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BEHAVIORAL EFFECTS OF NOVEL TREATMENTS FOR PAIN USING DIFFERENT PATHWAYS

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by

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Dedication

This thesis is dedicated to my parents, Mohammed Aldaghma and Dalal Alzubi, whose support and guidance have been invaluable throughout my academic journey. Their practical wisdom, encouragement, and belief in my abilities have been constant sources of motivation. I am grateful for their presence in my life and for the lessons they have taught me, which have been instrumental in reaching this milestone. This accomplishment is as much theirs as it is mine. Additionally, I extend my heartfelt thanks to my siblings, Sally, Waseem, Layth, and Mohaned, whose companionship, support, and encouragement have enriched this journey in countless ways. Their unwavering faith in me and their invaluable perspectives have been a source of strength and inspiration. To my entire family, thank you for everything.

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Abstract

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Pain is defined as an unpleasant sensation that is mostly caused by a stimulus from our surroundings. This sensation has the potential to become a significant concern, disrupting daily activities, and diminishing overall quality of life. However, it could also hold significant importance as it serves as a protective mechanism. Pain acts as an alarm system for the human body, alerting it to potentially harmful situations where tissues may be at risk of damage[1]. Despite the considerable advancements in pain treatment and the extensive knowledge scientists possess regarding the pathophysiology and pathways of pain, numerous medications aimed at alleviating pain often carry significant side effects that could limit their use. This thesis aims to elucidate the diverse pain pathways and explore several potential therapeutic targets for pain management. The main goal of these investigations is to find therapeutic targets to relieve pain. This involves developing non-opioid pain-relieving medications or finding new candidate medications that can be co-administered with opioid analgesics to selectively enhance analgesia. This approach will lead to reducing overall opioid exposure and the risks of opioid-induced side effects.

Table of Contents

Table of Contents (Continued)

Table of Contents (Continued)

List of Figures

List of Figures (Continued)

Figure Page

List of Tables

Chapter 1

Pain Pathways and Key Neurotransmitters in Pain Regulation

Introduction

Nociceptors are receptors that are associated with pain sensation. These receptors are located in the primary afferent fibers, encompassing both the unmyelinated C-fiber and the myelinated A-fiber[2]. Under normal conditions of homeostasis, the receptors remain dormant and inactive, resulting in the absence of pain. However, upon exposure to any harmful stimulus, these receptors become activated.

Following the activation of nociceptors by noxious stimuli, the brain undergoes a sequence of sensory events comprising three stages to perceive and respond to the pain stimulus effectively. The initial stage is sensing the pain which generates signals. These signals will be transferred from the periphery to the dorsal horn (DH) in the spinal cord facilitated by the peripheral nervous system (PNS). In the final stage, these signals will be transmitted to the higher brain via the central nervous system (CNS).

There are two pathways that control the transmission of pain signals, the ascending pathway and the descending pathway. The ascending pathway carries sensory information from the body, moving upwards through the spinal cord toward the brain. On the other hand, the descending pathway refers to the nerves that travel from the brain to reflex organs via the spinal cord, in a downward direction.

Pain is primarily categorized based on symptoms, mechanisms, and syndromes[3]. Consequently, it has been systematically classified into three distinct types internationally: nociceptive pain, neuropathic pain, and inflammatory pain. Nociceptive pain is the pain

experienced when the body's specialized receptors (nociceptors) detect and transmit signals in response to harmful or potentially harmful stimuli, alerting the central nervous system to potential tissue damage or injury. Neuropathic pain is chronic pain caused by nervous system damage or dysfunction. It's characterized by sensations like burning or tingling and is often resistant to standard pain medications. Inflammatory pain occurs due to inflammation in the body, triggered by injury, infection, or irritation. It involves pain, swelling, redness, and heat at the affected site[4].

Both the PNS and CNS have important roles in transmitting and translating signals that are related to all three pain perceptions. The main function of PNS, composed of ganglia and nerves situated outside the brain and spinal cord, is to form a connection between the CNS and various organs and limbs throughout the body. Whereas the CNS, which involves the brain and the spinal cord, translates the signals received from the PNS, and according to that, it will arrange all the functions in our body.

Mechanisms of Pain Signaling

There are 4 major events in the process of pain sensation following exposure to a noxious stimulus: transduction, transmission, modulation, and perception. As shown in **Figure 1**, transduction is when the tissue-harming stimulus activates nerve endings in peripheral tissues. In these tissues, there are 3 types of pain that activate nociceptors: heat, chemical, and mechanical pain (pinch or pressure). Mechanical and heat stimuli typically occur briefly, whereas chemical stimuli tend to be of longer duration. The mechanisms through which these stimuli activate nociceptors remain largely unknown. Various pain-inducing chemicals activate or sensitize primary afferent nociceptors. Some of these substances, such as histamine, potassium, or serotonin, can potentially be released either

by damaged tissue cells or by circulating blood cells that enter the affected tissue area. Other compounds, including prostaglandins, bradykinin, and leukotrienes, are produced by enzymes activated in response to tissue damage. Elevated concentrations of all these pain-inducing compounds are typically detected in areas experiencing inflammation as well as pain[5]. During transduction, stimulus events transform into chemical tissue events; following that, these chemical tissue events along with the synaptic cleft events undergo conversion into electrical signals within the neurons; and ultimately, these electrical signals within the neurons are transmuted back into chemical events at the synapses[3].

Once transduction is completed, the next stage involves transmission. During this process, the electrical signal with the nociceptive message is transmitted from the periphery (the site of tissue damage) to the central nervous system through the axon of the primary afferent nociceptor. The cell body (soma) of the neuron is located in the dorsal root ganglion, with a single axon that extends in two branches, to the peripheral tissue (distal process) and to the spinal cord (proximal process); importantly, axonal signals can progress directly from the periphery to the spinal cord while bypassing the soma in the DRG[5]. Meanwhile, neurotransmitters within the synaptic cleft transmit information between the post-synaptic terminal of one cell and the pre-synaptic terminal of another cell.

Pain modulation refers to the process of altering or adjusting the perception and transmission of pain signals within the nervous system. Pain modulation involves complex interactions among various neural pathways and neurotransmitters, resulting in either an enhancement or a reduction of the perceived pain intensity[6]. The sensation of "pain" is encoded within the pattern and frequency of impulses traveling along the axons of primary afferent nociceptors. There is a direct correlation between the strength of the stimulus and

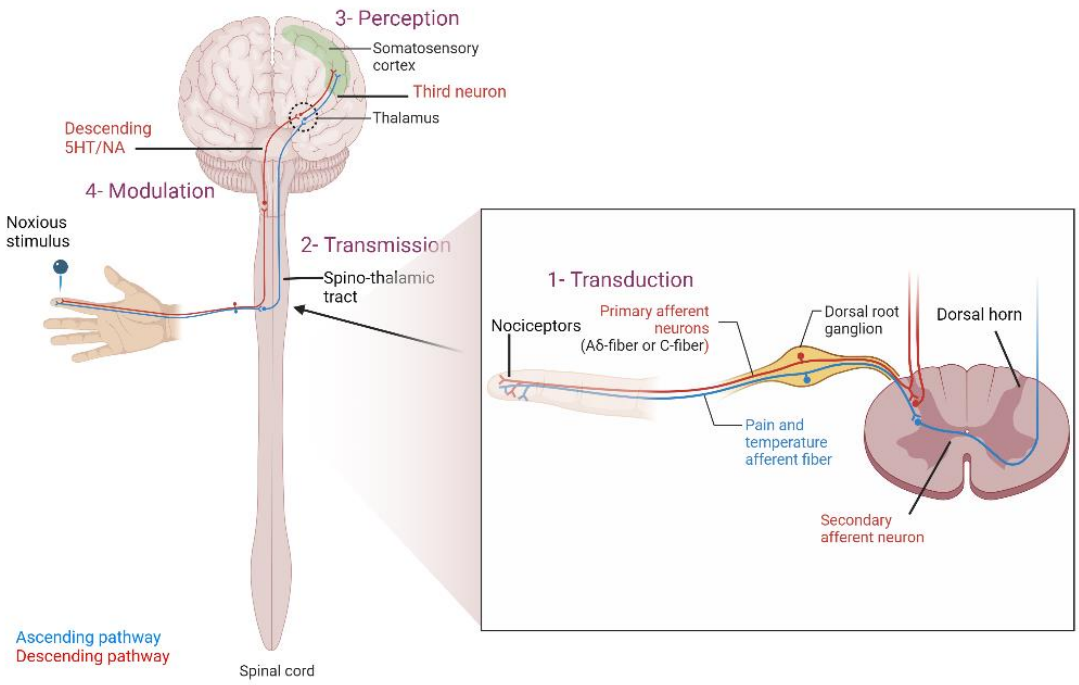
the rate of Nociceptor discharge. Preventing the transmission of signals through the small-diameter axons of nociceptors effectively alleviates pain, whereas blocking the activity of the larger-diameter axons in a peripheral nerve does not. Therefore, these primary afferent nociceptors are essential for the detection of harmful stimuli[5]. Pain modulation is a complex and dynamic process that can be influenced by a variety of factors, including psychological and emotional factors, as well as the presence of chronic pain conditions. Because of pain modulation, individuals respond to the same stimulus differently[7].

Finally, perception which is the individual's personal awareness resulting from sensory signals. It includes the synthesis of numerous sensory inputs into a consistent and meaningful experience. Perception is a complex process influenced by various factors, including attention, anticipation, and interpretation. Attention, which involves the selection and integration of sensory input from both external and internal sources, holds significant influence over the pain experience. A fundamental aspect of pain is its exceptional capacity to capture and maintain one's focus. For example, a patient who has experienced a substantial injury or illness and has concerns about the condition's return may remain alerted, constantly monitoring for any physical indications or discomfort linked to the feared disorder[5]. Another aspect of pain is that when attention is focused on it, its unpleasantness tends to be magnified[8]. When individuals focus on their pain, it tends to intensify and become more disabling. Conversely, engaging in distraction and meaningful activities is likely to diminish the intensity of pain sensations. when considering anticipation of pain regarding the cause of symptoms, individuals take into account a combination of psychosocial and cultural factors[5]. This complex state may affect the distress of pain. This could be due to cognitive factors where pain modulation depends on

prior experience with illness or the known information about the painful stimulus[9]. The interpretation of pain can specify how to deal with the injury. Different interpretations could determine the extent of the response, which will affect the decision to seek help and gain recovery[5].

Figure 1

The Major Neural Structures Relevant to Pain



Note. When an area is injured due to a noxious stimulus, immune cells are activated, and damaged cells release chemicals like prostaglandins, glutamate, and substance P. These chemicals bind to nociceptors on the primary afferent neurons, starting a signal to the spinal cord's dorsal horn (Transduction). The primary afferent neuron connects with a secondary neuron in the spinal cord and transmits the signal through the spinothalamic tract to the

thalamus, which acts as a relay station (Transmission). The thalamus relays the signal thorough a third neuron to the somatosensory cortex in the brain, where pain is perceived (Perceptions). Neurons from PAG connect with those in the nucleus raphe magnus, descending to the spinal cord to control the pain signal. They release serotonin and noradrenaline, inhibiting substance P release and activating endorphins, which modulate and control pain signal transmission (Modulation). Adapted from Marian O. et al. (1987). The anatomy and physiology of pain[5].

Primary Neurotransmitters Involved in Pain Perception

The human body relies on precise chemical signals transmitted by messengers called neurotransmitters. These messengers play a vital role in everyday functions like movement, sensation, and learning by carrying messages between nerve cells, enabling communication throughout the nervous system[10]. Pain neurotransmitters can be categorized into two groups: inflammatory neurotransmitters, including ATP, proton, BK, and adenosine, and non-inflammatory neurotransmitters, such as GABA, cannabinoids, and opioid peptides. In the following discussion, I will elaborate on some of the key non-inflammatory mediators and their roles in the pain pathway[3].

GABA

Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter. Widely distributed, GABA signaling occurs at approximately 40% of inhibitory synapses in our brain[11]. GABA is synthesized from the precursor glutamate in the cytoplasm of the presynaptic neuron by the enzyme glutamate decarboxylase. This enzymatic process relies on vitamin B6 (pyridoxine) as a cofactor[12].

GABA signaling is mediated by binding to ionotropic receptors, $GABA_A$ and $GABA_C$, which are ligand-gated chloride channels, and the metabotropic receptor $GABA_B$. The physiological, pharmacological, and molecular characteristics of $GABA_A$ receptors, have been extensively studied and documented, whereas our knowledge regarding $GABA_B$ receptors is comparatively limited[13]. However, a study has demonstrated that $GABA_B$ receptors in the spinal cord modulate pain by reducing the transmission of pain signals. Research has shown that activation of these receptors inhibits nerve terminals and neurons, leading to a decrease in the release of pain-related neurotransmitters like substance P and glutamate. However, alterations in receptor levels in conditions like neuropathic pain impact their efficacy in controlling pain perception[14].

$GABA_A$ is a ligand-gated ion channel, which binds to GABA, leading to the flow of chloride ions across the neuronal membrane. This activation results in the inhibition of neuronal activity, essentially acting as an "off switch" for nerve cells. As shown in **Figure 2**, the $GABA_A$ receptor has a pentameric structure composed of five subunits, typically arranged with two α subunits, two β subunits, and one γ subunit. These subunits are arranged around a Cl^--permeable pore. $GABA_A$ receptors most commonly contain α1, α2, α3, or α5 subunits ($\alpha1GABA_A$, $\alpha2GABA_A$, $\alpha3GABA_A$, and $\alpha5GABA_A$ receptors, respectively)[15].

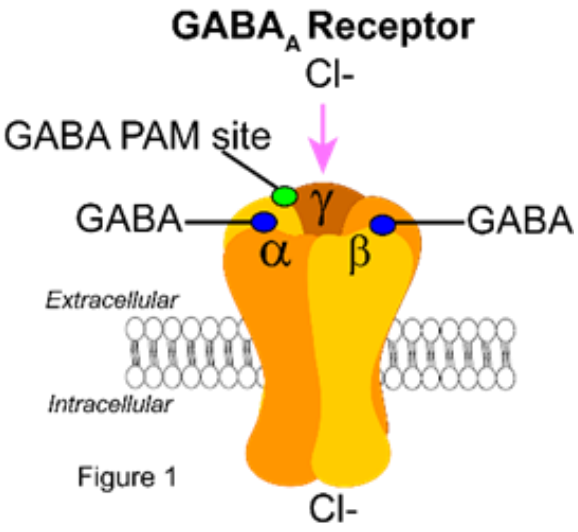
GABA-$GABA_A$ receptor activation can have an analgesic effect by inhibiting the transmission of pain signals along the pain pathways in the spinal cord and brain. When GABA binds to $GABA_A$ receptors on certain neurons, it hyperpolarizes the cell membrane, making it less likely for the neuron to generate and transmit pain signals to the brain[16]. The GABA binding site is located at the junction of the α and β subunits (α/β), while the binding

site for benzodiazepines is situated at the interface of the α and γ subunits (α/γ). Benzodiazepines are positive allosteric modulators (PAMs) since they bind to a distinct site from GABA's binding site and enhance GABA-mediated channel opening[17].

Several drugs that enhance the activity of GABA at GABAA receptors, such as benzodiazepines and barbiturates, are used in pain management. These drugs can help alleviate pain by enhancing the inhibitory effects of GABA on neurons, thereby reducing the perception of pain. However, it's important to note that these drugs have potential side effects and risks, including the risk of addiction and tolerance with long-term use[18,19]. GABAA α1 receptors are mainly responsible for the sedative effect of benzodiazepines, as well as the effect related to abuse and physical dependence[20].

Figure 2

The Structure of GABAA Receptor



Note. The GABAA receptor functions as a ligand-gated ion channel, activated by binding to GABA, which in turn allows chloride ions to pass through the neuronal membrane. This

activation leads to a reduction in neuronal activity, effectively serving as a neural "off switch." Structurally, the $GABA_A$ receptor is pentameric, consisting of five subunits, usually two α, two β, and one γ subunit, organized around a chloride-permeable central pore.

Cannabinoids

Cannabinoids are a group of chemical compounds that act as neurotransmitters in the endocannabinoid system (ECS) in the human body. While endocannabinoids are not traditionally classified as neurotransmitters, like serotonin or dopamine, they play a crucial role in neural communication and regulation of various physiological processes. The role of cannabinoids in neural function is often debated within scientific discourse. While their direct categorization as primary transsynaptic effectors remains contentious, there is strong evidence to support their function as neuromodulators. Rather than acting conventionally to initiate synaptic transmission, cannabinoids intricately modulate synaptic signaling and neuronal excitability. This modulation occurs through the manipulation of neurotransmitter release and the intricate regulation of neural network activity, underscoring their significant impact within the complex framework of the endocannabinoid system[21].

Cannabinoids primarily bind to and activate $G\alpha_{i/o}$-coupled GPCRs known as the cannabinoid type 1 (CB1) receptor and the cannabinoid type 2 (CB2) receptor. The former are found primarily in the CNS, while the latter is primarily located in the immune system. Activation of CB1 and CB2 receptors significantly decreases intracellular cAMP formation. In neurons, this leads to a substantial reduction in neuronal firing, including the suppression of signals encoding pain responses. CB2 activation in the immune system

reduces mast cell degranulation and the release of pro-inflammatory mediators, also contributing reduction in pain perception, particularly for inflammatory pain[3].

Endocannabinoids function as neuroregulatory modulators involved in retrograde neurotransmission. Following synaptic transmission, the postsynaptic neuron releases endocannabinoids that primarily bind to CB1 receptors situated on the presynaptic neuron. This binding inhibits the activation of presynaptic calcium channels, ultimately resulting in the reduction of neurotransmitter release from the presynaptic terminal[22]. The diverse psychotropic effects of cannabinoids mainly depend on their receptor-binding location. For example, cannabinoid receptor activation in the hippocampus will result in impaired short-term memory, while in the amygdala, it leads to panic and paranoia. Moreover, the activation of CB1 receptors in the spinal cord will cause analgesia[23,24]. Previous studies demonstrated the analgesic effect of cannabinoids on acute and chronic pain through spinal and supraspinal pathways[25]. However, they also induce unwanted side effects, such as mood change, impaired body movement, and impaired memory/learning, which limit their use in human[26].

Anandamide, known by its chemical name N-arachidonoylethanolamine, was the first endogenous cannabinoid that was discovered in 1992[27]. After the feeling of pain, anandamide binds to CB1, blocking pain signaling. However, its effectiveness is limited, and it doesn't last long due to its rapid metabolism within the body[27,28]. One of the most common types of naturally occurring cannabinoids is Δ_9-tetrahydrocannabinol (THC). It is a psychoactive substance found in the *Cannabis sativa* plant (commonly known as marijuana). It has a longer duration of action which makes it better than anandamide[28]. Some studies demonstrate the effect of THC compound as an analgesic[29,30], however, due

to its non-selective binding to both CB1 and CB2 receptors, it induces various undesirable effects, such as anxiety and paranoia[31].

Opioids

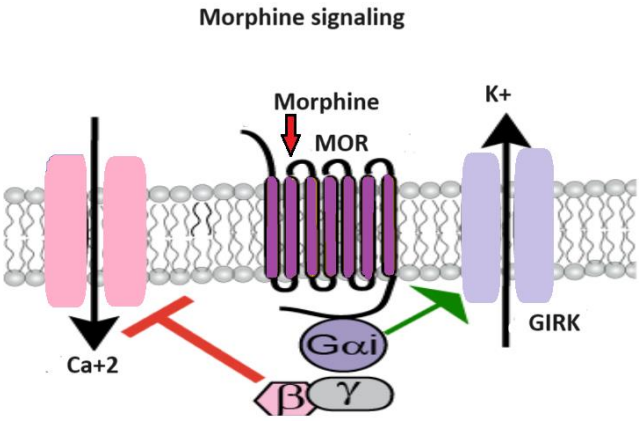
Opioid peptides are the endogenous ligands that bind to opioid receptors, such as μ-opioid receptors (MOR), δ-opioid receptors (DOR) and κ-opioid receptors (KOR). All types of opioid receptors belong to the Gi-protein-coupled receptor family, indicating that their activation leads to the inhibition of AC/cAMP activity. Opioids receptors are largely distributed in both primary afferent neurons and the dendrites of postsynaptic neurons. Enkephalin and Dynorphin are two of the most common endogenous peptides. These peptides are mainly released into the interneurons of the dorsal horn (DH). These peptides have a significant role in inhibiting the release of excitatory neurotransmitters from the afferent terminals, thereby diminishing neuronal excitability and ultimately leading to a reduction in pain perception[3]. Opioid peptides and their receptor subtypes are mainly targeted for pain treatment, due to their location in different regions in the brain and spinal cord, where they inhibit the transmission of pain signals and alter the perception of pain[32]. Morphine is one the most common opioids used for managing moderate to severe pain. This drug is often prescribed in medical settings or administered under careful supervision due to its serious adverse effects, such as constipation respiratory depression, dehydration, and abuse[33].

The analgesic effect of morphine primarily results from its binding to the μ-opioid receptors located in GABAergic terminals in the spinal cord and the periaqueductal gray (PAG). Morphine-MOR binding will inhibit the release of GABA neurotransmitter which will inhibit nociceptive signals[34]. In addition, it will activate dopaminergic neurons in the

nucleus accumbens region (NAc) which will induce reward pathway[35]. In a molecular level **Figure 3**[35], morphine-MOR will lead to the activation of Gi proteins. This will trigger changes in molecular signaling within the cell, including β-arrestin binding. G protein consists of three subunits: α, β, and γ. After morphine binds to MOR, GTP will bind to α subunit forming α-GTP. This complex will dissociate from βγ dimer, which will lead to the suppression of adenylate cyclase activity and subsequently decrease in cellular cyclic adenosine monophosphate (cAMP) levels. Both α-GTP and ßγ dimer contribute to receptor signaling. α-GTP initiates the activation of phospholipase-C (PLC), which subsequently hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 promotes the release of calcium from the endoplasmic reticulum, thereby activating calcium-dependent signaling pathways. Additionally, IP3 activates potassium channels, specifically the G-protein gated inward rectifying potassium channel (GIRK-3), resulting in increased cellular hyperpolarization and indirectly reducing cell excitability. On the other hand, the ßγ dimer directly blocks calcium channels, such as the P/Q-type, N-type, and L-type channels, leading to a decrease in intracellular calcium concentration. This reduction in calcium concentration suppresses the release of other neurotransmitters[35].

Figure 3

Morphine-MOR Signaling to Reduce Pain



Note. After morphine binds to MOR, Gai will dissociate from ßγ and activate potassium channel GIRK. This will increase potassium release. At the same time, ßγ dimer directly blocks the calcium channel which will reduce calcium concentration in the cell. This will cause a decrease in neuron excitability and neurotransmitter release which will eventually reduce pain[35]. Adapted from Listos, J et al, (2019). The Mechanisms Involved in Morphine Addiction: An Overview.

Research Goal

Chronic pain is a significant public health issue in the United States, affecting a substantial portion of the adult population. Data from the Centers for Disease Control and Prevention (CDC) reveals that during 2021, an estimated 20.9% of U.S. adults (approximately 51.6 million individuals) experienced chronic pain. Among these, 6.9% (around 17.1 million people) suffered from high-impact chronic pain, which substantially restricts daily activities. The prevalence of chronic pain and high-impact chronic pain is

notably higher among specific population groups, including older adults, females, veterans, adults living in poverty, and those with public health insurance. These disparities in the prevalence of chronic pain among certain populations are significant and highlight the need for tailored interventions and strategies addressing chronic pain in these groups[36].

Pain serves as an essential warning system crucial for our survival. It involves complex pathways associated with electrical signals and neurotransmitter release[37]. Understanding these pain pathways is essential to develop a therapeutic strategy to treat pain. While various drugs have been developed to alleviate pain, many of them come with significant side effects that could have a negative impact on patient's health. Opioids are one of the main potent class of drugs that have been used to treat pain. However, the opioid epidemic in the United States has emerged as a major public health crisis, with its roots in the late 1990s when there was a significant rise in the prescribing of opioids for pain management. This shift led to increased misuse and addiction, both of prescription and non-prescription opioid drugs. The epidemic is characterized by a dramatic increase in opioid-related overdoses. In 2021, the number of overdose deaths involving opioids was ten times higher than in 1999, with more than 80,000 people succumbing to opioid overdoses that year, nearly 88% of which involved synthetic opioids like fentanyl[38].

The opioid epidemic in the United States has evolved through three distinct waves, as outlined by the Centers for Disease Control and Prevention (CDC). The first wave began in the 1990s, marked by increased prescribing of opioids, with a rise in overdose deaths involving prescription opioids since at least 1999. The second wave started in 2010, characterized by a rapid increase in heroin-related overdose deaths. The third wave commenced in 2013, featuring significant increases in overdose deaths involving synthetic

opioids, particularly illicitly manufactured fentanyl. This epidemic has resulted in nearly 645,000 deaths from opioid overdose from 1999 to 2021[39].

This draws an important need to find a safe therapeutic strategy that could manage pain without negatively impacting patients' lives with unwanted side effects. Aside from the severe side effects mentioned above, morphine is considered to be one the most potent analgesics that treat moderate to severe pain. Therefore, many studies aim to find a therapeutic intervention that could mimic the potency and efficacy of morphine with less side effects or develop a compound that could synergize with morphine, enhancing its effectiveness at lower doses and consequently reducing side effects. The potential outcomes of these studies will significantly help patients struggling with long-lasting pain. This will potentially improve their overall quality of life by enhancing their mobility and improving their mood and mental well-being.

Chapter 2

The Effects of MP-III-024 Co-Administration on Morphine Conditioned Place Preference Test and Morphine Tolerance

Introduction

Pain is defined as an unpleasant feeling that could interrupt the quality of life. There are so many drugs, such as opioids that are very effective in treating pain. Opioids are potent analgesics commonly used to relieve acute to chronic pain. These drugs are originally derived from the opium poppy plant (*Papaver somniferum*)[40]. So far, researchers have identified five opioid receptor types, including mu receptor (MOR), kappa receptor (KOR), delta receptor (DOR), nociception receptor (NOR), and zeta receptor (ZOR)[41]. Opioid analgesia is mainly mediated by activating μ-opioid receptors (MORs) in both central and peripheral nociceptive pathways. While these medications are highly effective, they also come with adverse effects that can have a negative impact on one's well-being. For instance, morphine, an opioid widely recognized for its efficacy and potency in chronic pain relief, is associated with adverse effects such as drowsiness, nausea, respiratory depression, tolerance, physical dependence, and addiction. These side effects can sometimes impose limitations on the use of such drugs[33]. Therefore, it is very important to identify a new therapeutic intervention that could be effective to treat pain with limited side effects.

GABA, known as γ-Aminobutyrate, is an inhibitory neurotransmitter in the adult mammalian brain. It is a multifunctional molecule that has different functions in the central nervous system, peripheral nervous system, and certain non-neuronal tissues. The GABA neurotransmitter primarily activates $GABA_A$ and $GABA_C$ receptors, which are ionotropic

receptors. It also activates $GABA_B$ receptors, which are metabotropic receptors[13]. $GABA_A$ receptor is the major inhibitory neurotransmitter receptor. It has a significant role in providing rapid inhibition within the basal ganglia. It is part of the superfamily of "cys-cys loop" ligand-gated ion channels. The $GABA_A$ receptor has a pentameric structure composed of five subunits, two α subunits, two β subunits, and one γ subunit. These subunits are arranged around Cl^- permeable pore. Each subunit performs a different signaling pathway. The activation of $GABA_A$ receptors by GABA molecules or GABA-like compounds, such as benzodiazepines, triggers the opening of the ion channel, allowing the flow of negatively charged ions, specifically chloride ions (Cl^-) from extracellular space into the cell. This process leads to inhibitory hyperpolarization. The binding site where $GABA_A$ binds is located at the junction of the α and β subunits (α/β), while the binding site for benzodiazepines is situated at the interface of the α and γ subunits (α/γ)[17]. Benzodiazepines (a sedative drugs) are considered to be a positive allosteric modulator (PAM) since they bind to a distinct site from $GABA_A$'s binding site. These receptors contain α1, α2, α3, and α5 subunits (α1$GABA_A$, α2$GABA_A$, α3$GABA_A$, and α5$GABA_A$ receptors, respectively). They enhance GABA-receptor binding by increasing channel opening frequency. As a result, they enhance the inhibition of excitatory neurotransmitters[15]. $GABA_A$ α1 receptors are mainly responsible for the sedative effect of benzodiazepines, as well as the effect related to abuse and physical dependence. However, studies have shown the presence of $GABA_A$ receptors, specifically α2- and α3-containing $GABA_A$ receptors in spinal nociceptive circuits. These receptors play a significant role in transmitting pain sensory signals from the periphery to higher centers with minimal negative side effects, in comparison to $GABA_A$α1-associated PAMs[15,42].

As reported in Fischer *et al.*, 2017, a therapeutic strategy had been established to combine a benzodiazepine positive allosteric modulator (PAM), MP-III-024 with μ-opioid agonist, morphine. By doing so, the two drugs worked synergistically to produce antinociceptive and anti-hyperalgesic effects. This approach led to administering lower doses of morphine, which minimized the adverse effects, yet achieved the desired outcome. MP-III-024 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 a selective $\alpha 2GABA_A/\alpha 3GABA_A$ imidazodiazepine that has been introduced as benzodiazepine site positive allosteric modulator (PAM). In rodent models, this compound demonstrates a time course of action similar to morphine, and it shows minimal affinity for opioid receptors. These attributes make it an excellent candidate for combined therapy with morphine[42,43]. Several behavioral assays were performed including, von Frey assay, tolerance test, open field assay, food self-administration, and conditioned place preference, to assess the efficacy and safety of MP-III-024 individually and as a combination with morphine.

Prior Research Work Conducted by the Keck Lab

Researchers in Keck's lab have tackled several open questions regarding the novel drug MP-III-024. These results have been previously published, but are presented here for useful context[25]. The follow-up study shows whether MP-III-024 co-administration would enhance the analgesic effect of morphine on mechanical and thermal pain. My work contributed to the extension of this study by evaluating whether MP-III-024 co-administration altered morphine abuse liability or the development of morphine tolerance.

This study was conducted by Mohammad Atiqur, an alumnus of the Keck Behavioral Lab, to test the interactive effect of the μ-opioid agonist, morphine and the α2/

α3 $GABA_A$ positive allosteric modulator, MP-III-024, on mechanical and thermal stimuli. By using rodent pain models, a significant interaction between α2/α3 $GABA_A$ receptor and μ-opioid receptor has been demonstrated, indicating the effective combining therapy of morphine and MP-III-024 to treat pain-related disorders. Initially, the assessment focused on evaluating the analgesic and antinociceptive effects of morphine and MP-III-024 when administered separately at cumulative dosing of 1, 3.2, 10, and 32 mg/kg. Subsequently, fixed-ratio mixtures of MP-III-024/morphine combinations were examined (0.31:1, 0.94:1, and 2.8:1 MP-III-024 to Morphine). In this study, two behavioral assays were used: The von Frey assay to assess drug effect on mechanical pain and the hot plate assay to test drug effect on thermal pain. Drug mixtures ratio data were analyzed and identified through isobolographic and dose-addition analyses[25].

Mechanical Hyperalgesia. To induce inflammation, mice were given an injection of zymosan A in their right hindpaw. Zymosan A is a polysaccharide derived from the cell wall of Saccharomyces cerevisiae. It primarily consists of glucan and mannan residues, functioning as an inflammatory agent[44]. The left hindpaw wasn't injected and served as a control. After 24 hours, the von Frey assay was used to examine the effectiveness of morphine, MP-III-024, and their combination in reducing the pain response to mechanical pressure. This assay involved applying filaments of different stiffness to the mice's hindpaws and observing reactions like paw withdrawal, which indicates pain. The pain response was measured 30 minutes after the drugs were given. After analyzing the data, the mechanical threshold, which is the minimum force needed to elicit a positive withdrawal response, was identified, and standardized to the baseline measurement of the

non-injected left paw[25]. Subsequently, the percentage of the maximal positive effect (%MPE) for each mouse was calculated using this formula:

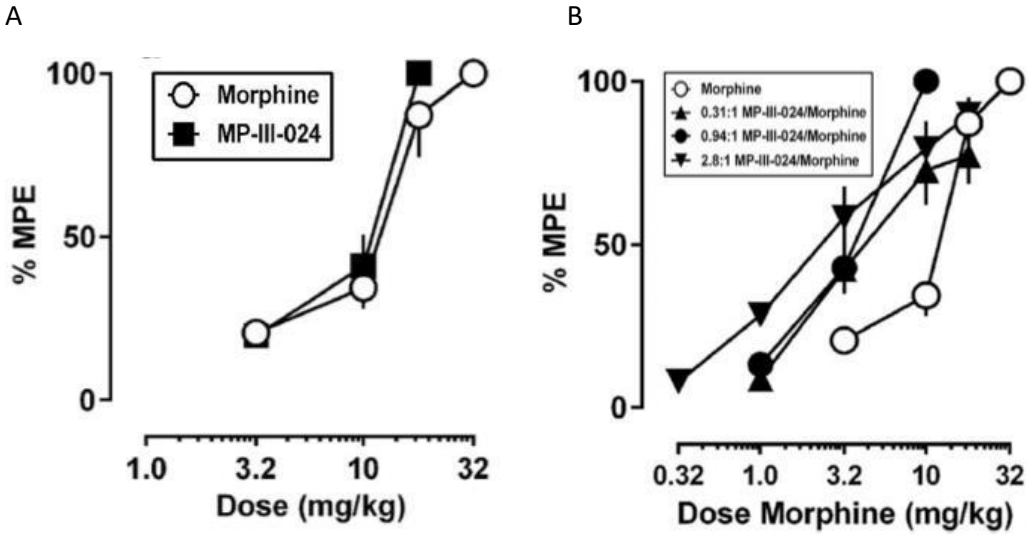
%MPE = [post drug right paw threshold (g)–baseline right paw threshold (g)]/[baseline left paw threshold (g)–baseline right paw threshold (g)][25].

As a result **(Figure 4)**, morphine, MP-III-024, and their fixed-ratio combinations exhibited dose-dependent antihyperalgesic effects in the mechanical hyperalgesia assay. The activity of the fixed-ratio mixtures occurred with either an additive or a supra-additive (synergistic effect), depending on the proportions of each component. According to the von Frey data, the mean ED_{50} values (± SEM) were 9.96 (8.81-11.26) mg/kg for MP-III-024 and 10.72 (9.68-11.86) mg/kg for morphine. The relative ED_{50} values obtained from the von Frey assay were used to establish the proportions of the compounds within each MP-III-024/morphine mixture[25].

In the isobolographic analysis for mechanical hyperalgesia testing **(Figure 5)**, the combination of morphine and MP-III-024 at a ratio of 1.0:0.31 demonstrated additive effects. This was indicated by the Effective Dose 50 (ED_{50}) values being near the additivity line. However, when the drug ratios were adjusted to 1.0:0.94 and 1:2.8 for morphine and MP-III-024, respectively, the results indicated synergistic effects, as evidenced by the ED_{50} values falling below the line of additivity[25].

Figure 4

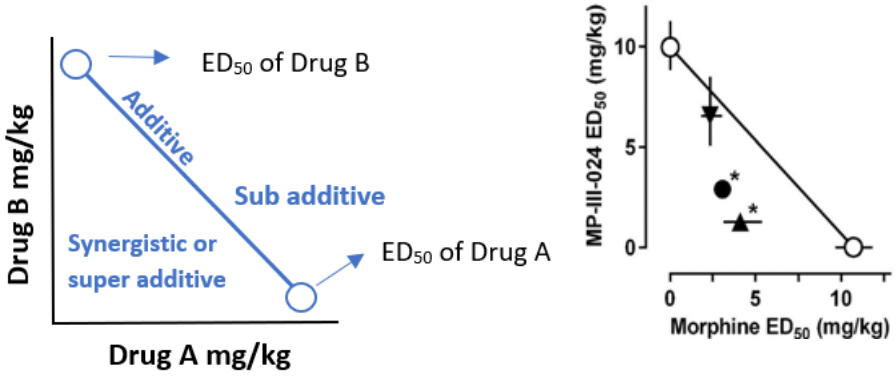
The Individual and Combined Administration of Morphine and MP-III-024 Attenuated Inflammatory Pain



Note. the dose-response curves for individual and combined administration of morphine or MP-III-024 in von Frey mechanical hyperalgesia. (a) The data obtained from the von Frey assay showed a dose-dependent increase in the antihyperalgesic effect of both morphine and MP-III-024 when were given separately. (B) Each mixture ratio led to a dose-dependent increment in the antihyperalgesic %MPE. The presence of MP-III-024 induced concentration-dependent shifts towards the left in the morphine dose-effect curve (synergistic effect). Data point represents the mean (± S.E.M.) obtained from a sample of 8 to 10 mice. Rahman A. et al. (2021).

Figure 5

Isobolographic von Frey Analysis



Note. The diagram illustrates the interaction between two drugs and their combined impact on efficacy, depicted by an additivity line derived from the Effective Dose 50 (ED_{50}) values of each drug. When dose pairs appear below and to the left of this line, it suggests that a lower quantity of the drugs was needed to achieve the ED_{50}. In a detailed analysis, the isobolograms for combinations of morphine and MP-III-024 were assessed at different ratios (1.0:0.31, 1.0:0.94, and 1.0:2.8). Rahman A. et al. (2021)[25,45].

Thermal Nociception. To evaluate the thermal nociception, a hot plate assay was performed. The hot plate was set at 56 ± 0.1 °C. Each mouse was positioned on the hot plate surface and the latency to hindpaw licking, shaking, or jumping is recorded. A cutoff time of 20 seconds was set to avoid tissue damage. Before drug administration, two baseline measurements were taken at 30 and 15 minutes in advance. The data were combined to calculate a single average baseline value. The percentage of the maximum possible effect was calculated from the recorded latencies following drug administration

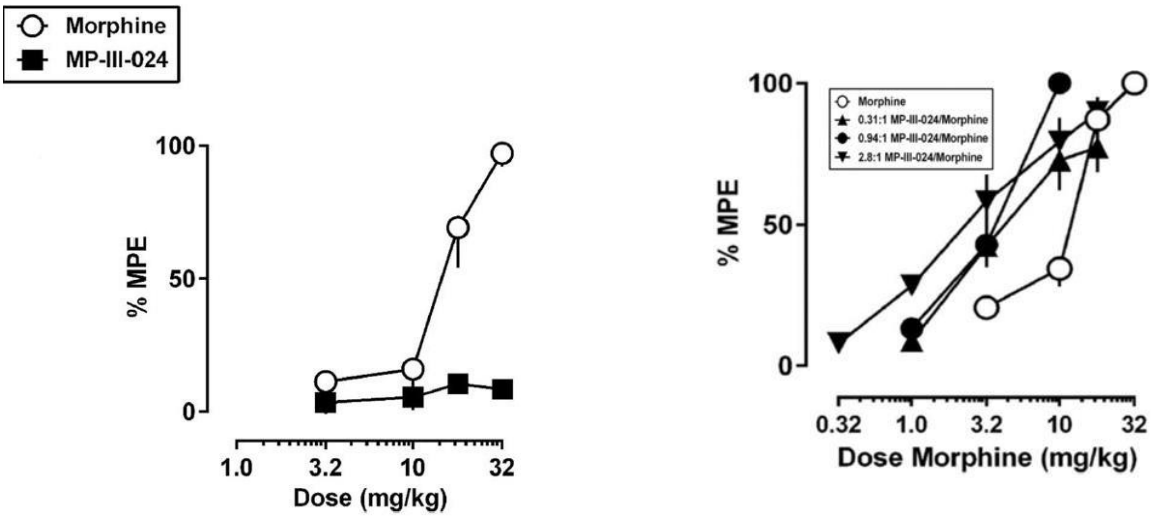
using this equation:

%MPE=[postdruglatency(s)−baselinelatency(s)]/[20−baselinelatency(s)].

As a result, MP-III-024 did not elicit any effects on acute thermal nociception. This aligns with previous studies illustrating that positive allosteric modulators (PAMs) targeting α2GABA$_A$ and α3GABA$_A$ receptors produce an effect mainly in pain models induced by chemical stimuli[42,46]. However, morphine and its combination mixtures with MP-III-024 had an effect on thermal nociception **(Figure 6)**[25]. According to the thermal nociception assay, the ED_{50} (± SEM) value of morphine is 14.98 (14.39-15.60) mg/kg. Since MP-III-024 exhibited no impact in the thermal nociception assay, the von Frey ED_{50} values that were previously applied in combination studies conducted in the hotplate test.

Figure 6

The Combined Administration of Morphine and MP-III-024 Reduced Thermogenic Pain



Note. The dose-response curves show the thermal nociception effect of morphine alone and in combination with MP-III-024 at different ratios. (A) In the thermal nociception procedure, only morphine had an antinociceptive effect in a dose dependent manner. (B) Each mixture ratio led to a dose-dependent increment in the thermal antinociception %MPE. The morphine dose-effect curve shifts leftward after the addition of MP-III-024. All results are presented as means ± SEM (n=8). Rahman A. et al. (2021).

The supra-additive behavioral data that were observed upon the co-administration of MP-III-024 and morphine in both assays indicate that there is a novel interaction between μ-opioid receptors and α2/α3 subunit-containing $GABA_A$ receptors, leading to an antinociceptive synergistic effect. A further investigation was conducted to evaluate if the synergistic effect of both compounds is applicable to other behavioral and physiological measures, specifically the adverse effects among the various ratios tested, the 1.0:0.94 ratio of morphine and MP-III-024 demonstrated the most substantial impact on antinociceptive synergy[25]. The Keck Lab conducted several behavioral assays to evaluate the safety of mixing MP-III-024 with morphine. These assays included the conditioned place preference test, which assesses the potential for drug dependency, and the tolerance test, to evaluate whether tolerance develops with the morphine/MP-III-024 combination.

Materials and Methods

Drugs

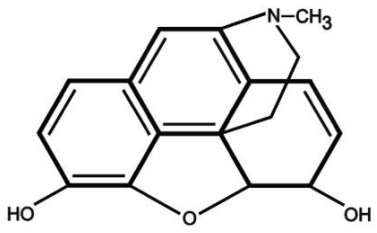
Morphine is a non-synthetic narcotic that is commonly used to treat pain. It is derived from the poppy plant, Papaver somniferum. It is a highly potent analgesic with a high potential for abuse. The scientific name of morphine is (4R,4aR,7S,7aR,12bS)-3-methyl-

2,4,4a,7,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7,9-diol.

Morphine and other opiates bind to opioid receptors, such as such as μ-opioid receptors (MOR), δ-opioid receptors (DOR) and κ-opioid receptors (KOR). The activation of these receptors will lead to pain alleviation and analgesia[47]. Morphine is an alkaloid that mainly dissolves in 0.9% NaCl[33]. In these experiments, the vehicle that has been used to dissolve this drug is 0.5% methylcellulose and 0.9% NaCl.

Figure 7

Chemical Structure of Morphine[48]

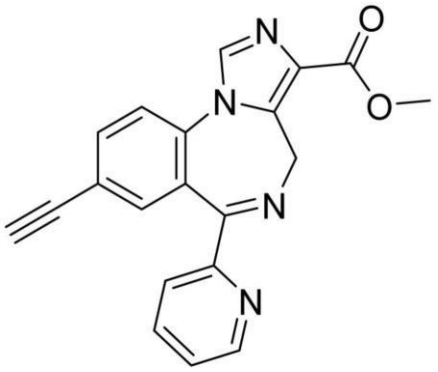


MP-III-024 with the scientific name of methyl 8-ethynyl-6-(pyridin-2-yl)-4Hbenzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate (MP-III-024), is a novel imidazodiazepine analog of benzodiazepines. This drug was acquired from the Department of Chemistry and Biochemistry at the University of Wisconsin-Milwaukee, where it is synthesized. The drug was suspended in a vehicle that contains 0.5% methylcellulose and 0.9% NaCl. MP-III-024 is a positive allosteric modulator (PAM) that selectively binds to α2/α3GABAA-receptor. It exhibits strong selectivity for specific subtypes of opioid

receptors, and similar time course of action as morphine[42]. It also exhibits minimal affinity for opioid receptors, which makes it a great selection for this study[43].

Figure 8

Chemical Structure of MP-III-024[42]



Animals

Drug-naïve CD1 male mice have been used for these studies. They were albino with white fur and red eyes. Their weight was between 30 and 45 grams. These mice were obtained from Charles River Laboratories. Once mice arrived at the vivarium located in Cooper Medical School of Rowan University (CMSRU), they were housed in a standard Plexiglas cage as four mice in each cage. The housing room was set to a 12 h light/dark cycle with a controlled environment, with a stable temperature (21-23°C) and humidity (45-50%). These mice were habituated for 2 weeks before the experiment with full access to food and water. The animals used in the subsequent experiments were attended to in accordance with the guidelines set forth by Rowan University's Institutional Animal Care and Use Committee.

Behavioral Assays

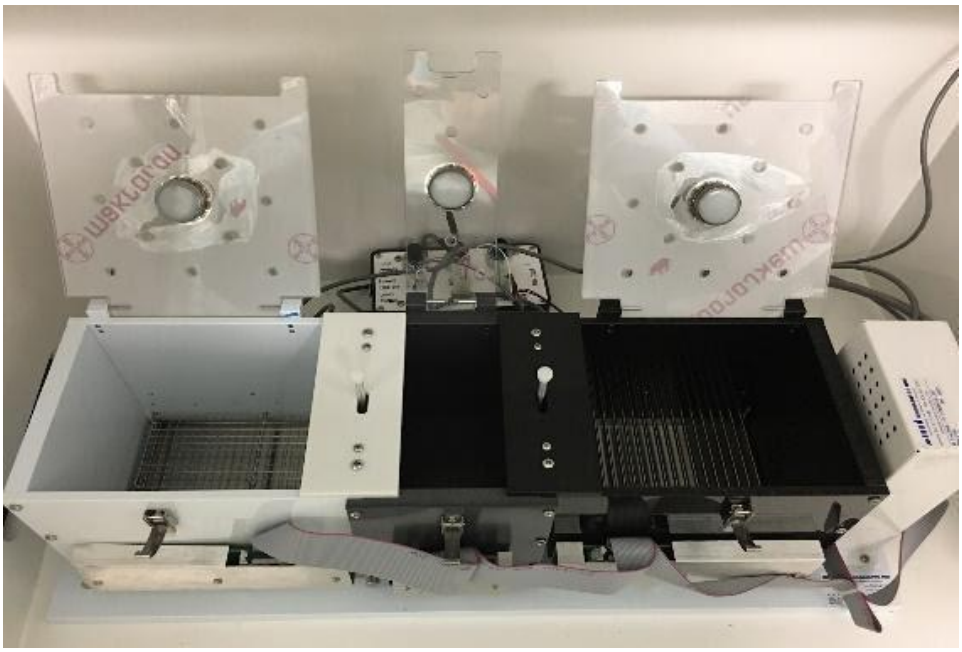
Conditioned Place Preference

The conditioned place preference paradigm (CPP) is a preclinical behavioral model employed to investigate the rewarding effect of drugs. The main role of this model is to study the link between a specific environment with drug treatment followed by associating another environment with the lack of drug presence (drug's vehicle). As shown in **Figure 9**, the CPP apparatus is mainly composed of three-compartment chambers (the white chamber, the black chamber, and the grey chamber). The white and black chambers are attached by a different stainless-steel grid flooring. Usually, one of them is associated with a specific drug and the other with a vehicle (no drug). The grey chamber which is in the middle is not associated with any drug and has no special features. There are two gates that separate the chambers which could be opened to enable the animals to move between the three compartments. Gates should always be closed during the training days and opened during the preference test day. Each drug/vehicle is paired to a specific compartment. On day 1, mice undergo an initial preference test. Establishing this test is crucial for removing any potential biases from the experimental procedure. Mice are placed in the center compartment with open gates, enabling them to move freely through the apparatus for 30 minutes. During this period, the time each mouse spent in each compartment is measured to assign each animal a drug-paired side and a vehicle-paired side through a random process. In case there was an initial side preference greater than 65%, mouse is excluded from the experiment. After that, mice undergo experimental training for 10 days. During this period mice were injected with the drug of interest and then placed in one of the outer compartments for 30 minutes. Next day mice will be injected with vehicle and placed in

the opposite compartment for the same period. This alternating pattern continues for a total of 10 days. On the 12th day, a final preference test is conducted. During this test the mouse is placed in the center compartment (grey chamber) while gates are opened. The time of each mouse spends in each of the outer compartment is recorded. Abuse drugs will produce conditioned place preference (CPP). This is when animals spend more time in the drug-paired compartment compared to the vehicle paired compartment. However, if animals spend more time in the vehicle-pair compartment, it is called conditioned place aversion (CPA). This is when a drug produce an aversive effect[49].

Figure 9

The Conditioned Place Preference Apparatus



Note. The conditioned place preference apparatus is composed of three chambers. The white chamber, black chamber, and grey chamber. Throughout the experiment, one

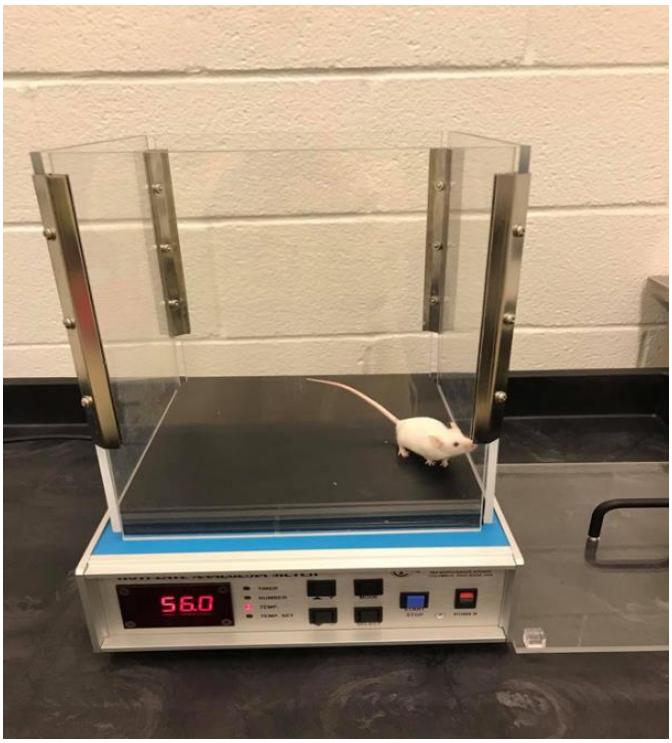
chamber is linked to a specific drug, another to a vehicle. The middle grey chamber is the neutral compartment connecting the black and white chambers via two gates. These gates are closed during the training days and opened during the initial and finale preference test days. Image from Sara Uribe's M.S. thesis (2023)[45]. Used with permission.

Hot Plate Assay

The hot plate assay is one of the most common methods used to test the sensitivity of mice against thermal pain (**Figure 10)**. Through this assay we can measure the effectiveness of specific drugs that are intended to elicit an antinociceptive response. This essay was presented by Eddy and Leimbach in 1953[50]. First, the hot plate temperature is set at 50-56°C. Before drug administration, two baseline measurements are taken in advance, then the data are combined to calculate a single average baseline value. Following that, mice are injected with a specific drug/vehicle, and then they are sequentially placed on the surface of the hot plate. Once the mouse is on the surface, a stopwatch should record how long the mouse has been on the surface until it reacts. This reaction could be a hindpaw licking and/or shaking, or jumping. Once the mouse reacts, it should be removed right away. The longer the latency for mice to react, the greater the analgesic effect produced by the drug[51].

Figure 10

The Hot Plate Apparatus



Note. The hot plate is set to a temperature ranging between 50 to 56°C. After drug administration, mice are placed on the hot plate until they show a nociceptive reaction. This reaction includes any response indicating the mouse's discomfort with the high temperature, such as a hindpaw licking and/or shaking, or jumping. Image from Sara Uribe's M.S. thesis (2023)[45]. Used with permission.

Experimental Procedures

Conditioned Place Preference

The main objective of this assay is to determine whether MP-III-024 exhibits any potential for drug abuse on morphine. More specifically, our goal is to determine whether MP-III-024 could lead to drug abuse, both when taken synergistically with morphine and when taken alone. This assay is categorized into three distinct tests: the initial preference test, the training test, and the final preference test. The first test is the initial preference test. Establishing this test is crucial for removing any potential biases from the experimental procedure. Mice are placed in the center compartment with open gates, enabling them to move freely through the apparatus for 30 minutes. During this period, the time each mouse spent in each compartment is measured to assign each animal a drug-paired side and a vehicle-paired side through a random process. In case there was an initial side preference greater than 65%, mouse is excluded from the experiment. Therefore, some groups of mice were less than 12 mice even though original plan specified a group size of 12 animals in each drug group with a total of 120 mice (morphine, MP-II-024, and combination of morphine and MP-III-024). This initial group size was established based on assessments of preliminary data from our laboratory and relevant literature. Therefore, these tests were conducted over an extended duration with 4 available CPP chambers, resulting in inadvertent errors in recording the number of subjects in each category until our conclusive analyses. By the end of the study, 117 animals were analyzed **(Table 1)**.

Secondly, the training test: Animals receive i.p injections of either the drug or vehicle, and then confined to a specific compartment with alternating exposures taking place on a daily basis throughout 10 training sessions. On the 12^{th} day, the finale preference

test is performed. Here each mouse will be placed in the center compartment with open gates allowing them to move freely through the three compartments. Based on the final preference test, we can ascertain whether mice will exhibit a preference for one compartment over the other. This determination will indicate whether MP-III-024, either alone or in combination with morphine, induces a conditioned place preference (CPP), suggesting a potential for abuse liability. Place preference was measured at 3, 10, 17.8, and 32 mg/kg of morphine, MP-III-024, or a combination of morphine and MP-III-024 at the 1.0:0.94 ratio.

Table 1

Conditioned Place Preference Drug Dosing & Groups

Morphine	**MP-III-024**	**Morphine + MP-III-024 (1:0.94)**
3 mg/kg (n=14)	3 mg/kg (n=6)	3 mg/kg (n=10)
10 mg/kg (n=10)	10 mg/kg (n=10)	10 mg/kg (n=12)
17.8 mg/kg (n=13)	17.8 mg/kg (n=14)	17.8 mg/kg (n=10)
	32 mg/kg (n=18)	

Note. This table shows doses that have been used in the experiment for morphine, MP-III-024, and a combination of morphine + MP-III-024 at a ratio of 1:0.94. It also shows the number of animals that were assigned to each dose. It is important to note that these doses were assigned to animals randomly and were administered alternately with the vehicle. The initial plan is to have a total of 120 mice to test the different doses of morphine, MP-II-

024, and their combination. However, some animals showed an initial side preference greater than 65%, leading to their exclusion from the experiment. In addition, due to the experiments being conducted at different times, we ended up with uneven group sizes, resulting in more animals in certain groups and fewer in others.

Morphine Tolerance Assay

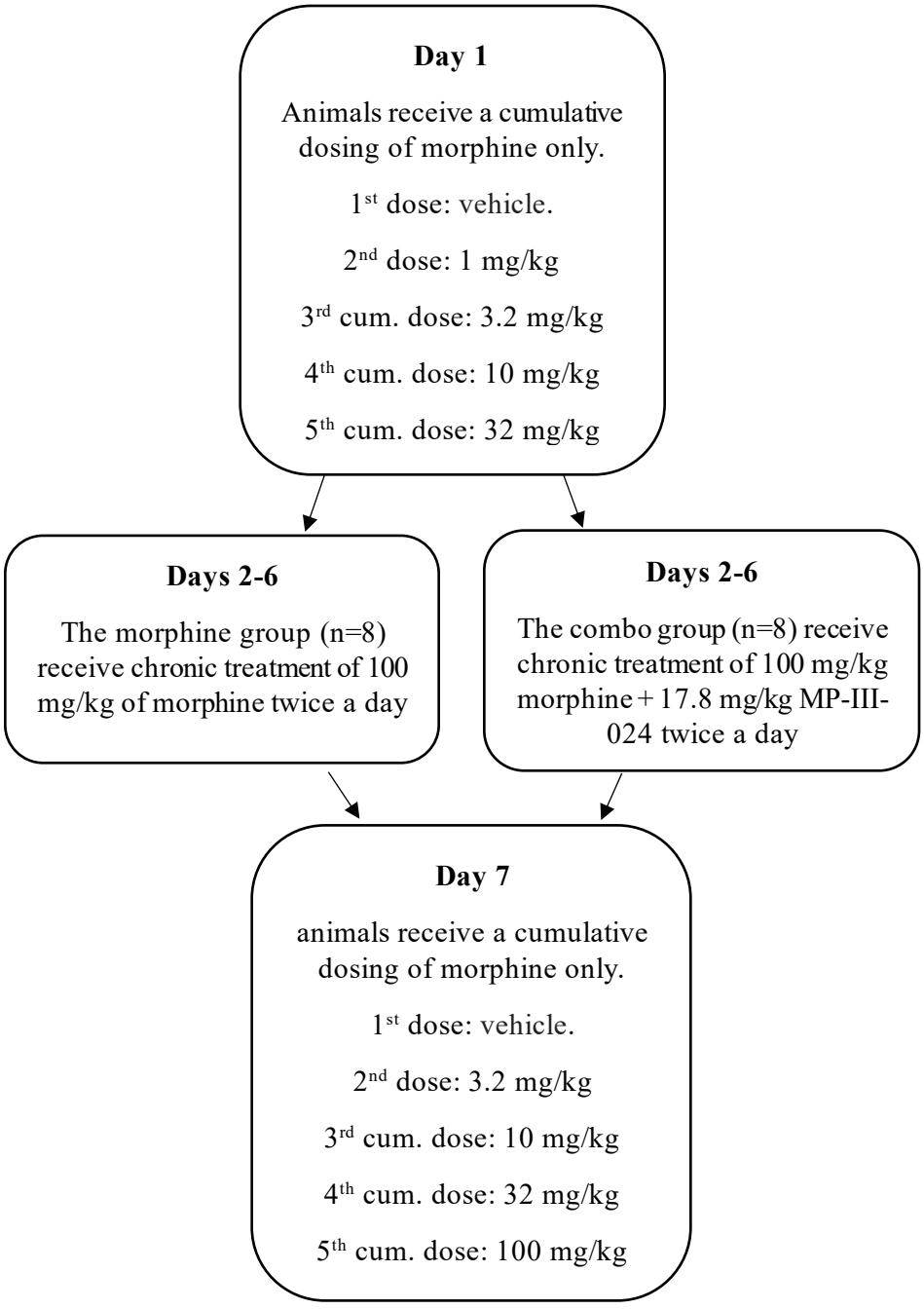
It is well known that prolonged use of morphine can lead to the development of tolerance[52]. In this study, we used the hot plate test to assess whether administering morphine in combination with MP-III-024 would result in similar, reduced, or enhanced tolerance effects. According to preliminary data in our lab and published literature, a group size of 16 male CD1 mice was selected for this study. These mice were categorized into two groups. One group was administered morphine (n=8), while the other group received a combination of morphine and MP-III-024 (n=8).

As shown in **Figure 11**, the tolerance assay is a 7-day experiment. Prior to the cumulative dosing, the hot plate was set to 56 ± 0.1 °C, and two baseline measurements were taken for each animal. On day one, mice received an i.p injection of a morphine cumulative dosing (0, 1, 3.2, 10, and 32 mg/kg) **(Table 2).** After each injection, mice were returned to their cages, and placed on the hot plate for testing after 30 minutes. The latency to hindpaw licking and/or shaking, or jumping, was recorded. Each mouse was removed immediately after any kind of reaction. No mice were allowed to remain on the hot plate for more than 20 seconds to avoid any risk of tissue damage. Throughout days 2-6, one group of mice received a chronic treatment of 100 mg/kg morphine while the other group received a combination of 100 mg/kg morphine + 17.8mg/kg MP-III-024 twice a day at

10:00 am and 4:00 pm. On day 7, all mice had another cumulative dosing of morphine, same as day one, except having an extra dose of 68 mg/kg to reach a maximum cumulative dose of 100 mg/kg.

Figure 11

Tolerance Test Experimental Design



Note. This experimental plan was designed to test the impact of MP-III-024 on morphine-induced tolerance. On days 1 and 7, two baseline measurements were taken for each mouse before they received cumulative doses of morphine. Thirty minutes following each vehicle or morphine injection, mice were placed on a hot plate with a temperature of 56.0 ± 0.1 °C to test their nociceptive latency. During days 2-6, one group of mice (n=8) received 100 mg/kg of morphine and another group (n=8) received 100 mg/kg morphine + 17.8 mg/kg MP-III-024 twice a day. These injections were performed at 10:00 AM and 4:00 PM.

Table 2

Tolerance Assay Experimental Drug Dosing

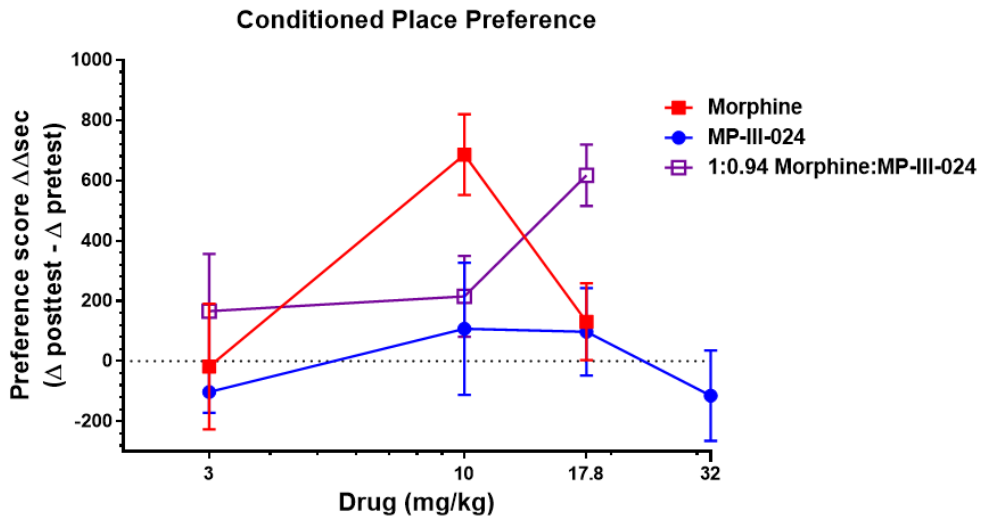
	Day 1	Day 2-6	Day 7
Drug	• Morphine (n=16)	• Morphine (n=8) • Morphine + MP-III-024 (n=8)	• Morphine (n=16)
Dose	• 0, 3.2, 6.8, 22 mg/kg (32 mg/kg total)	• 100 mg/kg morphine twice a day • 100 mg/kg morphine + 17.8 mg/kg MP-III-024 twice a day	• 0, 1, 2.2, 6.8, 22, 68 mg/kg (100 mg/kg total)

Note. This table shows doses that have been used in the experiment for morphine, MP-III-024, and a combination of morphine + MP-III-024 at a ratio of 1:0.94. It also shows the number of animals that were assigned to each dose. On day 1, all animals received repeated injections of 0, 1, 2.2, 6.8, and 22 mg/kg morphine, to reach a total cumulative dose of 32 mg/kg morphine. From day 2 to 6, group 1 received 100 mg/kg of morphine. Group 2 received 100mg/kg morphine + 17.8 mg/kg MP-III-024. These two doses were given twice a day at 10:00 AM and 4:00 PM. On day 7, mice also received repeated injections of 0, 1, 2.2, 6.8, and 22 mg/kg morphine with an extra dose of 68 mg/kg to reach a total cumulative dose of 100 mg/kg morphine.

Data & Results

Figure 12

MP-III-024 Does Not Enhance Morphine Abuse Liability in The Conditioned Place Preference Test

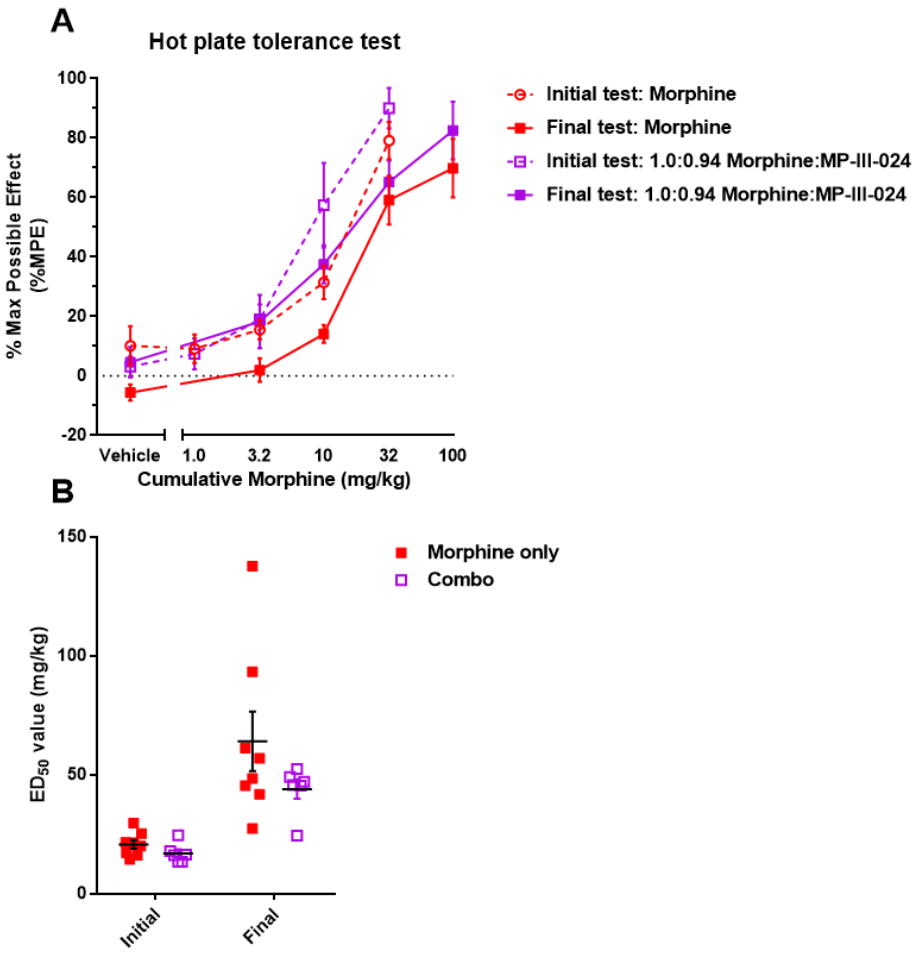


Note. To test whether MP-III-024 affects the abuse liability of morphine, a conditioned place preference (CPP) test was conducted with morphine, MP-III-024, and their combination at 1.0:0.94 ratio. Through the one-way ANOVA test, we found a difference in the preference scores among the different doses of morphine, MP-III-024, and morphine/MP-III-024 combination. According to the above graph there is a notable difference between 3 mg/kg and 10 mg/kg of morphine with a ($p = 0.0201$). In addition, the ratio of 1.0:0.94 could potentially lead to a shift towards the right in the curve, given that MP-III-024 does not elicit conditioned place preference (CPP). All results are presented as means ± SEM.

According to the results of the conditioned place preference experiment, MP-III-024 does not increase the CPP or the abuse liability of morphine. Based on the graph above **Figure 12,** we can see that MP-III-024 alone did not induce CPP. However, at 10 mg/kg, there was a significant peak for morphine, whereas in the combination of Morphine+MP-III-024, the peak was observed at 17.8 mg/kg. This data illustrates that in the presence of MP-III-024, a higher morphine dose is required to reach CPP compared to morphine alone. This means that the rightward shift might suggest a sub-additive effect (anti-synergistic) resulting from the interaction of the drug combination.

Figure 13

The Effect of MP-III-024 on Morphine Tolerance Development



Note. The hot plate assay was conducted to assess the tolerance effect of morphine with and without MP-III-024. This assessment involved comparing the dose-effect of the drug based on initial (before morphine chronic treatment) and final (after morphine chronic treatment) hot plate test. (A) There is a rightward shift of both morphine and morphine+MP-III-024 combination after the chronic treatments. Morphine alone produced

tolerance effect on the analgesic efficacy. (B) Using mixed ANOVA analysis on morphine and morphine+MP-III-024 combination to find the individual ED_{50} before and after the chronic treatments showed a significant effect of the chronic morphine treatment ($F_{(1, 12)}$ = 24.75, P = 0.0003) but no significant effect of MP-III-024 co-administration ($F_{(1, 12)}$ = 2.110, P = 0.1720). All results are presented as means ± SEM (n=8/group).

By comparing the initial and finale hot plate test data of morphine alone and morphine+MP-III-024 in **Figure 13a**, we can see rightward shift of the morphine dose-effect curve before and after the chronic treatment. This indicates the tolerance effect that was induced by the chronic administration of morphine. However, this graph also shows that the co-administration of MP-III-024 did not increase the progression of morphine tolerance. In addition, In **Figure 13b,** an ED_{50} in each animal was obtained before and after the chronic treatment, and a mixed ANOVA was used to better evaluate morphine's effect. There is a significant difference in ED_{50} value before and after the chronic treatment, but no difference between the two drugs (Morphine alone and Morphine+MP-III-024). In this experiment, we performed several t-tests: one for morphine alone, another for the combination of morphine and MP-III-024, and a vehicle test. These tests indicated that the presence of MP-III-024 did not elevate the development of tolerance to morphine.

Discussion

Opioids are widely used as analgesics for pain management. However, they are associated with significant drawbacks that limit their use. These drawbacks are mainly undesirable side effects such as respiratory depression, drug abuse, and tolerance. As a

result, it is very important to explore alternative therapeutic pathways that could treat pain in the safest way. Our primary goal in investigating MP-III-024 is to find a novel approach to combine it with morphine. Through this approach, we will enhance the potency of morphine, which means that we will be able to give lower doses of morphine with the same desirable effect. Therefore, this will minimize the occurrence of unwanted side effects associated with traditional morphine administration.

Benzodiazepine-like drugs primarily target $GABA_A$ receptors containing α1 subunits, which are distributed widely throughout the central nervous system (CNS). This distribution is implicated in inducing sedative effects and intensifying issues related to abuse and physical dependence. Because of that, there are concerns regarding the co-administration of benzodiazepine-like drugs with an opioid agonist. However, according to the research conducted by our collaborator Fischer *et al.*, 2017[42], MP-III-024 selectively binds to α2 and α3 subunit-containing $GABA_A$ receptors, which are predominantly present in spinal nociceptive circuits. These receptors appear to be free from the negative side effects associated with positive allosteric modulators (PAMs). Hence, this benzodiazepine-like compound holds the potential to exhibit promising outcomes when used in combination with morphine.

After testing multiple ratio mixtures of morphine and MP-III-024, the antinociceptive synergy demonstrated the most significant effect at the 1.0:0.94 ratio of morphine and MP-III-024[25]. From a clinical perspective, if the combined therapy yields a synergistic and leftward impact on pain yet results in an antagonistic and rightward effect on locomotor activity, self-administration, and CPP assays, it could potentially serve as a valuable therapeutic intervention. According to food self-administration and locomotor

results, the co-administration of morphine and MP-III-024 showed a potential sub-additive or anti-synergistic effect at the 1.0:0.94 ratio[45]. According to these findings we can say that the combination of morphine and MP-III-024 does not show universal synergism. Instead, the interactive effect of drug combinations depends on their relative proportions. Consequently, this approach could potentially expand the therapeutic window, differentiating between desired and undesirable effects induced by opioids.

The conditioned place preference test was mainly conducted to test if MP-III-024 has any abuse liability when it is taking with morphine. Based on the findings of the CPP studies, in the presence of MP-III-024, more morphine drug is needed to reach preference. This could lead to a reduction in the opioid dosage within medications, which will lower the likelihood of patients developing addiction.

It is well known that the long-term use of morphine could lead to tolerance. Therefore, the hot plate assay was mainly conducted to test the tolerance effect of MorphineMP-III-024 at a ratio of 1.0:0.94. According to our findings, MP-III-024 did not exacerbate morphine tolerance. Therefore, a combined MOR-α2/α3GABA$_A$ pharmacotherapy approach might offer advantages in addressing analgesia with reduced tolerance effect compared to using opioids alone.

This study had certain limitations: firstly, the two groups of animals that underwent testing in the open field assay were assessed at different time points. Secondly, only male mice were used in these investigations. Exploring the differences in opioid/GABA$_A$ signaling between male and female mice could provide valuable insights in the future.

Lastly, in the tolerance test, the animals were not randomized across diverse treatment groups within their housing units, which might have potentially influenced their behavior.

One of the primary contributors to mortality associated with opioid usage is respiratory depression[53]. An ongoing study is exploring the impact of MP-III-024 on morphine-induced respiratory depression. This investigation will employ plethysmography, a technique used to measure pulmonary function in mice[54]. Another potential side effect that will be subject to future investigation regarding the combination of MP-III-024 and morphine is constipation. Opioids reduce bowel motility, leading to the occurrence of constipation[55]. In addition, other opioid receptors will be tested to assess the effectiveness of this dual-pharmacological approach with different receptor types.

As previously mentioned, the combination of benzodiazepine-like drugs with opioids has raised significant concerns among scientists due to the substantial risk of disrupting behavioral equilibrium. This disruption could potentially lead to drug abuse, respiratory depression, and drug overdose. Although the precise role of $GABA_A$ α-subunits in the antinociceptive pathway is not extensively studied, our collaborator Fischer *et al*., 2017[42] has demonstrated the remarkable selectivity of MP-III-024, which selectively binds to $GABA_A$ receptors containing only α2 or α3 subunits. This selectivity limits other behavioral effects commonly associated with other $GABA_A$ α-subunits. Exploring innovative compounds that exhibit selective binding to $GABA_A$ α-subunits holds great significance. By exploring these compounds, we would be able to limit some of the side effects that come with benzodiazepine drugs[42].

Chapter 3

Uncovering the Role of The Novel G Protein-Coupled Receptor GPR83 in Nociception

Introduction

Over the past 20 years, there has been a surge in opioid overdose cases, which can be traced back to the higher prescription rates of opioid drugs for the management of both acute and chronic pain. Although opioid medications, like morphine, are highly potent for pain relief, they also have severe side effects including drowsiness, nausea, respiratory depression, tolerance, physical dependence, and addiction[39]. Nociception, which involves the detection of harmful or noxious stimuli, is a significant aspect of pain sensation. The dysregulation of this pathway can lead to chronic pain in many people[56].

PEN (peptide sequence SVDQDLGPEVPPENVLGALLRV) is one of the most common neuropeptides in the hypothalamus. It mainly regulates neurobiological functions that are related to reward, memory, feeding, and pain. The main receptor for PEN has been always a question until recently in 2016 when GPR83, a G protein-coupled receptor has been identified as the receptor for PEN[57]. Previous studies demonstrated the high expression of GPR83 in a region in the brain called the nucleus accumbens. This area has a significant role in reward, feeding, pain, and stress-related pathways[58]. The discovery of neuropeptide PEN as an endogenous ligand for GPR83 opens an opportunity to investigate the neurobiological function of this receptor[57,59]. Recent studies have shown the expression of GPR83 in a specific spinal tract responsible for transmitting information about noxious stimuli to the brain[60]. However, it is yet to be determined whether GPR83 plays an active

role in nociception itself. Nonetheless, the evidence suggests that GPR83 may be a potential target for reducing pain perception[60].

Introduction to Neuropeptide PEN

Peptide neuromodulator known as neuropeptide is a small substance made of proteins. It is synthesized by neurons and exerts its effect on neural target through the activation of neuropeptide receptors[61]. Targeting these receptors therapeutically offers a promising approach for addressing various pathophysiological conditions such as pain, obesity, and addiction[58,62]. Significant technological progress during the 1980s, characterized by enhanced sensitivity in peptide purification methods and the development of single-neuron mRNA sequencing techniques, has resulted in a surge of newly identified neuropeptides. However, up until now, the receptors for a considerable portion of these neuropeptides remain largely undisclosed[58]. PEN is a neuropeptide derived from the precursor protein proSAAS. The PCSK1N gene (located on the Xp11.3 of the human chromosome) encodes for proSAAS, a 26-kDa protein. This protein is highly expressed in human, rats, and mice. Research showed that many neuropeptide proteins are originally derived from proSAAS. Cleaving this protein from the N-terminal region will produce peptides GAV, littleSAAS, and KEP, while cleaving this protein from the C-terminal region will give PEN and BigLEN. Although these peptides were released from the same precursor protein, they do not share a similar sequence with PEN, therefore, they do not bind or activate GPR83 receptor[58]. According to a prior study, PEN has a role in regulating food intake and metabolism. This was evidenced by administering anti-PEN antibody to a group of fasting mice, resulting in a significant reduction in food intake for a duration of 14 hours subsequent to the antibody injection[63].

Introduction to GPR83 Receptor

GPR83 was first discovered in murine thymoma cells when it was highly expressed after the addition of glucocorticoid dexamethasone. Therefore, it was named the Glucocorticoid Induced Receptor (GIR). It is also referred to as GPR72 and JP05. Several studies showed the high expression of this receptor in mice and human brains. In human GPR83 has one isoform which is encoded by *Gpr83* gene. The specific location of this protein is in chromosome 11 (q21 region). This gene is translated into 423 amino acid protein with ~48 kDa. In mice, up to four isoforms were discovered, isoform 1 is similar to human's isoform. Therefore, most GPR83 studies focus on this isoform in mice. Isoform 2 has a deletion in exon 2 and it does not have the third transmembrane domain, therefore, it is expected to be nonfunctional. Isoforms 3 and 4 differ in the number of insertions in the second cytoplasmic loop. Isoform 3 has 68 amino acids, while isoform 4 have 20 amino acids[58]. GPR83 has a role in the regulation of feeding and stress. The deletion of exons 2 and 3 will cause a deletion of this receptor. Previous studies showed an alteration in food intake and anxiety-stress pathways due to the absence of GPR83[64,65].

PEN is the Endogenous Ligand for GPR83

Previous studies show the identification of neuropeptide PEN as the endogenous ligand of GPR83 by using the expression/distribution match strategy, where they match the expression of different peptide precursors in specific areas to different GPCRs. Firstly, they confirmed that PEN's receptor in the hypothalamus shares properties with a receptor present in Neuro2A cells. After that, they screened orphan GPCRs that are highly expressed in both the hypothalamus and Neuro2A cells, to identify if there are any that could be activated by PEN signaling[57]. From there, GPR83 was able to generate a signaling response

sufficiently, which ultimately led to the conclusion of GPR83 as the receptor for PEN. To test this precisely, they transfected Chinese Hamster Overy cells (CHO) with GPR83 (a cell line that does not endogenously express GPR83) and chimeric $G\alpha_{16/i3}$ protein. After that they test whether these cells produce signaling activity in response to neuropeptide PEN. They were able to test that by measuring the intracellular calcium level which indicates the activation of GPR83. They also test the signaling of other receptors including GPR19, GPR108, GPR165, and GPR171 by neuropeptide PEN. Eventually, they found that PEN is a selective and potent endogenous ligand for GPR83. In addition, scientists wanted to determine if GPR83 is required for the signaling of PEN. They used either Nero2A cells (a cell line that endogenously express GPR83 receptor) which modified by shRNA (knockdown) to express less GPR83 or used tissue samples that do not express the receptor (knockout). As a result, with knockdown cells there was a reduction in signaling of neuropeptide PEN and with knockout cells, there was a significant loss of PEN signaling[57,58].

Regional Expression Patterns of PEN-GPR83

In mice, GPR83 mRNA is highly expressed in the limbic structures of the forebrain, in specific areas of the hypothalamus, and the striatum, with a significant presence in the nucleus accumbens. It has relatively low expression levels in the periphery, excluding the thymus. Due to the expression of GPR83 in these regions, it has an important role in several neurological functions, including reward pathways, food intake, and immunological reactions. Since GPR83 is distributed throughout the thymus, it has a role in the immune system. Previous studies demonstrated the increase of GPR83 expression within regulatory T-cells (T-reg), a specific type of T-cell subgroup

responsible for inhibiting immune reactions against the body's own antigens. Additionally, they play a role in limiting excessive responses that could be detrimental to the host[66]. In addition, given that Gpr83 is present in hypothalamic nuclei which mainly controls energy metabolism and because its possible influence on the hypothalamus–pituitary–adrenal axis, which has a role in systemic metabolism, Gpr83 plays a part in centrally regulating energy metabolism[64].

In humans, GPR83 mRNA demonstrates significant expression levels in the brain and spinal cord, while being present in relatively lower amounts in the thymus and other tissues. In the human brain, GPR83 is mainly expressed in the hippocampus, amygdala, hypothalamus, striatum, and prefrontal cortex. This distribution implies a potential involvement of GPR83 in processes related to stress, reward, anxiety, fear, as well as learning and memory. In addition, within the human cerebellum, GPR83 is highly expressed in the cerebellar granular cell layer. This observation implies a potential function of GPR83 in controlling motor learning and coordination processes in humans[67].

Humans, mice, and rats showed a highest GPR83 expression through PEN binding in the striatum. Specifically, GPR83 expression is more significant in the nucleus accumbens (NAc) compared to the dorsal striatum[68]. This expression demonstrates the role of this receptor in learning and reward pathway[69].

Gq-Mediated Signaling Pathway by GPR83

Drawing from previous studies indicating the interaction of GPR83 with $G\alpha_q$ proteins, Phospholipase C (PLC) activity was examined in CHO-GPR83 cells (Chinese Hamster Ovary cells with overexpressed GPR83). PEN induced PLC activation in a dose-dependent manner with remarkable potency. This is significant because PLC triggers the

generation of IP3 (inositol 1,4,5-triphosphate), ultimately leading to the release of intracellular calcium[57]. This shows the activity of GPR83 through $G\alpha_q$-signaling. While in certain regions, such as hypothalamic membranes, GPR83 is activated through $G\alpha_q$ signaling, studies have demonstrated that the activation of GPR83 by PEN in other areas, such as hippocampal membranes, does not affect PLC (phospholipase C). In these cases, the pathway is activated through Gi proteins leading to a significant decrease in adenylyl cyclase activity. These findings indicate that PEN-GPR83 signaling varies depending on the specific brain region[57].

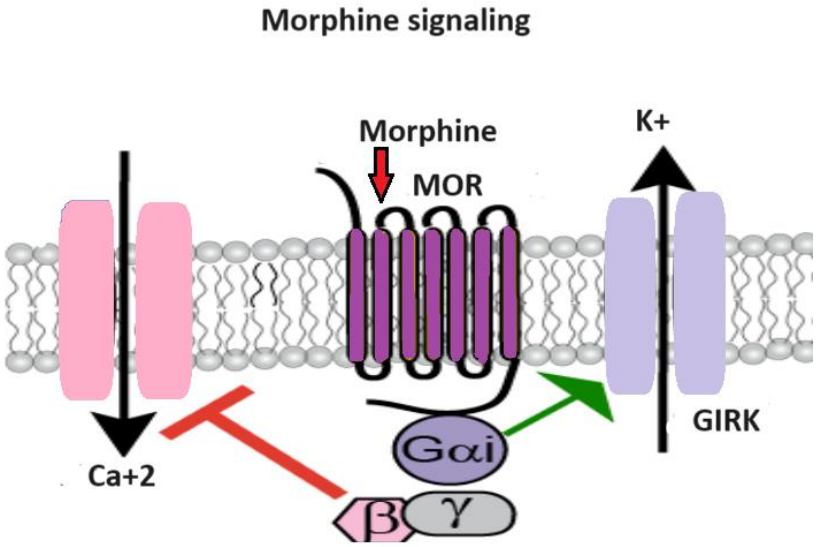
Morphine and MOR (Gαi) Signaling Pathway

The analgesic effect of morphine primarily results from its binding to the μ-opioid receptors located in GABAergic terminals in the spinal cord and the periaqueductal gray (PAG). Morphine-MOR binding will inhibit the release of GABA neurotransmitter which will inhibit nociceptive signals[34]. In addition, it will activate dopaminergic neurons in the nucleus accumbens region (NAc) which will induce reward pathway[35]. In a molecular level **Figure 14**[35], morphine-MOR will lead to the activation of Gi proteins. This will trigger changes in molecular signaling within the cell, including β-arrestin binding. G protein consists of three subunits: α, β, and γ. After morphine binds to MOR, GTP will bind to α subunit forming α-GTP. This complex will dissociate from βγ dimer, which will lead to the suppression of adenylate cyclase activity and subsequently decrease in cellular cyclic adenosine monophosphate (cAMP) levels. Both α-GTP and ßγ dimer contribute to receptor signaling. α-GTP initiates the activation of phospholipase-C (PLC), which subsequently hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2), producing inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 promotes the release of calcium from

the endoplasmic reticulum, thereby activating calcium-dependent signaling pathways. Additionally, IP3 activates potassium channels, specifically the G protein-gated inward rectifying potassium channel (GIRK-3), resulting in increased cellular hyperpolarization and indirectly reducing cell excitability. On the other hand, the ßγ dimer directly blocks calcium channels, such as the P/Q-type, N-type, and L-type channels, leading to a decrease in intracellular calcium concentration. This reduction in calcium concentration suppresses the release of other neurotransmitters[35].

Figure 14

Morphine Signaling



Note. After morphine binds to MOR, $G\alpha_i$ will dissociate from ßγ and activate potassium channel GIRK. This will increase potassium efflux, resulting in neuron hyperpolarization. At the same time, the ßγ dimer directly blocks calcium channels, reducing the cytosolic calcium concentration, causing a decrease in neuron excitability and neurotransmitter

release. Overall, these result in a reduction of nociception. *Adapted from Listos, J et al, (2019). The Mechanisms Involved in Morphine Addiction: An Overview.*

Recent studies have provided evidence that GPR83 is expressed in a specific ascending spinal tract that delivers information about noxious stimuli to the brain but did not determine whether GPR83 function itself played an active role in the detection of noxious stimuli[60]. Here, I will present recent findings from Dr. Fakira that illuminate the involvement of the GPR83 receptor in the pain pathway. Furthermore, the study will explore roles of its endogenous ligand (PEN), agonist (CPD1), and antagonist (CPD25), both individually and in combination with morphine.

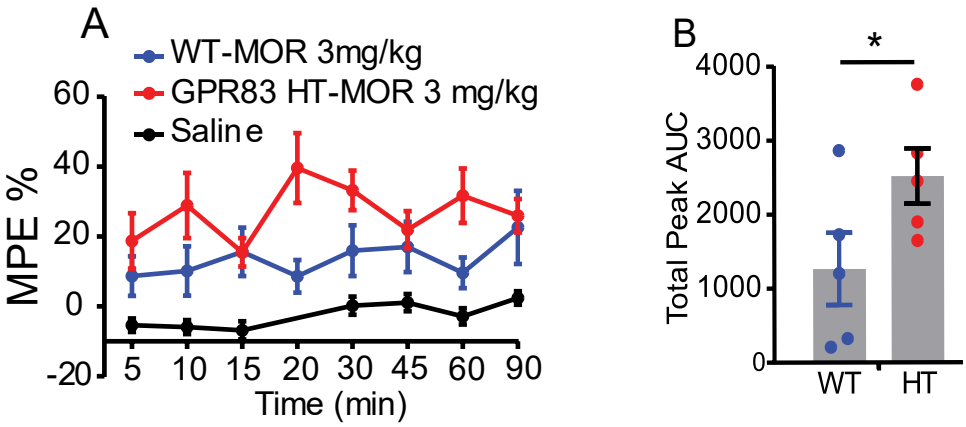
The Effect of GPR83 on Morphine Analgesia

To investigate the role of GPR83 on the antinociceptive effect of morphine, the tail flick assay has been conducted. This assay is mainly employed to measure heat-induced pain in animals following the administration of a specific analgesic medication. In this procedure, an intense light beam is directed onto the tail of mice, and a timer is initiated. The timer is stopped when the mouse flicks its tail in response to the heat, and the latency of the tail flick is recorded. A cut-off of 20 seconds will be imposed to prevent tissue damage, ensuring the safety of the animals. The longer it takes for the mouse to flick its tail, the more effective the analgesic effect of the drug[70]. For this test, a dose of 3 mg/kg of morphine was given (i.p) to 2 sets of mice: wild type mice (n=5) with (the normal amount of GPR83) and heterozygous mice (n=5) with (half the normal amount of GPR83). Subsequently, the tail flick assay was conducted at multiple time points within a 90-minute duration starting 30 minutes after morphine administration[71].

The data were analyzed by measuring the maximal possible effect of morphine using this formula $\%MPE=((post\ \ drug\ \ right\ \ paw\ \ threshold\ \ (g)-baseline\ \ right\ \ paw\ \ threshold(g))/(baseline\ \ left\ paw\ threshold\ (g)-baseline\ right\ paw\ threshold\ (g)))\ X\ 100$[72]. According to the data, morphine antinociceptive effect was increased in heterozygous mice compared to wild-type mice **Figure 15**. This indicates the increase of morphine analgesic effect in the absence of GPR83[71].

Figure 15

The Absence of GPR83 Enhanced the Analgesic Effect of Morphine



Note. The tail flick assay was used to examine analgesia in GPR83 wild-type mice (WT) and Heterozygous (HT) mice. Following the administration of 3 mg/kg of morphine (i.p). analgesia was measured over a 90-minute period. (A) individual time course curves were constructed for each mouse. The maximal possible effect of morphine was higher in heterozygous mice compared to wild type mice. (B) the area under the curve was calculated. Heterozygous mice had a higher AUC peak compared to wild type mice. The study involved a sample size of 5 mice per group, with statistical significance denoted as

*p<0.05. Fakira, et al, Mu-GPR83 analgesia. Cooper Medical School of Rowan University (CMSRU). Grant Application. (2022)[71].

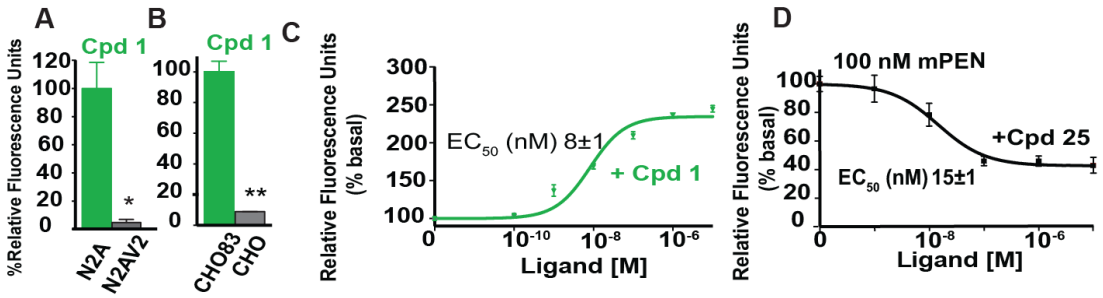
Identification of GPR83 Agonist and Antagonist

By using a crystal structure of a related receptor, a homology model was constructed. Virtual screening of a publicly available library of small molecule compounds was conducted, resulting in the identification of 50 hits predicted to interact with the binding pocket. These 50 hits were then subjected to testing in a cell-based assay. As a result of this assay, 2 agonists and 3 antagonists have been identified and are currently undergoing further characterization[73]. In Fakira's laboratory, CPD1, a GPR83 agonist, and CPD25, a GPR83 antagonist, have undergone evaluation using various cell lines to assess GPR83 expression. Calcium fluorescence has been used as an indicator, where the increase in intracellular calcium release reflects the upregulation in the expression of this receptor. Cell lines that have been used for this essay are: Neuro2A cells (a cell line that endogenously expresses GPR83 receptor), shRNA treated Neuro2A cells (knockdown which express less GPR83), Chinese hamster ovary cells (tissue samples that do not express the receptor 'knockout'), and a GPR83 transfected CHO **(Figure 16)**. According to the presented data, CPD1 (10 μM) increases calcium intracellular level in Neuro2A cells and GPR83 transfected CHO. This indicates the increase in the expression of GPR83. However, there was no increase in GPR83 expression in shRNA treated Neuro2A cells and CHO after the addition of the same compound. Using (1-10 μM) CPD25 there was a dose dependent reduction in the expression of GPR83 in Neuro2A cells that have been pre-

treated with 100nM neuropeptide PEN. Therefore, CPD1 is GPR83 agonist which activates the receptor and CPD25 is GPR83 antagonist which blocks the activity of the receptor[71].

Figure 16

Small Molecules, CPD1 and CPD25 are the Agonist and Antagonist of GPR83, Respectively



Note. (A) at a concentration of 10 µM, CPD 1 induces intracellular calcium release in Neuro2A cells (cells that naturally express GPR83). However, this effect is diminished in N2A cells where GPR83 has been knocked down using lentiviral shRNA (N2AV2). (B) at a concentration of 10 µM, CPD 1 did not induce GPR83 expression in CHO cells (which naturally lack GPR83), but it did trigger intracellular calcium release in CHO cells that were artificially transfected with GPR83. (C) CPD 1 demonstrates a dose-dependent induction of intracellular calcium release across a range of concentrations from 0 to 10 µM. (D) when N2A cells that naturally express GPR83 were treated with 100 nM mouse PEN (mPEN) in the presence of varying concentrations of CPD 25 (0-10 µM), the measurement of intracellular calcium release showed that CPD 25 effectively inhibits mPEN signaling. The values obtained with mPEN were considered as 100%. The data represents mean ±

SEM (n = 3). *p<0.05, **p<0.01. Fakira, et al, Mu-GPR83 analgesia. Cooper Medical School of Rowan University (CMSRU). Grant Application. (2022)[71].

The Effect of the GPR83 Agonist and Antagonist on Morphine's Activity

To assess the impact of GPR83 ligands on the antinociceptive effect of morphine, the tail flick assay has been conducted. Four different groups, each consisting of 12 mice (comprising both females and males), received different intraperitoneal injections (i.p.) as shown in **Table 3**. Following a 30-minute interval, the tail flick assay was conducted to evaluate the analgesic effect of morphine. After graphing the data and calculating the maximal possible effect (%MPE) for each mouse **(Figure 17)**, female mice that received 3 mg/kg of morphine with 5 mg/kg of CPD25 had a higher antinociceptive effect of morphine compared to mice who received 3 mg/kg of morphine with vehicle. On the other hand, female mice who received 3 mg/kg of morphine with 5 mg/kg of CPD1 had a lower effect of morphine compared to mice who received 3 mg/kg or morphine/vehicle. However, in male mice there was no change in morphine antinociceptive effect after CPD25 **(Figure 18).** Two potential reasons explained the lack of CPD25 effect in male mice. Firstly, it's possible that male mice inherently possess diminished GPR83 signaling, thus, additional inhibition is ineffective in demonstrating an antinociceptive boost. Secondly, a previous study conducted by Dr. Fakira showed that 3 mg/kg morphine dosage proves more efficacious in eliciting antinociception in male mice compared to female mice, it's possible that the dosage of CPD25 administered in these investigations (5 mg/kg) might not be adequate to further amplify the response[71].

Overall, GPR83 has a bidirectional effect on morphine analgesia. The antagonist (CPD25) enhanced the antinociceptive effect of morphine, while the agonist (CPD1) blunted the antinociceptive effect of morphine. This was observed only in female mice, with no alteration in morphine activity observed in male mice. One hypothesis that could account for this outcome is the presence of an interaction, either functional or physical, between PEN-activated GPR83 and μ-opioid receptor which led to the antinociceptive regulation of morphine. To test this hypothesis, an experiment was conducted to initially confirm the co-localization of MOR and GPR83 in the same brain region, specifically in the periaqueductal gray (PAG), a brain region rich in μ-opioid receptor, is known to play a critical role in antinociception[71].

Table 3

Summary of Administered Doses[71]

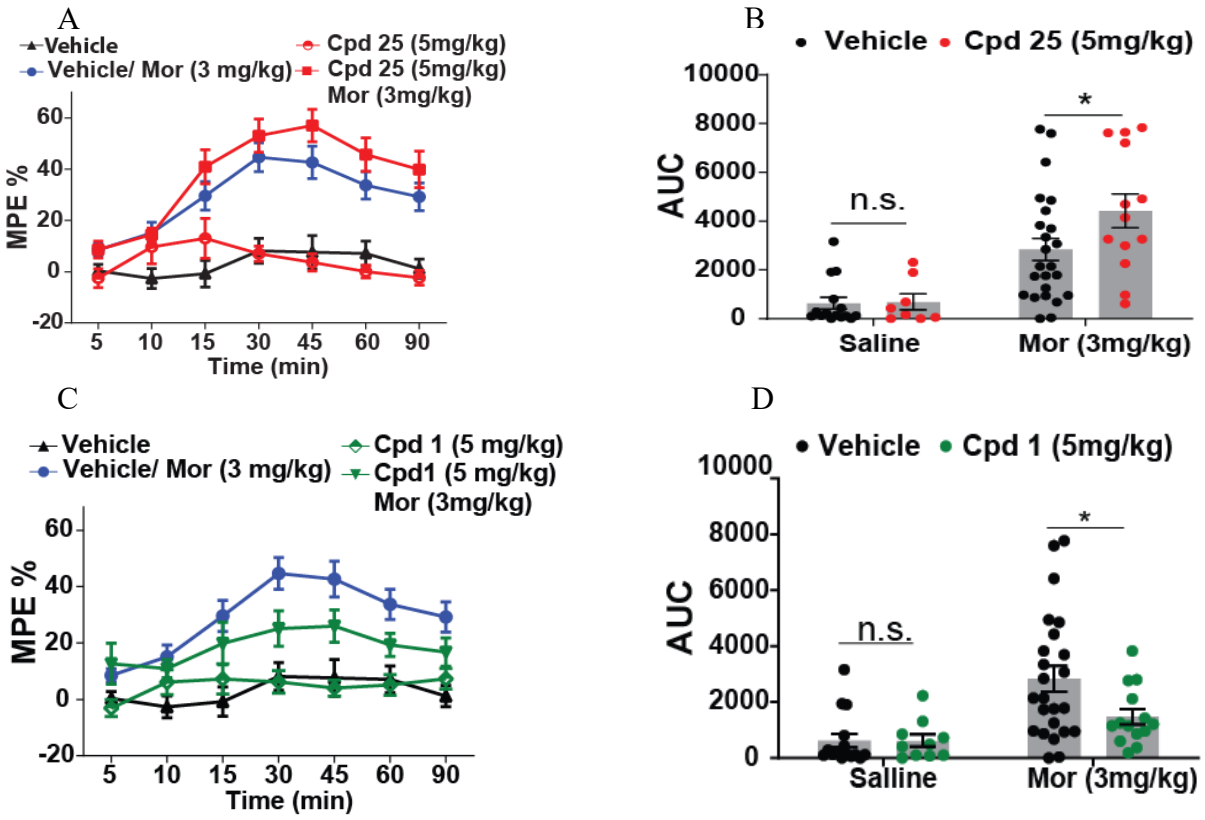
Group #	Treatment
(1)	Vehicle (10% DMSO, 5% Kolliphor 85 % Saline).
(2)	Vehicle/Morphine (3mg/kg).
(3)	CPD1 OR CPD25 (5mg/kg).
(4)	CPD1 OR CPD25 (5mg/kg)/ Morphine (3mg/kg).

Note. The tail flick assay was conducted to test the analgesic effect of morphine and CPD25 or CPD1 when taken at the same time. There were 4 groups of mice in each experiment (CPD1 or CPD25 experiments). Group 1 received vehicle, which composed of 10% DMSO, 5% Kolliphore, and 85 % Saline. Group 2 received vehicle and morphine

(3mg/kg). Group 3 received CPD1 or CPD25 (5mg/kg). Group 4 received CPD1 OR CPD25 (5mg/kg) with morphine (3mg/kg)[71].

Figure 17

CPD25 Attenuated the Analgesic Effect of Morphine While CPD1 Reduced It, in Female Mice

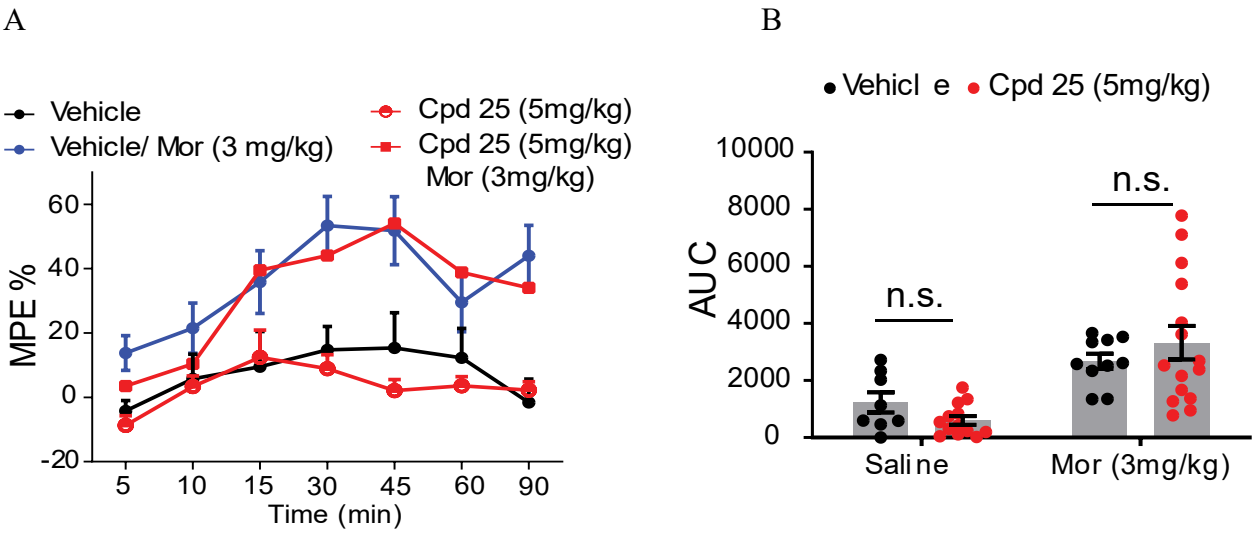


Note. A time course and the area under the curve were measured after testing CPD25 and CPD1 individually and combined with morphine in female mice. (A) CPD25/Morphine has a higher antinociceptive effect compared to Vehicle/Morphine. (C) CPD1/morphine has a lower antinociceptive effect compared to Vehicle/Morphine. (B) combining CPD25 with morphine increased the area under the curve compared to when morphine was taking

with vehicle.), Interaction F(1, 56) = 1.98, p=0.16; Morphine F(1, 56) = 30.34, p<0.0001; CPD 25 F(1, 56) = 2.33,p=0.13; (D) the area under the curve was reduced when CPD1 was given with morphine compared to morphine/vehicle.), Interaction F(1, 59) = 2.67, p=0.11; Morphine F(1, 59) = 13.51, p=0.0005; CPD 1 F(1, 59) = 2.66, p=0.11; * p<0.05, ** p<0.01, n=9-23 mice/gp. Fakira, et al, Mu-GPR83 analgesia. Cooper Medical School of Rowan University (CMSRU). Grant Application. (2022)[71].

Figure 18

CPD25 Had No Effect of Morphine's Analgesia in Male Mice



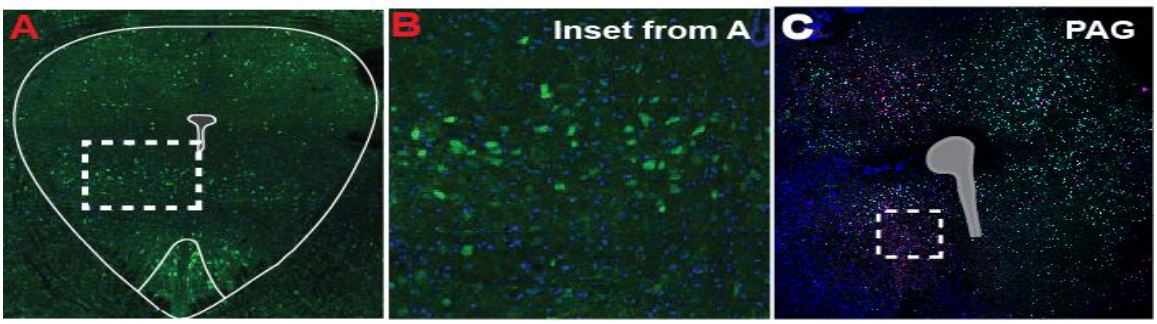
Note. (A) Time course and (B) the area under the curve were measured after testing CPD25 individually and combined with morphine in male mice. There was no change on morphine analgesia with the presence of CPD25. Interaction F(1, 42) = 2.10, p=0.15; Morphine F(1, 42) = 21.93,p<0.0001; CPD 25 F(1, 42) = 0.0004962, p=0.9823, n=8-10 mice/gp. Fakira, et al, Mu-GPR83 analgesia. Cooper Medical School of Rowan University (CMSRU). Grant Application. (2022)[71].

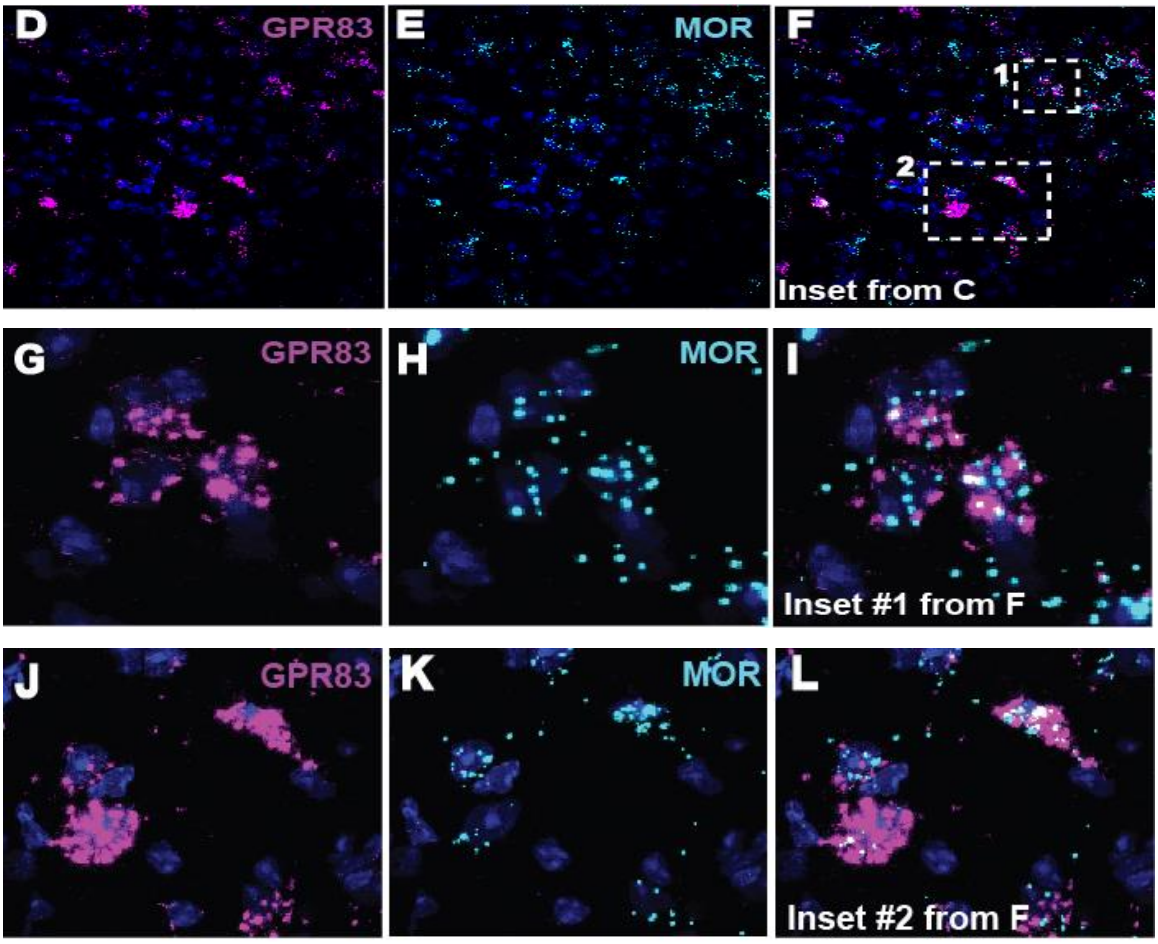
Co-Localization of GPR83 and μ-opioid Receptor (in PAG)

To investigate the presence of GPR83 in the same cells as MOR, neuroanatomical studies were conducted to map the expression pattern of GPR83 and MOR by crossing GPR83-eGFP mice with MOR-mCherry mice. RNA in situ hybridization was done to indicate the presence and location of GPR83 and μ-` using antibodies. MCherry and eGFP mice at the age of 2-3 months were perfused in 4% formaldehyde. After that, brains were removed, fixed, and 50 μM coronal brain slices were prepared to visualize the desired brain region under the microscope. According to the data demonstrated below in **Figure 19**, GPR83 was found to be co-expressed in the same neuronal cells as MOR in the PAG area. The GPR83 expression in GABAergic neurons also confirmed by previous studies showing its expression within parvalbumin-positive GABAergic neurons in two different brain regions: the amygdala and nucleus accumbens[59]. This will open a wide window to further investigate the presence of physical or functional interaction between GPR83 and μ-opioid receptor MOR, which will explain the antinociceptive regulatory effect of activated GPR83 receptor on morphine[71].

Figure 19

Co-Localization of GPR83 and μ-opioid Receptor (in PAG)[71]





Note. (A) GPR83 expression in the PAG in GPR83-eGFP reporter mice from GENSAT. (B) a magnified image of (A). (C) Low magnification image of GPR83 and MOR mRNA expression in the PAG. Higher magnification image from (C) GPR83 (magenta, D) and MOR (cyan, E) merged imaged in (F). (G-I) and (J-L) Larger images from box #1 and #2 in (F) respectively. Fakira, et al, Mu-GPR83 analgesia. Cooper Medical School of Rowan University (CMSRU). Grant Application. (2022)[71].

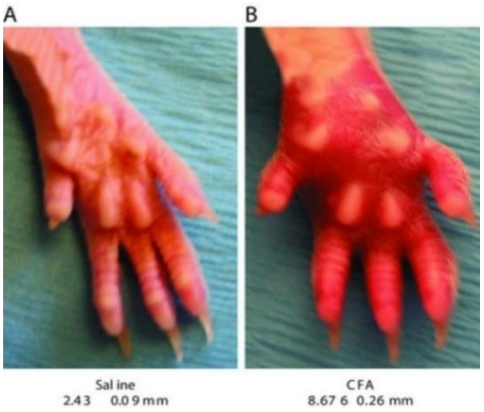
GPR83 Expression During Chronic Inflammatory Pain

Using the inflammatory pain model, the Complete Freud's Adjuvant (CFA), a study was conducted to test the impact of chronic inflammatory pain on GPR83 expression, specifically within two regions rich in GPR83 receptors: the nucleus accumbens and the Periaqueductal grey area (PAG). To induce pain in mice, the CFA, a mycobacterium suspension in oil, is administered into the plantar surface of the hind paw which will induce inflammation and tissue necrosis (**Figure 20)**. For this experiment, a group of 12 mice received CFA injections, while another set of 12 mice received saline injections (serving as the control group). The von Frey assay was conducted to indicate the sensitivity of mice due to mechanical stimulation. After that, mice were euthanized and their brain were collected, fixed, and sliced to measure the mRNA expression of GPR83 in the nucleus accumbens and the Periaqueductal grey area PAG**.**

As a result **(Figure 21)**, Male and female mice who received CFA injections had lower mechanical threshold compared to mice who received saline. After measuring GPR83 mRNA expression in the same mice, CFA group had a lower GPR83 expression compared to saline group in both brain regions, nucleus accumbens, and PAG. However, the reduction was more significant in the nucleus accumbens. This observation is in line with expectations, given that the nucleus accumbens typically exhibits higher levels of GPR83 expression compared to the periaqueductal gray[69].

Figure 20

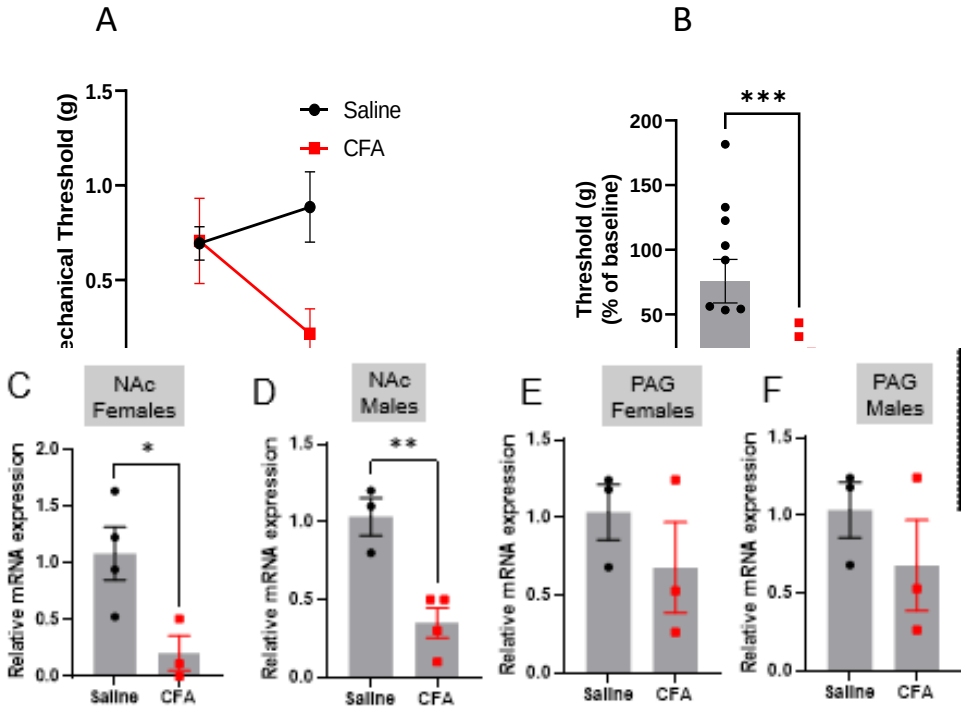
Saline Versus CFA Paw Injections



Note. Mice hind paw injected with (A) saline, or (B) CFA. *McCarson et al. 2015*[74].

Figure 21

GPR83 Expression was Reduced Following Chronic Inflammatory Pain



Note. (A) and (B) Injection of CFA (50µL) into the hind paw results in a decreased threshold for detecting mechanical stimuli as determined by the von Frey assay. (n= 11-13) per group (including both males and females), T-test $p<0.0001$. (C) GPR83 mRNA expression shows a reduction in the nucleus accumbens of female mice treated with CFA, and (D) a similar reduction is observed in male mice. However, in the periaqueductal gray, GPR83 mRNA expression is not significantly affected in either sex as shown in panels (E) and (F). T-test, with n=3-4 mice per group. *$p<0.05$; ** $p<0.01$.

Materials and Methods

Animals

Drug naïve adult male and female C57BL/6J mice obtained from Jackson laboratories were used for these studies. Upon arrival to the vivarium at Cooper Medical School of Rowan University 'CMSRU', animals were grouped as fours and housed in plexiglass cages equipped with bedding, nestlets, enviropaks, and constant access to water and food. They were kept in a room with constant temperature and humidity, under a 12h light/dark cycle (lights on at 7:00 AM). The Mice were 45 days old and weighed approximately 22-25 g. First, they were habituated in the room for 2 weeks (no experiments were conducted). After that, they were handled 4-5 days before starting the experiment. The animals involved in these experiments are within the guidelines set forth by the Institutional Animal Care and Use Committee of Rowan University. All experimentation was conducted following the "Guide for the Care and Use of Laboratory Animals." (National Research Council, National Academy of Sciences, Washington, D.C., USA, 2011). GPR83/eGFP transgenic mice and wild-type Swiss Webster mice were also used in this study. They were generated by the

GENSAT project and were obtained from The Mouse Biology program at the University of California, Davis. The arrangement involved placing the coding sequence of enhanced green fluorescent protein (eGFP) along with a polyadenylation signal into a bacterial artificial chromosome (BAC). This integration occurred at the GPR83 gene's ATG transcription codon. Consequently, cells that produce GPR83 mRNA simultaneously exhibit the expression of eGFP[75].

Drugs

Morphine is a potent pain-reliving medication, 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. It is a naturally extracted alkaloid from a plant called opium poppy, Papaver somniferum. It is part of a class of medications called opioids. Morphine binds selectively to μ-opioid receptor and activate it to inhibit pain signals that are sent from nociceptors. It is a highly potent analgesic with a high potential for abuse. This drug is fully dissolved in 0.9% NaCl sterile saline[47].

CPD1 and CPD25. Compound 1 (CPD1) and compound 25 (CPD25) are GPR83 agonist and antagonist, respectively. We get these compounds from a Dr. Lakshmi's lab in Icahn School of Medicine at Mount Sinai. These compounds are dissolved in 10% DMSO, 5% Kolliphore, 85% sterile saline[73].

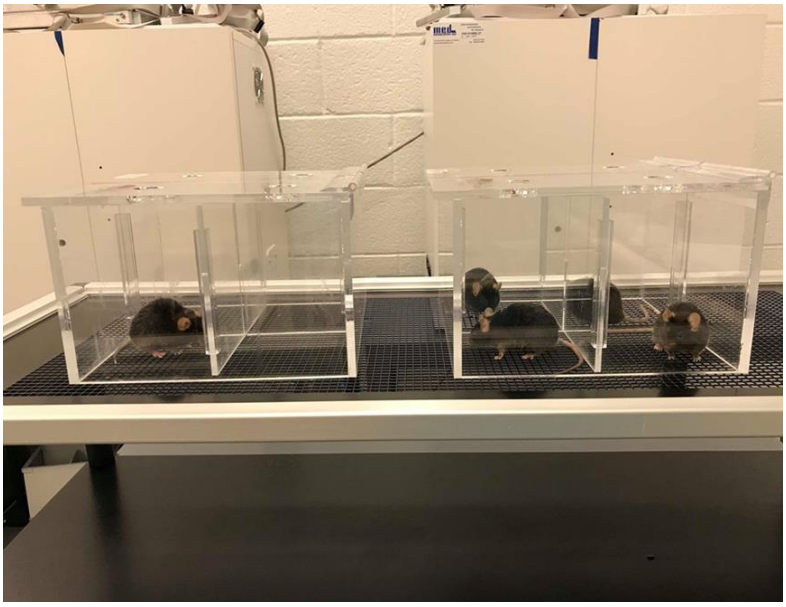
Behavioral Assays

von Frey Test

The 'up-down' von Frey assay is a method for evaluating mechanical allodynia in mice and rats[76]. Individual mice are placed in transparent plexiglass containers on a raised wire mesh platform, enabling access to the underside of their hind paw **(Figure 22)**. As shown in **Figure 23,** the von Frey hairs are used to measure withdrawal response frequency. These hairs are gradually graded filaments categorized by their stiffness (5-26 g). Each filament is applied to the mid plantar surface of the hindpaw until it bends. If mice sense the filament, they will react to it by withdrawing, licking, or shaking their paw. Any paw withdrawal behavior demonstrates a positive response. The animals are given at least one hour to acclimate prior to the test, and mechanical hypernociception is assessed at multiple time points. The withdrawal threshold of naïve mice is determined before any intervention or treatment, and this value serves as the baseline (B) for comparison in the graphs. The initial filament application is expected to elicit a 50% withdrawal threshold response. If there is no observable response, the subsequent filament with a higher force is employed. Conversely, if a response is detected, the next filament with lower force is used. This is stopped after four measurements following the first change in response (when transitioning from response to no-response or from no-response to response)[77,78].

Figure 22

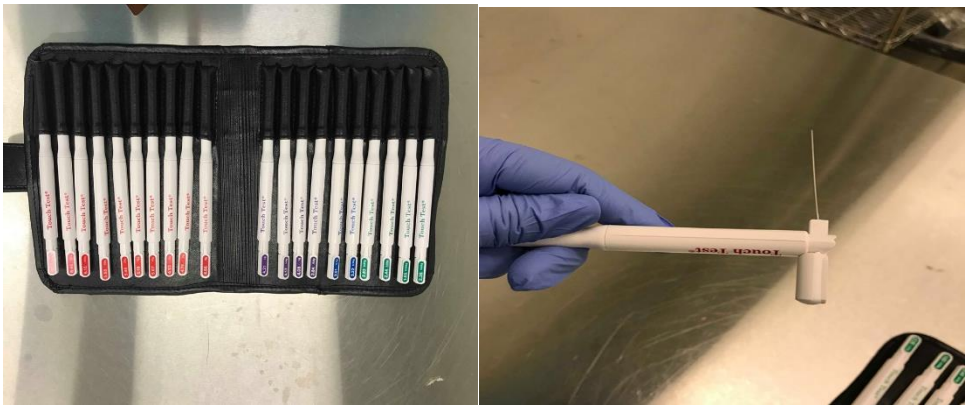
von Frey Setup



Note. Image of the von Frey setup from our research lab. The setup is composed of 3 transparent plexiglass containers. Each container is divided into 4 chambers. A mesh surface is located under these chambers where mice can move freely. The mesh is essential for the filament hair to go through. Picture from Rahman M. (2021). Used with permission[79].

Figure 23

von Frey Filaments



Note. Image of von Frey filaments from our research lab[79]. The von Frey hairs are used to measure withdrawal response frequency. These hairs are gradually graded filaments categorized by their stiffness (5-26 g). Each filament is applied to the mid plantar surface of the hindpaw until it bends. If mice sense the filament, they will react to it by withdrawing, licking, or shaking their paw. Any paw withdrawal behavior demonstrates a positive response. Picture from Rahman M. (2021). Used with permission.

Experimental Procedures

The von Frey Assay with CPD25

As previously demonstrated, CPD25, GPR83 antagonist, plays a crucial role in enhancing the antinociceptive effect of morphine[71]. Nevertheless, it was necessary to evaluate the influence of CPD25 on mechanical pain in a non-inflammatory context, and to achieve this, the von Frey assay was conducted. A group of 12 male mice underwent a two-day habituation process, consisting of two-hour sessions each day within transparent plexiglass containers. On the third day, von Frey measurements were taken to establish the baseline

for comparison. After 48 hours, mice were administered an i.p injection of 10mg/kg CPD25 or vehicle. von Frey measurements were taken 20 minutes after the drug administration. The same procedure was repeated the following week with a separate group of 12 female mice.

The von Frey Assay with CPD1/CFA

The previous data indicated the influence of CPD1 in diminishing the activity of morphine[71]. In this particular test, CPD1 was administered alongside the Complete Freud's Adjuvant (CFA) to investigate its effect on mice experiencing inflammatory pain. A group of 12 male mice underwent a two-day habituation process, consisting of two-hour sessions each day inside transparent plexiglass chambers for von Frey testing. On the third day, von Frey measurements were conducted to establish a baseline for comparison, immediately followed by paw injections of CFA or saline (0.9% NaCl). During the CFA/saline injections, each mouse was initially anesthetized with 3% Isoflurane, followed by a subcutaneous injection of 50 µl either CFA or saline into its right or left paw. After the injection, the mouse was returned to its respective cage. After a 48-hour interval from the CFA/saline injections, the mice received an intraperitoneal (i.p.) injection of either 10mg/kg CPD1 or vehicle. von Frey measurements were performed 20 minutes after the administration of the drug. The same procedure was repeated the following week using a group of 12 female mice. The injection regimen was evenly distributed in a way where each mouse that received vehicle/CPD1 would receive CFA or saline injections in either the right or left paw, as shown in **Table 4**.

Table 4

von Frey Assay with CPD1 Experimental Drug Dosing Regimen

ANIMAL ID	PAW INJ.	SAL/CFA	COMPOUND
C1A1	R	Sal	Vehicle
C1A2	R	CFA	Vehicle
C1A3	L	Sal	CPD1
C1A4	L	CFA	CPD1
C2A1	L	CFA	Vehicle
C2A2	L	Sal	Vehicle
C2A3	R	CFA	CPD1
C2A4	R	Sal	CPD1
C3A1	R	Sal	CPD1
C3A2	L	Sal	Vehicle
C3A3	R	CFA	CPD1
C3A4	L	CFA	Vehicle

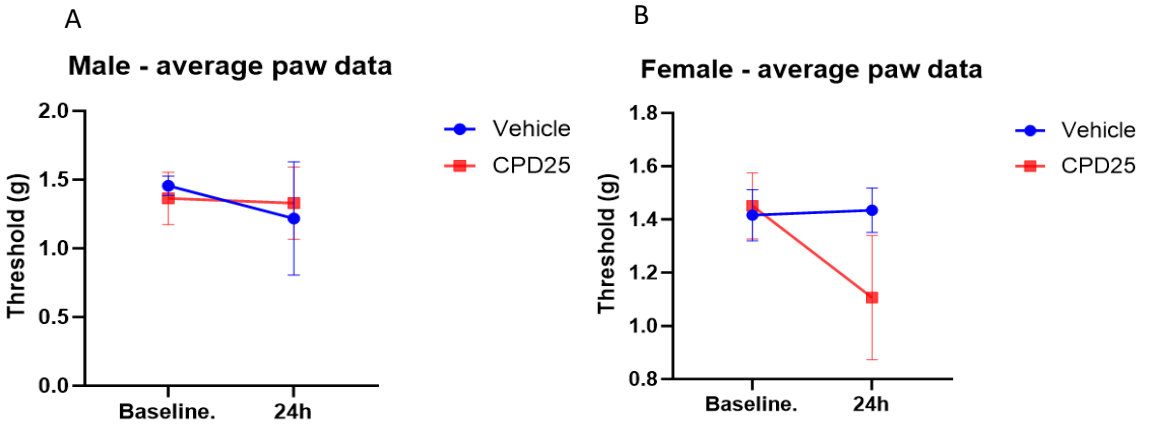
Note. Mice were assigned randomly to receive CPD1or vehicle and Saline or CFA. Half of the animals received the CFA injection on their right paw and the other half on their left paw.

Data & Results

von Frey Assay Results

Figure 24

The Threshold for Detecting Mechanical Stimuli After CPD25 in Male and Female Mice



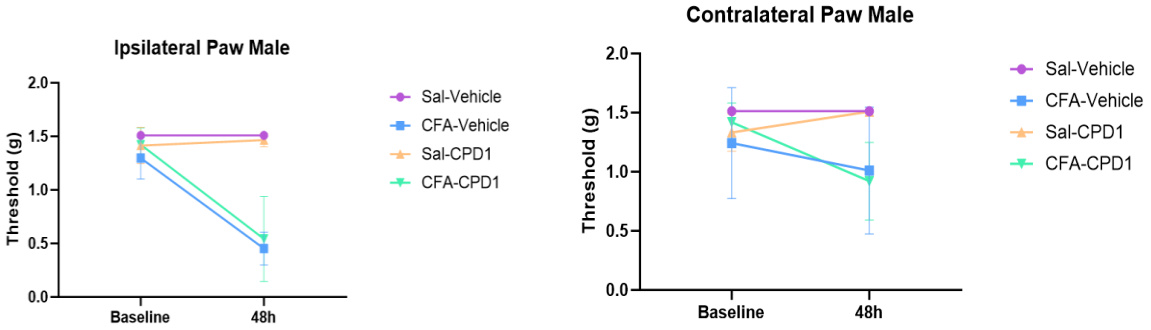
Note. Both male and female mice received injections of either Cpd 25 (10 mg/kg, i.p) or a vehicle (i.p). (A) and (B) illustrate the average withdrawal threshold for both the contralateral and ipsilateral paws. 2-way ANOVA, (A) the mechanical thresholds were similar after CPD25 and vehicle administration, indicating that CPD25 had no effect on male mice. interaction: F(1, 10), P=0.3290 CPD25: F(1, 10), p= 0.9635. Time: F(1, 10), P=0.1985. (B) CPD25 significantly decreased the mechanical threshold in female mice, interaction F(1, 9), P=0.0120 CPD25: F(1, 9), p= 0.0042 Time: F(1, 9), P=0.0205. (n=12)

To detect the effect of CPD25 on mechanical sensitivity, mice received either 10mg/ml of CPD25 or vehicle. As shown in **Figure24**, there was no significant change in mechanical sensitivity among male mice following the administration of CPD25 **(A)**.

However, there was a significant reduction in mechanical threshold in female mice after receiving CPD25, which indicates their higher sensitivity to the mechanical stimuli compared to vehicle administered mice **(B)**. This shows a sex-difference in the observed effects.

Figure 25

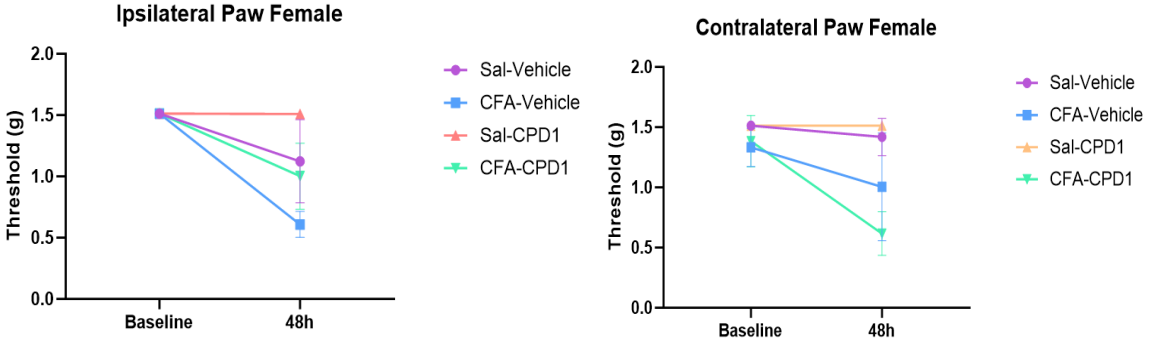
The Threshold for Detecting Mechanical Stimuli After CPD1 in Male Mice



Note. This graph mainly focuses on the mechanical threshold, or the minimum force required to evoke a positive withdrawal response after receiving CPD1 during inflammation. Male mice received i.p injections of either 10 mg/kg CPD 1 or vehicle, after CFA/saline injections (A) the ipsilateral paw of male mice (the CFA injected paw) had lower mechanical threshold after receiving CFA compared to saline. No effect observed in the CFA injected paw after CPD1 administration. This indicates that CPD1 had no effect on inflammatory pain. 2-way ANOVA, interaction: F(1, 8), P=0.0076 CPD1: F(1, 8), P= 0.0163. Time: F(1, 8), P=0.0001. (B) no effect observed in the contralateral paw (non-injected paw) after CPD1 administration. 2-way ANOVA, interaction: F(1, 8), P=0.0076 P=0.0001 CPD1: F(1, 8), p= 0.0163. Time: F(1, 8), P=0.0001.

Figure 26

The Threshold for Detecting Mechanical Stimuli After CPD1 in Female Mice



Note. the effect of CPD1 was tested on female mice after receiving CFA or saline on their right or left paw. (A) the threshold of the ipsilateral paw (the CFA/saline injected paw). CFA injected mice had a lower mechanical threshold compared to saline. However, CPD1 had no effect on the inflammation that was caused by CFA. 2-way ANOVA, interaction: F(1, 8), p= 0.0076 CPD1: F(1, 8), p= 0.0163. Time: F(1, 8), P=0.0001. (B) the threshold of the contralateral paw (The non-CFA/saline injected paw). There was a reduction in the mechanical threshold after receiving CPD1 in CFA injected mice. 2-way ANOVA, interaction: F(1, 8), p= 0.0055 CPD1: F(1, 8), p= 0.0005. Time: F(1, 8), p=0.0007. (n=12).

As shown in **Figure 25** and **Figure 26,** Female and male mice that received the Complete Freud's Adjuvant (CFA) in either paw, experienced a significant reduction in their mechanical threshold indicating their high sensitivity to the mechanical stimuli. This is due to the painful inflammation that was caused by the CFA. After GPR83 agonist, CPD1 administration, there was no effect on the mechanical sensitivity that was caused by the CFA injection. However, in female mice, there was a slight reduction in mechanical threshold in animals who received vehicle after saline injection. In addition, although CFA

induces local inflammation, there was a reduction in the mechanical threshold of the contralateral paw in mice that received CFA, and this reduction was more notable after CPD1 treatment.

Discussion

GPR83 is a G protein-coupled receptor that has been recently deorphanized by its endogenous ligand PEN[57]. The expression of this receptor can be observed in numerous regions, with a greater concentration in the striatum, amygdala, hippocampus, and hypothalamus. Therefore, many investigations from different laboratories are working on understanding this receptor and its relation to different neurological pathways, including feeding, pain, reward, and stress.[64,65,69]. The above-mentioned data demonstrates the significant role of GPR83 in pain pathway. The initial assay aimed to evaluate the influence of GPR83 presence or absence on the pain pathway. Morphine was administered to both heterozygous and wild-type mice, and thermal pain responses were assessed using the tail flick assay. The data revealed that heterozygous mice exhibited a significant antinociceptive response to morphine in comparison to the wild type following the tail flick assays. This suggests an enhancement of morphine's analgesic effect in the absence of GPR83. To ideally test the effect of this receptor on thermal and mechanical pain, small molecules known by CPD1 and CPD25 have been detected as GPR83 agonist and GPR83 antagonist, respectively. To prove the effectiveness of these small molecules, multiple cell lines have been used, including Neuro2A and CHO, to detect the expression of GPR83 after CPD1 and CPD25 treatments. CPD1 increased the expression of GPR83 while CPD25 inhibited the expression of this receptor in a dose-dependent manner.

Morphine is one of the most effective opioid analgesics that is highly used to treat pain. However, due to its severe side effects, such as respiratory depression, drug abuse, and tolerance, the use of this drug comes with a lot of risks in the long-term use. Therefore, it is necessary to develop a drug that could be as effective as morphine with fewer side effects or could be used synergistically with morphine to lower the needed administered morphine doses. This approach will reduce adverse effects while achieving the desired outcome. The tail flick assay has been conducted to test the effect of GPR83 agonist, CPD1 and antagonist, CPD25 on morphine's antinociceptive effect. GPR83 antagonist (CPD25) enhanced the antinociceptive effect of morphine, while GPR83 agonist (CPD1) blunt the antinociceptive effect of Morphine. This was only noticeable in female mice. Male mice have no change in morphine activity. Two potential reasons explain for the absence of an effect from CPD25 in male mice. First reason, it's possible that male mice already display diminished GPR83 signaling, which could explain why additional inhibition doesn't result in an increase in antinociception. An experiment was conducted to assess the difference in the antinociceptive effect of morphine between males and females, using a 3 mg/kg dose of morphine and performing the tail flick assay. The results revealed that the 3 mg/kg morphine dose is more effective in producing antinociception in male mice compared to female mice. Therefore, the second explanation could be that the dosage of CPD25 utilized in these studies (5 mg/kg) may not be sufficient to further enhance the response[71]. This significant effect of GPR83 small molecules on morphine's activity was illustrated by the co-localization of GPR83 with MOR in the PAG area (an area rich with opioid receptors). This indicates the possibility of a physical/functional interaction between these two receptors. This kind of interaction will lead to the formation of heterodimeric structure

which will cause GPR83 to dimmish MOR signaling by the activation of $G\alpha_q$ signaling (this will limit MOR-mediated GIRK channel activation) or by a conformational change between the receptors **(see Figure 27)**. It's important to note that this hypothesis has not been confirmed yet, and ongoing research is aimed at its validation.

It was essential to examine the expression of GPR83 during inflammation. Reduced levels of GPR83 expression were observed in the nucleus accumbens and the PAG regions in CFA prior treated mice. The reduction was more significant in the nucleus accumbens. This observation is in line with expectations, given that the nucleus accumbens typically exhibits higher levels of GPR83 expression compared to the periaqueductal gray[69]. This could be an interesting finding that has not yet been examined for its significance. CFA is Complete Freund's Adjuvant which is a solution composed of inactivated Mycobacterium tuberculosis suspended in non-metabolizable oils. This solution cause an intense inflammatory reaction[80]. Previous studies showed the long duration of action of CFA inflammation which could last for more than 19 days[81]. One of the planned future studies is to examine GPR83 expression in the spinal cord and its relevance to pain pathway.

The GPR83 antagonist CPD25 was tested individually to examine its effect on mechanical sensitivity in the absence of inflammation. Acute treatment with a GPR83 antagonist increased the sensitivity to mechanical stimuli in the von Frey assay in a sex-dependent manner. Male mice showed no change in their mechanical sensitivity when administered CPD25 or vehicle. Female mice were more sensitive to mechanical stimuli when they received CPD25 compared to the vehicle.

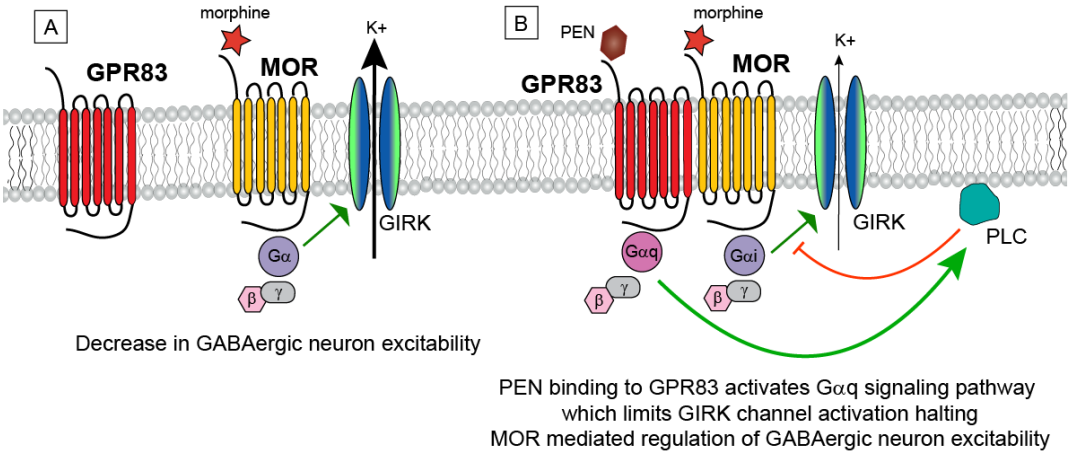
On the other hand, when examining the effects of the GPR83 agonist on chronic inflammatory pain, CPD1 had no effect on pain that has been induced by complete

Freund's adjuvant (CFA). However, there was a reduction in mechanical threshold in female mice that received vehicle after saline injection. Therefore, future studies will focus on repeating this experiment with a stable control group to confirm and validate the effect of CFA. In addition, CFA is known to induce a local inflammation, however, a reduction in the mechanical threshold of the contralateral paw in female mice that received CFA was observed, and this reduction was amplified after CPD1 treatment.

These effects on mechanical sensitivity that were induced by CPD1 and CPD25 in female mice could represent an important interaction with previously reported impacts of estrous cycle on pain. A previous study demonstrated mechanical sensitivity variations across different stages of the estrous cycle. The activation of progesterone receptor (PR) can induce pain. This study showed that Progesterone and PR agonist, segesterone, lower the mechanical threshold in mice, and this effect was blocked after the removal of PR in neurons and glia. They found that the response threshold is elevated in the diestrus cycle stage compared to the estrus cycle stage, which makes mice more sensitive to mechanical stimuli during the estrous cycle[82]. Therefore, a future study will be conducted to investigate the chronic exposure to CPD1 and assess its effect on pain.

Figure 27

Physical/Functional Interaction Between GPR83 and μ-opioid Receptor



Note. GPR83 interacts with MOR by creating heterodimers, which leads to a reduced MOR signaling. This reduction could be due to changes in the receptor structure or through the initiation of Gαq signaling that hampers the activation of GIRK channels managed by MOR. When PEN, also known as Compound 1, attaches to GPR83, it promotes the creation of more GPR83:MOR heteromers, which in turn provides a more precise regulation of MOR activity through a mechanism controlled by PLC that affects GIRK channel activation. Fakira, et al, Mu-GPR83 analgesia. Cooper Medical School of Rowan University (CMSRU). Grant Application. (2022).

Chapter 4

The Analgesic Effect of DS-II-48 and CP55,940 on Thermal and Mechanical Pain, and Sex Differences

Introduction

Nociceptors are the nerve endings that are responsible for the sensation of pain[2]. These are sensory receptors that get activated by noxious stimuli (mechanical, thermal, chemical, etc.). These stimuli could be dangerous and harmful to the body's integrity[83]. In the skin, there are four major types of nociceptors: mechanosensitive nociceptors, mechanothermal nociceptors, chemical nociceptors, and polymodal nociceptors. This chapter will focus on the first two types mentioned. Mechanonociceptors are receptors that only respond to severe mechanical stimulation, such as pinching, stretching, or cutting. When receptors respond to mechanical stimuli and thermal stimuli, it is called mechanothermal nociceptors[84]. Despite advances in pain treatment, many medications have limitations and side effects. The goal is to develop non-opioid medications or complementary treatments to enhance pain relief while reducing opioid-related risks and overall exposure. Our main approach involves combining two drugs to treat pain, aiming to maximize efficacy while minimizing side effects. Therefore, this study was mainly focused on determining the antinociceptive effect of DS-II-48 and CP55,940 on thermal and mechanical pain using different cumulative doses of each drug. After that, designing a combination therapy according to the ED_{50} values. This is to achieve a synergistic effect using lower doses of each drug, thereby minimizing potential adverse effects.

CP55,940 is a synthetic non-selective agonist cannabinoid receptor. This drug is more potent and efficacious than Δ9-tetrahydrocannabinol (THC), a naturally accruing cannabinoid agonist mainly extracted from the cannabis plant[85]. Cannabinoids generally stimulates cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) to induce multiple pathways, including pain[86], appetite[87], memory[88], and movement[89]. Most of cannabinoid activity, including the psychotropic effects, primary results from the activation of CB1 receptors, while CB2 receptors play more significant roles in immune and inflammatory functions.

Naturally, endocannabinoids act as neuroregulatory modulators that play a role in retrograde neurotransmission. After synaptic transmission, endocannabinoids are released by the postsynaptic neuron, binding primarily to CB1 receptors located on the presynaptic neuron. This binding leads to the inhibition of presynaptic calcium channel activation, subsequently reducing presynaptic neurotransmitter release. The diverse psychotropic effects of cannabinoids mainly depend on their receptor binding location. For example, cannabinoid receptor activation in the hippocampus will result in impaired short-term memory, while in the Amygdala, it leads to panic and paranoia. Moreover, the activation of CB1 receptors in the spinal cord will cause analgesia[23,24]. Previous studies demonstrated the analgesic effect of cannabinoids on acute and chronic pain through spinal and supraspinal pathways[25]. However, they also induce unwanted side effects, such as mood change, impaired body movement, and impaired memory/learning, which limit their use in human[26]. Cannabinoid agonists, specifically THC induce four major effects in rodents: hypolocomotion, hypothermia, catalepsy, and analgesia[90].

DS-II-48 is the salt form of MP-III-024 which is a positive allosteric modulator (PAM). This drug is a benzodiazepine-like compound that selectively binds to α2- and α3-containing $GABA_A$ receptors. These receptors are predominantly present in spinal nociceptive circuits. According to the previous data in chapter 2, MP-III-024 has demonstrated potential for promising outcomes when used in combination with morphine[25,42]. GABA, known as γ-Aminobutyrate, is an inhibitory neurotransmitter in the adult mammalian brain. It is a multifunctional molecule that has different functions in the central nervous system, peripheral nervous system, and certain non-neuronal tissues. The GABA neurotransmitter is primarily activated by $GABA_A$ and $GABA_C$ receptors, which are ionotropic receptors. It is also activated by $GABA_B$ receptors, which are metabotropic receptors[13]. $GABA_A$ receptor is the major inhibitory neurotransmitter receptor. It has a significant role in providing rapid inhibition within the basal ganglia. It is part of the superfamily of "cys-cys loop" ligand-gated ion channels. The $GABA_A$ receptor has a pentameric structure composed of five subunits, two α subunits, two β subunits, and one γ subunit. These subunits are arranged around Cl^--permeable pore. Each subunit performs a different signaling pathway. The activation of $GABA_A$ receptors by GABA molecules triggers the opening of the ion channel, allowing the flow of negatively charged ions, specifically chloride ions (Cl^-) from extracellular space into the cell. This process leads to inhibitory hyperpolarization. The binding site where $GABA_A$ binds is located at the junction of the α and β subunits (α/β), while the binding site for benzodiazepines is situated at the interface of the α and γ subunits (α/γ)[17].

Benzodiazepines and MP-III-024 are positive allosteric modulators (PAMs) since they bind to a distinct site from $GABA_A$'s binding site and enhance GABA-mediated activation of the receptor. These receptors contain α1, α2, α3, and α5 subunits ($\alpha1GABA_A$, $\alpha2GABA_A$, $\alpha3GABA_A$, and $\alpha5GABA_A$ receptors, respectively). They enhance GABA-receptor binding by increasing channel opening frequency. As a result, they enhance the inhibition of excitatory neurotransmitters[15]. $GABA_A$ α1 receptors are mainly responsible for the sedative effect of benzodiazepines, as well as the effect related to abuse and physical dependence. However, studies have shown the presence of $GABA_A$ receptors, specifically α2- and α3-containing $GABA_A$ receptors in spinal nociceptive circuits. These receptors play a significant role in transmitting pain sensory signals from the periphery to higher centers with minimal negative side effects, in comparison to $GABA_A$α1-associated PAMs[15,42]. Two main assays were conducted in this study to examine the effect of these two drugs in response to mechanical and thermal stimulation, the von Frey, and the hot plate assay, respectively.

Materials and Methods

Animals

Drug-naïve adult male and female C57BL/6J mice obtained from Jackson laboratories were used for these studies. Upon arrival to the vivarium at Cooper Medical School of Rowan University (CMSRU), animals were grouped as fours and housed in plexiglass cages equipped with bedding, nestlets, enviropaks, and constant access to water and food. They were kept in a room with constant temperature and humidity, under a 12h light/dark cycle (lights on at 7:00 AM). The Mice were 13 months old and weighed

approximately 25-30 g. First, they were habituated in the room for 2 weeks (no experiments were conducted). After that, they were handled 4-5 days before starting the experiment. The animals involved in these experiments are within the guidelines set forth by the Institutional Animal Care and Use Committee of Rowan University. All experimentation was conducted following the "Guide for the Care and Use of Laboratory Animals." (National Research Council, National Academy of Sciences, Washington, D.C., USA, 2011).

Drugs

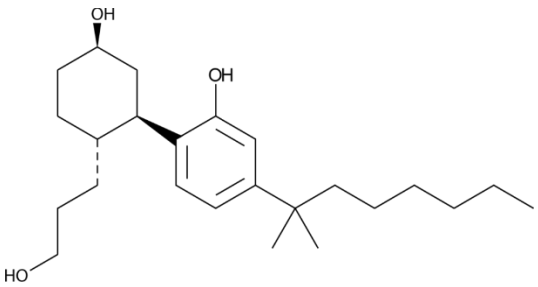
DS-II-48 is the salt form of MP-III-024 which is an analog of benzodiazepines, known by its scientific name methyl 8-ethynyl-6-(pyridin-2-yl)-4Hbenzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate (MP-III-024). It is a positive allosteric modulator (PAM) that selectively binds to α2/α3GABA$_A$-receptor. It also has a high subtype selectivity for opioid receptors[42]**.** The DS-II-48 was primarily developed to enhance the drug's solubility and absorption rate. This drug was acquired from the Department of Chemistry and Biochemistry at the University of Wisconsin-Milwaukee, where it is synthesized. The drug was dissolved in a vehicle that contains 5% DMSO and 95% Methylcellulose.

CP55,940 is a synthetic cannabinoid that mimic the effects of naturally occurring THC, an anti-psychoactive compound present in cannabis. It is a potent non-selective full agonist that acts on both cannabinoid receptors 1 and 2. It is known by its scientific name rel-5-(1,1-dimethylheptyl)-2-[(1R,2R,5R)-5-hydroxy-2-(3 hydroxypropyl)cyclohexyl]-phenol[91]. Pfizer developed CP 55,940 in 1974; however, it was never brought to the market. This drug is primary used as a research tool to investigate the endocannabinoid system for the purpose of treating various conditions related to pain, seizures, nausea, and

schizophrenia[92–94]. In this study, CP55,940 was dissolved in a vehicle that contains 5% DMSO and 95% Methylcellulose.

Figure 28

Chemical Structure of CP55,940[91]



Behavioral Assays

von Frey Test

The 'up-down' von Frey assay is a method for evaluating mechanical allodynia in mice and rats[76]. Individual mice are placed in transparent plexiglass containers on a raised wire mesh platform, enabling access to the underside of their hind paw. The von Frey hairs are used to measure withdrawal response frequency. These hairs are gradually graded filaments categorized by their stiffness (5-26 g). Each filament is applied to the mid plantar surface of the hindpaw until it bends. If mice sense the filament, they will react to it by withdrawing, licking, or shaking their paw. Any paw withdrawal behavior demonstrates a positive response. The animals are given at least one hour to acclimate prior to the test, and mechanical hypernociception is assessed at multiple time points. The withdrawal threshold of naïve mice is determined before any intervention or treatment, and this value serves as

the baseline (B) for comparison in the graphs. The initial filament application is expected to elicit a 50% withdrawal threshold response. If there is no observable response, the subsequent filament with a higher force is employed. Conversely, if a response is detected, the next filament with lower force is used. This is stopped after four measurements following the first change in response (when transitioning from response to no-response or from no-response to response)[77,78].

Hot Plate Assay

The hot plate assay is one of the most common methods used to test the sensitivity of mice against thermal pain. Through this assay we can measure the effectiveness of specific drugs that are intended to elicit an antinociceptive response. This essay was presented by Eddy and Leimbach in 1953[50]. First, the hot plate temperature is set at 50-56°C. Before drug administration, two baseline measurements are taken in advance, then the data are combined to calculate a single average baseline value. Following that, mice are injected with a specific drug/vehicle, and then they are sequentially placed on the surface of the hot plate. Once the mouse is on the surface, a stopwatch should record how long the mouse has been on the surface until it reacts. This reaction could include hindpaw licking and/or shaking or jumping. Once the mouse reacts, it should be removed right away. The longer the latency for mice to react, the greater the analgesic effect produced by the drug[51].

Experimental Procedures

Thermal Nociception

In this assay, the hot plate apparatus has been used to examine the individual and additive nociceptive effect of DS-II-48 and CP55,940 on thermal pain. A group size of 8 male and 8 female C57/Bl6 mice was used in this study. 4 of each sex were administered DS-II-43, and the other 4 received CP55,940. The first experiment was done to test DS-II-48, and a week after the same experiment was done to test CP55,940.

Prior to the cumulative dosing, the hot plate was set to 56 ± 0.1 °C, and two baseline measurements were taken for each animal. After baseline measurements, a total of 5 cumulative doses of DS-II-48 have been administered i.p. for each mouse: vehicle, 1, 3.2, 10, 32 mg/kg. Thirty minutes after each dose injection, each mouse is placed on the hot plate surface for testing. The latency to hindpaw licking and/or shaking, or jumping, was recorded. Each mouse was removed immediately after any kind of reaction. No mice were allowed to remain on the hot plate for more than 20 seconds to avoid any risk of tissue damage. After a week, the same experiment was repeated with 5 cumulative doses of CP55,940: vehicle, 0.1, 0.3, 0.56, 1.0 mg/kg.

Mechanical Hyperalgesia

This assay aimed to investigate both the individual and combined effects of DS-II-48 and CP55,940 in response to inflammation induced by the subcutaneous injection of 0.06 mg zymosan A. This compound was suspended in 20 µl of 0.9% NaCl and administered into the plantar surface of the right hindpaw. The left hindpaw, which did not receive an injection, served as the control. First, von Frey baseline measurements were recorded, followed by the administration of zymosan A injections. After a 24-hour period,

each mouse received a total of 5 cumulative doses of DS-II-48 via intraperitoneal injection, including vehicle, 3.2, 10, 17.8, and 32 mg/kg. Thirty minutes following each dose injection, the mice were placed on a wide-gauge wire mesh surface, and von Frey measurements were taken. After a week, the same experiment was repeated with 5 cumulative doses of CP55,940: vehicle, 0.1, 0.3, 0.56, 1.0 mg/kg.

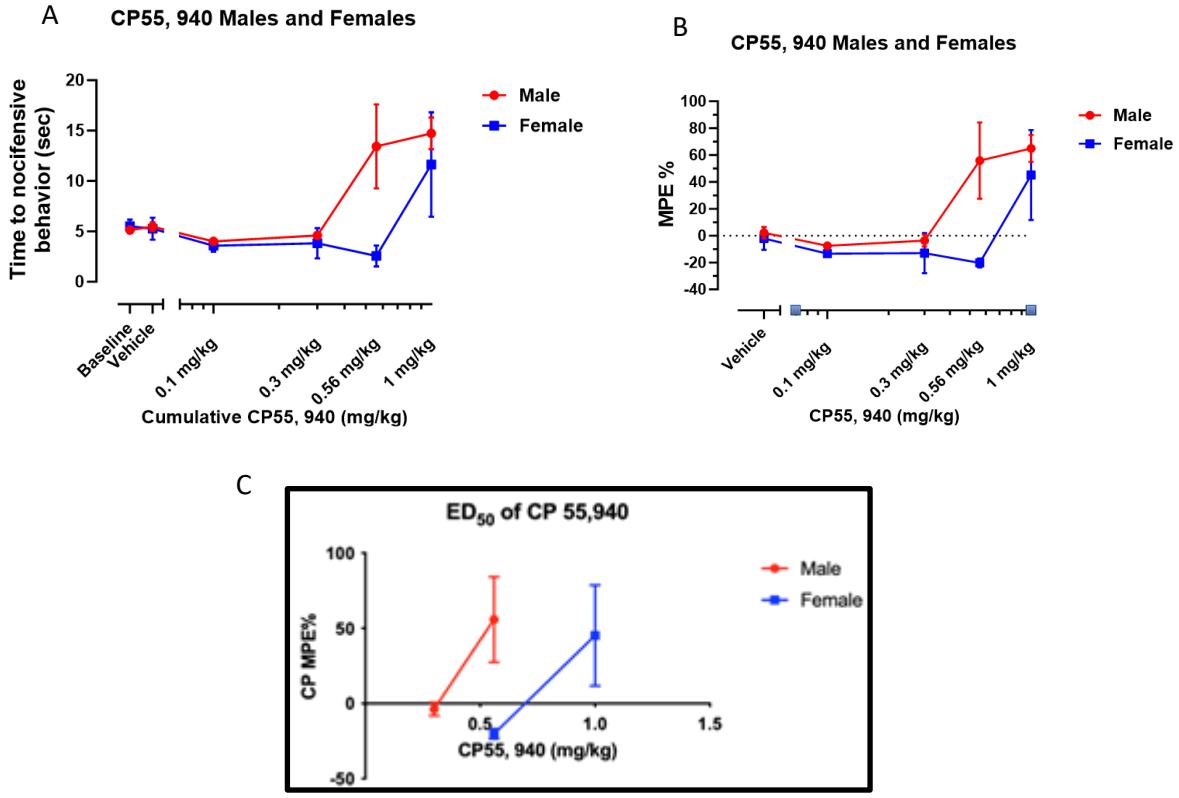
In the von Frey test, the up-down method was used[76]. The following filaments are used in mice: 2.44, 2.83, 3.22, 3.61, 3.84, 4.08, and 4.31. The cut-off values for the minimal filament is 2.44 and for the maximal filaments is 4.31. The test began with a filament weighing 3.61 g. If the animal responded, the strength of the subsequent filament was reduced; if there was no response, the force was increased. Even if there was no paw withdrawal at the maximal force of 4.31 g, the upper limit value is recorded as a positive response. The measurements stopped 4 cycles after the first change in animal response, whether transitioning from a response to no response or vice versa. The ipsilateral and contralateral hind paws were alternately tested whenever feasible. The filaments were applied at intervals of at least 2 minutes to prevent hypervigilance or sensitization between consecutive filament applications. Filaments were bent fully before assessing responses, and up to 4-5 seconds were allowed to consider a negative response. Any clear paw withdrawal, shaking, or licking is regarded as a nociceptive-like response[76].

Data & Results

The Hot Plate Assay Results

Figure 29

Cumulative Dosing Effect of CP-55,940 on Thermal Pain

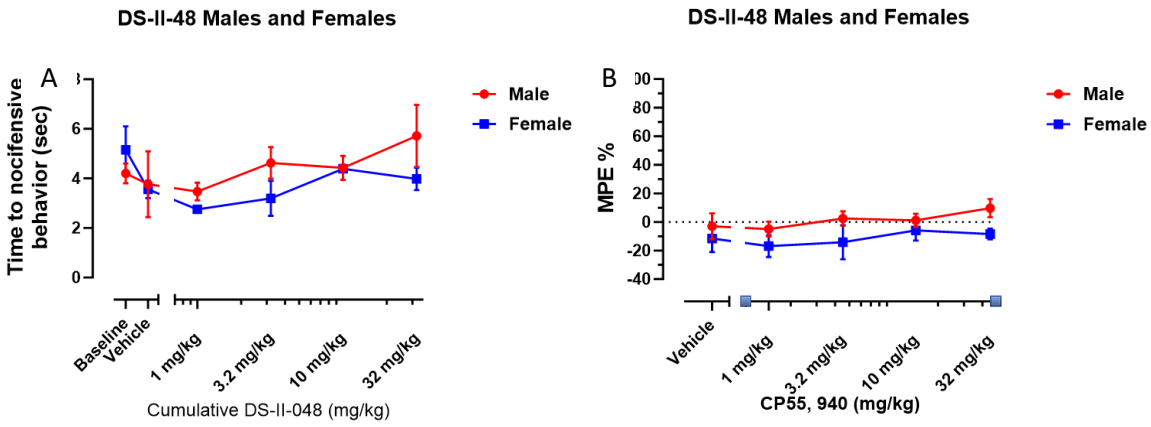


Note. These graphs illustrate the cumulative doses of CP55,940 (0, 0.1, 0.3, 0.56, and 1 mg/kg) used to induce antinociceptive effects in male and female mice in the hot plate assay. Thirty minutes after each dose, mice were placed on a hot plate with a temperature of 56.0 ± 0.1 °C to test their nociceptive latency. According to (A) the time for nocifensive behavior and (B) the maximal possible effect data, CP55,940 had a significant antinociceptive effect on male and female mice only at doses starting at 0.56

mg/kg and 1 mg/kg, respectively. (C) shows the ED_{50} values for both male and female mice, indicating that female mice had a higher ED_{50} (1.03 mg/kg) compared to male mice (0.53 mg/kg). (n=4).

Figure 30

Cumulative Dosing Effect of DS-II-48 on Thermal Pain



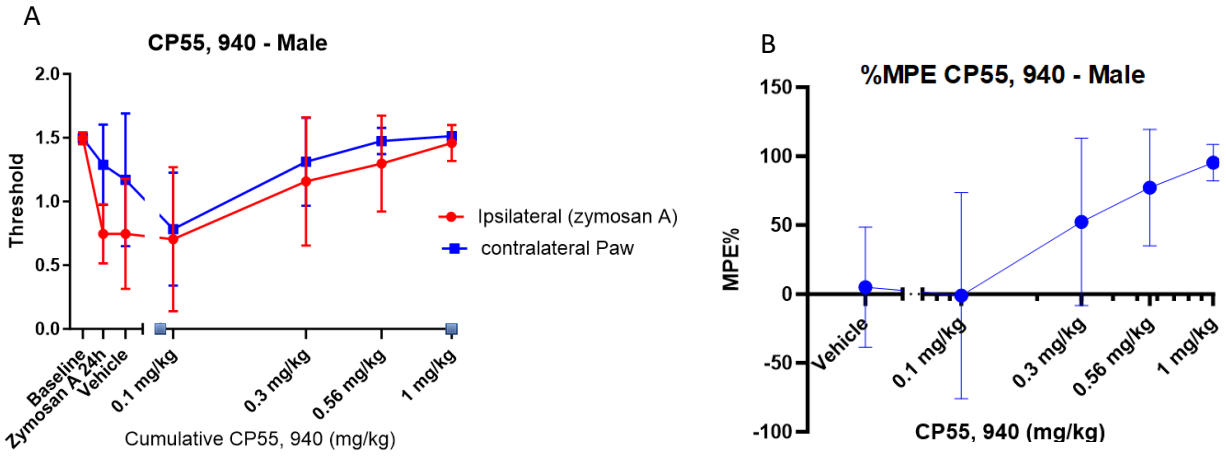
Note. These graphs illustrate (A) the time for nocifensive behavior, and (B) the maximal possible effect of DS-II-48 at various cumulative doses when administered to both male and female mice. Following the injection of mice with cumulative doses of vehicle, 1 mg/kg, 3 mg/kg, 10 mg/kg, and 32 mg/kg, no impact on nociception was observed when using the hot plate assay for both males and females. (n=4/group)

As shown in **Figure 29A, B**, both male and female mice responded to the analgesic effect of CP55,940 only at high doses. In females, the response was shown at 1 mg/kg whereas in males it was 0.56 mg/kg. Therefore, the analgesic effects of CP-55,940

required a significantly higher dose to develop in female mice compared to male mice. The dose of the half-maximal effect of both drugs ED_{50} was measured by calculating individual ED_{50} for each mouse using Prism. As indicated **in Figure 29C,** females had a higher ED_{50} of 1.033 mg/kg, compared to males which was 0.5345 mg/kg. On the other hand, after testing all 5 doses of DS-II-48 (vehicle, 1 mg/kg, 3 mg/kg, 10 mg/kg, and 32 mg/kg), there was no difference in the effect between vehicle and the rest of doses (**Figure 30)**. This indicates that DS-II-48 did not produce a significant effect on thermal nociception in both female and male mice.

Figure 31

Cumulative Dosing Effect of CP-55, 940 on Mechanical Pain

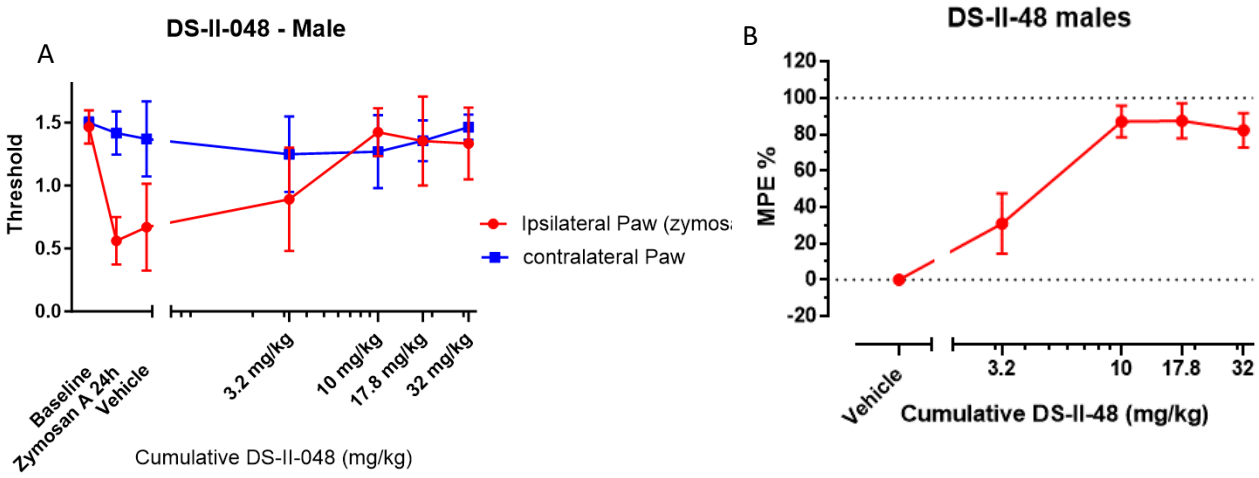


Note. The mechanical pain sensitivity was evaluated in male mice using the von Frey method. Prior to zymosan A injections, a baseline measurement was taken for each mouse. The first post-injection von Frey measurement was conducted 24 hours after

zymosan A administration. After that, four measurements were taken after each cumulative dose (vehicle, 0.1, 0.3, 0.56, and 1 mg/kg). (A) shows the threshold for both the ipsilateral (zymosan A injected) and the contralateral (zymosan A non-injected) paws. After the subcutaneous injection of zymosan A, there was a significant decrease in the threshold for both the ipsilateral and the contralateral paw, indicating induced inflammation by zymosan A. The antinociceptive effect of CP55,940 was observed after receiving a cumulative dose of 0.3 mg/kg, where there was a continuous increase in the threshold for detecting mechanical stimuli. (B) The maximum possible effect of CP55,940 was increased by 50% following the dosage of 0.3 mg/kg. This antinociceptive effect kept rising after 0.56 mg/kg and 1 mg/kg. After calculating the regression of each animal's curve and averaging all of the ED_{50} values, the analgesic effect of CP55,940 reached a half-maximal possible effect (ED_{50}) at 0.645 mg/kg in male mice. (n=7/group).

Figure 32

Cumulative Dosing Effect of DS-II-48 on Mechanical Pain



Note. The mechanical pain sensitivity was evaluated in male mice using the von Frey method. Prior to zymosan A injections, a baseline measurement was taken for each mouse. The first post-injection von Frey measurement was conducted 24 hours after zymosan A administration. After that, four measurements were taken after each cumulative dose (vehicle, 3.2, 10, 17.2, and 32 mg/kg). (A) illustrates the threshold for both the ipsilateral (zymosan A injected) and the contralateral (zymosan A non-injected) paws. After the subcutaneous injection of zymosan A, there was a significant reduction in the threshold of the ipsilateral paw, indicating induced inflammation by zymosan A. The antinociceptive effect of DS-II-48 was observed after receiving the first dose of 3.2 mg/kg with a continuous increase in the threshold for detecting mechanical stimuli. (B) The maximum possible effect of DS-II-48 was at its highest following the dosage of 10 mg/kg. After calculating the regression of each animal's curve and averaging all of the

ED_{50} values, the analgesic effect of DS-II-48 reached a half-maximal possible effect (ED_{50}) at 5.05 mg/kg in male mice. (n=8/group).

Figure 31 shows the threshold for mechanical stimuli detection and the maximal possible effect of CP55,940 treatment in male mice. After injecting mice subcutaneously with zymosan A, there was a significant decrease in the threshold for both the ipsilateral and contralateral paw which shows the inflammatory effect that was caused by zymosan A. After the injection of the second cumulative dose (0.3 mg/kg), there was an attenuation of pain. This effect continued to increase after the third and fourth dose of 0.56 and 1 mg/kg, respectively. Therefore, 0.1 mg/kg of CP55,940 did not demonstrate an antinociceptive effect on the mechanical pain induced by zymosan A. After conducting simple linear regression, the analgesic effect of CP55,940 reached a half maximal possible effect at 0.645 mg/kg in male mic (ED_{50}= at 0.645).

In **Figure 32**, there was also a reduction in the mechanical threshold due to the zymosan A injection. However, the pain was attenuated after the first dose of DS-II-48 (3.2 mg/kg). This was only observed in the ipsilateral paw, while the contralateral paw remained unaffected by both the zymosan A-induced inflammation nor the antinociceptive effect of DS-II-48, indicating it served as the baseline condition. After conducting simple linear regression, the analgesic effect of DS-II-48 reached a half-maximal possible effect (ED_{50}) at 5.05 mg/kg in male mice.

Discussion

Pain, an unpleasant sensation primarily triggered by external stimuli. It can significantly disrupt daily life but also serves as a protective mechanism by alerting the body to potential harm. Despite advances in pain treatment, many medications have limitations and side effects. The goal is to develop non-opioid medications or complementary treatments to enhance pain relief while reducing opioid-related risks and overall exposure. Our main approach involves combining two drugs to treat pain, aiming to maximize efficacy while minimizing side effects. Therefore, this study was mainly focused on determining the antinociceptive effects of DS-II-48 and CP55,940 on thermal and mechanical pain using different cumulative doses of each drug. After that, designing a combination therapy according to the ED_{50}, in order to use lower doses from CP55,940 to get the desirable synergistic effect with lower adverse effects.

The hot plate assay was performed to determine the antinociceptive effect of DS-II-48 and CP55,940 on thermal pain. Animals that received CP55,940 showed a significant effect on male and female mice only at higher doses starting at 0.56 mg/kg and 1 mg/kg, respectively. This indicates that female mice had a higher ED_{50} at 1.03 mg/kg compared to male mice, 0.53 mg/kg. According to the ED_{50} values, there is a sex difference in responding to CP55,940. Females needed a higher dose to reach half of the maximal effect compared to males. This could be due to the estrous cycle effect, where in specific stages female mice tend to be more sensitive to pain. A previous study in female rats showed a significant drop in paw licking latency during the hot plate assay in the metestrus and diestrus stages in comparison to the proestrus and estrus phases. The significant reduction in the paw licking latency is demonstrated due to the high sensitivity

to pain during the metestrus and diestrus cycles[95]. Therefore, during these cycles, female mice would be expected to require higher doses of CP55,940 to reduce the thermal pain that was caused by the hot plate. In addition, our findings align with earlier clinical studies, affirming that men tend to experience more pronounced pain relief from cannabis compared to women when they smoke it[96,97]. Our results also are consistent with a previous study conducted on female mice, indicating that the pain-relieving impact of WIN 55,212-2, a selective CB1 agonist, was heightened in ovariectomized mice[98]. We did not determine the estrous phase of the female mice in this experiment.

DS-II-48 did not significantly affect female or male mice in the hot plate assay. DS-II-48 is a salt formulation of MP-III-024 and these data is align with prior research conducted by Rahman et al. (2021) in which MP-III-024 alone also did not significantly affect thermal nociception[25]. An important limitation of this study is that the study cohort sizes were very small, comprising only 4 males and 4 females for each test. This underpowers our statistical comparison between sexes. Follow-up studies are underway with more animals in each group. Another critical limitation to consider is that the mice used in the study were approximately 13 months old. While there is no available data indicating that older mice exhibit differential sensitivity to pain, it is noteworthy that they do demonstrate behavioral alterations in other assays, such as anxiety, locomotor activity, and memory/ learning abilities[99,100]. Therefore, in order to ensure that the influence of old age is negligible on the results, there is a future plan for the repetition of these experiments using a larger and younger group of mice. This step is essential to enhance the reliability of the findings.

The von Frey assay was conducted to evaluate the mechanical sensitivity of mice after receiving CP55,940 and DS-II-48. Following subcutaneous zymosan A injections in mice, a significant reduction in the ipsilateral paw threshold was observed, indicating the inflammatory impact of zymosan a. However, despite zymosan A's induction of localized inflammation, some mice showed sensitivity in their contralateral paw. The exact mechanism behind the contralateral paw effect is not fully understood. It could be due to several reasons: Zymosan A injection could involve a phenomenon called neurogenic inflammation or cross-excitation[101]. The is when the localized inflammation triggered by zymosan A may activate nerve pathways, causing signaling molecules to spread and sensitize nerves on the opposite side. This process could lower the threshold for detecting stimuli in the contralateral paw. Another explanation could be due to central sensitization. When an area experiences inflammation, it can heighten the sensitivity of nerves in the central nervous system. This increased sensitivity might impact neighboring nerves, reducing the threshold for sensing stimuli in the contralateral paw[102]. As an example, someone with persistent pain in their right hand might develop increased sensitivity in their left hand over time, even though it's not injured. The continuous pain signals from the right hand can affect how their nervous system processes sensations on the opposite side of the body.

The administration of CP55,940 resulted in the attenuation of the lowered pain threshold induced by zymosan A-mediated inflammation. This effect was observed at the 0.3 mg/kg dosage. This pain-alleviating effect kept rising following subsequent doses of 0.56 and 1 mg/kg. However, the 0.1 mg/kg dosage of CP55,940 did not show any significant antinociceptive impact on zymosan A-induced mechanical pain. Furthermore,

through the application of simple linear regression analysis, it was determined that the analgesic effect of CP55,940 achieved a half-maximal efficacy at a dosage of 0.4364 mg/kg in male mice (ED_{50}=0.4364). Therefore, CP55,940 reduced the inflammatory pain in a dose-dependent manner.

The same experiment has been conducted to test the antinociceptive effect of DS-II-48 on treating the zymosan A-induced inflammatory pain. The first effect has been observed starting from the lowest dose of 0.1 mg/kg. After that, the von Frey assay showed a dose-dependent increase in the antihyperalgesic effect of DS-II-48 in male mice. This data matches the findings of a previous study conducted by Rahman et al. in 2021, confirming the evidence of the analgesic efficacy of MP-III-024 in attenuating mechanical inflammatory pain as observed in the von Frey assay. Therefore, this current study further supports the fact that MP-III-024 exhibits a dose-dependent response in alleviating inflammatory pain, consistent with the earlier research by Rahman et al. in 2021[25].

The von Frey assay was only conducted on male mice. However, upcoming experiments will include female mice showing sex differences in pain alleviation. In addition, considering the lowered threshold in the contralateral paw following zymosan A injection, it is necessary to repeat the CP55,940 von Frey test to make sure whether the sensitivity in the contralateral paw post-zymosan A administration is a consistent phenomenon or potentially influenced by other factors. Furthermore, after determining the ED_{50} values for the CP55,940 and the DS-II-48, our future plan involves designing a combination therapy approach. This approach seeks to evaluate the synergistic effect of both drugs on both thermal and mechanical pain.

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