is known to affect rodents' responsiveness toward experimental stimuli. In regard to self-administration, fasting helps to manipulate animals to work for rewards and establishes levels of motivation.

2.3.5.2. Open Field Procedure. Similar to food self-administration, eight CD-1 mice were used. Before the initiation of the open field testing, drug solutions were prepared. For the first day of testing, the mice were given an acclimation day. Each mouse

was placed in an open field chamber for 15 minutes and allowed to explore the testing apparatus. On the second day of testing, the mice received six i.p. injections of just vehicle. After each injection, the mice were placed into the open field chambers for 30 minutes and their locomotor function was recorded and processed by the cameras placed over top of the chambers and the ANY-maze tracking software installed on one of our computers. For the last day of testing, each mouse again received six injections, this time consisting of the incremental drug doses of morphine alone, MP-III-024 alone, or morphine + MP-III-024, starting with 1.0 mg/kg and ending with 14.0 mg/kg (cumulatively 32 mg/kg). After each injection, the mice were placed into the open field chambers for 30 minutes and their behavior and activity were analyzed in order to determine if their locomotor function was disrupted due to the introduction of morphine, MP-III-024, and/or the drug combination, morphine + MP-III-024.

2.4. Data and Results

2.4.1. Food Self-Administration Reward Values

Table 10

Morphine Reward Values

	Cage 1 Animal 1	Cage 1 Animal 2		Cage 1 Animal 4
Vehicle	100	82		100
3.2 mg/kg	100	60		100
10 mg/kg	100	45		68
32 mg/kg	32	8		8
	Cage 2 Animal 1	**Cage 2 Animal 2**	**Cage 2 Animal 3**	**Cage 2 Animal 4**
Vehicle	81	87	100	100
3.2 mg/kg	36	66	100	100
10 mg/kg	0	34	91	94
32 mg/kg	0	0	0	15

Table 11

Morphine + MP-III-024 (Ratio → 1.0:0.31) Reward Values

	Cage 1 Animal 1	Cage 3 Animal 4		Cage 3 Animal 2
Vehicle	100	78		100
3.2 mg/kg	100	61		66
10 mg/kg	100	15		88
32 mg/kg	0	0		0
	Cage 2 Animal 1	**Cage 2 Animal 2**		**Cage 3 Animal 3**
Vehicle	27	60		60
3.2 mg/kg	29	62		100
10 mg/kg	75	69		43
32 mg/kg	0	0		0

Table 12

Morphine + MP-III-024 (Ratio → 1.0:0.94) Reward Values

	Cage 1 Animal 1	Cage 1 Animal 2		Cage 1 Animal 4
Vehicle	100	100		32
3.2 mg/kg	100	100		27
10 mg/kg	100	100		100
32 mg/kg	62	38		29
	Cage 2 Animal 1	**Cage 2 Animal 2**	**Cage 2 Animal 3**	**Cage 2 Animal 4**
Vehicle	2	65	100	100
3.2 mg/kg	81	5	100	100
10 mg/kg	24	100	31	100
32 mg/kg	0	0	100	5

Table 13

Morphine + MP-III-024 (Ratio → 1.0:2.8) Reward Values

		Cage 3 Animal 4		Cage 3 Animal 2
Vehicle		100		100
3.2 mg/kg		90		100
10 mg/kg		68		56
32 mg/kg		11		0
	Cage 2 Animal 1	**Cage 2 Animal 2**		**Cage 3 Animal 3**
Vehicle	59	100		100
3.2 mg/kg	28	100		49
10 mg/kg	29	100		55
32 mg/kg	0	0		42

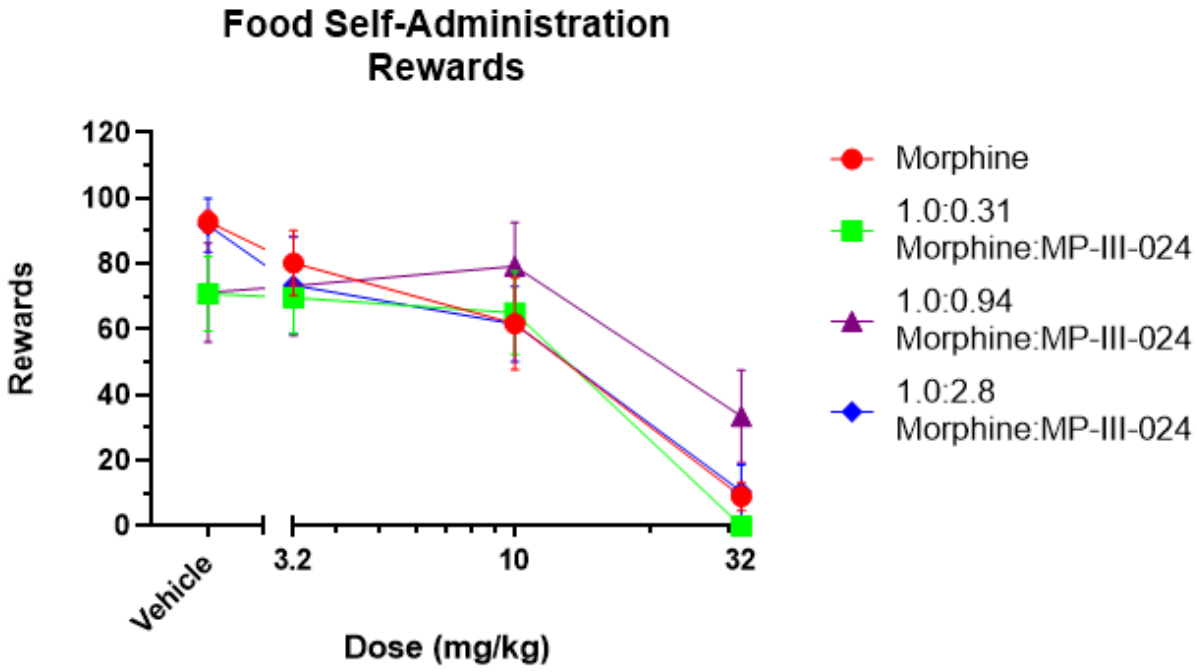
Note. **Tables 10-13:** Self-administration reward values for morphine alone and morphine in combination with MP-III-024 at varying ratios. The data collected for the vehicle, which acted as our control, was compared to the animals' reward values at 3.2, 10, and 32 mg/kg doses of morphine or morphine + MP-III-024 in order to observe the behavioral disruptions

induced by morphine and/or the drug mix. The greyed areas on the tables represent deceased animals or animals removed from the study.

2.4.2. Food Self-Administration Rewards Graph

Figure 8

Morphine and MP-III-024 Self-Administration Rewards Graph



Note. The graph above depicts the food self-administration reward values for morphine and morphine + MP-III-024, our combination drug therapy. We wanted to know if MP-III-024 would enhance or reduce morphine's behavioral effects at varying ratios. The x-axis represents the injected dose of morphine alone or morphine + MP-III-024 (3.2, 10, or 32 mg/kg), as well as our vehicle control, while the y-axis is the number of earned rewards. Two-way ANOVA revealed that morphine significantly reduced the number of earned

palatable food rewards, but the drug mix at every tested ratio (1.0:0.31, 1.0:0.94, and 1.0:2.8) did not significantly affect earned rewards in comparison to morphine alone. All results are presented as means ± SEM.

2.4.3. Food Self-Administration Response Rate Values

Table 14

Morphine Response Rate Values

	Cage 1 Animal 1	**Cage 1 Animal 2**		**Cage 1 Animal 4**
Vehicle	0.092	0.046		0.261
3.2 mg/kg	0.105	0.033		0.321
10 mg/kg	0.094	0.025		0.039
32 mg/kg	0.018	0.004		0.005
	Cage 2 Animal 1	**Cage 2 Animal 2**	**Cage 2 Animal 3**	**Cage 2 Animal 4**
Vehicle	0.045	0.049	0.089	0.061
3.2 mg/kg	0.020	0.037	0.067	0.058
10 mg/kg	0.000	0.019	0.051	0.053
32 mg/kg	0.000	0.000	0.000	0.009

Table 15

Morphine + MP-III-024 (Ratio → 1.0:0.31) Response Rate Values

	Cage 1 Animal 1	**Cage 3 Animal 4**		**Cage 3 Animal 2**
Vehicle	0.337	0.044		0.158
3.2 mg/kg	0.286	0.035		0.038
10 mg/kg	0.059	0.009		0.049
32 mg/kg	0.000	0.000		0.000
	Cage 2 Animal 1	**Cage 2 Animal 2**		**Cage 3 Animal 3**
Vehicle	0.016	0.034		0.034
3.2 mg/kg	0.016	0.035		0.057
10 mg/kg	0.010	0.038		0.024
32 mg/kg	0.000	0.000		0.000

Table 16

Morphine + MP-III-024 (Ratio → 1.0:0.94) Response Rate Values

	Cage 1 Animal 1	Cage 1 Animal 2		Cage 1 Animal 4
Vehicle	0.266	0.057		0.018
3.2 mg/kg	0.528	0.061		0.015
10 mg/kg	0.231	0.096		0.216
32 mg/kg	0.034	0.021		0.016
	Cage 2 Animal 1	**Cage 2 Animal 2**	**Cage 2 Animal 3**	**Cage 2 Animal 4**
Vehicle	0.002	0.038	0.103	0.202
3.2 mg/kg	0.046	0.003	0.073	0.170
10 mg/kg	0.013	0.074	0.017	0.081
32 mg/kg	0.000	0.000	0.115	0.003

Table 17

Morphine + MP-III-024 (Ratio → 1.0:2.8) Response Rate Values

		Cage 3 Animal 4		Cage 3 Animal 2
Vehicle		0.087		0.251
3.2 mg/kg		0.052		0.084
10 mg/kg		0.041		0.043
32 mg/kg		0.006		0.000
	Cage 2 Animal 1	**Cage 2 Animal 2**		**Cage 3 Animal 3**
Vehicle	0.033	0.071		0.062
3.2 mg/kg	0.016	0.080		0.027
10 mg/kg	0.017	0.061		0.031
32 mg/kg	0.000	0.000		0.023

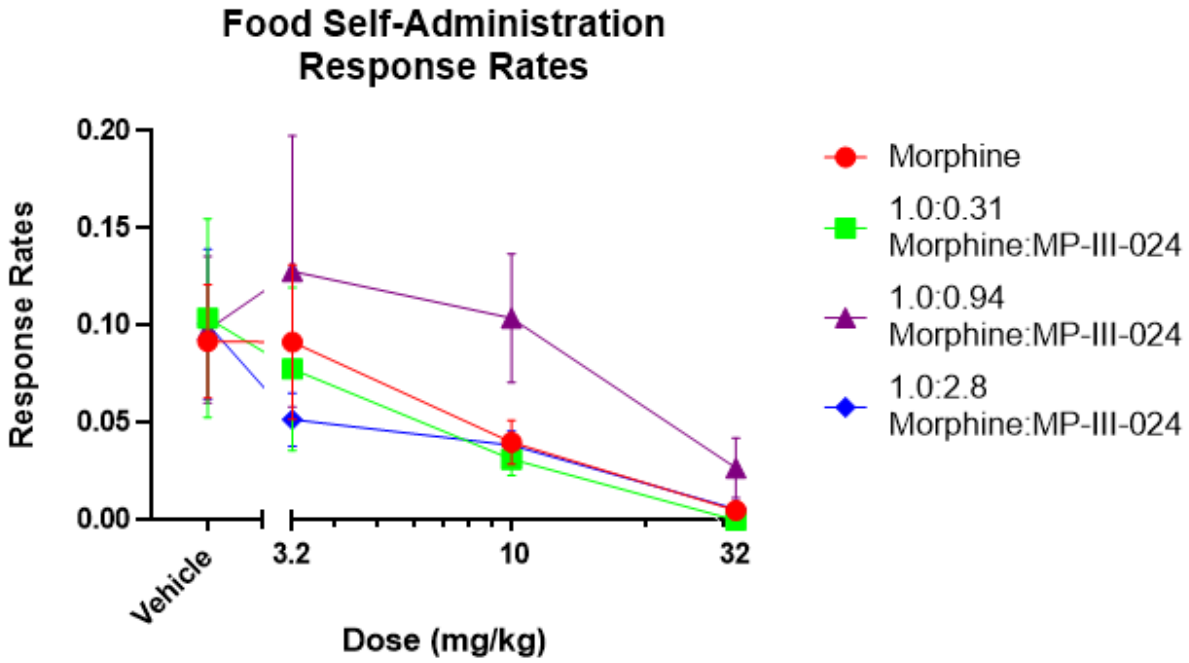
Note. **Tables 14-17:** Self-administration response rate values for morphine alone and morphine in combination with MP-III-024 at varying ratios. The data collected for the vehicle, which acted as our control, was compared to the animals' response rate values at 3.2, 10, and 32 mg/kg doses of morphine or morphine + MP-III-024 in order to observe the

behavioral disruptions induced by morphine and/or the drug mix. The greyed areas on the tables represent deceased animals or animals removed from the study.

2.4.4. Food Self-Administration Response Rates Graph

Figure 9

Morphine and MP-III-024 Self-Administration Response Rates Graph



Note. The graph above depicts the food self-administration response rate values for morphine and morphine + MP-III-024, our combination drug therapy. We wanted to know if MP-III-024 would enhance or reduce morphine's behavioral effects at varying ratios. The x-axis represents the injected dose of morphine alone or morphine + MP-III-024 (3.2, 10, or 32 mg/kg), as well as our vehicle control, while the y-axis represents the animals' response rates. Two-way ANOVA revealed that morphine significantly reduced the

animals' operant responding, but the drug mix at every tested ratio (1.0:0.31, 1.0:0.94, and 1.0:2.8) did not significantly reduce response rates in comparison to morphine alone. Therefore, it can be concluded that morphine co-administered with MP-III-024 has statistically indistinguishable effects from morphine alone but adding MP-III-024 to morphine does not make morphine more disruptive. If anything, there is a slight rightward shift at the 1.0:0.94 ratio, which is indicative of a possible subadditive (anti-synergistic) effect. All results are presented as means ± SEM.

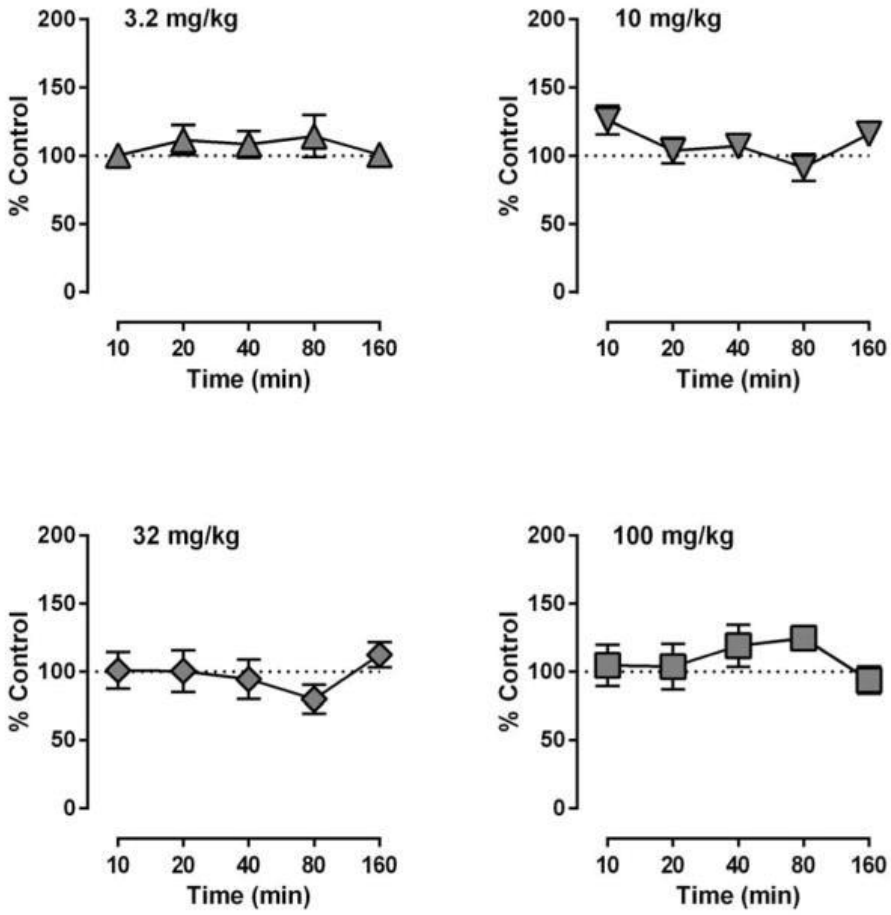
From the data collected from the MED-PC program, as well as the two-way analysis of variance (ANOVA), it was concluded that morphine induced disruption in food self-administration for the mice, especially the higher the dose. Comparingly, when morphine was given in combination with MP-III-024, we found the effects to be statistically indistinguishable from morphine alone. However, adding MP-III-024 to morphine did not make morphine more disruptive. In fact, we found that the disruption in food-self administration caused by morphine was somewhat restored when co-administered with MP-III-024, as indicated by the upper, rightward shift on the dose-response curves, most notably at the 1.0:0.94 morphine:MP-III-024 ratio. This behavioral restoration was most evident when analyzing morphine + MP-III-024's response rates in comparison to morphine's response rates. Two-way ANOVA demonstrated that the drug mix at every tested ratio (1.0:0.31, 1.0:0.94, and 1.0:2.8) did not significantly reduce the mice's motivation to nose poke for palatable food rewards. Decreases or increases in animals' response rates (in our study, how fast or slow the mice nose poked) are more indicative of true behavioral changes in comparison to other outputs, like earned rewards.

Seemingly, at an almost 1:1 ratio, morphine co-administered with MP-III-024 does not induce behavioral disruptions in food self-administration, which is suggestive of a subadditive (anti-synergistic) effect.

2.4.5. Fischer et al.'s Findings

Figure 10

Fischer et al.'s MP-III-024 Food Self-Administration Graphs



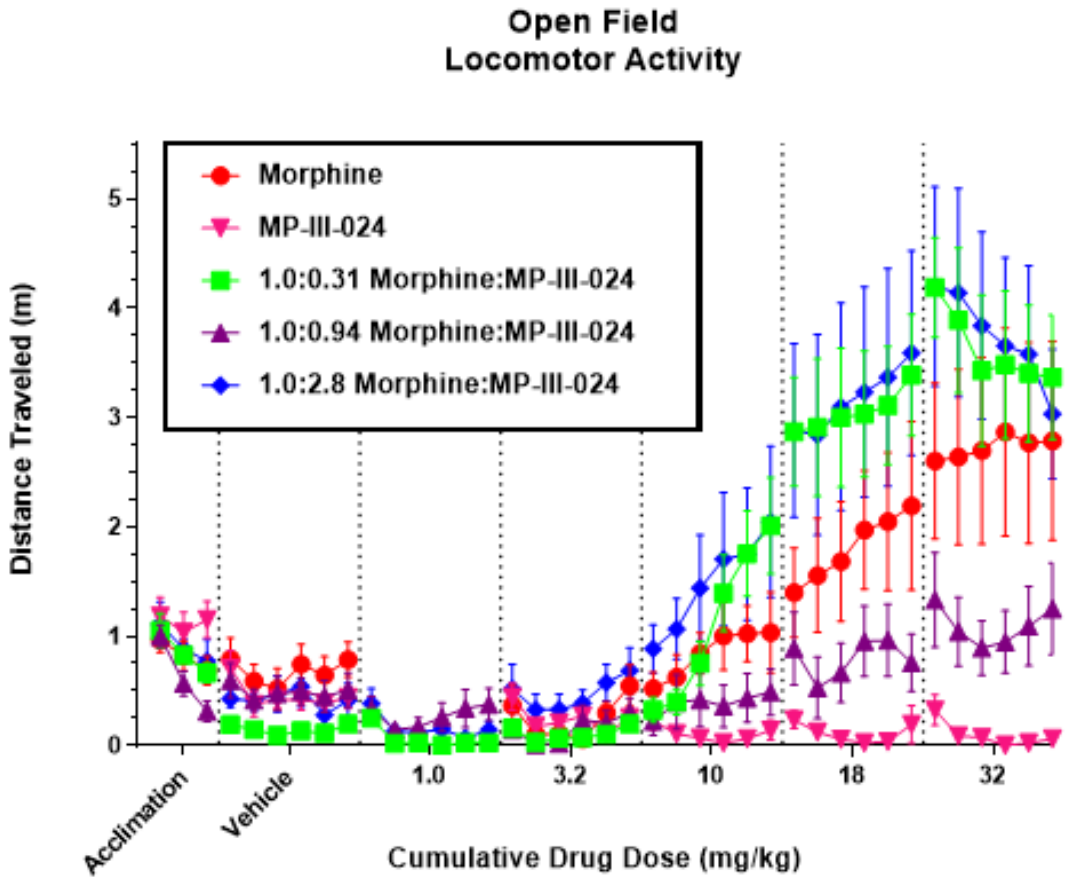
Note. Each graph represents the effects of different doses (3.2, 10, 32, and 100 mg/kg) of MP-III-024 on operant behavior. The x-axis depicts the time-course in minutes of each individual dose following i.p. injections. The behavior of the mice was accessed at 10, 20, 40, 80, and 160 minutes post-injection. Likewise, the y-axis depicts the percent control of the animals' response rate post-injection in comparison to their baseline rate, that being 0.91 ± 0.03 responses per second. Each data point is the average of 8-10 mice. This set of self-administration testing was conducted by Fischer *et al.* at Cooper Medical School of Rowan University (CMSRU) in 2017.[45]

According to Fischer *et al.*'s research paper titled *Pharmacological and antihyperalgesic properties of the novel α2/3 preferring $GABA_A$ receptor ligand MP-III-024*, MP-III-024 did not decrease the animals' operant response rates across any dose.[45] Therefore, an ED_{50} value could not be determined.[45] This is a good indication that when given in combination with morphine, a drug that is known to negatively affect operant behavior, MP-III-024 may help to neutralize morphine's side effects and fully or somewhat restore response rates to their baseline state.

2.4.6. Open Field Data and Results

Figure 11

Morphine and MP-III-024 Open Field Graph

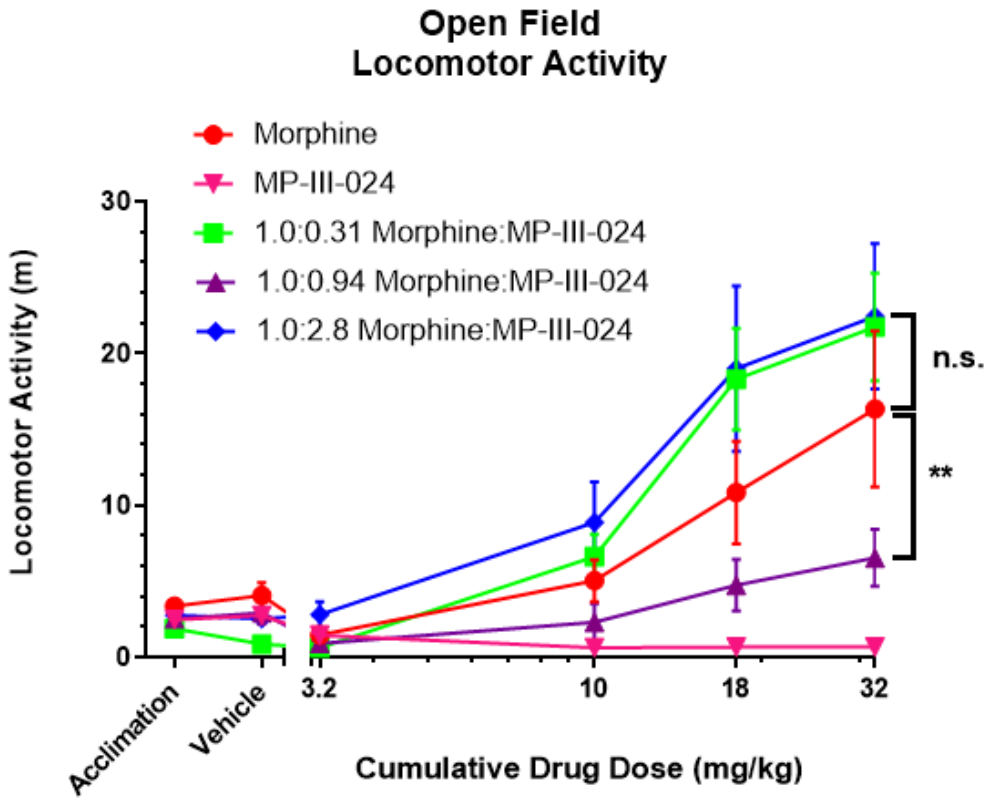


Note. Graph for open field testing which depicts the locomotor activity of the mice using a cumulative dosing procedure (n = 8 per group). The x-axis represents the cumulative drug dose each animal received every 30 minutes, while the y-axis represents the distance the mice traveled in meters while in the open field chambers during each test session. Injections consisted of morphine or MP-III-024 alone or different drug mix ratios of morphine + MP-III-024. Morphine alone induced a clear hyperlocomotive effect, while MP-III-024 alone

did not. At the 1.0:0.94 ratio of morphine in combination with MP-III-024, a rightward shift in the activation of locomotor activity was demonstrated, a subadditive (anti-synergistic) effect. Contrastingly, two-way ANOVA revealed there was not a significant difference between morphine alone and the 1.0:0.31 and 1.0:2.8 drug mix ratios, despite what looks to be an increase in locomotion at both those ratios. All results are presented as means ± SEM.

Figure 12

Morphine and MP-III-024 Open Field Graph Showing Significance



Note. Additional graph depicting the open field locomotor activity of the mice (n = 8 per group). The x-axis represents the cumulative drug dose each animal received every 30

minutes, while the y-axis represents the distance the mice traveled in meters while in the open field chambers during each test session. Two-way ANOVA determined a significant difference between morphine alone and the 1.0:0.94 ratio, as indicated by the asterisks (** means $p < 0.01$). In other words, the 1.0:0.94 ratio was statistically less than morphine alone, meaning it had a subadditive (anti-synergistic) effect. Two-way ANOVA also determined that there was not a significant difference between morphine alone and the 1.0:0.31 and 1.0:2.8 ratios, even though it looks like there could have been a synergistic effect at those two ratios when just analyzing the graph itself. All results are presented as means ± SEM.

When morphine alone was administered to the mice during open field testing, locomotor function was increased dose-dependently. In other words, morphine produced dose-dependent increases in locomotor activity. Contrastingly, when MP-III-024 alone was administered to the mice, no effect was observed, something that Fischer *et al.* also noticed back in 2017. Therefore, the effects of morphine and MP-III-024 were said to be heteroergic, with morphine having an effect and MP-III-024 lacking an effect. When given in combination, we expected to continue to see dose-dependent increases in locomotor activity, meaning more movement from the mice the higher the dosage of morphine + MP-III-024. At first, we thought this was true for two out of our three drug mix ratios, 1.0:0.31 and 1.0:2.8; but, after two-way ANOVA, it was revealed that there was not a significant difference between morphine alone and the 1.0:0.31 and 1.0:2.8 ratios. However, for the 1.0:0.94 ratio, two-way ANOVA determined there was a significant difference between morphine alone and that particular ratio. More specifically, the 1.0:0.94 ratio was

statistically less than morphine alone, as demonstrated by the rightward shift for that proportion on the open field locomotor activity graphs (Figures 11 and 12). Along with our inferences from the statistical analysis, this rightward shift in the activation of locomotor activity is indicative of a subadditive (anti-synergistic) effect. Together, with our previous findings, it can be concluded that by adding a $\alpha2/\alpha3GABA_A$ PAM to a MOR agonist, the analgesic-like effects of the MOR agonist can be potentiated without simultaneously increasing (and possibly even decreasing) effects unrelated to analgesia, like locomotor function, as witnessed through our open field testing.

2.5. Discussion

In conjunction with the data and results collected throughout the duration of this study and the insight provided by Fischer *et al.*'s publication regarding MP-III-024, it was concluded that the drug mix, morphine + MP-III-024, at all three tested ratios (1.0:0.31, 1.0:0.94, and 1.0:2.8) was statistically indistinguishable from morphine alone; however, adding MP-III-024 to morphine did not make morphine more disruptive during food self-administration. This means that unlike Rahman *et al.*'s findings, morphine + MP-III-024 are not always synergistic when administered in combination. If anything, there was a slight rightward shift at the 1.0:0.94 ratio, most obvious when looking at the food self-administration response rate graph (Figure 9). This is indicative of a possible subadditive (anti-synergistic) effect. Subadditivity regarding drug combinations "occurs when one drug interferes with the action of the other to decrease its effect."[2] The effect of morphine in combination with MP-III-024 at an almost 1:1 ratio would be subadditive rather than antagonistic because each individual drug is working on a different site of action, morphine

being μ-opioid receptors (MORs) and MP-III-024 being α2/α3GABA$_A$ receptors. When looking at each drug alone, morphine negatively impacted food self-administration, something that we expected to happen based on opioids' known side effects. Contrastingly, MP-III-024, when administered by itself, did not disrupt rodent operant response rates across any dose when compared to the vehicle control, thus leading Fischer *et al.* to conclude that MP-III-024 was ineffective at inducing behavioral toxicity.[45] With the results of this study, we now know that morphine + MP-III-024 is not universally synergistic; therefore, this combination drug therapy may be able to fully promote analgesia without posing a lot of harmful health risks.

Furthermore, in regard to open field testing, we found that morphine + MP-III-024 also has a subadditive (anti-synergistic) effect when it comes to locomotor function at a 1.0:0.94 morphine to MP-III-024 ratio. This conclusion was based on our statistical analysis (i.e., two-way ANOVA), as well as the rightward shift that was produced when morphine was co-administered with MP-III-024 at this ratio. Solely, morphine is known to increase locomotion in rodent models, especially at higher doses. However, MP-III-024 alone was found to be ineffective.

So far, we now know that morphine + MP-III-024 has a synergistic effect regarding analgesia and a subadditive (anti-synergistic) effect regarding behavioral disruption and locomotion at approximately a 1:1 ratio. These findings suggest that unlike morphine, which dampens pain signals and responses in the central and peripheral nervous system, MP-III-024 may only dampen pain signals, not necessarily pain responses. In other words, MP-III-024 may only impact the PNS, not the CNS, therefore causing a combination therapy of morphine and this imidazodiazepine PAM to be void of a synergistic side effect

profile, especially if MP-III-024 is not interacting with the CNS, which controls most functions of the body and mind[91]. Additionally, one of the reasons we might not have seen a synergistic side effect profile in this drug combination is because MP-III-024 is $\alpha 2/3GABA_A$ selective; Fischer *et al.*'s study showed that there are little to no side effects associated with the $\alpha 2/3GABA_A$ receptors.[45] Rather, the negative side effects associated with benzodiazepines, such as sedation and respiratory depression, as discussed previously, are linked to the $\alpha 1GABA_A$ receptors.[45] Thus, a dual MOR-$\alpha 2/\alpha 3GABA_A$-acting pharmacotherapy that treats pain with minimal to no side effects is achievable.

Nevertheless, in order to determine the full therapeutic window of this dual pharmacology approach, additional testing will need to be carried out; tolerance will be measured by repeated hot plate testing, constipation will be assessed by a charcoal transit assay, respiratory depression will be evaluated by plethysmography, and abuse liability will be analyzed using conditioned place preference (CPP) tests, which are currently underway. If successful, these studies will identify a new method to enhance opioid analgesia without requiring high doses of opioid medications to be taken, in turn reducing the likelihood of patients developing opioid dependence and addiction.

It is important to acknowledge that there were some limitations in regard to this experiment. The most notable limitation is the fact that this study only evaluated male mice, not female mice. This is something to consider, especially since there are noteworthy sex-related differences in male and female mice, which can influence behavior and responses to drugs of abuse.[52] For example, there are sex-mediated differences in opioid receptor expression and signaling.[46] This could potentially have an impact on the drug mix results. Perhaps, the data would be variable if only female mice or a mixture of male and female

mice were utilized. Moreover, it is important to address the fact that prolonged and/or repeated intraperitoneal administration can cause a degree of stress and discomfort in mice (this is especially true for cumulative dosing); injection retraining techniques may also add a level of stress.[92] When the drug mix injections were initiated, the animals had already been poked a handful of times from the morphine and/or MP-III-024 injections and additional injections may have become a stressor to the animals, thus causing some of the mice to underperform. Additionally, a few of our mice used for self-administration were found to have pre-existing health issues, which we discovered after initiating testing. This could also have impacted some of the animals' operant responding.

CHAPTER 2: KEY TAKEAWAYS

Self-Administration Conclusions:

- ❖ Morphine induced behavioral disruptions in the mice, especially when administered at high doses, thus negatively impacting food self-administration
- ❖ MP-III-024 did not disrupt the animals' operant response rates across any dose when compared to the vehicle control, thus producing negligible effects regarding food self-administration
- ❖ MP-III-024 did not alter morphine-induced behavioral disruption; in fact, MP-III-024 may have produced a subadditive (anti-synergistic) effect at the 1.0:0.94 morphine:MP-III-024 ratio

Open Field Conclusions:

- ❖ Morphine increased locomotor activity, whereas MP-III-024 had no effect on locomotor function
- ❖ The 1.0:0.31 and 1.0:2.8 morphine:MP-III-024 ratios produced a non-significant enhancement of morphine-induced hyperlocomotion
- ❖ The 1.0:0.94 morphine:MP-III-024 ratio demonstrated a statistically significant subadditive (anti-synergistic) effect

Overall Conclusions:

- Morphine + MP-III-024 enhances analgesia-like effects
- MP-III-024 does not enhance morphine induced disruptions
- Therefore, MP-III-024 is NOT universally synergistic with morphine

NOTE: Interactive effects of drug mixes depend on their relative proportions; there are key differences in the 1.0:0.31, 1.0:0.94, and 1.0:2.8 morphine:MP-III-024 ratios' effects

Chapter 3

The Effects of the Dopamine D4 Receptor Antagonist CAB-01-019 on Alcohol and Palatable Food Self-Administration

3.1. Abstract

Alcohol use disorder (AUD) is characterized as an uncontrollable drinking problem as a result of physical and/or emotional dependence on alcohol. According to the National Survey on Drug Use and Health (NSDUH), nearly 15 million people ages 12 and over suffer from AUD in the United States. This chronic disease continues to be a major health issue with little relief from current pharmacotherapeutic treatments. Therefore, AUD requires the identification of new targets for developing alternative treatment options. In this study, the dopamine D4 receptor (D_4R) full antagonist, CAB-01-019, was explored as a potential therapy for AUD. This experimental compound was tested on eight CD-1 mice to see how its effects impact food self-administration. Over a two-month period, the mice were trained to nose poke for palatable food rewards in the form of vanilla Ensure and water. After the animals were fully trained, they were injected with either a vehicle control or CAB-01-019 in order to observe the drug's effect on the mice's behavioral responses in comparison to the control. Through one-way ANOVA, it was demonstrated that CAB-01-019 did not significantly reduce operant responding at any of the three tested doses (10, 17.8, and 30 mg/kg) when looking at reward and response rate values. Since CAB-01-019 did not evoke any type of behavioral disruption, we can continue on with our testing and see its effect on rodent models of alcohol addiction. We propose CAB-01-019 will reduce alcohol-taking and -seeking behaviors during ethanol self-administration.

3.2. Introduction

Just like opioid use disorder (OUD) is a major health problem around the globe, so is alcohol use disorder (AUD), sometimes known as alcoholism. AUD is medically characterized as a brain disorder that impairs an individual's ability to discontinue or control alcohol consumption regardless of adverse social, occupational, and/or health consequences.[57] Lasting changes in the brain as a result of alcohol misuse often perpetuate AUD and make those who readily abuse alcohol more susceptible to relapse.[57] According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), a person's risk of developing AUD is directly correlated to how much, how often, and how quickly one consumes alcohol.[57] Nevertheless, there are other factors that increase the risk of AUD, such as drinking from an early age, genetics and family history of alcohol issues, mental health conditions (e.g., depression, PTSD, and ADHD), and a history of trauma, especially trauma stemming from childhood.[57]

The 2019 National Survey on Drug Use and Health (NSDUH) estimated that 414,000 adolescents between the ages of 12 to 17, 1.7% of this age group, suffer from AUD.[93] Underage drinking not only interferes with normal adolescent brain development, but contributes to a variety of acute consequences, including injuries (e.g., falls, burns, and drownings), sexual assaults, alcohol overdoses, and deaths, especially from motor vehicle crashes, suicides, and homicides.[93] In comparison, the NSDUH estimated that 14.5 million people ages 12 and older, 5.3% of this age group, suffer from AUD.[93] This number incorporates 9.0 million men and 5.5 million women.[93] Each year, approximately 95,000 people succumb to alcohol-related causes, thus making alcohol the third-leading preventable cause of death in the United States behind tobacco use and poor diet and

physical inactivity, respectively.[93] For example, in 2019, alcohol impaired driving fatalities were responsible for 10,142 deaths, accounting for 28.0% of the overall driving fatalities for that year.[93] The World Health Organization (WHO) considers the harmful use of alcohol to be a causal factor in the development of more than 200 diseases and injury related health conditions, which may lead to premature death and disability.[93]

Alcohol has short- and long-term effects on the body. After substantial oral absorption has taken place following consumption of an alcoholic beverage, alcohol enters the bloodstream.[94] The more a person drinks, the higher the blood alcohol concentration (BAC) climbs.[94] As an individual's BAC elevates, a greater degree of intoxication and impairment occur.[94] These effects include but are not limited to slurred speech, motor impairment, confusion, and memory and concentration problems.[94] Alcohol's immediate effects appear within 10 minutes of consumption.[94] Excessive alcohol use, whether on a single occasion (binge drinking) or over time (chronic drinking), can negatively impact one's health and lead to long-term health problems, most notably associated with the brain, heart, liver, pancreas, and immune system.[95] Heavy drinking, especially, takes a serious toll on the liver and can lead to a variety of complications, like steatosis (or fatty liver), alcoholic hepatitis, fibrosis, and cirrhosis.[95] Likewise, drinking alcohol increases the risk of a person developing several kinds of cancers, such as head and neck (i.e., mouth, pharynx, and larynx), esophagus, liver, breast, and colorectal.[96]

Although alcohol affects many different aspects of the body, the area we are most concerned about in this study is the brain, specifically its dopamine receptors. It is no secret that alcohol has a profound effect on the complex structures of the brain.[97] Alcohol blocks chemical signals between brain cells (i.e., neurons), thus resulting in immediate symptoms

of intoxication, as discussed previously.[97] If excessive drinking persists over an extended period of time, the brain begins to adapt to these blocked signals by over activating specific brain chemicals (i.e., neurotransmitters).[97] This, in turn, may result in neurotoxicity; neurotoxicity occurs when neurons overreact to neurotransmitters for too long, eventually causing certain neurons to "burn out."[97] Along with pathway damage, heavy drinking has the potential to harm brain matter itself.[97] Individuals with alcohol dependence oftentimes experience brain shrinkage, a reduction in the volume of gray and white matter that compose the brain.[97] Gray matter consists primarily of cell bodies, whereas white matter is associated with the cell pathways of the central nervous system (CNS).[97]

Formerly, scientists believed that alcohol acted as a membrane disruptor that inflicted a generalized effect all over the brain, especially since its small molecules have the ability to freely diffuse and penetrate the blood-brain barrier (BBB).[64] However, scientists now know that this notion is not entirely true; rather, there are particular structures in the brain that alcohol targets, the most notable being γ-aminobutyric acid (GABA) receptors, glutamate receptors, and the nucleus accumbens.[64] GABA is known to be the major inhibitory neurotransmitter in the brain.[64] Alcohol is thought to imitate GABA's inhibitory effect by binding to GABA receptors and hindering neuronal signaling.[64] Likewise, alcohol has been found to inhibit glutamate, the major excitatory neurotransmitter in the brain, especially at the N-methyl-D-aspartate (NMDA) glutamate receptor.[64] Both GABA and glutamate are often associated with the sedative effects of alcohol.[98] The nucleus accumbens, on the other hand, plays a major role in the brain's reward pathway.[99] This important middle brain structure helps to maintain motivation, pleasure, satiety, and memories.[99] When consumed, alcohol tends to activate the brain's

whole reward system.[64] Consumption, even in small doses, enhances the amount of dopamine, the neurotransmitter that mediates pleasure in the brain, released by the nucleus accumbens.[64] Since the nucleus accumbens is part of the neuronal circuit that regulates reward-seeking behavior and alcohol produces feelings of euphoria and well-being by intensifying the release of dopamine throughout the body, the brain is easily tricked into thinking that alcohol is a system of positive reinforcement; this is one of the main reasons why alcohol is so addictive and provokes relapse.[64] Along with dopamine, alcohol also affects serotonin and acetylcholine activity.[98]

Due to alcohol's profound effect on the brain's dopaminergic receptors, it is plausible to hypothesize that medications that target dopamine receptors may be up-and-coming treatment options for people suffering from AUD. Previous studies have shown that antagonism of the dopamine D4 receptor (D_4R) reduces drug-taking and -seeking behaviors in rodent models of cocaine addiction, therefore representing a new, explorable area of medication development for various substance use disorders.[66] Our drug of interest for this study, CAB-01-019, is a full antagonist of D_4R.[66] D_4Rs are G protein-coupled receptors (GPCRs) and are a member of the D_2-like subfamily of dopamine receptors.[66] In comparison to the other D_2-like receptors (i.e., D_2R and D_3R), D_4Rs exhibit the lowest level of expression in the brain but have a distinct distribution.[66] D_4Rs are mainly expressed in the prefrontal cortex and hippocampus, where they are involved with neuronal functions that affect attention and exploratory behavior, as well as performance in object recognition and inhibitory avoidance cognitive tasks.[66] Originally, budding medications aimed at targeting D_4Rs were thought to be good candidates for combating certain antipsychotic conditions, such as schizophrenia.[66] While D_4R proved to be an

unsuccessful target for schizophrenia treatment, recent studies utilizing the full antagonist, CAB-01-019, have shown D_4Rs to have potentiality when it comes to being a pharmacological target for the treatment of addiction.[66] Preliminary data using this drug demonstrated that it attenuated cocaine self-administration in rats (personally communicated to Dr. Keck by Takato Hiranita and Scott Hemby); cocaine is a highly addictive stimulant drug that, similar to alcohol, has a profound effect on the dopaminergic receptors of the brain.[66] In conjunction with these findings and the commonalities between cocaine and alcohol's effect on dopamine, we propose that CAB-01-019 will also attenuate drug-taking and -seeking behaviors in animal models of AUD and prove to be a promising new avenue for AUD medication development.

To date, there are three FDA-approved medications on the market for the treatment of AUD: disulfiram, naltrexone, and acamprosate.[59] Disulfiram was the first to be approved by the FDA in 1951.[63] This drug works by inhibiting the metabolism of alcohol, thus causing acetaldehyde, a highly toxic substance, to build up in the body.[63] As a result of this, when a person drinks even the smallest amount of alcohol, unpleasant symptoms (e.g., nausea, heart palpitations, and flushing) rapidly arise.[59,63] Therefore, disulfiram is said to be a psychological deterrent to alcohol use.[63] Naltrexone, on the other hand, was approved by the FDA in 1994 as an oral medication and then again in 2006 as an extended-release injectable.[63] Unlike disulfiram, this drug is a pure opioid receptor antagonist, meaning it blocks the pleasurable effects of alcohol by inhibiting the μ-opioid receptor.[59,63] Alcohol consumption is known to stimulate endogenous opioid release and enhance dopamine transmission throughout the body.[59] If naltrexone makes alcohol ingestion less rewarding by interfering with its euphoric effect, then it is expected that

heavy drinking habits will eventually decrease.[63] Likewise, the last drug to be approved by the FDA in 2004 as a potential treatment option for people suffering from AUD was acamprosate.[63] Although its mechanism of action remains uncertain, this drug is believed to help restore the homeostasis between neuronal excitation (glutamatergic) and inhibition (GABAergic) that heavy drinking, as well as withdrawal, are known to dysregulate through interactions with NMDA receptors.[59,63] Research has shown acamprosate to be most effective at maintaining abstinence in patients experiencing alcohol dependence.[59] Even though there are several treatment options out there when it comes to treating AUD, as just discussed, there is still room for new pharmacotherapies to be explored that offer more precise receptor selectivity, less adverse effects, and strong compliance rates. We hypothesize that CAB-01-019, due to its D_4R antagonism, will attenuate alcohol-taking and -seeking behaviors and thus could be a new AUD therapeutic.

In order to determine whether CAB-01-019 affects alcohol-taking and -seeking behaviors, we proposed to determine its effects on alcohol self-administration in mice. Herein, we report the effects of CAB-01-019 on palatable food self-administration in mice, a test that serves as 1) a control for alcohol tests, separating alcohol-specific effects from non-specific behavioral and appetitive effects, and 2) as a training precursor for ethanol self-administration; animals are trained to self-administer ethanol only after learning to self-administer food. The future directions of this experiment seek to determine CAB-01-019's effects on operant alcohol self-administration in rodent models of AUD.

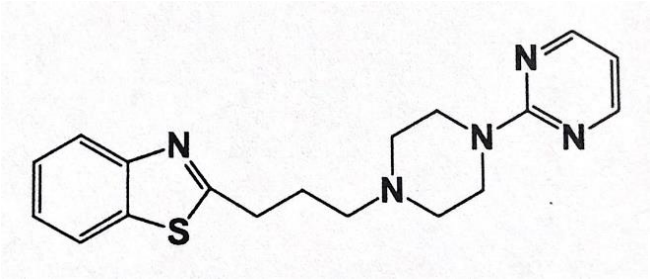
3.3. Materials and Methods

3.3.1. Drug

3.3.1.1. CAB-01-019. Our drug of interest for this study was CAB-01-019, a full antagonist of the dopamine D4 receptor (D_4R), as measured by $G\alpha_{i/o}$-mediated signaling and β-arrestin2 recruitment (manuscript in preparation).[66] CAB-01-019 was synthesized by our collaborators at High Point University. This drug has been found to dose-dependently attenuate intravenous cocaine self-administration in rats (personally communicated to Dr. Keck by Takato Hiranita and Scott Hemby).[66] Importantly, studies by our collaborators have determined that 17.8 mg/kg of CAB-01-019 significantly reduces cocaine intake; non-specific effects (e.g., reduced food self-administration, reduced internal body temperature) are evident only at 32 mg/kg or higher.[66] Additionally, CAB-01-019 has facile membrane permeation.[66] During central nervous system multiparameter optimization (CNS MPO) testing, which helps evaluate whether certain drugs will be able to cross the blood-brain barrier (BBB), CAB-01-019 scored a 4.5; scores ≥4 have demonstrated a correlation to CNS penetrance.[66,100] When compared to the known brain-penetrant CNS ligand, buspirone, CAB-01-019's permeability (27×10^{-6} cm/s) surpassed buspirone's previously established permeability value (25×10^{-6} cm/s), thus suggesting that CAB-01-019 should be able to easily penetrate the BBB and produce a therapeutic effect.[66]

Figure 13

Chemical Structure of CAB-01-019



[66]

3.3.2. Drug Dosing

CAB-01-019 was administered at three doses, 10, 17.8, and 30 mg/kg, during this experiment, using a Latin square design. A vehicle composed of 5% Tween 80 and 5% propylene glycol in 0.9% NaCl (saline) was also used as our control. Both CAB-01-019 and the vehicle were administered to the mice via intraperitoneal (i.p.) injections. The amount of injected solution was based on each animal's body weight; weights were determined on the morning of designated injection days.

3.3.3. Animals

The animals used for testing were drug-naïve adult male CD-1 mice obtained from Charles River Laboratories. Similar to our morphine and MP-III-024 addiction and pain study, the animals were not exposed to any kind of behavioral or pharmacological manipulation prior to this experiment. Each mouse weighed between 30-45 grams; weights often fluctuated due to daily fasting. The animals were cared for in accordance with the

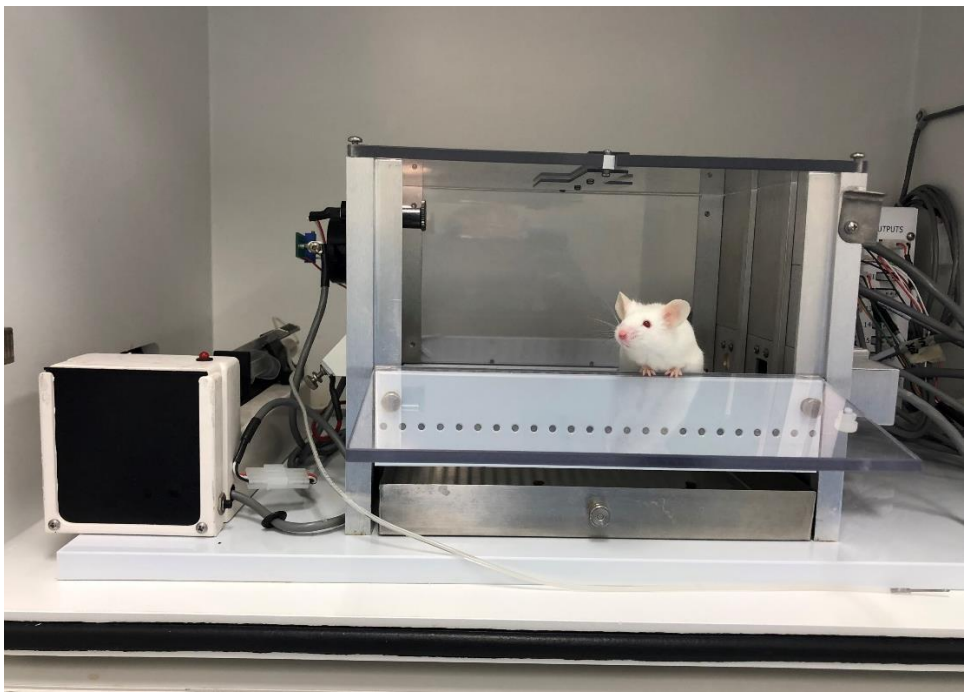
guidelines of the Institutional Animal Care and Use Committee of Rowan University and all testing followed the “Guide for the Care and Use of Laboratory Animals.”

3.3.4. Equipment

3.3.4.1. Operant Chambers. The apparatuses used for this behavioral study were operant chambers. As previously mentioned in the subsequent chapter on morphine and MP-III-024, each operant chamber was equipped with a reward receptacle located between two nose poking response holes. The left nose poke hole was designated the correct hole and when poked, the animals received a food reward, that being a 50/50 combination of vanilla Ensure and water. Contrastingly, the right nose poke hole was designated the incorrect hole and when poked, did not render a food reward for the animal. Over a period of about two months, the mice were trained to nose poke using a fixed ratio (FR) system. The animals were started at an FR 1 and gradually increased to an FR 4.

Figure 14

Animals and Testing Apparatus for CAB-01-019 Self-Administration



Note. Picture of a CD-1 mouse in an operant chamber. This type of animal and testing apparatus was used for our CAB-01-019 food self-administration experiment.

3.3.5. Experimental Procedure

3.3.5.1. Food Self-Administration Procedure. Eight CD-1 mice were trained to nose poke for rewards for approximately two months. Each testing session was 120 minutes, or two hours, and was carried out seven days a week. Once the mice reached a consistent reward response at an FR 4, the animals were injected on designated injection days with CAB-01-019 and placed in the operant chambers. Once in the chambers, the MED-PC program analyzed the animals' disruption in food self-administration caused by

the drug. This analysis included important information, such as number of rewards received and response rates. A "rest" day was placed in between each injection day, which consisted of regular self-administration testing (i.e., training). The animals were allowed to eat for one hour after daily testing sessions, then fasted overnight for ~21 hours for the following, next day experiment.

3.4. Data and Results

3.4.1. Food Self-Administration Reward Values

Table 18

CAB-01-019 Reward Values

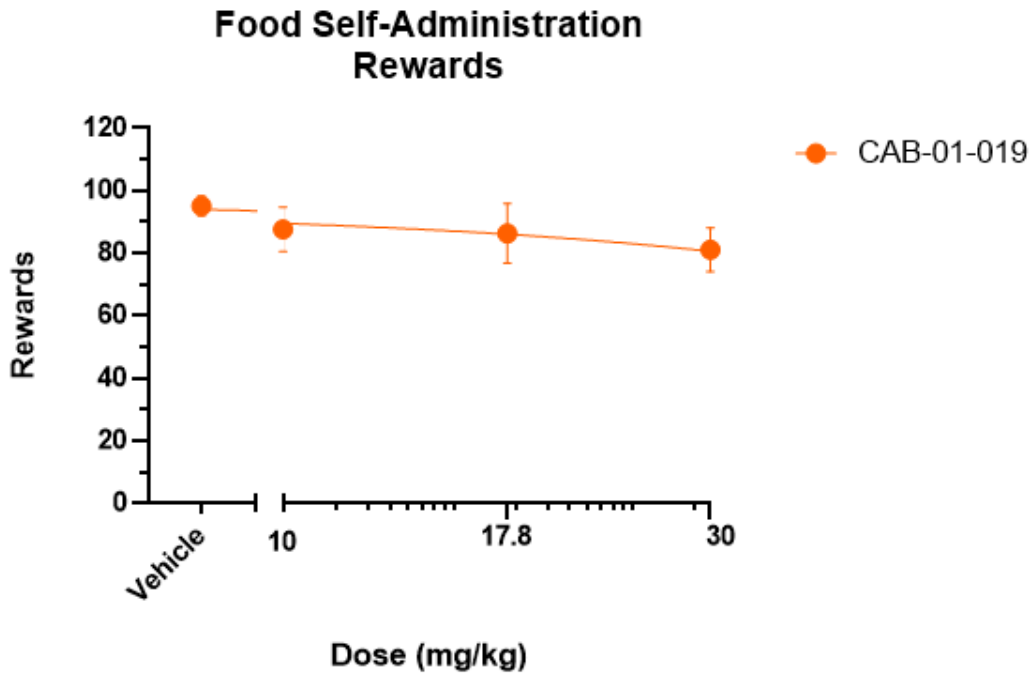
	Cage 1 Animal 1	Cage 1 Animal 2	Cage 1 Animal 3	Cage 1 Animal 4
Vehicle	100	100	100	78
10 mg/kg	100	77	100	44
17.8 mg/kg	100	98	95	75
30 mg/kg	81	92	100	56
	Cage 2 Animal 1	**Cage 2 Animal 2**	**Cage 2 Animal 3**	**Cage 2 Animal 4**
Vehicle	100	100	83	100
10 mg/kg	100	100	80	100
17.8 mg/kg	100	100	23	100
30 mg/kg	98	72	50	100

Note. The chart above displays the food self-administration reward values for the full D_4R antagonist, CAB-01-019, at 10, 17.8, and 30 mg/kg (n = 8). The rewards earned at these three doses were compared to each animal's vehicle reward value. A vehicle composed of 5% Tween 80 and 5% propylene glycol in 0.9% NaCl (saline) was used as our control. Our goal was to see if CAB-01-019 induces behavioral disruptions.

3.4.2. Food Self-Administration Rewards Graph

Figure 15

CAB-01-019 Food Self-Administration Rewards Graph



Note. The graph above depicts the food self-administration reward values of CAB-01-019 (n = 8). The x-axis represents the injected dose of CAB-01-019 (10, 17.8, or 30 mg/kg), as well as our vehicle control, while the y-axis is the number of earned rewards. One-way ANOVA revealed that CAB-01-019 did not significantly reduce palatable food self-administration at any of the three doses. All results are presented as means ± SEM.

3.4.3. Food Self-Administration Response Rate Values

Table 19

CAB-01-019 Response Rate Values

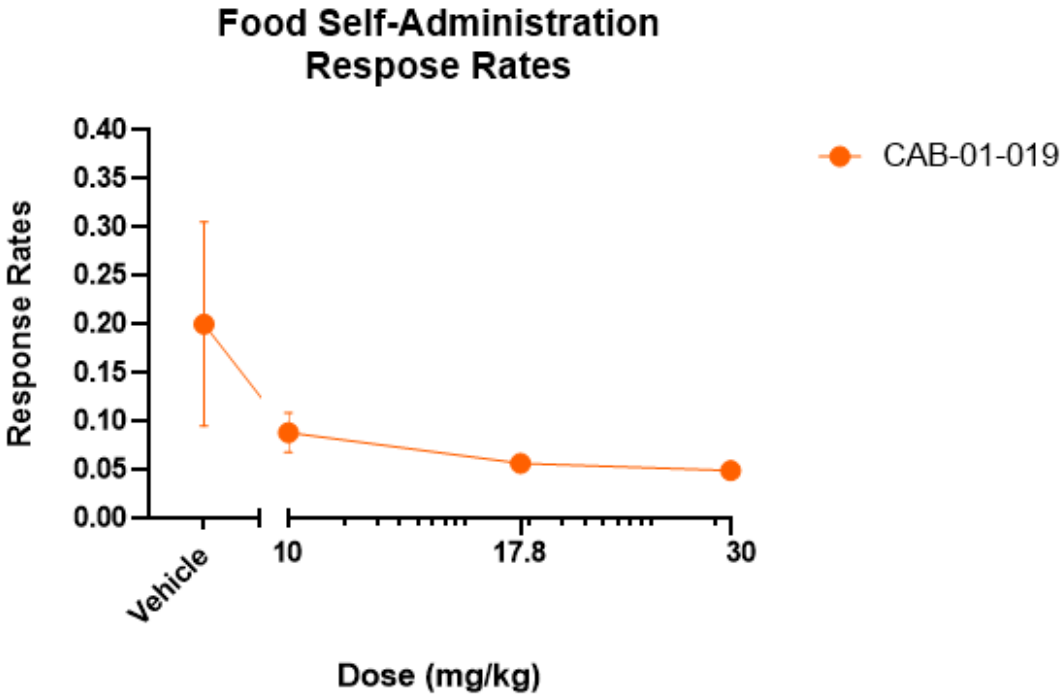
	Cage 1 Animal 1	Cage 1 Animal 2	Cage 1 Animal 3	Cage 1 Animal 4
Vehicle	0.187	0.110	0.059	0.044
10 mg/kg	0.096	0.044	0.087	0.025
17.8 mg/kg	0.069	0.055	0.053	0.042
30 mg/kg	0.045	0.052	0.082	0.031
	Cage 2 Animal 1	**Cage 2 Animal 2**	**Cage 2 Animal 3**	**Cage 2 Animal 4**
Vehicle	0.921	0.060	0.047	0.176
10 mg/kg	0.189	0.070	0.044	0.157
17.8 mg/kg	0.106	0.059	0.013	0.061
30 mg/kg	0.054	0.040	0.028	0.066

Note. The chart above displays the food self-administration response rate values for CAB-01-019, at 10, 17.8, and 30 mg/kg (n = 8). The response rates at these three doses were compared to each animal's vehicle response rate, which acted as our control.

3.4.4. Food Self-Administration Response Rates Graph

Figure 16

CAB-01-019 Food Self-Administration Response Rates Graph



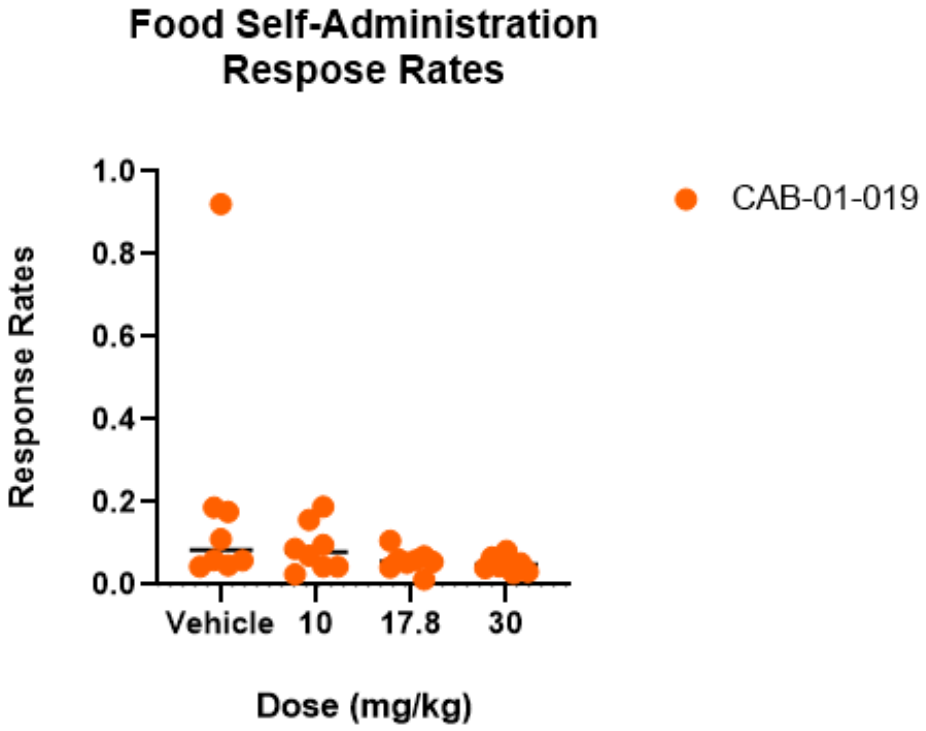
Note. The graph above depicts the food self-administration response rate values of CAB-01-019 (n = 8). The x-axis represents the injected dose of CAB-01-019 (10, 17.8, or 30 mg/kg), as well as our vehicle control, while the y-axis represents the animals' response rates. One-way ANOVA revealed that CAB-01-019 did not significantly reduce operant responding at any of the three doses. Therefore, it can be concluded that CAB-01-019 does not induce behavioral disruptions in mice trained to self-administer food. All results are presented as means ± SEM.

From the rewards and response rates data collected from the MED-PC program, as well as the one-way ANOVA, it was revealed that CAB-01-019 did not significantly attenuate food self-administration at any of the three tested doses (10, 17.8, or 30 mg/kg). This means that this dopamine D4 receptor (D_4R) full antagonist may be able to reduce drug-taking and -seeking behaviors with few disruptive side effects.

3.4.5. Food Self-Administration Response Rates Graphs Taking into Consideration the Outlier Vehicle Animal

Figure 17

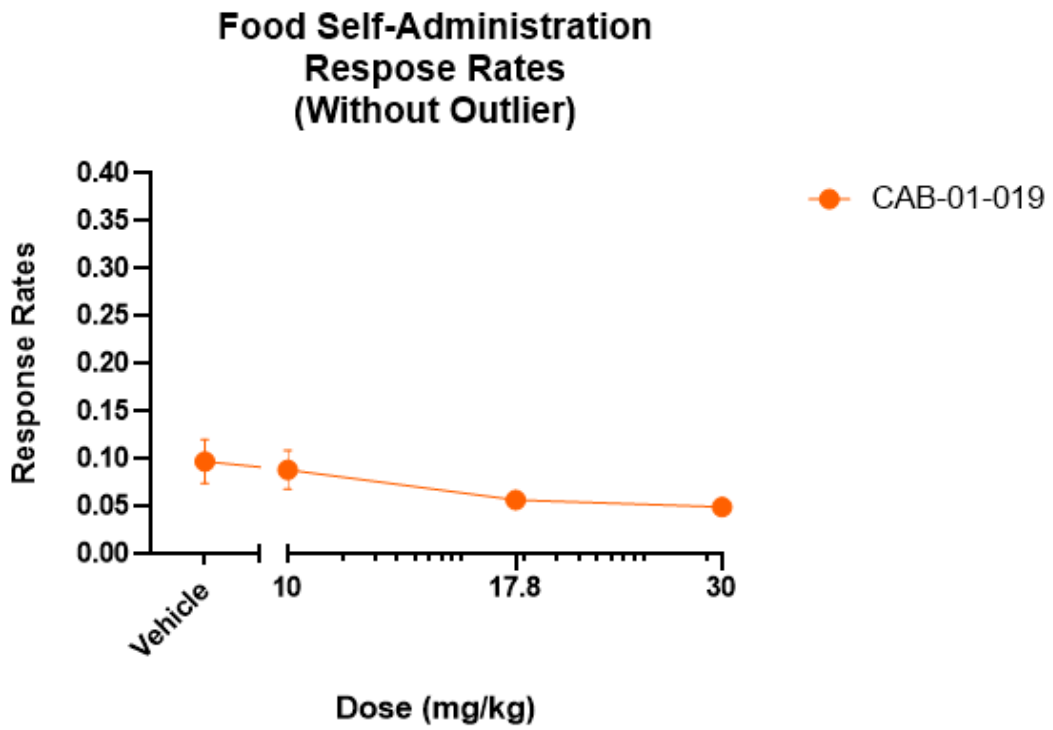
Response Rates Graph Focusing on the Outlier Vehicle Animal



Note. Food self-administration response rate graph for CAB-01-019 depicting the outlier animal in the vehicle group, which contributed to the long error bars in the preceding response rate graph (Figure 16). When it comes to response rates during self-administration, a normal response rate is considered to be around 0.1. However, this particular outlier had a response rate of 0.921. A number of factors could contribute to this above average value, such as the animal being really well-trained or extremely hungry on that specific test day.

Figure 18

Response Rates Graph Without the Inclusion of the Outlier Vehicle Animal



Note. The graph above depicts the food self-administration response rate values of CAB-01-019 without the outlier (n = 7 for vehicle group). The x-axis represents the injected dose of CAB-01-019 (10, 17.8, or 30 mg/kg), as well as our vehicle control, while the y-axis represents the animals' response rates. What is notable in this graph is the length of the error bars for the vehicle dose in comparison to the preceding food self-administration response rate graph (Figure 16) with the outlier still averaged in. Without the outlier animal, the margin of error for the vehicle is much smaller. All results are presented as means ± SEM.

3.5. Discussion

When it comes to alcohol use disorder (AUD), treatment options are scarce and the few available are often underused. According to the 2019 National Survey on Drug Use and Health (NSDUH), an estimated 7.2% of the people suffering from AUD received any treatment in the past year.[93] Likewise, less than 4% of individuals diagnosed with AUD were prescribed one of the three medications approved by the U.S. Food and Drug Administration (FDA) to treat their disorder.[93] This is concerning. Although disulfiram, naltrexone, and acamprosate are on the market to help people stop or reduce their drinking habits and prevent relapse, medication compliance tends to be poor, especially as a result of their side effects or intense daily dosing regimens (often requiring supervision).[59,63] In fact, disulfiram is no longer considered a first-line treatment option for AUD because of difficulties with adherence, as well as its toxicity.[62] The American Psychiatric Association (APA) recommends that disulfiram only be given to patients who are intolerant to or have not responded well to naltrexone or acamprosate.[62] Due to the low success rates

surrounding these treatments for AUD, there is a clear need for new therapeutics to be explored, one being the dopamine D4 receptor (D_4R) full antagonist, CAB-01-019.

Thus far, our food self-administration study with CAB-01-019 has been successful and we are hopeful that this success will continue on into our ethanol self-administration study (which is currently underway), with this drug proving to be a new potential treatment option for AUD. Our results demonstrated that CAB-01-019 did not attenuate food self-administration at any of the three tested doses (10, 17.8, and 30 mg/kg). This is a significant finding because we are looking for a drug that not only has strong receptor selectivity but causes minimal disruptive side effects. In our sample set of mice not yet addicted to alcohol, we were able to see that CAB-01-019 does not seem to interrupt the animals' desire and motivation to eat. We can now use these results as a critical control for our ongoing alcohol tests, helping us to separate alcohol-specific effects from non-specific behavioral or appetitive effects.

Furthermore, if CAB-01-019 proves to be successful in rodent models of alcohol addiction, this will be the first drug to offer precise receptor selectivity, targeting the dopaminergic receptors in the brain, which alcohol is known to profoundly affect. When it comes to disulfiram, naltrexone, and acamprosate, their mechanisms of action in regard to alcohol are still not fully understood. This is problematic and shows that there is no proof that these three drugs effectively treat AUD. A drug needs to be discovered that completely and efficiently targets areas in the brain associated with AUD, like the dopamine receptors in the nucleus accumbens, a major component of the brain's reward system. This in turn will affect alcohol-taking and -seeking behaviors and hopefully reduce alcohol consumption in those suffering from alcoholism.

Just like our study with morphine and MP-III-024, it is important to acknowledge that there were some limitations in regard to our CAB-01-019 experiment. The most notable limitation is the fact that this study only evaluated male mice, not female mice. This is something to consider, especially since there are noteworthy sex-related differences in behavioral and physiological responses to drugs in rodents, including efficacy and potency.[3] Therefore, it is often necessary to test both sexes. Perhaps, the data would be variable if only female mice or a mixture of male and female mice were utilized. Additionally, there is a possibility that food restriction may have an effect on D_4R receptor expression and/or signaling; however, there is currently no published data regarding this. It would be interesting to see how CAB-01-019 affects the operant responding of mice who are not food restricted and compare the results to our food self-administration mice who are fasted on a daily basis.

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