Investigating The Acute and Chronic Effects of Known and Novel Opioid Ligands

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Pharmacology)
in the University of Michigan
2021

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Dedication

This is dedicated to those who have made the ultimate sacrifice and had their lives cut short in the line of duty and weren't able to flourish in this world. I promise to carry this torch and pass it on to the generation that follows. Knowledge is power and we can become as powerful as we want with the proper mindset. Semper Fidelis.

Acknowledgements

First and foremost, I'd like to thank my incredible mentor, Dr. Emily Jutkiewicz. The dedication, patience, and drive you have instilled in not only me but the lab as a whole was incredibly admirable, and you made my graduate career a welcoming and enjoyable one. I would also like to thank my committee members, Dr. Traynor, Dr. Mosberg, Dr. Kemp, and Dr. Birdsong. Your knowledge and understanding were undoubtedly helpful in aiding me to reach my goals.

To Dr. Ram Kandasamy, you've been an incredible colleague and friend, and I'm grateful to have gained and continued a friendship with you.

To Dr. Rick Harris, words can't describe how helpful you have been over the past few years. From talking about neuroscience and pharmacology to acupuncture, and the stressors of life, you have made a mark on my life that I will truly never forget. You're an incredible inspiration, and the insights I have gained from you are ones I plan to pass on to my kin.

Lastly, I would like to thank my family. This past year has been a rough one, with the pandemic impacting just about every aspect of our life. It's great to have a support system like you when times get tough, and I cannot thank you enough.

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Abstract

Acute and chronic pain are widespread and debilitating diseases that, for a large population, cannot be adequately managed with current pain treatments. Opioid analgesics, such as morphine, have long been used to treat pain and exert their effects by activating the mu-opioid receptor (MOR). While effective, these MOR agonists produce on-target adverse effects such as tolerance, physical dependence, and euphoria. Overall, the experiments described in the current thesis evaluated ways of minimizing opioid tolerance by targeting multiple opioid receptor types simultaneously (chapters 1 and 2) or different populations of opioid receptors *in vivo* (chapter 3).

Previous studies demonstrated that simultaneous modulation of both the MOR and the delta-opioid receptor (DOR) could improve the therapeutic profile of opioid ligands. These experiments sought to further characterize mixed-efficacy opioid ligands, specifically ligands binding to both MORs and DORs with nearly equal affinity. These studies utilized multiple pre-clinical pain models to determine antinociceptive properties of the mixed efficacy opioid ligands, AMB67 and AAH8, following acute and chronic administration.

To evaluate the antinociceptive effects of AMB67 and AAH8, we used models of thermal, chemical, and mechanical nociception. In C57BL/6 mice, AMB67 produced dose-dependent antinociceptive effects in all three models of nociception. Mice chronically administered AMB67 failed to develop tolerance to the antinociceptive effects. Chronic administration of AMB67 produced physical dependence as mice exhibited naltrexone-precipitated withdrawal-like behaviors; however, these effects were significantly less than morphine. AMB67 was also less potent than morphine in multiple models assessing abuse potential. In contrast to AMB67, AAH8

significantly attenuated chemical-induced visceral pain following system administration but failed to produce antinociceptive effects in other pain models. Repeated administration of small doses of AAH8 failed to produce tolerance to the antinociceptive effects of AAH8; however, chronic treatment with more frequent, larger doses produced tolerance to the antinociceptive effects of AAH8. These data provide support that mixed-efficacy MOR-DOR ligands may offer improvement over current pharmacotherapies for pain management.

Additionally, this study assessed the involvement of peripheral opioid receptors in the development of tolerance to the centrally-mediated antinociceptive effects of MOR agonists. This study used a model of acute, peripherally-mediated visceral pain, acetic acid stretch assay (AASA) and a centrally-mediated thermal reflex assay, warm water tail withdrawal (WWTW). Morphine and the peripherally restricted MOR agonist loperamide produced acute antinociceptive effects in the AASA, and NLX, a non-selective opioid receptor antagonist, blocked these effects. However, only morphine produced opioid-receptor mediated antinociceptive effects in the WWTW. Chronic administration of morphine (3x/day for five days) shifted the ED_{50} of the morphine dose-effect curve 2.5-fold to the right in the WWTW. Naloxone-methiodide pretreatments to chronic morphine prevented the rightward shift in the morphine dose-effect curve. Conversely, chronic administration of a peripherally active dose of loperamide (3.2 mg/kg, 4x per day for five days) shifted the morphine dose-effect (DE) curve to the right. Pretreatments with naloxone-methiodide completely reversed loperamide-induced cross-tolerance to the antinociceptive effects of morphine. Overall, these data suggest the involvement of peripheral opioid receptors in tolerance development to centrally acting opioids.

Overall, the work presented in this thesis furthers our understanding of the *in vivo* mechanisms of opioid tolerance and potentially identifies novel, safer opioid analgesics.

Minimizing the adverse effects of chronic opioid use would significantly improve opioid-based treatment, improve the lives of those who require opioids for everyday function, and help fight the current opioid epidemic.

Chapter I: General Introduction

History of Opioids and Their Clinical Utility:

It is impossible to speak about opioid use disorder (OUD) without the mentioning of Papaver somniferum, or the "poppy plant." The earliest dated use of the poppy plant spans back to 3,400 B.C., where the Sumerians referred to opium poppy as *Hul Gil*, or the "joy plant," for the incredible cerebral effects associated with the ingestion of the plant[1]. As the demand for opioid poppy increased, many countries throughout Asia, mass production ultimately catalyzed the Opium Wars. Soon after, immigration to the United States brought opium to the Americas[1].

In 1803, a brilliant German pharmacist and pioneer in alkaloid chemistry, Friedrich Sertürner, was the first to isolate morphine from the opium poppy[2]. Hailed as a miracle drug for its pain-relieving, cough suppressing, and sedative effects, morphine was widely prescribed by physicians throughout the majority of the 1800s[3]. However, the abuse potential of morphine, its use in the American Civil War, and the production of the hypodermic needle all became major components in the production of America's opioid epidemic[4,5]. With clinicians at a standstill for treating pain, Charles Romley Alder Wright, an English chemist, and physicist, attempted to create a non-addictive form of morphine, diacetylmorphine, commonly known as heroin[6].

In 1898, Bayer Pharmaceuticals introduced heroin to the medical community for patient use. However, in the early 1900s heroin use reached soaring levels throughout the United States, leading to its illegalization in 1924[7]. Heroin would eventually become classified as a schedule 1

drug under the Controlled Substances Act of 1970, and the search for improved opioid analgesics continues to this day.

Acute and Chronic Pain Management

Current opioid therapeutics have been incredibly effective for treating acute, moderate to severe pain. Opioids are often prescribed for postoperative pain and unpleasant conditions requiring emergency department or primary care services. However, chronic pain is an intractable problem with devastating consequences. Nearly one-third of the United States population suffers from pain which has become a serious concern that encompasses massive financial burdens including increased medical treatment and decreased work productivity[8]. Understanding the essential need for improved pain care, in 1995, Dr. James Campbell addressed the American Pain Society and prodded healthcare workers to include pain as a "5th vital sign"[9]. Unfortunately, opioid prescriptions rose from nearly 110 million opioid prescriptions in the United States in 1992, to nearly 260 million by 2012[10]. It is quite possible that the increased demand for treating pain, fueled the increased demand for prescription opioids and the increase in OUD. This resulted in people not only suffering from pain, but also opioid addiction. With no better alternative to pain management, opioids continue to be the number one prescribed drug class for pain therapy.

Clinically-used opioids used for pain therapy target and activate a specific subset of opioid receptors, the mu-opioid receptor (MOR). Activation of MORs produces pain relief but also adverse effects. These adverse effects include tolerance, physical dependence, respiratory depression, addiction, and constipation[11,12], and these often lead to diminished life quality, OUD, and increases in overdose/death[13]. Further investigation into the interactions between opioid receptor subtypes may be crucial for potentially improving opioid-based pain therapy.

Opioid Receptor System

The opioid receptor system consists of 4 opioid receptor types, MOR, delta-opioid receptor (DOR), kappa-opioid receptor (KOR) and the (nociceptin-opioid receptor)NOP. Opioid receptors belong to the family of G protein-coupled receptors (GPCRs), which have seven-transmembrane domains and interact with intracellular heterotrimeric G proteins[14]. Their sensitivity to pertussis toxin, the ability to activate inwardly rectifying potassium channels[15], inhibition of adenylyl cyclase[16], and inhibition of calcium conductance[17,18], indicates that opioid receptors couple to inhibitory Gi/o proteins.

Heterotrimeric G proteins are composed of three subunits, one Gα subunit, and a heterodimer containing a β and γ subunit. Upon agonist binding, a conformational change in the receptor occurs, leading to the exchange of guanosine diphosphate (GDP), for guanosine triphosphate (GTP) on the Gα subunit, leading to the dissociation of the βγ subunit from the Gα subunit leading to downstream signaling. The Gα -GTP and βγ subunit complex interact with downstream intracellular signaling molecules to generate physiological effects, including antinociception, nausea, constipation, miosis, and respiratory depression. Opioid receptors can be activated by both exogenous opioids, such as morphine, or endogenous opioids that play an important role in mechanisms of supraspinal, spinal, and peripheral analgesia, reward-mediated food intake and drug addiction, and modulation of emotion and stress responses[19].

Four predominant endogenous opioid peptides were discovered in 1975, [Leu^5]Enkephalin, [Met^5]Enkephalin, β_h-Endorphin, and Dynorphin A[20,21]. [Leu^5]Enkephalin and [Met^5]Enkephalin are derived from the precursor preproenkephalin, β_h-Endorphin is derived from proopiomelanocortin (POMC), and Dynorphin A is derived from prodynorphin. For many decades it was proposed that each endogenous opioid demonstrates preferences for individual opioid

receptor types. Studies suggested [Leu^5]Enkephalin and [Met^5]Enkephalin bound to the DOR, β_h-Endorphin bound to the MOR and Dynorphin A bound to the KOR[22]. However, recent studies established promiscuous opioid receptor activity for endogenous opioid ligands and their putative fragments, as each fragment displayed agonist activity at all three opioid receptors, MOR, DOR, and KOR[23].

MORs are highly concentrated within brain regions involved in the reinforcing properties of drugs of abuse, including the vental tegmental area (VTA) and the nucleus accumbens (NAc)[24,25]. The VTA is a dopamine-rich brain region that sends projections to the NAc and makes up a dominate feature of the mesolimbic pathway, also commonly referred to as the reward pathway[26]. MOR activation in the reward pathway produces robust dopamine release from the VTA and the NAc[27], which increases the risk of drug addiction following prolonged use of opioids.

Expression of opioid receptors within the brain and spinal cord are important for modulating pain processing[28]. MORs are densely expressed in peripheral nerve terminals within the dorsal root ganglion, the dorsal horn of the spinal cord, the periaqueductal gray, rostral ventral medulla, thalamus, and cingulate cortex[29-32]. MOR agonists are effective analgesics because of their actions on both the ascending and descending pain pathways (Figure 1.2)[33]. The ascending pain pathway is crucial for carrying sensory information from the periphery to the brain. Peripheral nerve endings residing outside of the spinal cord are activated by noxious stimuli. This causes transduction of electrical impulses that are then transmitted to a cluster of neurons in the dorsal root of the spinal nerve (DRG), into the spinal cord of the dorsal horn, and eventually into supraspinal regions for processing. Regulation of spinal nociceptive processing occurs, either via facilitation or inhibition, and the final perception of pain occurs[33]. For example, if one were to break a bone in the leg, it requires an electrical impulse to send this information to the brain to

perceive the pain. The descending pain pathway is vital for incorporating information processed in the nervous system's central structures, projecting this information to the dorsal horn of the spinal cord. Processed information is sent down the descending pain pathway, resulting in behaviors to minimize further damage to the broken leg, such as limiting the amount of pressure placed on the leg. Proper functioning of the descending pain pathway is crucial in human health. It acts as a safety mechanism to deter the continuation of a presenting noxious stimuli, and studies suggest alterations in the functioning of the descending limb are present in patients suffering from chronic pain. Expression of MOR in the peripheral nervous system (PNS) is a viable target for treating diseases such as some bowel diseases[34]. However, MORs are also important for decreasing the transmission of painful stimuli, leading to analgesia. The impact of peripheral MORs and their clinical utility as viable targets for pain treatment is still being investigated.

MORs are located in both enteric neurons and mucosal endocrine cells within the gastrointestinal (GI) tract[35]. The enteric nervous system is comprised of excitatory ascending motor neurons which control peristaltic contractions, and inhibitory descending motor neurons that control the circular muscular layer of the GI tract[36]. For proper peristaltic movement, acetylcholine and Substance P activate their corresponding receptors on ascending motor neurons, resulting in muscle contraction and forward movement of fecal boli. Concurrently, nitrous oxide inhibits descending motor neuron function, resulting in circular smooth muscle relaxation, allowing fecal boli to pass. MORs located in the GI tract, upon activation, inhibit the release of the excitatory neurotransmitters, acetylcholine and substance P resulting in decreased peristaltic contractions[37], and also inhibit the release of the inhibitory neurotransmitter, nitrous oxide. The net effect is diminished excitatory signaling of excitatory ascending motor neurons and decreased inhibitory effects of inhibitory descending motor neurons, resulting in decreases of gastric emptying,

increases of pyloric muscle tone, and ultimately delayed fecal transit through the small and large intestine[38]. Unsurprisingly, targeting MORs in the GI tract has been highly useful in treating patients with irritable bowel syndrome (IBS)[39,40,41], accompanied by frequent diarrhea, where constipation effects of MOR agonists are beneficial for alleviating diarrhea associated with IBS. However, constipation continues to be problematic in patients chronically using opioids and is often a reason for patients discontinuing their prescribed medication.

Alternative Approaches For Targeting MOR For Pain Therapy

For the past 100 years, selective MOR agonists have been the predominant approach for treating pain[42]. Adverse effects have hindered this clinical approach, enticing scientists to develop alternative approaches for pain therapy. Previously, scientists have tried to develop KOR, DOR, and NOPR selective agonists for treating pain, but activation of these receptors has not been shown to be as effective as MOR agonists in preclinical studies and have their own adverse effects[43].

KORs play a role in modulating stressful stimuli, and the activation of the KOR induces aversive and depressive-like states in a variety of species, including humans and laboratory animals[44-46]. Further, antagonism of the KOR suggests potential benefit in patients suffering from stimulant addiction[47,48]. With regards to pain relief, KOR activation induces modest antinociceptive effects[49-51], and there is a KOR agonists that is approved for use as an analgesic in Japan[52].

While the NOP shares ~60% sequence homology to the other opioid receptors, its classification as an opioid receptor was in question due it its insensitivity to naloxone[53]. The endogenous peptide for NOP is the nociceptin and orphanin FQ, or N/OFQ[54]. Distribution of NOP includes dense populations within the brain and spinal cord, as well as peripheral organs. Upregulation of the NOP-N/OFQ system has been shown in disease states including chronic pain

and depression[55]. Interestingly, Parkinsonian patients displayed elevated levels of N/OFQ in their cerebrospinal fluid, suggesting NOP may be a target for Parkinson patients[61,56]. NOP activation is also associated with suppression of basal and drug-induced dopamine release in the nucleus accumbens, suggesting NOP may be a useful target for patients suffering from substance abuse disorder[57]. Unfortunately, studies investigating distribution and localization of the NOP-N/OFQ system demonstrate vast differences between species, and these differences may play a significant role in translating the validation of the NOP-N/OFQ system as a potential target for different pathophysiological states[58,59].

Recent advances have suggested a role for the DOR in MOR-mediated behavioral effects following chronic administration of morphine[60,61]. DOR agonists, such as SNC80, have extensively been shown to induce antidepressant-like effects, as well as anxiolytic effects in a variety of species[62-64], improving the clinically beneficial profile of DOR. More impressively, DOR activation induces significant thermal antinociceptive effects[65]. The antinociceptive and antidepressant-like effects of DOR agonists are mediated through DOR activation and are independent of the convulsive effects, such that suppressing DOR-mediated convulsions (with anticonvulsant drugs) does not alter antidepressant effects produced by DOR agonists[66,67] Unfortunately, similar to the KOR, DOR activation also induces severe CNS-mediated adverse effects that deter clinical use of DOR agonists for therapy purposes. Both DOR agonists BW373U86 and SNC80 produce convulsions in mice, rats, and monkeys[66,68,69], limiting continued validation for selective DOR agonists as a therapeutic approach for pain management.

Another alternative approach under investigation for improving pain treatment is the use of biased MOR agonists. Traditional MOR agonists bind to the orthosteric site of the receptor. The orthosteric site is the site on the receptor to which endogenous ligands bind to produce their effects.

Biased agonism is when ligands bind to the orthosteric site, they can preferentially activate some downstream pathways over others, such as G-protein signaling over β-arrestin signaling[70]. Ligands stabilize specific receptor conformations once bound, which results in specific signaling profiles based on the ligan-receptor interaction[71-73]. The most predominant method of measuring bias is the differential activation of G proteins (G protein-bias) vs arrestin proteins[74]. The arrestin family of proteins consists of 4 members, 1-4. Arrestin 1 and 4 are exclusively expressed within rod and cone cells within the visual system[75,76]. Arresting 2 and 3 (also known as β-arrestin1 and β-arrestin2) are predominately expressed in tissues within the central nervous system and cells within the periphery, including the spleen and GI tract[77-80]. β-arrestins also regulate CB1R and MOR signaling, among many other receptor types. Upon GPCR phosphorylation, β-arrestins bind to phosphorylated MOR, ultimately diminishing G-protein signaling, even in the presence of saturating concentrations of agonist[81,82].

To assess the effects of β-arrestin on MOR-mediated antinociception, antisense oligonucleotides and mice lacking the β-arrestin2 protein have aided in elucidating these effects[83]. Specific inhibition of β-arrestin 2 with siRNA lentivirus microinjected in mice periaqueductal gray significantly enhanced the antinociceptive effects of morphine[84]. Similarly, mice lacking the β-arrestin2 protein display enhanced and prolonged morphine-induced antinociception in both supraspinal and spinal antinociceptive responses[85]. Further, β-arrestin2 KO mice failed to develop tolerance to the antinociceptive effect of morphine, suggesting β-arrestin2 has significant implications on the acute antinociceptive effects of morphine as well as the antinociceptive effects of morphine following repeated administration[83]. Therefore, selective MOR agonists displaying biased G protein signaling over β-arrestin signaling should exhibit improved antinociception with lessened tolerance development. Oliceridine (TRV130), a novel MOR agonist, is a biased MOR

agonist that preferentially activates G-protein vs. β-arrestin signaling pathways. Preclinical studies show TRV130 produces robust antinociception, and following 3x daily injections of 10mg/kg, TRV130 failed to induce tolerance[86]. Overall, these data suggest a biased agonism strategy at the MOR may provide benefit over current pharmacological interventions for pain therapy.

Allosteric modulation of the MOR is another approach implemented for improving pharmacologically-based pain treatment[87]. As previously mentioned, typical MOR agonists, such as morphine and fentanyl, bind to an orthosteric binding site on the receptor. Allosteric modulation is a ligand that binds to a site on the receptor that is distinct from the orthosteric site[88]. Allosteric ligands fall within a large spectrum of activity, ranging from negative allostery to positive allostery. A positive allosteric modulator (PAM) can enhance the binding affinity or efficacy of an orthosteric agonist, including endogenous opioid peptides. Noxious stimuli cause the release of endogenous opioids in many regions important for pain alleviation such as the DRG, spinal cord, and midbrain[89], and PAMs may be able to treat pain by enhancing the activity of endogenous opioids within these regions. It has been proposed that enhancing the activity of endogenous opioids in a spatially and temporally-controlled manner may minimize the development of tolerance and physical dependence that occurs during continuous treatment with exogenously administered MOR agonists that act at MORs throughout the body[90].

Tolerance To The Effects Of MOR Agonists

A significant complication of opioid use is the decrease in antinociceptive effects following chronic administration, such that larger doses of MOR agonist are required to produce the same antinociceptive effects (i.e., tolerance). Recapitulation of opioid-induced tolerance has been demonstrated in rodents, although studies suggest much of opioid tolerance involves opioid receptors within the CNS, and therefore requires drug accessibility to the CNS. The underlying

mechanisms for opioid tolerance still remain unclear, however early adaptive processes such as receptor downregulation or receptor desensitization are suggested to be key features in tolerance development. Receptor desensitization is characterized by a decrease in the coupling of the receptor to downstream signaling transduction pathways[91]. Receptor downregulation is the decrease in receptor number on the surface or total number of receptors for ligand binding, therefore decreasing the original baseline responses from the agonist[92]. Both receptor desensitization and downregulation results in decreased signaling of the agonist, and are thought to occur quite rapidly, within just a few minutes of agonist exposure. Therefore, it is relatively unknown if these processes contribute to long-term changes in receptor sensitivity following chronic exposure.

Many studies have evaluated opioid tolerance utilizing ICV or intrathecal administration methods, offering direct delivery of drug to the CNS. Mice chronically administered ICV morphine produce robust tolerance to the antinociceptive effects of morphine[93]. Further, intrathecal injection of morphine requires increasing doses in humans[94]. These studies highly suggest that opioid tolerance is a centrally mediated mechanism. Many proposed hypotheses for how tolerance develops include alterations in adenylyl cyclase inhibition, voltage-gate calcium channel inhibition, activation of GIRK channels, cAMP sensitivity, and MAP kinase activation[91]. All of which, however, are studied in either overexpressed cell cultures or *ex vivo* brain slices. A few studies have demonstrated that peripheral opioid receptor antagonism attenuates tolerance development to the antinociceptive effects of morphine[95]. Therefore, probing the involvement of peripheral opioid receptors is crucial for elucidating all mechanisms involved in opioid tolerance and potentially improving our approaches for treating clinical pain.

Compared to MOR agonists, DOR agonists are inadequate analgesics for acute pain, but are highly effective in models of chronic pain[96]. However, similarly to MOR agonists, rodents chronically administered systemic SNC80 or ARM390[97], developed tolerance to the antinociceptive effects[98]. Tolerance also limits the value of selective DOR agonists, and therefore other approaches are required for improving pain therapy.

Targeting Multiple Opioid Receptors

Since selective opioid receptor agonists have not been shown to be highly effective for treating pain without abuse liability, it has been suggested that simultaneously targeting multiple opioid receptor types (i.e., mimicking endogenous opioids) might produce pain relief with fewer side effects. Promising results have suggested mixed efficacy opioids may be highly beneficial for analgesic effects with attenuated adverse effects. In the early 1990s, it was shown that treatment with the DOR selective antagonist, naltrindole, prevented the development of tolerance to the antinociceptive effects of morphine[60]. In transgenic mice lacking DORs, the acute antinociceptive effects of morphine were preserved; however, the daily administration of morphine for eight days failed to induce tolerance[61]. These results were further validated with antisense DOP knockdown, which reduced tolerance development following prolonged morphine administration[99]. Collectively, these data suggest that DOR activation contributes to the development of tolerance to MOR agonists. Further, it may indicate that DOR signaling alters mechanisms that may contribute to opioid tolerance, such as receptor phosphorylation, desensitization, and/or downregulation. It is unclear whether the MOR-DOR interactions occur at the cellular level with MORs and DORs on the same cell or MOR-DOR heterodimers, or at the circuit level (i.e., MORs and DORs on different cells.

Designing a single compound that binds to both MORs and DORs would be advantageous over coadministration of multiple ligands[100]. This led to the synthesis of ligands that simultaneously targeted both MOR and DOR, in forms of peptides like DIPPψNH$_2$[101], bivalent ligands such as MDAN-21[102], and the multifunctional/mixed-efficacy opioid alkaloids such UMB425[103]. However, both DIPPψNH$_2$ and UMB425 induced significant tolerance and physical dependence following repeated administration. Recent studies have described a similar mixed-efficacy ligand, VPR26, which produced significant antinociception in the WWTW without acute tolerance development[104]. While progress is still needed, present research provides a proof of concept that dual mixed-efficacy ligands elicit a more desirable profile than traditional selective MOR agonists.

The synthesis of mixed-efficacy opioid ligands further utilized endogenous opioid peptide structures, such as Met-enkephalin. One feature of the endogenous peptides, such as Met-enkephalin, is that the chemical structures offer tremendous flexibility, increasing the probability of interactions with all three opioid receptors. Cyclic peptides derived from Met-enkephalin increased rigidity of the molecule, and led to the production of DPDPE, a prototypical DOR-agonist that displays high potency and affinity for DOR[105,106]. Further derivatization of DPDPE yielded a smaller, more drug-like peptide in JOM-13 that is roughly 600-fold-selective for DOR over MOR[106]. Computation studies were performed on JOM-13 to understand which pharmacophores within the JOM-13 structure were necessary for facilitating DOR activation. These studies resulted in the production of a more conformationally constrained peptidomimetic, KSKPP1E (Figure 1.1). Interestingly, KSKPP1E displayed nearly 200-fold selectivity for MOR over the parent compound, JOM-13, and an EC_{50} of 0.18nM measuring [35S]GTPyS stimulation

at the MOR[107]. This structure and *in vitro* profile are highly distinct from the selective DOR agonist it was based on.

KSKPP1E displayed antinociceptive effects in a model of spinal nociception in mice similar to that of morphine, however KSKPP1E displayed a slightly shorter duration of action[108]. Further, KSKPP1E showed slight DOR agonist activity and so further derivatization and optimization of the parent structure was required to eliminate the agonist activity at DOR[109,110]. Further evaluation and substitutions to KSKPP1E elucidate that substitutions N-substitutions to the THQ core of KSKPP1E further enhanced the binding affinity balance between MOR and DOR[111] (Figure 1.1). Following acute *in vivo* screening, two promising ligands were selected for further characterization, AAH8 and AMB67 (Figure 1.1). Both compounds displayed improved affinity balances compared to KSKPP1E. AAH8 is 5-fold selective for MOR over DOR, and displays DOR antagonist effects, whereas AMB67 is 24-fold more selective for MOR over DOR and displays a DOR agonist *in vitro* profile; both of which are improvements over the 42-fold affinity difference for KSKPP1E. Because early screening demonstrated both AMB67 and AAH8 induced antinociceptive effects, further evaluation of adverse effects including constipation, addiction liability and chronic treatment assessing physical dependence and tolerance development across a number of pain measurements was conducted.

Studying Opioid-Mediated Effects *In Vivo*

The use of preclinical models is highly important in translation research. Novel drug entities require preclinical evaluation and validation before being able to be tested in a human population. Therefore, various preclinical assays have been developed and are used to evaluate to the antinociceptive effects of novel ligands.

Assays used to evaluate novel drugs attempt to model four main types of pain: nociceptive, inflammatory, neuropathic, and idiopathic pain. Nociceptive and neuropathic pain encompasses both pain experienced in the skin, muscles and bones (somatic) or pain residing in organs or abdominal cavities (visceral). Neuropathic pain can occur due to either damage or disease that affects the somatosensory nervous system, typically resulting in pain from normally non-painful stimuli, also known as allodynia. However, neuropathic pain can also be idiopathic where the cause of the pain cannot be determined. The benefits of categorizing pain experiences are that they differ in severity, location, and duration of effect. For example, nociceptive pain is typically short-term, whereas neuropathic and idiopathic pain are typically long-lasting. Dozens of pain assays have been developed to improve the approach of studying pain therapy. Nevertheless, to measure antinociception in non-human animals, specific behavioral readouts have been established.

Neuropathic assays have become more prevalent in the pharmacology field. Neuropathic pain assays typically involve surgery, and the first neuropathic pain model was developed in 1979, named the neuroma model[112]. Newer models of neuropathic pain focus primarily on the innervation of the sciatic nerve. The sciatic nerve consists of three main branches, peroneal, sural, and tibial nerves. The spared nerve injury model involves injury to the common peroneal and tibial nerves, leaving the sural nerve intact[113,114]. This approach to target the sciatic nerve produces a long-lasting, robust hypersensitivity to both mechanical and thermal stimuli and have been useful in studying antinociceptive effects of currently used drugs, and comparing these results to clinical effects as well as assessing newly developed ligands for neuropathic pain therapy[115].

Inflammatory pain is a spontaneous hypersensitivity to pain in response to tissue damage and resulting inflammation. Inflammatory assays incorporate a state of inescapable pain and utilize agents such as formalin, capsaicin, carrageenan, and complete Freund's adjuvant. The initial use

of inflammatory assays mimics arthritic pain in humans; however, these approaches have failed to improve arthritic pain therapy in the clinical realm. However, the use of inflammatory assays still provides benefits for measuring the antinociceptive effects of novel ligands.

Recapitulating painful diseases has become a more prominent method for assay development. An overwhelming realization that current pain models fail to replicate existing conditions led researchers to develop models that directly model prevalent pain syndromes. Notable models include burn-related pain[116], cancer pain[117], chemotherapy-induced neuropathic pain, complex regional pain syndrome[118], and spinal cord related injury[119]. While these assays have been beneficial in assessing antinociceptive properties of drugs, it is also important to evaluate the behavioral responses as not only nociceptive vs antinociceptive, but also as pain elicited vs. pain depressed behaviors.

Pain Elicited Vs Pain Depressed Behaviors

Pain can stimulate nocifensive behaviors (behaviors evoked by noxious stimuli), but also suppresses many behaviors, such as feeding or locomotion. These have been referred to as pain-elicited behaviors or pain-depressed behaviors.

Pain-elicited behavior *increases* in rate, frequency, intensity, or duration in response to noxious stimuli[120]. Common examples and those used in these studies include measuring tail-flick latencies in response to thermal stimuli, paw withdrawal thresholds in response to mechanical stimuli, and stretching behavior in response to a chemical stimulus. Pain-elicited behaviors are widely used throughout drug development laboratories but can be problematic for various reasons. For example, drug-induced motor impairments and general sedation can be mistaken for, or interpreted as pain relieving effects because pain-elicited behaviors are decreased[121].

Pain-depressed behaviors can be defined as any behavior that *decreases* in rate, frequency, intensity, or duration in response to a noxious stimulus. Common examples include decreases in feeding behavior, locomotion, and operant behavior[122,123]. This approach provides several advantages over pain elicited behaviors as one goal of analgesic treatment is the restoration of pain-depressed behavior[124]. Pain depressed behaviors are not limited by the locomotor depressant effect of drugs, hopefully minimizing the potential for false-positive antinociceptive results. Measuring pain-depressed behaviors may be more clinically relevant since a goal in treating pain in humans is to restore normal function and activity[125]. Approaches utilizing both pain-elicited and pain-depressed behaviors may provide greater benefit over using a single approach.

Assessment of Adverse Effects In Pre-Clinical Models

Rewarding properties

Abuse liability of newly developed ligands within various animal models typically serves as the forerunner for subsequent clinical studies. These experiments are advantageous in that they provide preliminary information on abuse liability. The methods utilized within this thesis include drug discrimination procedures, conditioned place preference studies, and testing physical dependence development following repeated administration of the drug.

Drug discrimination is a paradigm in which animals are trained to respond for a food reinforcer (glucose pellets) and trained to use a drug's interoceptive effects as a cue (i.e., discriminative stimulus) to respond on the appropriate manipulanda

Many drugs can elicit discriminative stimulus effects, and all drugs of abuse produce discriminative properties that are thought to contribute greatly to their abuse potential[126]. For example, opioids, including morphine and fentanyl, fail to generalize to the discriminative effects

of amphetamine and vice versa[127], demonstrating that drug discrimination paradigms can separate interoceptive properties of different drug classes.

Focusing on the opioid system, MOR agonists are distinguishable from KOR agonists[128,129]. Studies have demonstrated that naloxone, and similar opioid antagonists such as naltrexone, can attenuate behavioral effects induced by both MOR and KOR agonists. Similarly, naloxone and other opioid antagonists can shift EC_{50} in animals trained to discriminate either morphine from saline or U50, 488 from water. However, in monkeys trained to discriminate a morphine, KOR agonist substitutions fail to generalize to the interoceptive properties of morphine[130]. In rats trained to discriminate 5.6 mg/kg U50, 488, the KOR agonist bremazocine fully generalizes to the discriminative effects of U50, 488, but, MOR agonists morphine and fentanyl fail to generalize to the discriminative stimulus effects of U-50,488[131]. In rats trained to discriminate 0.32mg/kg SNC80 from saline, both morphine and U-50,488 failed to substitute for SNC80. These results suggest that drug discrimination studies provide receptor-selective sensitivity and allow investigation of specific receptor-mediated interoceptive effects. Therefore, we wanted to assess mixed-efficacy opioids in similar drug discrimination paradigms.

Conditioned place preference (CPP) is a commonly used assay to assess rewarding properties of a drug[132,133]. Typically, drugs of abuse such as cocaine or morphine produce CPP, whereas drugs that elicit aversive effects, such as lithium chloride or the selective KOR agonist, U69, 488, induce conditioned place aversion (CPA)[134,135]. CPP has also been established in CNS depressant drugs such as ethanol and diazepam, cannabinoid receptor agonist delta-9-tetrahydrocannabinol (THC), and common opioids such as morphine and heroin[136-138]. Interestingly, studies demonstrate site-specific injections of morphine into either the ventral

tegmental area[139] or the nucleus accumbens[140] is sufficient for inducing CPP, and the authors suggest the MOR is important for mediating such behaviors.

Interestingly, mice lacking the DOR show loss of morphine reward[141]. This suggests that the DOR, in part, is involved in MOR-mediated CPP. These results have aided in developing mixed efficacy MOR agonist/DOR antagonist ligands in hopes that antinociceptive effects will be retained through activation of the MOR but will not induce CPP. However, improving the rewarding properties of opioid-mediated antinociception is only one component to discovering the "holy grail" analgesic. Therefore, assessing physical dependence development is a crucial component to improved pain pharmacotherapy.

Physical Dependence

A significant confounding variable involved in opioid use disorder (OUD) is the development of physical dependence, and many patients experience physical dependence at different stages of their pain therapy[142].

Following chronic exposure or use of an opioid, abstinence from drug can produce physical symptoms as well as affective symptoms of withdrawal[143]. Assessment of physical dependence development can be assessed in various ways and can be quantified by the type and severity of withdrawal signs that emerge after the discontinuation of drug treatment or the administration of a pharmacological antagonist, such as naltrexone[144,145]. In rodents, withdrawal-like effects include jumping, teeth chattering, piloerection, wet dog shakes, paw tremors, and soft stool. All of which are robust behaviors in rodents physically dependent on MOR agonists[146]. A global withdrawal score can be calculated by including all withdrawal-like effects to assess total physical dependence development.

In contrast to the well-characterized physical dependence development to the behavioral effects of MOR and KOR agonists[147,148], probing DOR-mediated dependence has been studied less extensively. Mice treated sub chronically with ICV DPDPE, a selective DOR agonist, exhibit no jumping behavior following naloxone administration[149]. Conversely, rats administered ICV DPDPE for 70h display precipitated withdrawal signs similar to those in rats receiving ICV morphine, although to a lesser degree[150]. However, physical dependence induced by ICV DPDPE may actually be due to some activity of DPDPE at MORs as the antinociceptive effects of DPDPE are less potent in mice lacking MORs[151].

SNC80 is a selective DOR agonist with 800-fold selective for DOR over MOR[97]. Rhesus monkeys treated chronically with SNC80 did not exhibit signs of precipitated withdrawal[152]. Interestingly, studies have suggested the DOR may be involved in morphine-induced physical dependence, as low dose naltrindole produced dose-dependence decreases in morphine-induced withdrawal-like effects following chronic administration of morphine[60,153]. Therefore, we wanted to assess if chronic administration of AMB67 induced naloxone precipitated withdrawal-like effects.

Experimental Objectives of the Current Dissertation

The overall goal of this research project is to find a safer opioid analgesic with less abuse liability and fewer side effects. The specific objective of the experiments described in this thesis are 1) to characterize the antinociceptive effects and the adverse effects of novel mixed-efficacy opioids ligands following acute and chronic administration in pre-clinical rodent models and 2) to evaluate novel mechanisms involved in opioid tolerance. These studies address downfalls of current pain management approaches, the adverse effects associated with chronic opioid use, and

elucidation of tolerance mechanisms in order to improve how pain management is conducted, hopefully leading to a decrease in the prevalence of OUD worldwide.

Aim 1: To Characterize the antinociceptive and adverse effects of AMB67, a mixed-efficacy opioid ligand

The first study in this thesis evaluated the antinociceptive effects of AMB67 following acute and chronic administration and elucidated adverse effects, including tolerance development, physical dependence, and rewarding properties. *It was hypothesized that a drug that is a MOR and DOR agonist would produce less tolerance and physical dependence than the MOR agonist morphine. In vitro* studies assessed binding affinity, potency, and efficacy of AMB67 at both the MOR and DOR. The present study also evaluated AMB67-induced chemical, thermal, and mechanical antinociception following acute and chronic administration, including assessing physical dependence development following repeated administration of AMB67. Antinociception studies included dose-effect comparisons with morphine, as well as antagonism studies following systemic administration. Additionally, the present study evaluated the addiction liability of AMB67 across multiple species and paradigms.

Aim 2: Evaluate behavioral effects of AAH8, a mixed-efficacy, MOR agonist/DOR antagonist following acute and chronic administration.

In the second study, acute and chronic antinociceptive effects of AAH8 were assessed. Based on previous studies, *it was hypothesized that chronic administration of AAH8 would not induce tolerance*. Previous studies demonstrated AAH8 induced acute antinociceptive effects compared to morphine[154]. The present study elaborated on the behavioral effects of acute and

chronic AAH8, including antinociception and constipation effects. Antinociception studies included dose-effect comparisons with morphine, as well as pharmacological antagonism using both peripherally restricted and non-restricted opioid antagonist, as well as assessment of AAH8-induced antinociception in mice lacking the MOR. Comparisons were made between the antinociceptive effects of AAH8 administered by intraperitoneal or intracerebroventricular routes of administration. Further, constipation effects of AAH8 were assessed and compared between intraperitoneal and subcutaneous routes of administration.

Aim 3: Elucidate the role of peripheral opioid receptors in the development of tolerance to MOR agonists.

The final study of this thesis evaluated the involvement of peripheral opioid receptors in developing tolerance to MOR agonists. Previous studies suggested that peripheral opioid receptors may be involved in the production of tolerance to the antinociceptive effects of morphine[95]. *It was hypothesized that antagonism of peripheral opioid receptors was sufficient for attenuating tolerance development to the centrally-mediated antinociceptive effects of MOR agonists*. For these studies, centrally-mediated antinociceptive effects of morphine were assessed following repeated administration in a thermal pain model. Comparison of central vs. peripheral antinociceptive effects of morphine were assessed following pretreatments of either peripherally restricted or non-restricted opioid antagonists. Further, naloxone-methiodide, a peripherally restricted MOR antagonist, was utilized to assess if peripheral opioid receptors' antagonism was sufficient for attenuating morphine-induced tolerance. Additionally, loperamide, a peripherally restricted MOR agonist, was assessed for acute and chronic antinociceptive effects, including evaluating cross-tolerance to morphine.

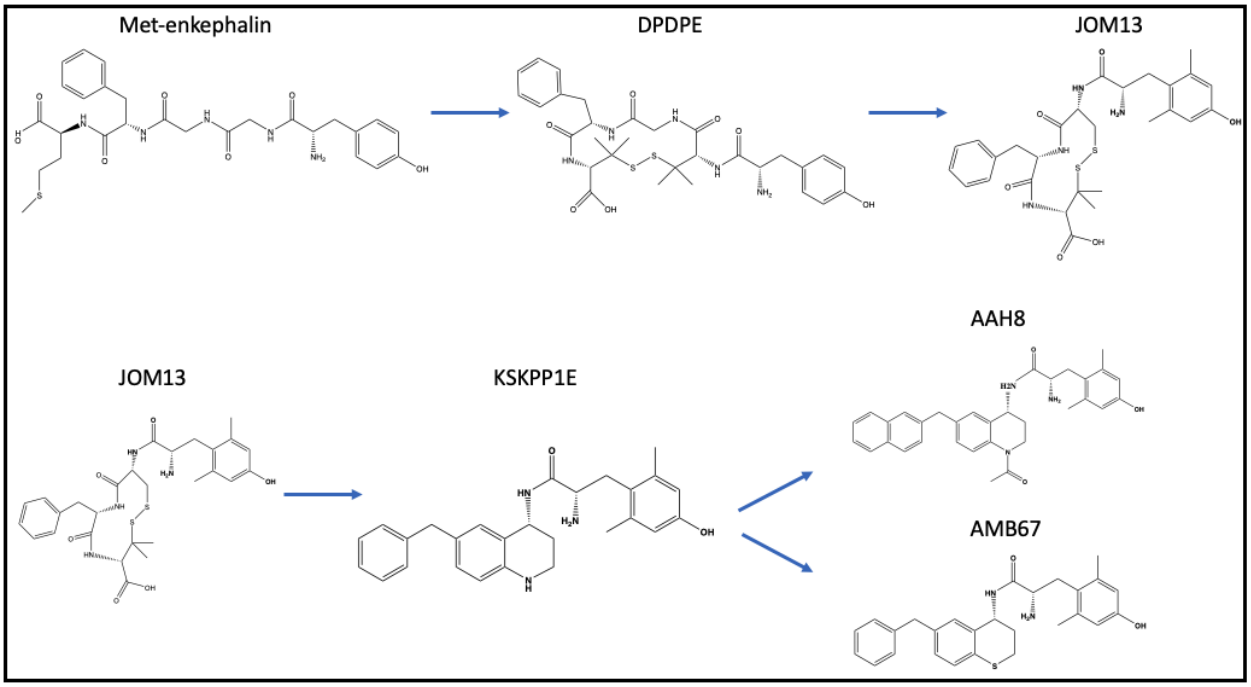

Figure 1.1 Structural Evolution of the Endogenous Peptide Met-enkephalin into KSKPP1E and Eventually the Lead Compounds AAH8 and AMB67

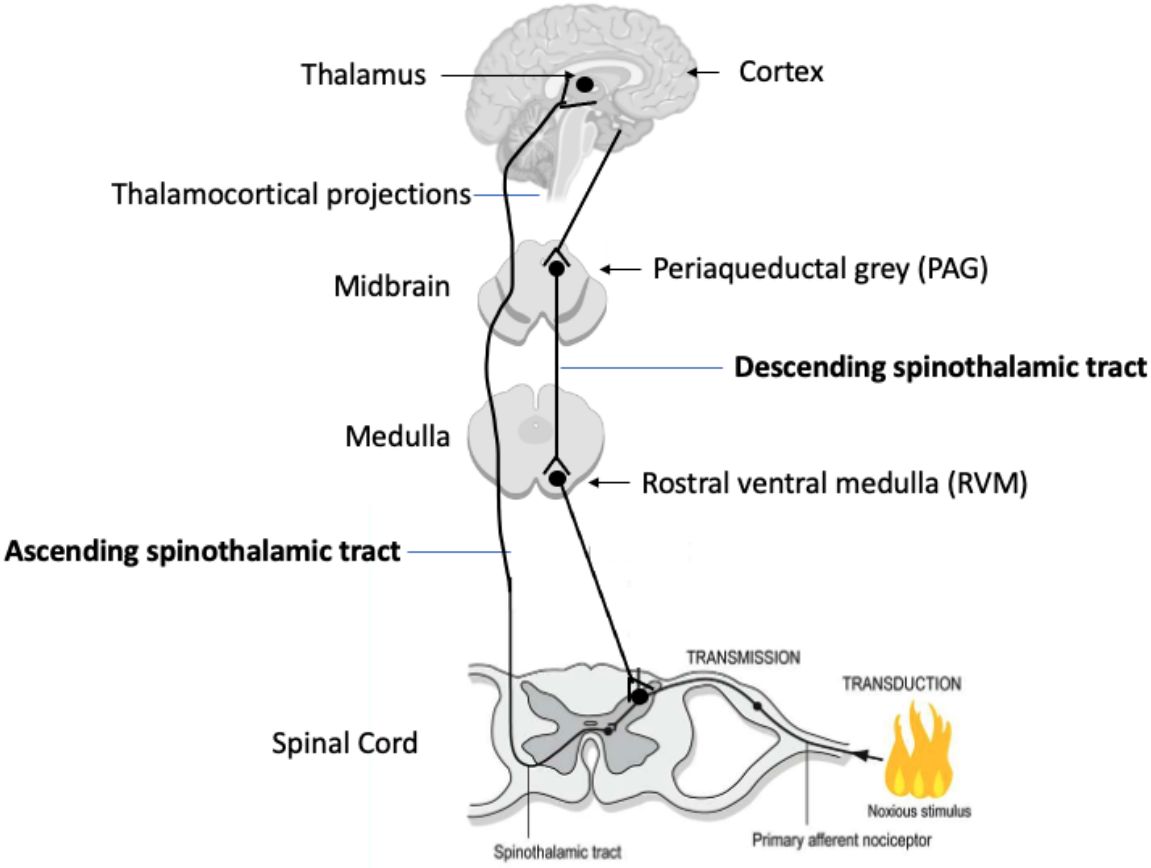

Figure 1.2 Ascending and Descending Limbs of Pain Transmission

Ascending and descending pain pathways. Primary afferent nociceptors respond to noxious stimuli, where transduction of noxious stimuli to a chemical signal occurs. Chemical signals from the periphery relay to the spinal cord. In the dorsal horn of the spinal cord, nociceptors synapse onto interneurons that signal to second order neurons. This information is sent up the spinal cord to the brain stem and thalamus. Third order neurons receive signals in the thalamus and relay them to the somatosensory cortex of the brain where the noxious stimuli are interpreted as pain. The descending pathway involves efferent signaling from the PAG to the RVM to the spinal cord and modulation of the ascending pathway Figure adapted from International Association of Pain

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Chapter II: Characterizing the Activity of A Novel, Multifunctional Opioid Ligand, AMB67

Abstract

Opioids, such as morphine, have long been used to treat pain. Opioids exert their effects through MORs. While effective, MOR agonists produce on-target adverse effects such as constipation, tolerance, physical dependence, and euphoria. Multiple studies have demonstrated that simultaneous modulation of both MORs and DORs could improve the therapeutic profile of opioid ligands. AMB67 is a peptidomimetic opioid ligand that binds to MORs and DORs with low nanomolar affinity. The present study characterized the acute effects of AMB67 *in vitro* and *in vivo* as well as the effects *in vivo* following repeated or chronic administration. To evaluate the antinociceptive effects of AMB67, we used models of thermal, chemical, and mechanical nociception. In C57BL/6N mice, AMB67 produced dose-dependent antinociceptive effects in all three pain models. The antinociceptive effects of AMB67 were attenuated with naloxone pretreatments and was absent in MOR KO mice. Following 5 days of administration with increasing doses, the ED_{50} for the AMB67 dose effect curve was unchanged in models of thermal and chemical nociception; however, following the same chronic dosing regimen, the dose effect curve for AMB67 in mechanical nociception was shifted 1.5-fold to the right. Naltrexone precipitated a greater withdrawal in morphine-treated mice as compared with AMB67-treated mice. After 5 days of conditioning, AMB67 was less potent than morphine in producing conditioned place and in morphine-like discriminative stimulus effects. Overall, AMB67 produced similar antinociceptive effects to morphine but produced less tolerance, physical dependence, and

rewarding effects than morphine. These findings highlight a compound that may have a safer profile of activity than typical opioid analgesics.

Introduction

Pain affects 100 million Americans and is a major contributor to national rates of morbidity, mortality, and disability[1]. Opioids, such as morphine, are useful for treating pain, but they also produce on-target, adverse effects, such as tolerance, physical dependence, respiratory depression, constipation and abuse liability[2,3]. Tolerance development to the pain-relieving effects of opioids contributes to the necessity for dose escalation, which may result in misuse of prescription opioids[4]. Further, continued use of opioids produces physical dependence, also increasing the risk of misuse[4-6]. Therefore, the development of ligands that are effective for treating pain but produce less tolerance and physical dependence will greatly improve the treatment of pain.

The analgesic effects and adverse effects discussed above of opioid analgesics occur via activation of the mu-opioid receptor (MOR). Other opioid receptor types (delta, kappa, and ORL1) alone or in combination with MOR agonists have also been studied as alternative targets for treating pain. The use of selective DOR agonists for treating pain is clinically limited because they produce DOR-mediated convulsive behavior in mice, rats, and monkeys[7,8,9]. Interestingly, inhibition of DORs does not alter acute MOR-mediated antinociceptive effects but attenuates the development of tolerance and physical dependence following MOR agonist administration[10-12]. Therefore, research has focused on synthesizing multifunctional opioid ligands exhibiting a MOR agonist/DOR antagonist *in vitro* profile[7,13-19] or MOR/DOR agonist activity profile[20-24]. There is

some evidence that multifunctional opioid ligands, such as SRI-22131, produce fewer adverse effects than selective MOR agonists following repeated administration[23,25-27].

We hypothesized, based on the literature above, that a multifunctional opioid ligand with nearly equal, sub nanomolar affinities at MORs and DORs would display antinociceptive properties similar to morphine, but lack tolerance development, physical dependence development, and rewarding properties. Therefore, we evaluated AMB67, a dual MOR/DOR agonist in mouse models of acute and chronic nociception, physical dependence, as well as evaluation of the rewarding properties of AMB67 using conditioned place preference and drug discrimination.

Methods

In Vitro Studies

All tissue culture reagents were purchased from Gibco Life Sciences (Grand Island, NY, USA). Radioactive compounds were purchased from Perkin-Elmer (Waltham, MA, USA). C6-rat glioma cells stably transfected with a rat μ(C6-MOR) or δ(C6-DOR) opioid receptor[28] and Chinese hamster ovary (CHO) cells stably expressing a human μ(CHO-MOR) or δ(CHO-DOR) opioid receptor[29] were used for all *in vitro* assays. Cells were cultured and membranes prepared as previously described[30]

Radioligand Binding Assays.

Radioligand binding assays were performed as previously described[30]. In brief, assays were performed using competitive displacement of 0.2 nM [3H] diprenorphine (250μCi, 1.85 TBq/mmol) by the test compound from membrane preparations containing opioid receptors. The assay mixture, containing membrane suspension (20μg of protein/well) in 50 mM Tris-HCl buffer

(pH 7.4), [^{3}H]diprenorphine, and various concentrations of test ligand, was incubated at room temperature for 1 h to allow binding to reach equilibrium. The samples were filtered through Whatman GF/C filters and washed three times with cold 50 mM Tris-HCl buffer (pH 7.4). The radioactivity retained on dried filters was determined by liquid scintillation counting after saturation with EcoLume liquid scintillation cocktail in a Wallac 1450 MicroBeta (Perkin-Elmer, Waltham MA, USA). Nonspecific binding was determined using 10μM naloxone. Ki values were calculated using nonlinear regression analysis to fit a logistic equation to the competition data using GraphPad Prism, version 5.01, for Windows. The results presented are the mean ± standard error from at least three separate assays performed in duplicate.

Stimulation of [^{35}S]GTPγS Binding.

Agonist-induced stimulation of [^{35}S]guanosine 5′-O-[γ-thio]triphosphate ([^{35}S]GTPγS, 1250 Ci,46.2 TBq/mmol) binding was measured as described previously[31]. Briefly, membranes (10−20μg of protein/well) were incubated for 1 h at room temperature in GTPγS buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2, pH 7.4) containing 0.1 nM [^{35}S]GTPγS, 30μM guanosine diphosphate (GDP), and varying concentrations of test peptides. Agonist-induced stimulation of [35S]GTPγS was compared with 10μM standard compounds [D-Ala2,N-MePhe4,Gly-ol]enkephalin(DAMGO) at MOR and D-Pen2,5-enkephalin (DPDPE) at DOR. The reaction was terminated by rapidly filtering through GF/C filters and washing 10 times with cold GTPγS buffer. Retained radioactivity was measured as described above. The results are presented as the mean ± standard error from at least three separate assays each performed in duplicate; maximal stimulation and EC_{50} values were determined using nonlinear regression analysis with GraphPad Prism, version 5.01, for Windows.

Animals and Solutions for *In Vivo* Administration

All animal care and experimental procedures complied with the US National Research Council's Guide for the Care and Use of Laboratory Animals[32]. Mice were group- housed with a maximum of five animals per cage in clear polypropylene cages with corn cob bedding and nestlets as enrichment. Mice had free access to food and water at all times. Animals were housed in pathogen- free rooms maintained between 68 and 79°F and humidity between 30 and 70% humidity with a 12 h light/dark cycle with lights on at 07:00 h. We conducted experiments in the housing room during the light cycle. All studies utilize wildtype male C57BL/6N mice from Envigo laboratories (formerly Harlan, Indianapolis, IN), or wildtype, heterozygous and homozygous male MOP-KO (B6.129S2- *Oprm1*^{tm1Kff}/J stock number 007559; Jackson Laboratory). All mice used for behavioral experiments weighed between 20-30 g at 7-15 weeks old. All drug solutions were injected at a volume of 10ml/kg. AMB67 was dissolved in 1:9 DMSO/saline solution. Morphine sulphate was dissolved in saline. Acetic acid was diluted in water to a 0.6% solution. All drugs were given either by intraperitoneal (IP) or subcutaneous (SC) injection, and the diluted acetic acid was given IP.

Acetic Acid Stretch Assay (AASA)

Antinociceptive effects were evaluated in the mouse acetic acid stretch assay[33]. Test drug or vehicle was given via a SC injections 30 min prior to an injection of 0.6% acetic acid (IP). Following acetic acid administration, mice were placed individually in clear plastic observation cages (10 x 6 x 8 in) with bedding. A 5 min latency period occurred prior to starting observations, and then the total number of stretches or writhes observed for 20 min was recorded. For antagonism

studies, a dose of 10 or 3.2 mg/kg naloxone or 10mg/kg naloxone-methiodide was administered IP 15 min before administration of test drug or vehicle.

For the tolerance studies, mice received increasing doses of morphine (1-5mg/kg),AMB67 (1-5mg/kg) or vehicle every 12 hours (7 am, 7 pm) for five days. No injections were administered the evening prior to test day.

Von Frey

For all von Frey experiments, mice were placed in a plastic box (4in x 4in x 4inch) with a mesh floor that was elevated sixteen-inches above the table. All mice and were habituated to the plastic box for two hours per day for three days and baseline (pre-CFA). mechanical withdrawal thresholds were assessed. To assess withdrawal threshold, each von Frey monofilament was applied perpendicularly to the ventral medial portion of the hind paw for approximately 3 sec. A withdrawal response was characterized by rapid removal of the paw from the filament within the 3 sec time limit. The Up-Down method was used to determine all withdrawal thresholds[34]. Complete Freund's Adjuvant (CFA, Catalog #77140, Thermo Scientific) was administered unilaterally in a volume of 15ul in a randomized manner. 24 hrs post CFA injections (test day), withdrawal thresholds were evaluated in the absence or presence of drug.

On test day, all mice were administered saline or vehicle, and withdrawal thresholds were evaluated 30 min later (post-CFA baseline withdrawal threshold). Increasing doses of morphine or AMB67 were administered every 30 minutes in a cumulative dosing fashion, and withdrawal thresholds were recorded.

For tolerance studies, on day 1 (24h post CFA administration), all mice were administered saline or vehicle, and withdrawal thresholds were evaluated 30 min later (post-CFA baseline

withdrawal threshold). Increasing doses of morphine or AMB67 were administered every 30 minutes in a cumulative dosing fashion, and withdrawal thresholds were recorded to assess antinociceptive effects. Mice were then administered morphine (10mg/kg), AMB67 (10mg/kg), or vehicle 3x per day for five days (Days 1-5) (7am, 1pm, 7pm). No injections were administered the evening prior to test day to ensure no circulating drug was present during mechanical threshold testing. On day 6, all mice were administered saline or vehicle, and withdrawal thresholds were evaluated 30 min. Increasing doses of morphine or AMB67 were administered every 30 minutes in a cumulative dosing fashion, and withdrawal thresholds were recorded.

Warm Water Tail Withdrawal (WWTW)

To determine tail withdrawal latencies, mice were placed briefly in a cylindrical plastic restrainer and the distal 2-3 cm of the tail were dipped into a water bath maintained at 50° C. The latency to tail withdrawal or rapid tail flicking, was recorded. If a mouse did not remove its tail by 20 sec, it was removed by the experimenter to prevent tissue damage. For time course experiments, morphine (10mg/kg) and AMB67 (10mg/kg) were given via SC administration, and tail-flick latencies were recorded over time. For dose effect curve evaluation, cumulative dosing of test compounds (1-32mg/kg) was given IP at 30 min intervals and tail-flick latencies were recorded at the end of each 30 min interval. For pharmacological antagonism studies, naloxone (3.2mg/kg) or naltrindole (3.2mg/kg) was administered via IP injection 15 min pretreatment prior to the agonist. For the tolerance studies, mice were administered morphine (10 mg/kg), AMB67 (10 mg/kg), or vehicle 3x per day for five days. No injections were administered the evening prior to test day to ensure no circulating drug was present for testing.

Physical Dependence

Wild-type C57BL/6N mice were treated for five days with either saline, vehicle, or escalating doses of the test compound (10-50 mg/kg, IP) every 12 h (7am, 7 pm). On day 6, mice were given a 50mg/kg dose of test drug or saline (IP) and returned to their home cage. Two hours later, each mouse was given 10mg/kg naltrexone IP and placed in an empty, individual plastic observation cage (10 x 6 x 8 in.). Mice were observed for 30 min after naltrexone administration for signs of opioid withdrawal including jumping behavior, teeth chattering, piloerection, wet dog shakes, paw tremors and soft stool. A modified global withdrawal score was calculated for each animal by assigning each individual physiological response a numerical value as previously reported[35]. Teeth chattering, piloerection, wet dog shakes, paw tremors and soft stool were all assigned a numerical value of 1 based on presence or no presence. Total number of jumps was weighted as follows and global withdrawal scores are the sum across each physiological response: 0 jumps = 0, 1-9 jumps = 1; 10-19 jumps = 2; 20-29 jumps = 3 etc.

Conditioned Place Preference

The apparatus contained two compartments separated by a single, vertically sliding door. Each compartment was 72x130x72mm (med associates, MED-CPP2-3013-2). One side of the apparatus contained white walls with parallel rod flooring; the other side of the apparatus contained black walls and grid flooring.

We utilized a modified biased design. On days 1 and 2, the mice were placed into one compartment (side start alternated across 2 days) and allowed to roam freely in both chambers for 30 min. The average time spent in each compartment was calculated across both days and was used to determine bias. If a mouse spent more than or equal to 70% of time during bias evaluation

in one compartment, then that mouse was discarded from the study (~5% occurrence rate). If mice showed a slight preference for one side (50-69% of time spent in one compartment), then drug was assigned to the least preferred side. If there was no bias (50% time spent in each compartment), drug-paired sides were assigned at random.

Following bias testing, mice underwent 5 conditioning days. On each conditioning day, animals received a SC injection of saline in the morning and immediately confined to the saline-paired chamber. Similarly, in the afternoon, animals received a SC injection of a single dose of morphine, AMB67, or vehicle and were immediately confined to the drug-paired chamber. On Day 8, each mouse was placed in a side of the chamber randomly and allowed access to both compartments of the apparatus. The amount of time spent in each compartment was recorded for 30 min. Time spent on the drug-paired side during bias testing was subtracted from time spent on drug-paired side during test day to calculate a Mean Place Preference Score.

Drug Discrimination Apparatus

Drug discrimination procedures were performed in twelve standard operant conditioning chambers with an area of 30.5 x 24.1 x 21.0 cm (Med Associates, ENV-018MD). Each chamber contained stainless-steel parallel floors (ENV-008; Med Associates, St. Albans, VT) and were equipped with stimulus light panels (Med Associates, 3 LED lights, ENV-222M) placed directly over two illuminated nosepoke devices (ENV-114BM). Between the nosepoke devices, the chambers contained a pellet dispenser (Med Associates, ENV-203M-45), and a food receptacle (Med Associates, ENV-200R2M). Each chamber was contained within ventilated, sound-attenuating boxes.

Operant Training

Rats were first trained to respond for a 45 mg sucrose pellet on a fixed ratio 1 (FR1) schedule of reinforcement during a 30 min session. The FR schedule gradually increased to FR10 until responding was stable (>30 pellets earned in each session). After an FR10 schedule was reached, the length of the session was gradually decreased to 10 min. To train drug discrimination, rats were injected with saline (SC) or 3 mg/kg morphine (SC) and placed immediately into the operant chamber. Following a 20-minute blackout period, stimulus lights turned on in each nose poke, and responding (FR10) on the injection-appropriate nose poke was reinforced with a 45 mg sucrose pellet. Incorrect nose poke responding was recorded but resulted in no scheduled consequences. Delivery of a sucrose pellet or completing a ratio on the incorrect nose-poke, resulted in a 10-s timeout (TO). Responding during the TO was recorded but had no scheduled consequence.

Discrimination Testing and Maintenance

Test sessions were only conducted when the following criteria were met: (1) the first response must be completed on the injection-appropriate nose poke for two consecutive training days, and (2) at least 85% of responses must be on the injection-appropriate nose poke. If, at any point, a training session occurred where criteria were not met, three subsequent days of meeting criteria was required to proceed to a test day. During test sessions, responding on either nose poke was reinforced with sucrose regardless of the type of injection received. Rats received no more than three tests per week. For tests sessions, rats were administered vehicle, saline or morphine (SC), or AMB67 (IP).

Data Analysis

All data analyses were performed using GraphPad Prism version 8.4.3. Level of significance (α) was set to 0.05. Repeated measures, two-way ANOVAs were conducted for all dose-effect curves unless otherwise stated. We conducted Tukey's post hoc analyses to correct for multiple comparisons. Post hoc analyses were only performed when F values achieved $p < 0.05$. Approximate ED_{50} values were calculated using GraphPad Prism version 8.4.3. The 50% maximum effect was interpolated from the straight-line analysis and then averaged within each treatment group. This included 2-3 points along the linear portion of the curve only. Standard errors of the mean were calculated for ED_{50} values where stated. However, in some studies, we could not calculate standard errors of the mean for ED_{50} values because different mice were present at each dose. Fold shifts in dose-effect curves were calculated by dividing ED_{50} value of interest by ED_{50} value calculated in wild-type mice or in the presence of the agonist alone.

Results

In Vitro Results:

Binding Affinity and Efficacy for AMB67

Binding affinity, potency, and efficacy for morphine and AMB67 were determined at both the rat and human MORs and DORs (Table 2.) At the rat MOR in C6 cells, morphine had a Ki of 6.5±1.9 nM and stimulated [^{35}S]GTPyS binding to 21.8% of DAMGO stimulation. At the rat DOR, morphine had a Ki of 94.6nM and showed 22.7% of DAMGO-induced G protein binding.

At the human MOR in CHO cells, morphine had an affinity of 4.2±1.3 nM affinity and 98% DAMGO stimulation. However, at the human DOR, morphine exhibited a 104±9.2 nM affinity and 56.3% DPDPE stimulation. The potency for morphine at both the rat and human MOR,

as well as the human DOR was in the triple digit nanomolar range, whereas at the rat DOR was in the micromolar range.

AMB67 exhibited low nanomolar affinity at both rat and human MOR and DOR. At rat MOR expressed in C6 cells, AMB67 displayed a Ki of 0.2±0.1 nM and 81% DAMGO stimulation. At the rat DOR, AMB67 displayed a Ki of 4.8±1.1 nM, however failed to stimulate [^{35}S]GTPyS. At the human MOR expressed in CHO cells, AMB67 exhibited a Ki of 0.1±0.01 nM and 83% DAMGO stimulation. However, at the DOR in CHO cells, AMB67 displayed a Ki of 2.4±0.4 nM and 34.5% DPDPE stimulation. Potency for AMB67 at both rat and human MOR was in the low nanomolar range. Whereas at the rat DOR, AMB67 exhibited no [^{35}S]GTPyS stimulation and double-digit nM potency at the human DOR.

***In Vivo* Results:**

Acute Antinociceptive Effects of Morphine and AMB67

The acute antinociceptive effects of AMB67 and morphine were assessed using the WWTW (thermal), AASA (chemical), and von Frey (mechanical) assays in male C57BL/6N mice. In the WWTW, 10 mg/kg AMB67 and morphine increased withdrawal latency as supported by a significant main effect of time [$F(5, 50) = 60.3$, $P<0.0001$] and no significant interaction [$F(5, 50) = 0.63$, $P=0.68$] (Figure 2.1A).

In the WWTW, dose effect curves of morphine and AMB67 were evaluated (Figure 2.1C). Both compounds produce dose dependent increases in tail withdrawal latency as supported by main effect of dose [$F(4, 50) = 137$, $P<0.0001$]. However, there were no significant differences between morphine and AMB67 [$F(1, 50) = 1.6$, $P=0.21$) and no significant interaction between

the two drugs [F (4, 50) = 0.9, P=0.5] Further, morphine had an EC_{50} of 6.1±0.3 mg/kg, while AMB67 was slightly less potent with an EC_{50} of 8.6±2.3 mg/kg, although this was insignificant.

In the AASA, morphine and AMB67 produced dose-dependent decreases in acid-induced stretching [F (3, 40) = 40.6, P<0.001], but there was no significant difference between the compounds [F (1, 40) = 2.4, P=0.13) nor a significant interaction between the drugs [F (3, 40) P=0.36). Similar to the results in the WWTW, AMB67 was slightly less potent than morphine a simple linear regression analysis revealed interpolated EC_{50} values for morphine (0.43mg/kg) and AMB67 (0.62 mg/kg) (Figure 2.1B). A two-way ANOVA revealed no interaction [F (3, 42) = 0.7, P=0.6] but there were significant effects for dose [F (3, 42) = 40.7, P<0.0001]. (Figure 2.1B).

In the model of mechanical nociception, morphine and AMB67 dose-dependently reversed CFA-induced mechanical hypersensitivity, with EC_{50} values of 4 mg/kg and 3.3 mg/kg, respectively (Figure 2.1D). Repeated measures, two-way ANOVA revealed no significant interaction [F (2, 24) = 1.4, P=0.3]. However, there was a significant effect of dose [F (2, 24) = 24.7, P<0.0001].

Receptor-Mediated Antinociceptive Effects of Morphine and AMB67

We next assessed the opioid receptor type mediating the antinociceptive effects of morphine and AMB67. In the WWTW, the acute morphine dose effect curve was shifted 2.3-fold to the right following naloxone pretreatment. A two-way ANOVA revealed a significant interaction [F (4,50) = 13.4, P<0.0001] (Figure 2.2A).

Similarly, In the WWTW (Figure 2.2B), naloxone pretreatment produced a rightward shift in the AMB67 (Figure 2.2B) dose effects curve. a two-way ANOVA revealed a significant interaction [F (4, 55) = 31.5, P<0.0001].

Previous studies demonstrated that the antinociceptive effects of morphine in the WWTW assay are mediated by the MOR[36]. Therefore, we wanted to evaluate the antinociceptive effects of AMB67 in transgenic mice lacking the MOR (The EC_{50} for AMB67 was shifted 2-fold in MOR heterozygous (Figure 2.2G). In wild-type littermates, AMB67 increased withdrawal latency in a dose dependent manner (EC_{50} = 6.7 mg/kg). In mice expressing about 50% of MORs as compared with wild-type littermates, AMB67 also produce dose-dependent increases in tail withdrawal latencies; however, the AMB67 dose effect curve was shift approximately 3-fold to the right (EC_{50} = 14.8 mg/kg) of that observed in wildtype littermates. In MOR homozygous knockout mice, AMB67 failed to increase withdrawal latencies up to a dose of 56 mg/kg.

Next, we evaluated the receptor-mediated antinociceptive effects of morphine and AMB67 in the AASA. Morphine decreased acid-induced stretching [one-way ANOVA, significant main effect [$F (3, 20) = 34.3$, $P<0.0001$] (Figure 2.2B). Naloxone significantly attenuated the antinociceptive effects morphine ($p<0.0001$); however, NTI did not attenuate morphine-induced antinociception (Figure 2.2B). AMB67 significantly decreased acid-induced stretches [$F (4, 25) = 5.2$, $P=0.004$], and these effects were block by naloxone ($p=0.02$), beta-funaltrexamine ($p=0.04$), and NTI ($p=0.01$) (Figure 2.2E).

Morphine and AMB67 dose-dependently reversed CFA-induced mechanical hypersensitivity (Figure 2.2C and D). Naloxone induced a ~3-fold shift in the morphine DE curve (Figure 2.2C) [interaction, $F (2, 42) = 4.9$, $P=0.01$]. Naloxone produced a ~5-fold shift was observed for the AMB67 DE curve [interaction, $F (2, 39) = 9.4$, $P=0.0005$] (Figure 2.2F).

Antinociceptive Effects of AMB67 and Morphine Following Five Days of Repeated Administration

To test our hypothesis that a dual MOR/DOR agonist would display limited tolerance development, we examined the antinociceptive effects of AMB67 or morphine in all three pain models after 5 days of repeated administration. In the WWTW, repeated administration of morphine, but not saline, produced a 3-fold, rightward, parallel shift in the morphine dose-effect curve (Day 1 EC_{50}=5.07±0.05 mg/kg, Day 6 EC_{50}=14.3±1.02 mg/kg) (Figure 2.3A). A two-way ANOVA revealed a significant interaction between the morphine DE curve day 1 vs day 6 [F (4, 40) = 22.7, P<0.0001] (Figure 2.3A). Repeated administration of AMB67 resulted in no significant interaction comparing day 1 vs day 6 [interaction, [F (4, 40) = 0.3, P=0.8], nor was there a main effect of day [F (1, 10) = 1.1, P=0.3]. Following five days of repeated administration, the EC_{50} for AMB67 was not significantly altered, 7.5±1.5 and 9.9±2.1 for day 1 and day 6, respectively.

In the AASA assay, following five days of twice daily, the EC_{50} for morphine on day 6 shifted ~4-fold as compared with the morphine dose effect curve on day 1 (Figure 2.3C). A two-way ANOVA revealed a significant interaction between the morphine dose effect curves on curve day 1 vs day 6 [F (2, 30) = 6.8, P=0.004] (Figure 2.3E). The EC_{50} for AMB67 was not shifted following 5 days of repeated administration (2.7 mg/kg and 5mg/kg for day 1 and day 6, respectively). A repeated measures, two-way ANOVA revealed a significant main effect of dose [F (2, 32) = 82.2], <0.0001, but no main effect of Day [F (1, 32) = 0.8] or interaction of Dose x Day [F (2, 32) = 1.07, 0.4] (Figure 2.3D)].

In von Frey, mice repeatedly administered 10 mg/kg morphine displayed significant decreases in morphine-induced mechanical hypersensitivity comparing day 1 to day 6 [F (2, 30) = 6.8, P=0.004]. For AMB67, a repeated measures two-way ANOVA revealed 3 and 10mg/kg AMB67 significantly attenuated CFA-induced mechanical hypersensitivity with a significant main

effect of dose, [F (2.3, 27.47) = 34.5, P<0.0001]. There was no effect of day [F (1, 12) = 0.07, P=0.8], nor a significant main effect [F (3, 36) = 1.7, P=0.2] (Figure 2.3F).

Physical Dependence Development Following Chronic Administration of AMB67

We tested the ability of AMB67 to produce physical dependence via naltrexone-precipitated withdrawal. Wild-type mice were treated repeatedly with increasing doses of morphine, or AMB67. Naltrexone, a non-selective opioid antagonist, precipitated withdrawal-like behaviors in mice chronically administered morphine, but not in mice chronically administered AMB67 (Figure 2.4) [F (2, 15) =17.5, P=0.0001]. In morphine treated mice, naltrexone precipitated significantly more withdrawal-like behaviors than mice treated with either saline (P=0.0001) or AMB67 (P=0.0024). The number of naltrexone-precipitated withdrawal-like behaviors in AMB67-treated mice was similar to those treated with saline. However, while AMB67 was not statistically different than saline, a small increase in global withdrawal score was observed.

AMB67 and Morphine-Induced Conditioned Place Preference

The rewarding properties of morphine and AMB67 were assessed using the CPP assay (Figure 2.5). Five days of conditioning with morphine produced a significant increase in time spent on the morphine-paired side compared to conditioning with saline [F (5, 25) = 6.3, P=0.0006]. Post-hoc comparisons revealed 1, 3.2 and 10 mg/kg morphine ($p < 0.01$) significantly increase mean place preference scores. A one-way ANOVA also revealed a significant increase in time spent on AMB67-paired chamber [F (4, 26) = 4.7, P=0.006]. 10 and 18mg/kg AMB67 (both $p<0.05$) induced significant increases in mean place preference score

Interoceptive Effects of AMB67 in Rats Trained To Discriminate Morphine vs Saline

Interoceptive properties of morphine, fentanyl, SNC80, and AMB67 were examined in rats trained to discriminate an injection of 3 mg/kg morphine sulfate from saline. During training sessions, morphine engendered responding (>95%) on the morphine appropriate nose poke (Figure 2.6A, open triangles), while saline primarily responded to the saline appropriate nose poke (Figure 2.6A, closed, inverted triangle). Substitution tests with various doses of the MOR agonists morphine or fentanyl produced dose-dependent, full substitution to the morphine training dose with an EC_{50} of 1.65mg/kg±0.17 and EC_{50} 0.016±0.001mg/kg, respectively (Figure 2.6A). SNC80, a selective DOR agonist, failed to generalize to the morphine discriminative stimulus at doses up to 18 mg/kg. Substitutions of AMB67 elicited dose-dependent substitution, morphine-appropriate responding with an EC_{50} of 9.02mg/kg±2.04.

We also assessed the response rate during the substitution tests. Both fentanyl and morphine produced dose-dependent decreases in response rates, at doses of 0.1mg/kg and 10mg/kg, respectively (Figure 2.6B). AMB67 also produced dose-dependent rate decreasing effects, but unlike fentanyl and morphine, AMB67 did not induce complete suppression of responding up to a dose of 18 mg/kg (Figure 2.6B).

Discussion

First, the affinity, potency, and efficacy of AMB67 was evaluated in vitro as compared with morphine in cells expressed with MORs or DORs. AMB67 had low nanomolar or sub nanomolar affinity for MORs and DORs at both the rat and human receptors. AMB67 had 20 to 40-fold greater affinity for MORs and DORs as compared with morphine, depending on the MOR type evaluated.

AMB67 demonstrated similar efficacy at rat and human MORs with approximately 80% stimulation of G protein activation relative to DAMGO. Interestingly, morphine was ~4-fold more efficacious at human MORs than rat MORs. This discrepancy in the efficacy or morphine could be due to the number of MORs expressed in each cell line, which did not seem to alter the efficacy of AMB67. These data might also suggest that the receptor reserve for AMB67 is greater than that for morphine. At both rat and human MORs, AMB67 was approximately 100-fold more potent than morphine.

At DORs, morphine was more efficacious in human DORs (56% of DPDPE) than at rat DORs (23%); however, morphine is not potent at either DOR with an EC50 of ~0.6-1.2 μM. The efficacy of AMB67 differed greatly between rat and human DORs. In rat DORs, AMB67 did not stimulate G protein activation; however, AMB67 produced ~35% of DPDPE-induced G protein stimulation in human DORs. The partial agonist activity of AMB67 in human DORs was potent with an EC50 of 38 nM. The efficacy differences in AMB67 and morphine across rat and human DORs are likely due to DOR expression levels and/or receptor-G protein coupling efficiency between these two cell lines. These data may suggest that AMB67 and morphine have small receptor reserves in both rat and human DORs.

The in vitro findings indicate AMB67 has greater affinity for both MORs and DORs than morphine. AMB67 is a potent, full agonist at MORs; however, its activity at DORs is less clear. AMB67 showed no significant activity at rat DORs but has potent, partial agonist activity at human DORs. These data might suggest that multiple downstream effectors should be evaluated to establish the activity of novel agonists. Together, these data suggest that AMB67 is a full agonist at MORs and a low efficacy agonist at DORs. Finally, these data highlight important differences

between receptor expression and cell lines that can have a critical impact on interpretations of potency and efficacy of novel compounds.

Earlier studies characterizing the in vivo effects of multifunctional MOR-DOR ligands demonstrated mixed results with regards to tolerance development after repeated administration[23,24]. Therefore, we chose to utilize a wide array of pain models to evaluate the acute effects of AMB67 and tolerance development following chronic administration

Both morphine and AMB67 induced dose-dependent antinociceptive effects in measures of thermal, mechanical, and chemical nociception. AMB67 was equi-potent and equi-effective as morphine in these assays. Similar to that previously observed with morphine[37,38], activation of MORs mediate AMB67-induced antinociception in the WWTW. Interestingly, the antinociceptive effects of AMB67 in the AASA were attenuated by both a MOR and DOR antagonist. Previously, it was shown that selective DOR agonists, such as SNC80, have also been shown to exhibit antinociceptive effects in the AASA[39]. Further, studies show the MOR agonist, loperamide, and the DOR agonist, oxymorphindole, produce antinociceptive synergy in a model of thermal nociception[40]. Further, SNC80 produced modest enhancement of the antiallodynic effects of methadone, morphine, and nalbuphine[41]. Together, these data suggest activity at both receptor types is required for the antinociceptive effects of AMB67 or that agonist activity at DORs potentiate the antinociceptive effects produced by MOR agonists. Future studies should further elucidate the contributions of MOR and DOR for the effects induced by AMB67 and elucidate whether the effects of AMB67 at both receptors are additive or synergistic. Consistent with some of *in vitro* activity profiles, these results suggest that AMB67 acts as a MOR and DOR agonist *in vivo*.

The next studies evaluated the development of tolerance to the antinociceptive effects of morphine or AMB67 in these three pain assays. The same dosing paradigms to assess tolerance development were used for both morphine and AMB67 since they had similar duration of antinociceptive effects and were nearly equip-potent and equi-effective in all pain assays. In both all three assays, repeated administration of morphine produced tolerance development to its antinociceptive effects (Figure 2.3A, 2.3C, and 2.3E). Interestingly, AMB67 failed to induce tolerance development to its antinociceptive effects in in each of the three pain models (Figure 2.3B, 2.3D and 2.3F). These data do not rule out the possibility that AMB67 could potentially induce tolerance to its antinociceptive effects if a more rigorous paradigm was utilized. Ultimately, we did not evaluate if the attenuated tolerance development following chronic AMB67 was due to its activity at the DOR, however one of the assumptions based on previous literature and the *in vitro* profile of AMB67, that the DOR activity may play a role. Other possibilities include differences in receptor reserve and G-protein bias. Studies have shown the intrinsic efficacy of opioid agonists is inversely related to the degree of tolerance development[42], and an increased receptor reserve for AMB67 compared to morphine may explain the lack of tolerance following chronic AMB67 administration. Further, studies have demonstrated that β-arrestin signaling may contribute to opioid tolerance[43-45], suggesting ligands biased toward G-protein activation may provide benefit over ligands that recruit β-arrestin. *In vitro*, morphine displays significant bias toward β-arrestin recruitment and produces significant tolerance development[46,47]. Conversely, AMB67 displays greater bias toward G-protein activation,[46] suggesting the lack of β-arrestin recruitment following acute administration of AMB67 may explain the lack of tolerance development. It would be interesting to assess chronic AMB67 administration in mice overexpressing β-arrestin to see if tolerance development occurs[48].

Physical dependence is thought to play a crucial role in opioid use disorder and occurs when opioids are administered chronically[7,23,49]. Physical dependence was measured via naltrexone-precipitated withdrawal-like behaviors including jumping behavior, teeth chattering, piloerection, wet dog shakes, paw tremors and diarrhea/watery stool, agonists[50]. After 5 days of increasing dose administration, repeated administration of AMB67 failed to induce physical dependence development compared to repeated morphine or repeated saline administration. One concern is that the dose of naltrexone used was not enough to displace AMB67 from MORs or that the affinity of naltrexone was not enough to displace AMB67 from MORs. However, further characterizing these effect before and after chronic AMB67 would aid in elucidating the differences between morphine and AMB67.

Rewarding properties are a major limitation is the development of MOR agonists for the treatment of pain. It has been well established that drugs interacting with MOR display strong rewarding properties[51,52] and have discriminative stimulus properties. AMB67 produced conditioned place preference and generalized to morphine-like discriminative stimulus effects; however, AMB67 was ~8-fold and 3-fold less potent than morphine, respectively. These data suggest that AMB67 produce less reward-related effects than morphine at antinociceptive doses. It is unclear if the activity of AMB67 at DORs is responsible for the differences. but the literature suggests it to be unlikely, as the DOR agonist TAN67 dose dependently enhanced morphine-induced place preference[53]. MMP2200, another previously reported dual MOR/DOR agonist, failed to induce conditioned place preference. Conversely, morphine-induced conditioned place preference is attenuated by DOR antagonists and in DOR knockout mice[54,55]. However, Together these data suggest that alterations in endogenous DOR signaling may be implicated in the rewarding properties of MOR agonists. Further studies are required to fully understand why dual

MOR/DOR agonists display attenuated CPP compared to morphine, and if the DOR, or other possibilities such as drug pharmacokinetics are responsible for the observed results.

In summary, AMB67 produced robust, acute antinociceptive effects in multiple assays, produced less tolerance and physical dependence following chronic administration, and has less potent reward-related effects than the MOR agonist morphine. These results provide evidence that mixed-efficacy dual MOR-DOR agonists may offer an improvement over current opioid analgesics, and further preclinical and clinical development of AMB67 or similar compounds for the treatment of pain is warranted. At this time, it is unclear whether the profile of activity is due to its concurrent activity at DORs or whether it is due to other pharmacodynamic properties of this compound, e.g., greater receptor reserve or differential downstream signaling.

Rat Receptor						
Compound	MOR Ki (nM)	MOR EC_{50} (nM)	MOR % Stim	DOR Ki (nM)	DOR EC_{50} (nM)	DOR % Stim
Morphine	6.5 (1.9)	145 (15)	21.8 (3.8)	94.6	1200	22.68 (12.1)
AMB67	0.2 (0.1)	1.36 (0.5)	81 (4.3)	4.8 (1.1)	N/A	DNS

Human Receptor						
Compound	MOR Ki (nM)	MOR EC_{50} (nM)	MOR % Stim	DOR Ki (nM)	DOR EC_{50} (nM)	DOR % Stim
Morphine	4.2 (1.3)	147 (48)	98 (3.8)	104 (9.2)	593	56.3
AMB67	0.1 (0.01)	0.9 (0.2)	83 (2.1)	2.4 (0.4)	38.4 (0.2)	34.5 (2.6)

Table 2.1 In Vitro Analysis of AMB67 and Morphine

Table 2.1: Binding affinities (Ki) were obtained by competitive displacement of radiolabeled [3H]diprenorphine in the absence of sodium chloride. Efficacy data were obtained using [^{35}S]GTPγS binding assay. Efficacy is presented as percent maximal stimulation relative to standard agonists DAMGO (MOR) and DPDPE (DOR) at 10μM. All values are expressed as the mean ± SEM of three separate assays performed in duplicate. dns = does not stimulate.

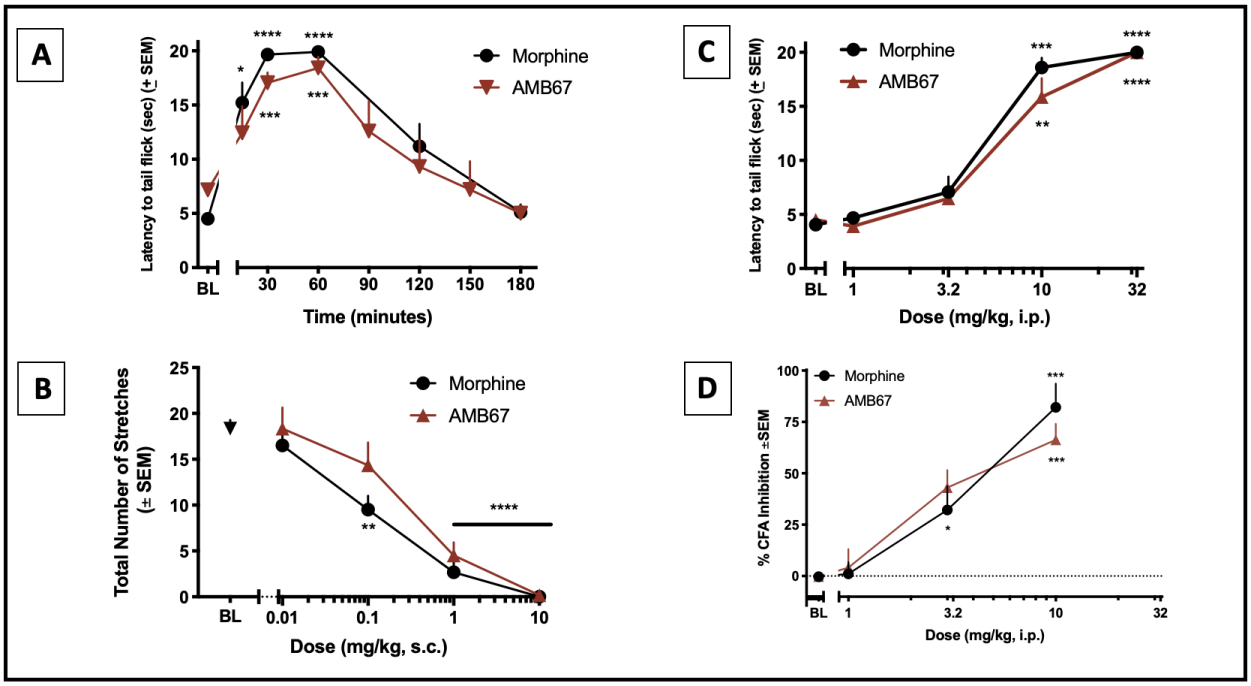

Figure 2.1 Acute Antinociceptive Effects of Morphine and AMB67

Figure 2.1: Antinociceptive effects and duration of action of AMB67 and morphine. C57BL/6 mice were injected IP with 10mg/kg bolus (A), a cumulative dosing fashion (C, D) or single dose of 0.01-10 mg/kg (B). Tail-flick latencies (A, C), total stretches (B) and mechanical thresholds (D) were evaluated for antinociception. N=6 per group. (B) Total stretches were recorded after single doses of drug was administered. (D) Mechanical thresholds were assessed after CFA injection.

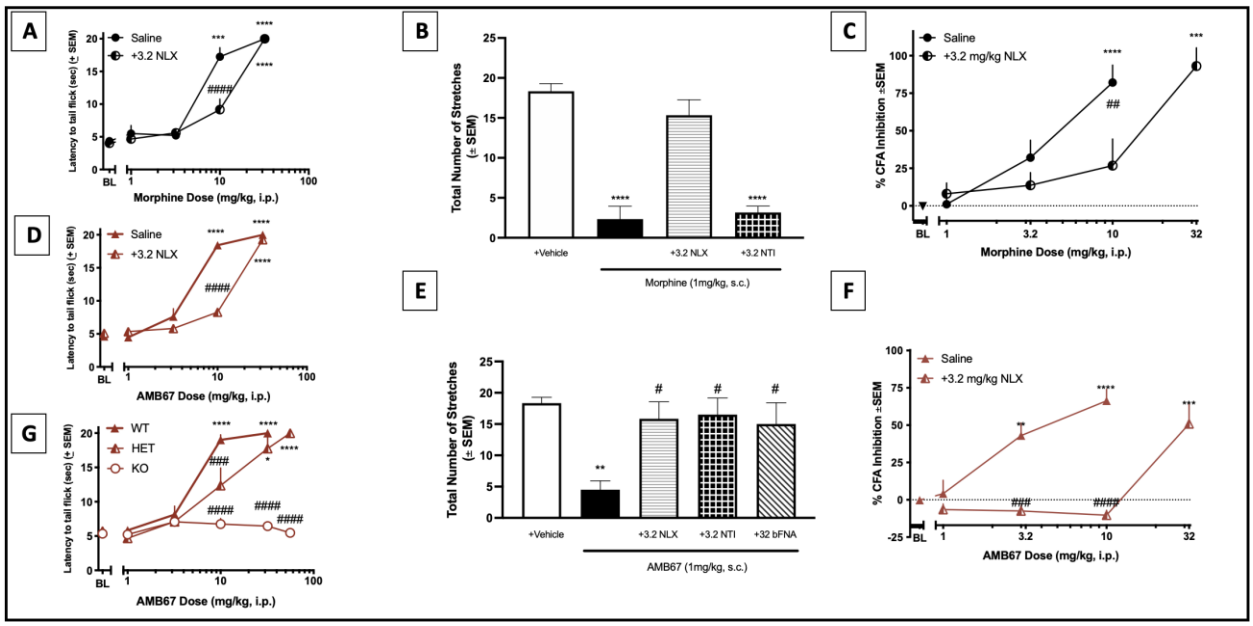

Figure 2.2 Receptor-Mediated Antinociceptive Effects of Morphine and AMB67

Figure 2.2. Antinociceptive effects were evaluated via tail-flick latencies (A,D,G), acid-induced stretches (B, E) and mechanical thresholds (C,F). Naloxone pretreatment to the antinociceptive effects of morphine in thermal (A), chemical (C), and mechanical (C) antinociception. Naloxone pretreatment to the antinociceptive effects of AAH8 in thermal (A), chemical (C), and mechanical (C) antinociception. AMB67-induced thermal antinociception in MOR WT, HET, and KO mice (D). N=5 or 6 per group. Naltrindole and b-FNA pretreatments to a 1mg/kg bolus dose of AMB67 (E). **** = P<0.0001 versus vehicle control. ### = P<0.001 versus AMB67.

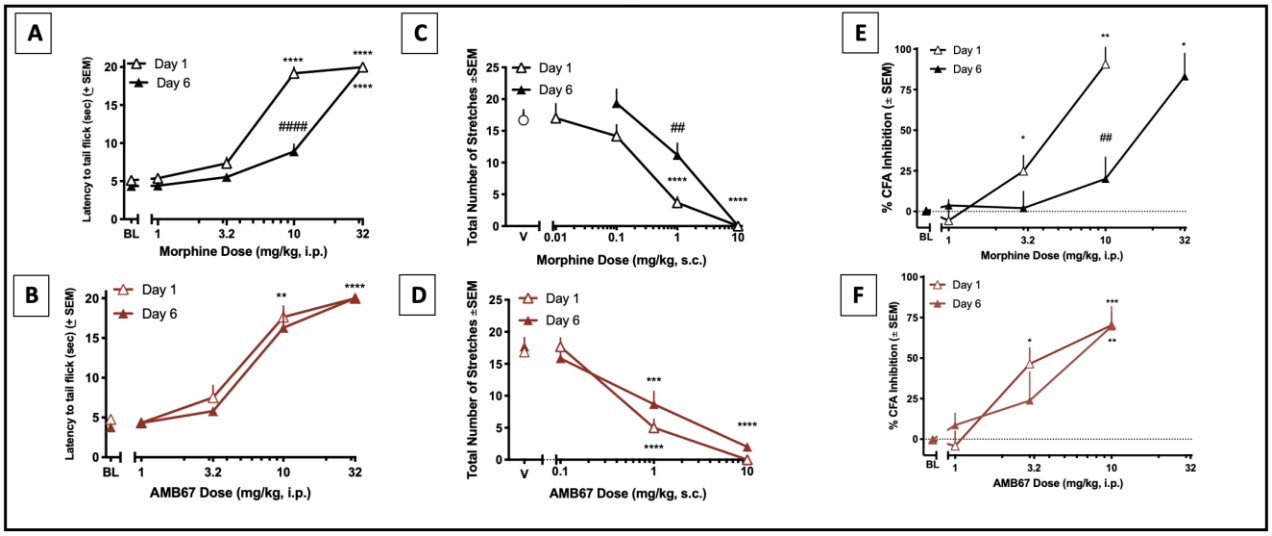

Figure 2.3 Antinociceptive Effects of AMB67 and Morphine Following Five Days of Repeated Administration

Figure 2.3: Morphine or AMB67-induced tolerance following repeated systemic administration. Cumulative dose effect curves of morphine, Day 1 vs Day 6, following 5 days of 3x daily administration of 10 mg/kg morphine (A, E). Cumulative dose effect curves of morphine, Day 1 vs Day 6, following 5 days of 2x daily administration of increasing doses (1-5 mg/kg) morphine (C). Cumulative dose effect curves of AMB67, Day 1 vs Day 6, following 5 days of 3x daily administration of 10 mg/kg morphine (B, F). Cumulative dose effect curves of AMB67, Day 1 vs Day 6, following 5 days of 2x daily administration of increasing doses (1-5 mg/kg) (D). N=6 per group. *** = P<0.001, ** = P<0.01, * = P<0.05 versus saline control. ### = P<0.001, ## = P<0.01 versus same dose of Day 1 results.

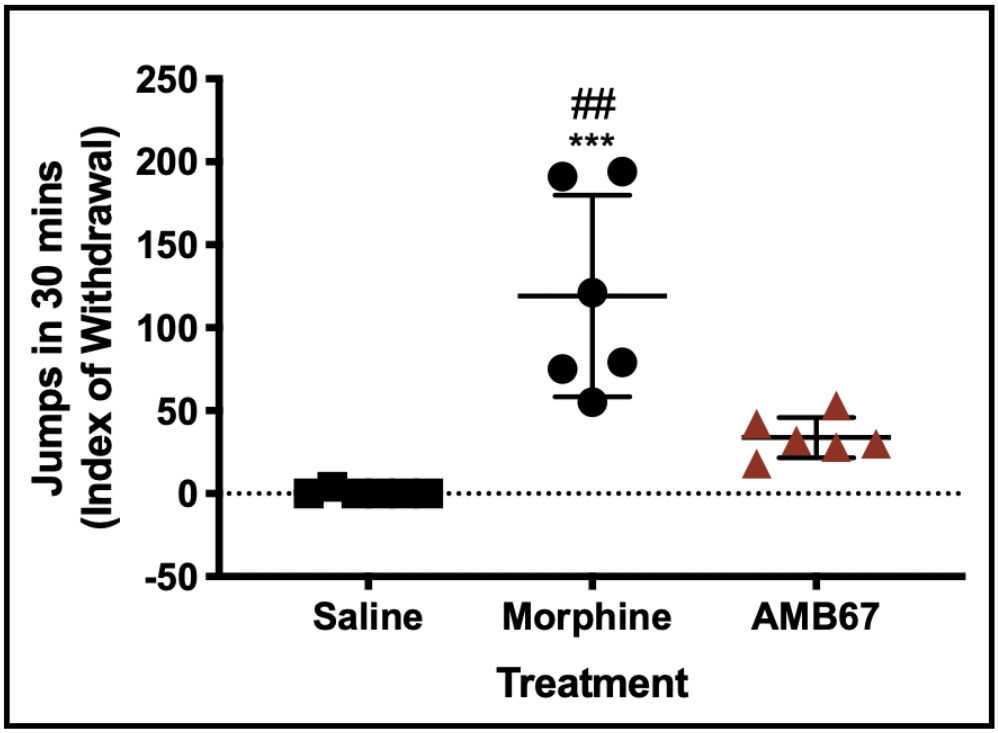

Figure 2.4 Physical Dependence Development Following Chronic Administration of AMB67

Figure 2.4: Naltrexone precipitated withdrawal on repeated administration of AMB67 or morphine. Drug or vehicle (IP) were administered to male C57BL/6 mice for 5 days in an increasing dosing paradigm. On day 6, mice were administered 10mg/kg naltrexone (IP) for withdrawal precipitation and jumping behavior, teeth chattering, piloerection, wet dog shakes, paw tremors and soft stool were recorded. N=6 per group. **** = P<0.0001 versus saline control. ### = P<0.001 versus AMB67.

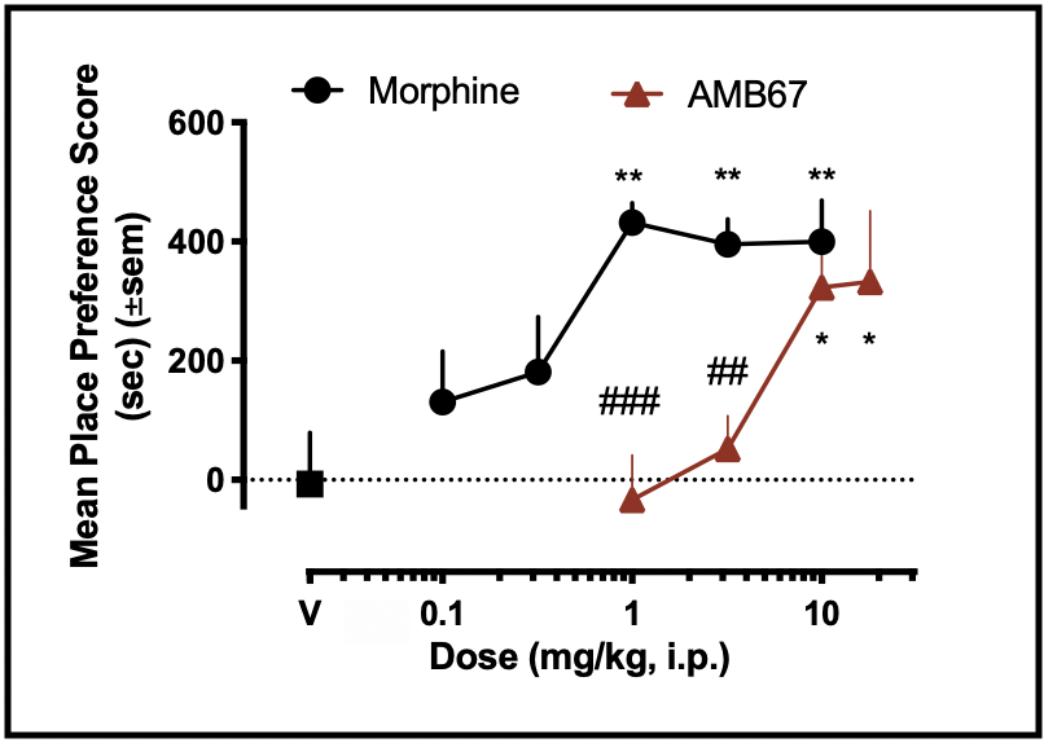

Figure 2.5 AMB67 and Morphine-Induced Conditioned Place Preference

Figure 2.5: AMB67 exhibits decreased potency in rewarding properties in the conditioned place preference assay. Drug or vehicle (IP) were administered to male C57BL/6 mice and the time difference of total time spent on drug paired chamber on test day was compared to total time spent on drug paired chamber during bias test. Morphine and AMB67 produce dose dependent increases in mean place preference with ED50 values of ~0.65mg/kg and ~5mg/kg, respectively. N=6-8 per condition

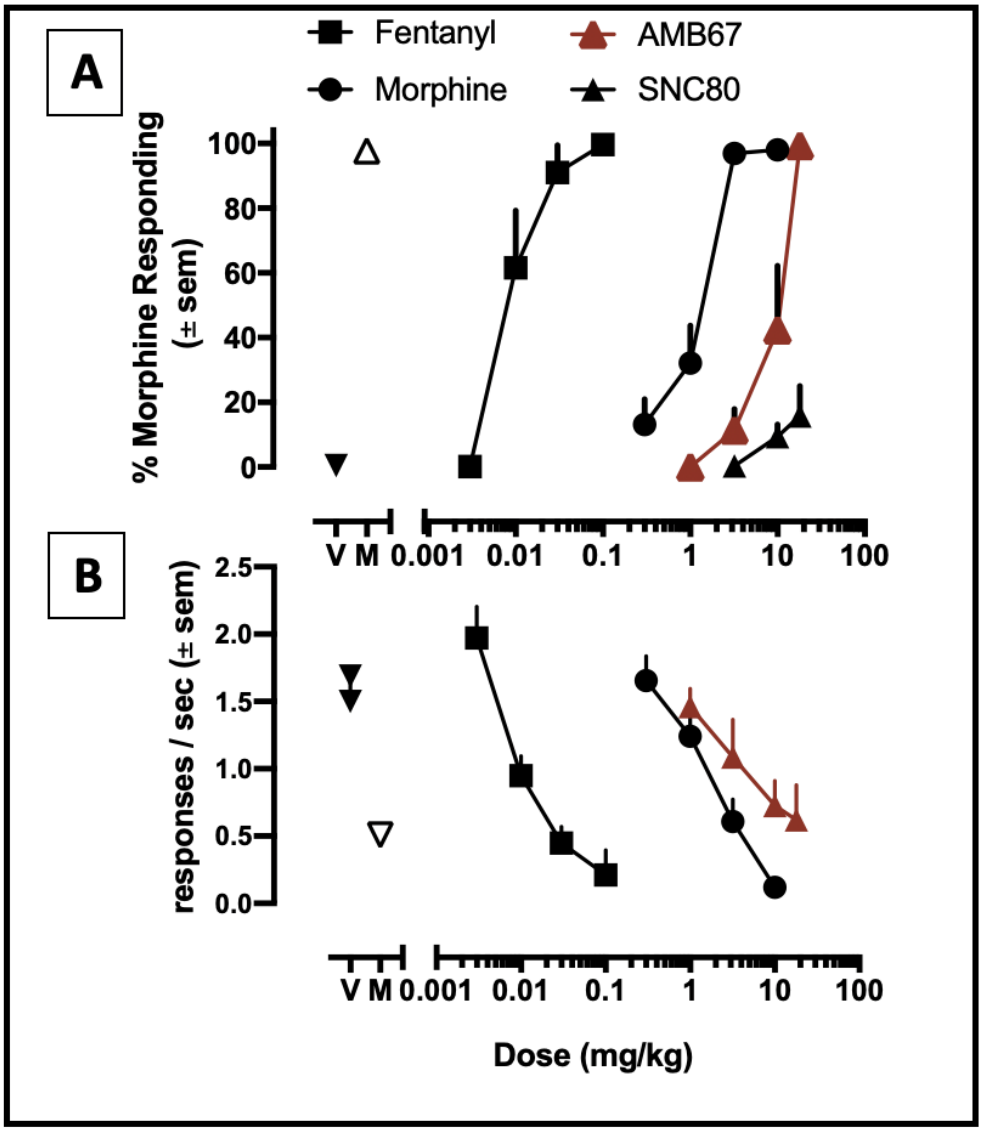

Figure 2.6 Interoceptive Effects of AMB67 in Rats Trained to Discriminate Morphine vs Saline

Figure 2.6: Male Sprague Dawley rats were trained to discriminate 3mg/kg morphine from saline SC. (A) Morphine, fentanyl and AMB67 substitutions (B) Rates of responding for morphine, fentanyl and AMB67. N=6-8 per condition – within subject.

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Chapter III: Characterizing the Acute and Chronic Effects of the Multifunctional Opioid Ligand, AAH8

Abstract

Opioids, such as morphine, have been used to treat pain for centuries and exert their effects through the MOR. While the analgesic effects of such opioids are effective, these traditional MOR agonists produce on-target adverse effects, such as constipation and tolerance development. Previous reports demonstrated that the mixed-efficacy MOR agonist/DOR antagonist, AAH8, produced antinociceptive effects without tolerance following repeated administration. We conducted a further evaluation of the properties of AAH8 after acute and repeated administration. AAH8 produced decreased bowel movements to a similar extent as selective MOR agonists and was more potent than morphine. AAH8 produced antinociceptive effects in a model of visceral pain but had little to no antinociceptive effects in the warm water tail withdrawal assay and the CFA-induced mechanical hypersensitivity model. However, intracerebroventricular administration of AAH8 produced dose-dependent increases in tail-flick latency with an EC_{50} of 6.6 mg/kg. Diluted acid-induced writhing or stretching (AASA) was evaluated following repeated administration of AAH8 or morphine. Following five days of repeated administration with increasing doses, the ED_{50} for the morphine dose effect curve was shifted ~3-fold, whereas the ED_{50} for AAH8 was unchanged. However, repeated administration of 10 mg/kg AAH8, three times per day for five days produced significant tolerance to the antinociceptive effects of 10 mg/kg AAH8. Together, this study suggests that AAH8 produces antinociception and constipation effects through activation of peripheral MORs, and tolerance development may be delayed following repeated administration, but it is not completely prevented.

Introduction

Opioids are a mainstay in pain management due to their robust analgesic effects[1-3]. Morphine is considered the prototypical opioid for pain management and provides antinociceptive effects through activation of mu-opioid receptors (MORs) within spinal and supraspinal regions[4-8]. MORs are found in many different areas along the pain-processing pathway, both in the peripheral nervous system (PNS) and in the central nervous system (CNS)[9-11]. Unfortunately, nearly all MOR agonists, including many clinically used opioids such as fentanyl, oxycodone, and methadone, produce unwanted, on-target adverse effects. Repeated administration of MOR agonists produces tolerance and physical dependence, which can contribute to or exacerbate the development of opioid use disorder (OUD)[12,13]. Previous studies demonstrated that morphine-induced tolerance and physical dependence development may be impacted by activity at delta-opioid receptors (DORs) because administration of naltrindole, a DOR antagonist, attenuated the development of tolerance to and physical dependence on morphine[14,15]. The co-administration of MOR and DOR agonists, and even DOR antagonists, retain MOR-mediated antinociception, yet display reduced adverse effects[15-19]. For example, mice lacking the DOR display reduced morphine-induced tolerance and reward, suggesting important interactions between the two opioid receptor types[20]. Therefore, one goal of recent research efforts is to target both MORs and DORs for treatment of pain. A single chemical entity with activity at both MORs and DORs[21-23] may provide greater benefit over co-administration of two different drugs, such as lack of drug-drug interactions and improved compliance.

We have previously published a series of peptidomimetic compounds exhibiting the desired MOR agonist/DOR antagonist profile[24,25]. Within this series, a small set of compounds were extensively characterized *in vivo*[26], one of which is AAH8. AAH8 was shown to be effective

in animal models of thermal nociception and failed to produce tolerance to its antinociceptive effects following repeated administration. AAH8 is a highly promising ligand and we wanted to further characterize the effects of AAH8 after acute and repeated administration.

Methods

Animals and Drug Solutions

All animal care and experimental procedures complied with the US National Research Council's Guide for the Care and Use of Laboratory Animals[27]. Mice were group- housed with a maximum of five animals per cage in clear polypropylene cages with corn cob bedding and nestlets as enrichment. Mice had free access to food and water at all times. Animals were housed in pathogen- free rooms maintained between 68 and 79°F and humidity between 30 and 70% humidity with a 12 h light/dark cycle with lights on at 07:00 h. We conducted experiments in the housing room during the light cycle. All studies utilize wildtype male or female C57BL/6N mice from Envigo laboratories (formerly Harlan, Indianapolis, IN), or male and female transgenic mice lacking MORs (MOP-KO,B6.129S2- *Oprm1*^{tm1Kff}/J stock number 007559; Jackson Laboratory) or DORs (DOP-KO,B6.129S2- *Oprd1*^{tm1Kff}/J stock number 007557; Jackson Laboratory, Sacramento, CA, USA). Mice used for behavioral experiments weighed between 20-30 g at 7-15 weeks old. All drug solutions were injected at a volume of 10 ml/kg. AAH8 was dissolved in 1:9 DMSO/saline solution, or 1:1:8 EtOH/DMSO/Water solution. Morphine sulphate was dissolved in saline. All drugs were given by either intraperitoneal (IP) or subcutaneous (SC) injection, and diluted acetic acid was given IP. For intracerebroventricular (ICV) injections, morphine and AAH8 were dissolved in DMSO.

Acetic Acid Stretch Assay (AASA)

Antinociceptive effects were evaluated in the mouse acetic acid stretch assay. Diluted acetic acid (0.6%) was used as the noxious stimulus to induce a stretching behavior, characterized by constriction of the abdomen, followed by extension of the hind limbs. Test drug or vehicle was administered SC 30 min prior to the injection of acetic acid. Immediately after administration of 0.6% acetic acid, IP, mice were placed individually in clear plastic observation cages (10 x 6 x 8 in) with bedding for 5 min prior to starting a 20 min observation period. No more than three mice were observed simultaneously. For antagonism studies, a dose of 3.2 or 10 mg/kg naloxone, 10mg/kg naloxone-methiodide, or 3.2 mg/kg naltrindole was administered IP 15 min before administration of test drug. βFNA (IP) was administered as a 48-hour pretreatment prior to test compounds.

Antinociceptive effects were assessed on day 1 and day 6 following repeated administration of morphine, AAH8 or vehicle. Two different within-subject dosing paradigms were utilized to evaluate tolerance development. In the first paradigm, increasing doses of morphine or AAH8 (1-5mg/kg) or vehicle were administered twice per day at ~7am and 7pm for five consecutive days. In the second paradigm, mice received either 1 mg/kg morphine or 10mg/kg AAH8 three times per day (at ~7am, 1pm and 7pm) for five consecutive days. In both paradigms, no drug administration occurred in the evening prior to test day, such that mice were 12 hours drug-free prior to evaluating acid-induced stretching on day 6.

Von Frey

For all von Frey experiments, mice were placed in a plastic box (4in x 4in x 4inch) with a mesh floor that was elevated sixteen-inches above the table. All mice and were habituated to the

plastic box for two hours per day for three days and baseline (pre-CFA). mechanical withdrawal thresholds were assessed. To assess withdrawal threshold, each von Frey monofilament was applied perpendicularly to the ventral medial portion of the hind paw for approximately 3 sec. A withdrawal response was characterized by rapid removal of the paw from the filament within the 3 sec time limit. Directly following the measurement of pre-CFA mechanical thresholds on day 3, an average of pre-CFA withdrawal thresholds was calculated. Animals with an average 50% withdrawal threshold < 0.8g was excluded from the study (~5%). The Up-Down method was used to determine all withdrawal thresholds[28]. Complete Freund's Adjuvant (CFA, Catalog #77140, Thermo Scientific) was administered unilaterally in a volume of 15ul in a randomized manner. 24 hrs post CFA injections (test day), withdrawal thresholds were evaluated in the absence or presence of drug.

On test day, all mice were administered saline or vehicle, and withdrawal thresholds were evaluated 30 min later (post-CFA baseline withdrawal threshold). Increasing doses of morphine or AMB67 were administered every 30 minutes in a cumulative dosing fashion, and withdrawal thresholds were recorded.

For tolerance studies, on day 1 (24h post CFA administration), all mice were administered saline or vehicle, and withdrawal thresholds were evaluated 30 min later (post-CFA baseline withdrawal threshold). Increasing doses of morphine or AMB67 were administered every 30 minutes in a cumulative dosing fashion, and withdrawal thresholds were recorded to assess antinociceptive effects. Mice were then administered morphine (10mg/kg), AMB67 (10mg/kg), or vehicle 3x per day for five days (Days 1-5) (7am, 1pm, 7pm). No injections were administered the evening prior to test day to ensure no circulating drug was present during mechanical threshold testing. On day 6, all mice were administered saline or vehicle, and withdrawal thresholds were

evaluated 30 min. Increasing doses of morphine or AMB67 were administered every 30 minutes in a cumulative dosing fashion, and withdrawal thresholds were recorded.

WWTW

Mice were placed in a cylindrical plastic restrainer and 2-3 cm of the tail tip was immersed into a 50° C water bath. Withdrawal latencies were recorded with a maximum cut off time of 20 seconds to prevent tissue damage. Dose-response curves were established via cumulative dosing of either morphine or AAH8 (1-32mg/kg) IP, and tail-flick latencies were recorded approximately 30 min after each cumulative dose was administered.

Intracerebroventricular (ICV injections were performed using the modified method of (Haley and McCormick, 1957)[29] as described by (Laursen and Belknap, 1986)[30]. Morphine (0.32-10nmol), AAH8 (1-10nmol), or DMSO was administered ICV in a volume of 3 µL and withdrawal latencies were recorded 30 min after drug administration. To perform ICV injections, mice were anesthetized in a cylindrical drop jar consisting of 0.2 ml isoflurane. Following adequate anesthetization, the head of the mouse was oriented in a manner to estimate the location of bregma based on ear orientation and distance between eyes and ears, about 1-3 mm rostral from the anterior base of the ears (Laursen & Belknap, 1986). After locating bregma, the Hamilton micro syringe (Model #1701, Reno, NV) fitted with a 26G needle was placed at a right angle to the skull and used to create a puncture point approximately 2 mm lateral to bregma with a depth of 4 mm (to the tip of the bevel) in order to deliver compounds directly into the lateral ventricle. Following assessment of antinociception in the WWTW, Fast Green FCF (F7252-5G, Lot # MKBX8539V) was delivered through the same puncture points under heavy anesthesia. Two to three minutes later, mice were euthanized by decapitation and the brain was removed. Coronal slices were made through the lateral, third, and fourth ventricles. The presence of Fast Green FCF within the lateral

and third ventricles confirmed the appropriate site of injection. Alternatively, if Fast Green was not observed in the ventricles, the ICV injection was considered a missed injection site, and these tail-flick latency values were excluded from the data (2 mice in total were excluded from the data set for missed ICV injections).

Constipation

Tinted chow was made in-house, consisting of 25 g of regular chow (PicoLab Laboratory Rodent Diet 5L0D, 0067138), mixed with 40 ml of tap water and 0.25 ml of blue food dye. After food pellets were softened, the tinted chow was mixed for even distribution of food dye. One week before each experiment, group-housed female C57BL/6 mice were habituated to the tinted chow for 24 h with continuous access to normal chow and water. Five days later, and at least 12h before a test, mice were placed in individual cages without bedding or food, with free access to water. On the morning of the experiment, mice were given 1h access to tinted food. Tinted food was then removed, the cages were wiped down, and the mice were given an injection of either drug or vehicle IP. Following access to tinted food, approximately 3 g of normal chow was made available for the remainder of the experiment. The weight of both the normal chow and the tinted chow was recorded both before and after the experiment. The number of tinted fecal boli were recorded every hr for 6 hrs.

Data Analysis

All data analyses were performed using GraphPad Prism version 8.4.3. Level of significance (α) was set at 0.05 for all statistical measures. A two-way ANOVA was conducted for all graphs containing dose-effect curves unless otherwise stated. Tukey's post hoc analyses

were used to correct for multiple comparisons and were only performed when F values achieved $p < 0.05$. Approximate ED_{50} values were calculated using GraphPad Prism version 8.4.3. The 50% maximum effect was interpolated from the straight-line analysis of 2-3 points along the linear portion of the curve only. When possible (e.g., within-subject experimental designs), individual ED_{50} values were averaged across mice and reported as the group mean ED_{50} ± standard error of the mean (SEM). However, in some studies (e.g., between-subject experimental designs), 50% maximum effects were calculated for the group averaged data, thus a single ED_{50} (without SEM) was determined for the DE curve. Fold shifts in dose-effect curves were calculated by dividing ED_{50} value of interest by ED50 value calculated in wild-type mice or in the absence of an antagonist.

Results

In Vivo Results:

Acute Antinociceptive Effects of AAH8 and Morphine in the Acetic Acid Stretch Assay

The acute antinociceptive effects of AAH8 were assessed in male C57BL/6N male mice in the AASA. In the absence of drug, 0.6% acetic acid-induced an average of ~18 total stretches within the 20 min observation period. Morphine produced dose dependent decreases in stretches, similar to previous reports with an EC_{50} of ~0.4mg/kg[31-33]. AAH8 produced dose-dependent decreases in acid-induced stretches with an EC_{50} of ~0.5mg/kg (Figure 3.1A). A Two-Way ANOVA revealed a significant effect of drug [$F(1, 30) = 11$, $P=0.002$] and dose [$F(2, 30) = 44.1$, $P<0.0001$]. As expected, there was no significant interaction between the two drugs [$F(2, 30) = 0.9$, $P=0.4$]. A bolus dose of 1 mg/kg dose of morphine produced significant decreases in stretches for up to three hours, whereas 10mg/kg morphine was effective for six hours (Figure 3.1B). AAH8

had a shorter duration of action as compared with morphine, such that a bolus of 1 or 10mg/kg AAH8 produced significant decreases in stretches up to 2 and 4hr respectively (Figure 3.1C).

Opioid Receptor-Mediated Antinociceptive Effects of AAH8 and Morphine

AAH8 (10 mg/kg) and morphine (1 mg/kg) significantly decreased acid-induced stretches in C56BL/6N mice (Figure 1A and B, respectively). Naloxone, a non-selective opioid receptor antagonist attenuated the antinociceptive effects of morphine [$F (4, 26) = 28.8$ $P<0.0001$] (Figure 3.2B). Similarly, naloxone pretreatments attenuated AAH8-induced antinociception [$F (3, 24) = 21.4$ $P<0.0001$] (Figure 3.2A). In transgenic mice lacking the MOR, AAH8-induced antinociception was abolished [$F (2, 16) = 28.3$, $P<0.0001$] (Figure 3.2B). Naltrindole, a DOR-selective antagonist, did not alter the effects of morphine (Figure 3.2C); however, naltrindole partially attenuated the effects of AAH8, but this effect was not statistically significant from 10 mg/kg AAH8 alone ($P= 0.2$) (Figure 3.2A).

Acute Mechanical, Thermal, and Chemical Antinociceptive Effects of AAH8 and Morphine

In Figure 3, the effects of morphine and AAH8 were evaluated in the von Frey and WWTW assay following peripheral administration and in the WWTW assay following central (ICV) administration. In the von Frey assay, a one-way ANOVA, repeated measures analysis showed a significant effect for morphine treatment [$F (1.6, 11.1) = 20.5$, $P = 0.0003$]. Morphine produced a dose-dependent reversal of CFA-induced mechanical hypersensitivity at 3.2 and 10 mg/kg (Figure 3.3A). However, larger doses of morphine (>10 mg/kg) could not be evaluated because of locomotor stimulating effects. AAH8 failed to significantly attenuate CFA-induced mechanical hypersensitivity up to 32 mg/kg [$F (3, 21) = 1.1$, $P=0.4$] (Figure 3.3A).

The effects of AAH8 and morphine in the WWTW were assessed (Figure 3.3B). A repeated measures, two-way ANOVA analysis revealed a significant interaction [F (4, 40) = 42.5, P=0.0001], main effect of dose [F (2.6, 25.7) = 104.3, P<0.0001], as well as main effect of drug [F (1, 10) = 36.8, P<0.0001]. In the WWTW, AAH8 produced minimal antinociceptive effects, with 32 mg/kg inducing a small, but significant, increase in tail flick latency (Figure 3.3B). Morphine produced a dose-dependent increase in withdrawal latency with an EC_{50} of 6 mg/kg +/- 0.2 mg/kg (Figure 3.3B).

Next, we assessed the antinociceptive effects of morphine and AAH8 when administered ICV (Figure 3.3C). Both morphine and AAH8 produced dose dependent increases in latency to withdrawal with EC_{50} of (1.6 +/- 0.3 nmol) and (6.6 +/- 0.001 nmol), respectively, demonstrating that AAH8 was 4-fold less potent than morphine. An ordinary Two-Way ANOVA reported a significant interaction [F (3, 40) = 7.9, P<0.0003], main effect of dose (F (3, 40) = 30.4, P<0.0001), and main effect of drug [F (1, 40) = 29.2].

Antinociceptive Effects of AAH8 and Morphine Following Five Days of Repeated Administration

We next sought to assess tolerance development after repeated drug administration in the AASA. Acute antinociceptive effects of AAH8 or morphine were assessed on day 1. Since acetic acid was the noxious stimuli, only a single dose was evaluated in each mouse. Following assessment of acute antinociceptive effects on day 1, mice received increasing doses of morphine or AAH8 (1-5mg/kg) every 12 h over 5 days, and assessment of antinociceptive effects was again conducted on day 6 following chronic treatment (Figure 3.4A and Figure 3.4C). A three-way ANOVA displayed a significant dose x day x treatment [F (2, 30) = 5.85, P=0.007] comparing the

acute antinociceptive effects of morphine following either chronic saline or chronic morphine treatment. There was also a significant main effect of dose [F (2, 30) = 103.9, P<0.0001], treatment [F (1, 30) = 12.8, P=0.0012], and day [F (1, 30) = 17.8, P=0.0002]. Conversely, repeated AAH8 administration failed to alter the EC_{50}, similar to previous reports (Figure 3.4C)[26]. A three-way ANOVA revealed no significant interaction between day x dose x treating [F (2, 30) = 0.12, P=0.89] (figure a significant main effect of dose (F (2, 31) = 35.4, P<0.0001), but no significant effect of chronic treatment comparing chronic AAH8 and chronic saline [F (2, 30) = 0.32, P<0.72] (Figure 3.4C).

Further, we wanted to assess tolerance utilizing a more-robust dosing paradigm, such that (1 mg/kg of morphine or 10 mg/kg AAH8 3 times daily at ~7am, 1pm and 7pm) was administered for 5 consecutive days. In the AASA, acute antinociceptive effects of 1 mg/kg morphine or 10 mg/kg AAH8 were assessed on day 1, and again on day 6 following chronic treatment (Figure 3.4B and 3.4D). As expected, there was a significant difference in number of stretches measured after an injection of 1 mg/kg morphine on day 1 vs day 6 (Figure 4C) [F (2, 15) = 59.1, P<0.0001]. A One-Way ANOVA also revealed a significant reduction in the antinociceptive effects of 10mg/kg AAH8 on day 6 as compared with day 1 (Figure 3.4D) [F (2, 15) = 36.3, P<0.0001].

AAH8-Induced Constipation

It is well established that MOR agonists produce constipation. Morphine given by IP or SC routes of administration produced a dose-dependent decrease in the number of fecal boli produced over 6 h [F (4, 26) = 32.8, P<0.0001] and [F (3, 20) = 30, P<0.0001], respectively. Similarly, IP or SC AAH8 administration produced a dose-dependent decrease in fecal boli and an ordinary two-way ANOVA revealed a significant interaction between AAH8 SC and AAH8 IP [F (3, 19) =

3.8, 0.03], as well as a significant main effect for dose [F (3, 24) = 53.7, <0.0001] and route of administration [F (1, 19) = 17.8, 0.0005].

Discussion

This study evaluated the effects of AAH8 as compared with morphine following acute and repeated administration. AAH8 displayed dose-dependent antinociceptive effects with similar potency to morphine in the acetic acid stretch assay. The antinociceptive effects of AAH8 were antagonized by naloxone, suggesting opioid-mediated effects. Antinociception was also abolished in mice lacking MOR, suggesting that these effects were mediated by MORs. However, naltrindole may also attenuate some of the antinociceptive effects of AAH8; although this effect was not a significant change from 10mg/kg AAH8 alone. AAH8 was previously reported to be both a MOR agonist and DOR antagonist[21,26,34], making these results intriguing. One explanation for these results is that AAH8 may be a high affinity, low efficacy partial agonist at the DOR. In the tail suspension test, 3.2 mg/kg AAH8 attenuated SNC80-induced decreases in immobility, suggesting DOR antagonist activity[26]. However, if AAH8 is a partial agonist at the DOR, then it could also attenuate SNC80 induced decreases in immobility if a partial agonist alone does not decrease immobility in the mouse forced swim test. It is possible that AAH8 displays low efficacy DOR agonist effects *in vivo*, and that in the AASA, both MORs and DORs are involved in the antinociceptive effects of AAH8.

We also assessed tolerance development to the antinociceptive effects of AAH8 or morphine in the AASA. Using an increasing dosing paradigm (1-5mg/kg), repeated administration of morphine shifted the dose-response curve 5-fold to the right (Figure 3.4A). However, repeated administration of AAH8 did not induce tolerance, similar to previous reports (Figure 3.4C)[26]. AAH8 may have failed to induce tolerance using this paradigm perhaps due to a slightly shorter

duration of action than morphine (Figure 3.1C). Therefore, we chose to use a more frequent dosing paradigm with larger doses of AAH8. Mice administered 1 mg/kg morphine, three times per day for five days produced tolerance to the antinociceptive effects of 1 mg/kg morphine (Figure 3.4B). Mice administered 10mg/kg AAH8, three times per day for five days also produced tolerance to the antinociceptive effects of 10mg/kg AAH8 (Figure 3.4D). Studies show that receptor occupancy is an essential factor for tolerance development[35,36,37]. Therefore, we conclude that it may be the shorter duration of action for AAH8 that contributes to the limited tolerance development and that when dose and injection frequency are increased, tolerance development occurred. These results suggest that tolerance development to the antinociceptive effects of AAH8 occurs when repeated dosing regiments are closely matched for dose and duration of action.

It is well established that MOR agonists can attenuate inflammatory pain in preclinical models[38,39], but do so with very little efficacy in the human population[40]. Morphine was effective in both thermal and mechanical nociception measures (Figure 3.3A and 3.3B) as previously reported[39,41,42]. Therefore, it was surprising that AAH8 lacked acute antinociceptive effects in animals with inflammation-induced mechanical hypersensitivity and in the WWTW following systemic administration (Figure 3.3A). It is unclear why the antinociceptive effects of AAH8 in the WWTW previously reported could not be repeated[26]. Perhaps this was due to an altered synthesis or different people/group making AAH8. However, the current data suggest that AAH8 may have limited ability to cross the blood brain barrier. Therefore, we chose to assess AAH8 in the WWTW following ICV administration. We show that in the WWTW, AAH8 (ICV) displayed dose-dependent increases in tail flick latency, demonstrating antinociceptive effects. We did assess concentrations of 100nmol, ICV, which elicited full antinociceptive effects. However, these mice also experienced convulsions, so further evaluation with this dose was terminated.

Finally, we evaluated the effects of AAH8 and morphine on gastrointestinal function by evaluating excretion of fecal boli over time. As expected, morphine produced constipation as demonstrated by a decrease in fecal boli excreted over 6 hours. The effects of morphine were similar whether morphine was given IP or SC. In general, AAH8 was more potent than morphine in producing constipation independent of route of administration; however, SC AAH8 was approximately 3-fold less potent than IP AAH8 (Figure 3.5). Overall, these data suggest that AAH8 has limited ability to activate MORs in the CNS, unless it is administered directly to the brain. It is well established in the literature that thermal nociception in the WWTW and mechanical nociception are predominately measures of spinal and supraspinal nociception[43,44]. It is unclear whether the lack of central effects of AAH8 are due to a limited ability to cross the blood brain barrier or if AAH8 is a substrate of P-glycoproteins. It is likely that there is delay in or limited absorption and/or distribution of AAH8 as suggested by the differences in potency between IP and SC administration to produce constipation. Future studies should directly measure AAH8 levels in the brain following systemic administration as AAH8 may not cross the BBB in significant concentrations or may be a substrate for P-glycoproteins resulting in active transport out of the CNS.

Overall, this study suggests that AAH8 produces antinociception and constipation effects through activation of peripheral MORs. This study also suggests that AAH8 may have potential pharmacokinetic issues deterring the distribution of AAH8 into central regions. Further, these data suggest repeated activation of peripheral opioid receptors produces tolerance to the peripherally-mediated antinociceptive effects of MOR agonists. Further studies are required to elucidate exact pharmacokinetic properties hindering the absorption, distribution, and CNS penetrability of AAH8

following systemic administration. Also, including tolerance to other peripherally mediated MOR-induced physiological effects such as constipation and the impact of drug route administration on potency differences of AAH8-induced constipation.

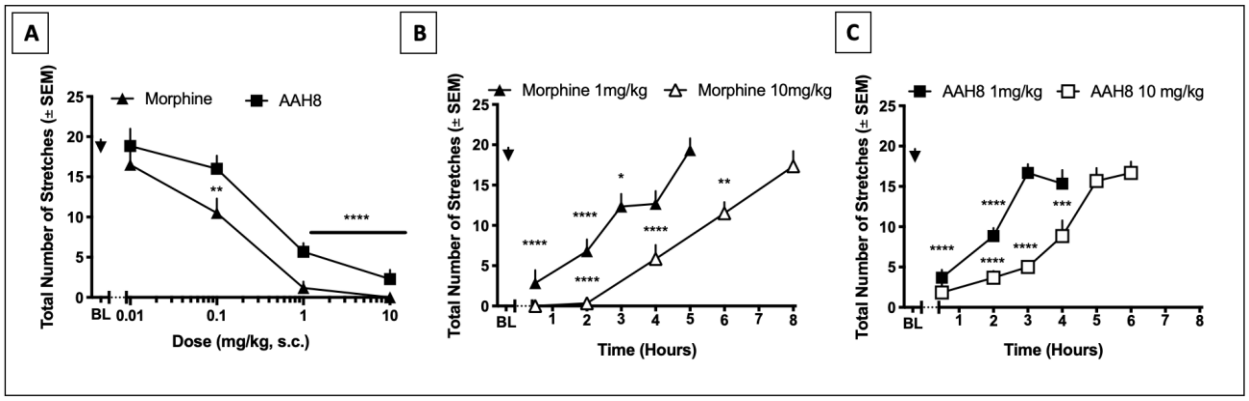

Figure 3.1 Acute Antinociceptive Effects of AAH8 and Morphine

Figure 3.1: Antinociceptive effects and duration of action of AAH8 and morphine in the acetic acid stretch assay. C57BL/6N mice were injected SC with drug. Total number of stretches were measured to evaluate antinociception (N=5-6 for each group). Between subject dose response curves measuring antinociception (A). Duration of action following bolus doses of 1mg/kg or 10mg/kg morphine (B). Duration of action following bolus doses of 1mg/kg or 10mg/kg AAH8 (C). **** = P<0.0001, *** = P<0.001, ** = P<0.01, * = P<0.05 versus vehicle control (BL).

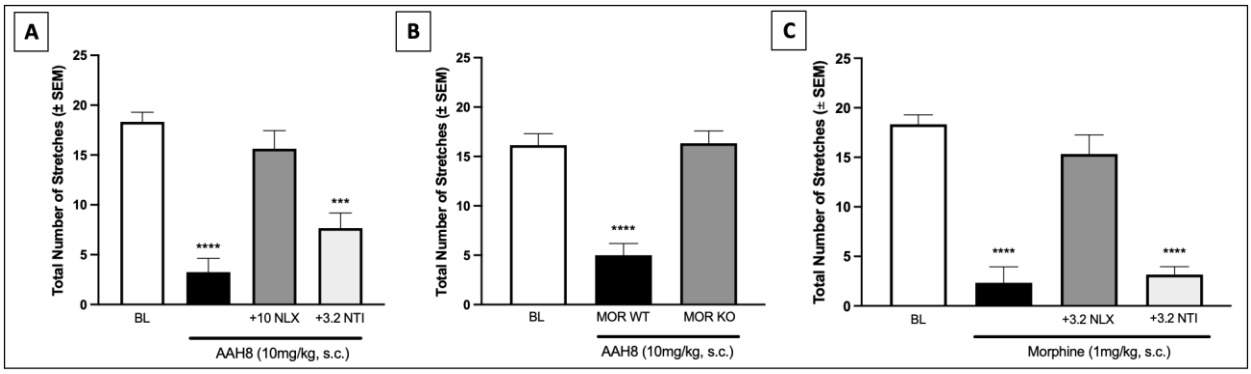

Figure 3.2 Opioid Receptor-Mediated Antinociceptive Effects of AAH8 and Morphine

Figure 3.2: Opioid receptor antagonists block the antinociceptive effects of morphine and AAH8 in the AASA assay. Naloxone and naltrindole pretreatments to 10mg/kg AAH8 (A). 10mg/kg AAH8 in MOR WT and KO mice (B). Naloxone and naltrindole pretreatments to 1 mg/kg morphine (B). (N=6 for each group) **** = P<0.0001, *** = P<0.001, versus vehicle control.

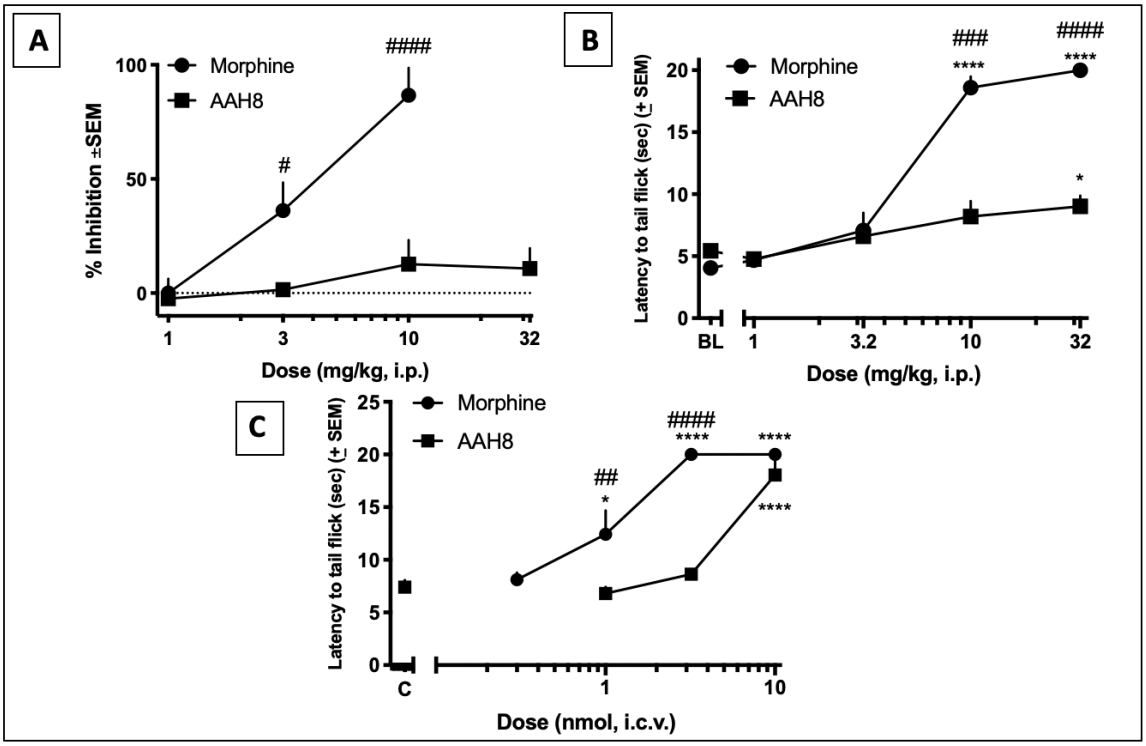

Figure 3.3 Acute Antinociceptive Effects of AAH8 Following Intraperitoneal or Intracerebroventricular Administration

Figure 3.3: Antinociceptive effects of AAH8 and morphine in von Frey and WWTW following IP administration and in the WWTW following ICV administration. (A) Cumulative dose effect curves for AAH8 and morphine. %Inhibition of CFA-induced allodynia is plotted on the y-axis. (B) Cumulative dose effect curves in the WWTW after IP administration. (C) Dose effect curves in the WWTW after ICV administration dose dependent mechanical antinociception. (N=6 for each group) **** = P<0.0001, * = P<0.05, versus vehicle control. #### = P<0.0001 ### = P<0.001, ## = P<0.01, #=P<0.05 versus same dose of AAH8.

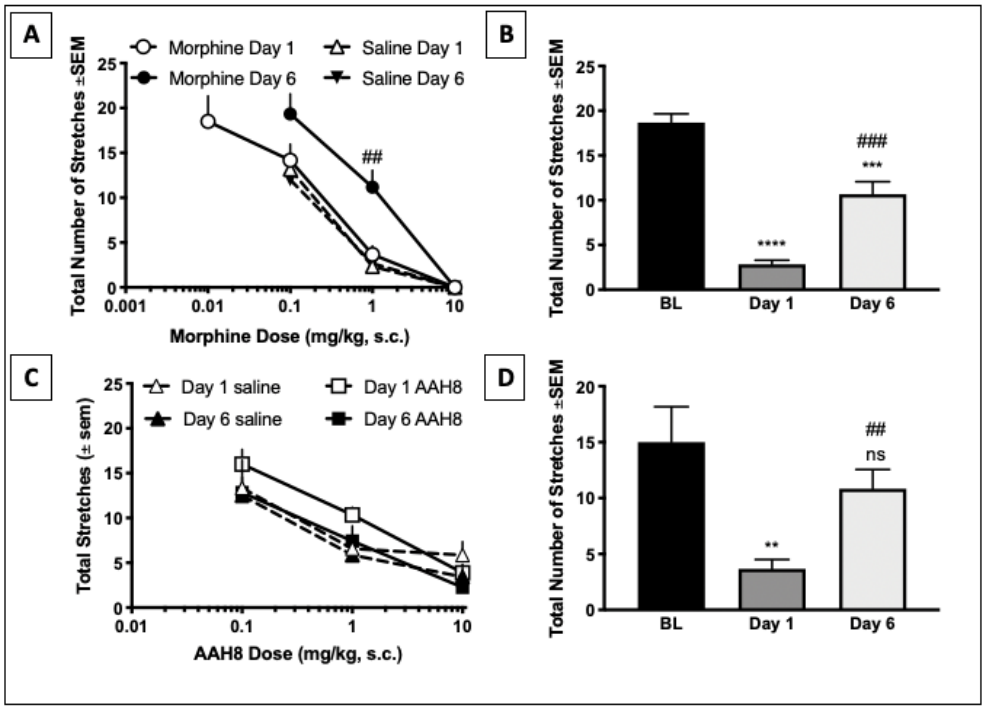

Figure 3.4 Antinociceptive Effects of AAH8 and Morphine Following Five Days of Repeated Administration

Figure 3.4: The effects of repeated administration of AAH8 and morphine on the antinociceptive effects of AAH8 and morphine. (A) 5-day increasing dosing paradigm of 2 injections/day (1-5mg/kg) of morphine. (B) 5-day paradigm of 3 injection/day of 1mg/kg morphine (C) 5 day increasing dosing paradigm of 2 injections/day (1-5mg/kg) of AAH8. (D) 5-day paradigm of 3 injection/day of 10mg/kg AAH8. (N=6 for each group) **** = P<0.0001, *** = P<0.001, ** = P<0.01 versus vehicle control (BL). ### = P<0.001, ## P<0.01 versus Day1

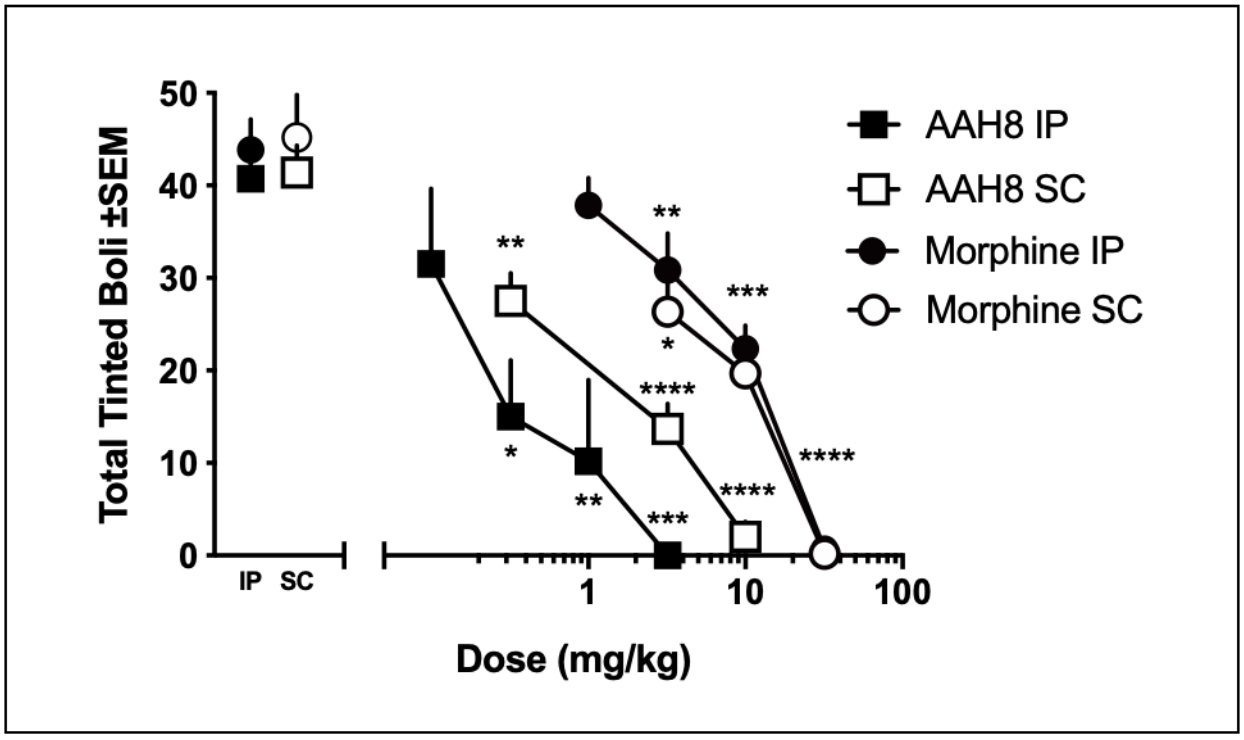

Figure 3.5 Opioid-Induced Constipation Effects of AAH8

Figure 3.5: Constipation of effect of AAH8 and morphine. Y-axis displays the total number of tinted boli collected during the 6 hours of collection. Acute antinociceptive effects after bolus injections (IP or SC) of either AAH8 or morphine (A). (N=6 for each group) **** = P<0.0001, *** = P<0.001, ** = P<0.01, * = P<0.05 versus vehicle control. (■ and □ = 10/10/80 vehicle), (● and ○ = saline vehicle

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Chapter IV: Involvement of Peripheral Opioid Receptors in the Development of Tolerance to μ-Opioid Receptor Agonists

Abstract

Opioids are regarded as the most effective drugs for pain therapy. The majority of opioid use is in managing severe, acute pain and is considered standard of care throughout most countries. However, the effectiveness of opioids is greatly hindered when treating chronic pain because of many adverse effects, including tolerance development. Elucidating mechanisms involved in tolerance development will improve our knowledge of opioid pharmacology and potentially improve our treatment of chronic pain. This study aims to evaluate the role of peripheral opioid receptors in tolerance development to centrally and peripherally-acting MOR agonists. In this study, we used a model of acute, peripherally-mediated visceral pain, the acetic acid stretch assay (AASA), and a centrally-mediated, thermal reflex assay, warm water tail withdrawal (WWTW). In the AASA, loperamide and morphine displayed acute antinociceptive effects with ED_{50} values of 0.62 and 0.15 mg/kg, respectively. In the WWTW, following 5 days of 3x daily administration of morphine, the ED_{50} of the morphine dose-effect curve was shifted 2.5-fold to the right. Interestingly, naloxone-methiodide pretreatments before each chronic morphine injection prevented the chronic morphine-induced rightward shift in the morphine dose-effect curve. Additionally, chronic loperamide (5 days of 4x daily administration) produced tolerance to the antinociceptive effects of morphine, as displayed by a rightward shift in the morphine dose-effect curve. Pretreatments of naloxone-methiodide completely reversed cross-tolerance to the antinociceptive effects of morphine. These results suggest that repeated activation of peripheral

opioid receptors is sufficient for tolerance production and that peripheral opioid receptors may be involved in developing tolerance to clinically used opioids.

Introduction

Transmission of painful stimuli begins with activation of nociceptors located on primary afferent neurons residing within the peripheral nervous system[1]. Nociceptor activation sends electrical signals along the ascending pain pathway, to the dorsal horn and eventually many brain structures including the medulla, midbrain, amygdala, thalamus and the anterior cingulate cortex (ACC)[2-4]. Processed pain signals within the somatosensory cortex send projections via the descending pain pathway to the periaqueductal gray (PAG), rostral ventral medulla (RVM), and dorsal horn, where significant facilitation or inhibition of nociceptive inputs occur[5].

Opioid agonists bind to and activate MORs within the substantia gelatinosa in the dorsal horn of the spinal cord and modify painful stimuli along the ascending limb[6]. Direct activation of MORs in the peripheral nervous system (peripheral nerve terminals and dorsal root ganglion [DRG]), as well as the spinal cord level and supraspinal regions have been shown to produce antinociceptive effects in rodent models[7,8]. For example, intrathecal (IT) administration of morphine also produces MOR-mediated antinociceptive effects[9,10]. Also, morphine administered directly into the rostral ventral medulla (RVM) or periaqueductal gray (PAG) inhibit responses to nociceptive stimuli in various animal models in a MOR-mediated manner[11-14].

Repeated administration of morphine systemically induced tolerance to the antinociceptive effects of opioids. *In vitro* and *in vivo* studies have identified various opioid receptor-mediated mechanisms thought to contribute to opioid tolerance. Canonically, MOR desensitization and downregulation was thought to mediate tolerance development[15,16]. Additionally, both the MOR agonist, DAMGO and morphine caused robust MOR agonist-induced desensitization of G protein-

coupled inwardly rectifying potassium channel (GIRK) channel activation following prolonged morphine exposure in HEK293 cells expressing rat MOR, elucidating additional mechanisms that may contribute to tolerance development[17,18]. However, MOR desensitization and down regulation *ex vivo* was not always observed following chronic MOR agonist administration *in vivo*[19]. For example, mice implanted with subcutaneous morphine pellets were tolerant to the antinociceptive effects of morphine but there was no significant downregulation of MOR in brain slices[20]. Repeated microinjections of morphine into the ventrolateral PAG produced robust tolerance development to ventrolateral PAG-mediated antinociceptive effects of morphine, and this was accompanied with significant decreases in morphine-induced adenylyl cyclase inhibition in the PAG[21-23]. Further, reductions in morphine-induced decreases in calcium current, as well as decreased GIRK activation have been demonstrated in PAG neurons isolated from morphine tolerant mice[24]; these effects have also been shown in thalamus neurons isolated from morphine tolerant rats[21]. These data collectively suggest chronic systemic administration of morphine induces robust tolerance development via receptor desensitization in multiple brains regions involved in opioid-mediated nociception. However, these data do not rule out other contributing brain regions or neuronal circuits that may be involved in tolerance development.

Chronic morphine administration into the PAG elicits tolerance development while chronic morphine administered into the RVM does not[22]; suggesting RVM neurons are relatively resistant to tolerance development and that morphine-induced tolerance may occur in neurons that precede the RVM along the pain pathway. Even with this understanding, the brain regions, population of MORs, or neuronal circuits are responsible for opioid tolerance remain unresolved. Tolerance development is one of the major driving forces resulting in decreased pain control and contributes

to opioid misuse, further elucidating mechanisms and receptor populations involved in opioid tolerance may help improve opioid-based pain treatments.

Recently, studies have alluded to significant involvement of MORs expressed on primary afferent neurons in opioid induced tolerance and opioid induced hypersensitivity (OIH)[25]. When MORs were removed from neurons of the TRPV1 lineage (neurons expressing the TRPV1 promoter), acute antinociceptive effects of morphine were unaltered, but opioid tolerance and OIH were prevented[25]. Further, the peripherally restricted MOR agonist, loperamide, produced robust tolerance to its antinociceptive effects in nerve-injured rats[26]. Collectively, these studies suggest peripheral MORs may significantly contribute to opioid tolerance. The present study aims to evaluate the involvement of peripheral opioid receptors in the development of tolerance to centrally-mediated antinociceptive effects of MOR

Methods

Animals and *In Vivo* Solutions

All animal care and experimental procedures complied with the US National Research Council's Guide for the Care and Use of Laboratory Animals (Kuwahara et al., 2012)[27]. Mice were group- housed with a maximum of five animals per cage in clear polypropylene cages with corn cob bedding and nestlets as enrichment. Mice had free access to food and water at all times. Animals were housed in specific pathogen- free rooms maintained between 68 and 79°F and humidity between 30 and 70% humidity with a 12 h light/dark cycle with lights on at 07:00 h. We conducted experiments in the housing or adjoining rooms during the light cycle. All studies utilize wildtype male C57BL/6 mice from Envigo laboratories. Mice weighed between 20-30 g at 7-15 weeks old at the time of use. All drug solutions were injected in a volume of 10ml/kg. Loperamide was dissolved in a 1:1:8 Ethanol/Castor oil/water solution. Morphine sulphate and 0.6% acetic

acid were dissolved in saline and water, respectively. All drugs were given by either IP or SC, as indicated, and the 0.6% acetic acid was given by IP injection.

Acetic Acid Stretch Assay (AASA)

This assay evaluated the effects of MOR agonists to alleviate stretching or writhing behaviors induced by intraperitoneal injection of diluted glacial acetic acid. Test drug or vehicle was administered SC. Thirty min later, mice were administered diluted acetic acid (IP, 0.6%) and were individually placed in clear plastic observation cages (10 x 6 x 8 in) containing corn cob bedding. Five min after acetic acid administration, total number of stretches were recorded over 20 min. For antagonism studies, naloxone (3.2 or 10 mg/kg) or 10mg/kg naloxone-methiodide was administered IP 15 min before administration of test drug.

Warm Water Tail Withdrawal

To assess tail withdrawal latencies, mice were briefly placed in a cylindrical plastic restrainer and 2-3 cm of their tails were immersed into a water bath maintained at 50° C. The latency to tail withdrawal or a rapid flicking motion of the tail was recorded with a maximum cut off time of 20 seconds to prevent any tissue damage from occurring. Morphine or loperamide were given via SC administration in a cumulative dosing fashion (1-32mg/kg) and tail-flick latencies were recorded every 30 min. In antagonism studies, naloxone (3.2mg/kg) or naloxone-methiodide (10mg/kg) was administered via IP administration as a 15 min pretreatment to cumulative doses of morphine.

For the tolerance studies, a within-subject design was used. Cumulative dose effect curves were established on day one in opioid naïve mice. Mice were administered morphine (10mg/kg) or vehicle 3x per day for five days (7am, 1pm, and 7pm) or loperamide (3.2mg/kg) 4x/ day (7am, 1pm, 7pm, and 1am). On day 5, a cumulative morphine dose effect curve was determined. In

another group of animals, 10 mg/kg NLX-M (IP) was administered as 15 min pretreatments to either each treatment of morphine or loperamide during repeated injections. No drug treatments were administered the evening prior to test day.

Data Analysis

All data analyses were performed using GraphPad Prism version 8.4.3. Level of significance (α) was set to 0.05 for all statistical measures. Two-way ANOVAs were conducted for all dose-effect curves unless otherwise stated. Post hoc analyses were conducted by Tukey's post hoc tests to correct for multiple comparisons and were only performed when F values achieved $p < 0.05$. In the absence of an interaction, we did post hoc comparisons on the main effect of dose. To calculate approximate ED_{50} values, the 50% maximum effect was interpolated from the straight-line analysis for each individual anima, and then the ED_{50} values were averaged across all in mice in the same treatment group. This included 2-3 points along the linear portion of the curve only. Standard errors of the mean were calculated for ED_{50} values where stated. Fold shifts in dose-effect curves were calculated by dividing the ED_{50} value of interest by the ED_{50} value calculated in wild-type mice or in the absence of the antagonist.

Results

Antinociceptive Effects of Morphine and Loperamide in the Acetic Acid Stretch Assay

The effects of acute morphine or loperamide were evaluated in the AASA. Morphine produced dose-dependent decreases in acid-induced stretches [$F (4, 25) = 40.4$, $P<0.0001$, Figure 4.1A] with significant decreases observed at 0.1 mg/kg morphine and larger. Loperamide also decreased acid-induced stretches in a dose dependent manner [$F (4, 26) = 34.1$, $P<0.0001$] with significant decreases at doses of 1 mg/kg and larger (Figure 4.1A). Both naloxone (3.2 mg/kg) and

the peripherally restricted, non-selective opioid receptor antagonist, NLX-M (10 mg/kg), attenuated the acute antinociceptive effects of morphine [$F(3, 21) = 23.1$, $P<0.0001$] (Figure 4.1B). Similarly, naloxone and NLX-M significantly attenuated the acute antinociceptive effects of 3.2mg/kg loperamide [$F(4, 26) = 34.1$, $P<0.0001$] (Figure 4.1C).

Antinociceptive Effects of Morphine and Loperamide in the WWTW

The acute antinociceptive effects of morphine and loperamide was evaluated in the WWTW assay. Morphine produced dose-dependent increases in withdrawal latencies [$F(4, 20) = 117$, $P<0.0001$] with significant effects observed at 10 and 32 mg/kg. In the WWTW, naloxone, but not naloxone-methiodide, shifted the morphine dose effect curve approximately 2.7-fold to the right. A repeated measures, two-way ANOVA revealed a significant interaction [$F(4, 50) = 13.4$, $P<0.0001$], main effect of dose [$F(4, 50) = 153.1$, $P<0.0001$], and a main effect of treatment [$F(1, 50) = 19.2$, $P<0.0001$] (Figure 4.2A). However, both naloxone and naloxone-methiodide failed to alter the acute antinociceptive effects of loperamide [$F(6, 51) = 1.02$, $P=0.4$]. There was a significant main effect of dose [$F(1.919, 32.63) = 124.4$, $P<0.0001$], and 32 mg/kg loperamide significantly increased tail-flick latencies (Figure 4.2B).

Effects of Naloxone-Methiodide on the Development of Tolerance Produced by Repeated Administration of Morphine on the WWTW.

Methylnaltrexone, a peripherally restricted non-selective opioid receptor agonist, was previously demonstrated to attenuate morphine-induced tolerance in a model of perioperative and chronic pain[25]. To assess which opioid receptor population was involved in morphine tolerance, morphine-induced antinociceptive effects were assessed following repeated administration of

morphine +/- pretreatments of naloxone-methiodide (10mg/kg). In the WWTW, a three-way ANOVA revealed tail flick latency was resistant to 10 mg/kg morphine following 6 days of morphine treatment, which was supported by a day x dose x treatment interaction [$F(4, 40) = 5.3$, $P=0.002$], as well as a significant main effects of dose [$F(4, 40) = 506.3$, $P<0.0001$], treatment [$F(1, 10) = 28.4$, $P=0.0003$], as well as day [$F(1, 10) = 7.8$, $P=0.02$]. This effect is supported by a 3.5-fold rightward shift in the morphine dose-effect curve following repeated morphine administration. following chronic morphine administration, the antinociceptive effects of morphine displayed an approximate 2.5-fold rightward shift in the morphine dose-effect curve (Figure 4.3A). In mice that received chronic administration of naloxone-methiodide and morphine, the day 6 morphine dose effect curve was unaltered compared to day one [$F(4, 40) = 0.7$, $P=0.6$] (Figure 4.3B). However, there was a main effect of dose [$F(4, 40) = 136.4$, $P<0.0001$] but no effect of treatment [$F(1, 10) = 0.03$, $P=0.9$]. Chronic administration of NLX-M as a pretreatment to chronic saline did not alter the acute antinociceptive effects of morphine as displayed by no shift in the morphine DE curve [$F(4, 50) = 0.5317$, $P=0.7130$] (Figure 4.3D). There was however a main effect of dose [$F(4, 50) = 143.2$, $P<0.0001$].

The Antinociceptive Effects of Morphine or Loperamide Following Repeated Administration of 3.2mg/kg Loperamide

To further assess the involvement of peripheral opioid receptors in opioid-mediated antinociception and tolerance production, we evaluated tolerance and cross-tolerance following repeated administration of loperamide. We initially assessed loperamide-induced cross tolerance to the antinociceptive effects of morphine. (Figure 4.4A and Figure 4.4B). Repeated administration of loperamide, 3.2mg/kg injection three times/day for five days, produced a modest rightward shift

in the morphine DE curve [F (4, 40) = 3.24, P=0.02] (figure 4.4A). There was no main effect of the day (1 vs 5) following repeated loperamide administration [F (2, 20) = 3.5, P=0.1] (Figure 4B). However, repeated administration of loperamide, 3.2mg/kg injection three times/day for five days, induced a significant rightward shift in the loperamide dose effect curve (Figure 4.4D). A two-way ANOVA revealed a significant interaction of day x dose [F (4, 40) = 12.1, P<0.0001], as well as significant main effect of day (1 vs 6) [F (1, 10) = 23.1, P=0.0007] and dose [F (4, 40) = 31.2, P<0.0001]. Therefore, we administered loperamide, 3.2mg/kg injection four times/day for five days and assessed the morphine dose effect curve on day 1 and day 6 (Figure 4.4B). Four daily injections of 3.2 mg/kg loperamide induced approximately a 3.5-fold rightward shift in the morphine DE curve (Figure 4.4B). A two-way ANOVA revealed a significant interaction [F (4, 50) = 2.960, P=0.03], a main effect of dose [F (4, 50) = 88.84, P<0.0001], as well as a main effect of day (1 vs 6) [F (1, 50) = 4.543, P=0.038]. To assess possible accumulation of loperamide in the CNS following repeated administration, baseline tail-flick latencies were assessed following the first and last 3.2mg/kg injections on day 1, and day 5, respectively (Figure 4.4C). There was no significant difference between baseline tail-flick latencies between day 1 and day 5 (t=1.2, df=5, p=0.29).

Effects of Chronic Naloxone-Methiodide on Loperamide-Induced Tolerance to the Antinociceptive Effects of Morphine

Chronic loperamide administration produced cross tolerance to the antinociceptive effects of morphine following repeated systemic administration(Figure 4.4B). To confirm that this effect was mediated by peripheral opioid receptors, mice received pretreatments of 10 mg/kg NLX-M in conjunction with repeated administration of 3.2 mg/kg loperamide, four times per day for five

days. 10 mg/kg NLX-M attenuated loperamide-induced tolerance to the antinociceptive effects of morphine in the WWTW assay as displayed by a lack of shift in the morphine dose effect curve. A two-way ANOVA revealed a main effect of dose [$F (4, 50) = 102.1$, $P<0.0001$], but no main effect of day (1 vs 6) [$F (1, 50) = 0.7491$, $P=0.4$] (Figure 4.5).

Discussion

The present study evaluated the contribution of peripheral ORs to the development of tolerance to the centrally-mediated, antinociceptive effects of morphine. Morphine produced antinociceptive effects in both the AASA and the WWTW assay as expected[28-30], and morphine was 10-fold more potent in the AASA as compared with the WWTW. Naloxone and the peripherally restricted NLX analog, NLX-M, blocked the antinociceptive effects of morphine in the AASA. However, only NLX attenuated the effects of morphine in the WWTW, consistent with previous studies[31,32]. Together, these data suggest that morphine produces antinociceptive effects in the AASA through activation of peripheral opioid receptors, but central opioid receptor activation is required for antinociceptive effects in the WWTW assay. While naloxone-methiodide is believed to be peripherally restricted, some NLX-M may cross into the CNS; however, based on our data, it would suggest that not enough NLX-M gets into the CNS to block the centrally-mediated antinociceptive effects of morphine measured in the WWTW. Further, these data suggest that the antinociceptive effects of opioids in the AASA are peripherally mediated following systemic administration, consistent with previous data[28].

The MOR agonist, loperamide, the active ingredient in Imodium, causes slowing of the GI tract to prevent diarrhea. It is primarily restricted to the gastrointestinal tract following oral administration because it is a major substrate for the efflux transporter P-glycoprotein (P-gp), limiting its ability to be absorbed into circulation and to cross the blood-brain barrier[33]. Morphine

is also a P-gp substrate, but to a much lesser degree. Mice lacking P-gp display between a 10-fold and 65-fold higher concentration of loperamide in the brain following IV and SC administration, respectively[34,35], further demonstrating the peripheral restrictiveness of loperamide in WT mice. In the same strain of mice, morphine concentrations in the CNS only increases 2-fold.[36] When systematically administered, acute loperamide produced antinociceptive effects in both the AASA and WWTW assays. The antinociceptive effects of loperamide in the AASA were blocked by NLX and NLX-M, indicating that peripheral ORs mediated these effects. However, only the largest dose of loperamide (32 mg/kg) produced antinociceptive effects in the WWTW assay; however, neither NLX nor NLX-M blocked these effects. These data suggest that large doses of loperamide produce non-opioid receptor-mediated antinociceptive effects. Larger doses of loperamide have been shown to directly bind to both N-type and L-type calcium channels[37]. Additionally, intrathecal administration of the N-type calcium channel blocker, omega-conotoxin GVIA, dose dependently increase tail-flick latencies in the WWTW[38], suggesting calcium channels may be the mechanism behind the antinociceptive effects of loperamide in the WWTW.

Next, we wanted to assess whether antagonism of peripheral opioid receptors would modulate the development of tolerance to the centrally-mediated antinociceptive effects of morphine. Systemic administration of morphine at 10 mg/kg, 3x daily dosing for five days, was sufficient for inducing tolerance to the antinociceptive effects of morphine (Figure 4.3A). Three times daily administration of NLX-M alone did not alter the acute antinociceptive effects of morphine, nor did it alter baseline tail-flick latencies. These data suggest that repeated administration of NLX-M did not lead to blockade of central MORs either through drug accumulation or changes in the integrity of the BBB. However, NLX-M pretreatments during daily morphine injections completely attenuated morphine-induced tolerance, as demonstrated by a lack

of shift in the morphine dose-effect curve following repeated administration (Figure 4.3B). These data are in agreement with those published by Corder et al., 2017, suggesting antagonism of peripheral opioid receptors is sufficient for attenuating morphine-induced tolerance development[25].

To further elucidate the involvement of peripheral opioid receptors, we chronically activated peripheral opioid receptors with 3.2mg/kg loperamide. Loperamide produced a dose-dependent attenuation of acid-induced stretches (Figure 4.1A). 3.2 mg/kg loperamide was fully effective and these effects were inhibited by NLX-M (Figure 4.1C). Further, this dose of loperamide was inactive in the WWTW assay. Together, these data suggest that a dose of 3.2 mg/kg loperamide only activates peripheral opioid receptors.

We next further assessed the ability of loperamide to induce cross-tolerance to the antinociceptive effects of morphine. Three times daily systemic administration of 3.2 mg/kg loperamide produced modest but non-significant cross-tolerance to the antinociceptive effects of morphine (Figure 4.1B). However, when the frequency of loperamide dosing was increased to four-times daily, administration of peripherally active doses of loperamide produced tolerance to the centrally-mediated antinociceptive effects of both morphine and loperamide. Together, these data suggest that activation of peripheral opioid receptors plays a role in developing tolerance to the centrally-mediated antinociceptive effects of morphine.

There have been many mechanisms proposed to be involved with opioid tolerance that incorporate peripheral mechanisms. Chronic administration of morphine produced robust tolerance development, and this effect was associated with a significant increase in brain P-gp. Increased P-gp concentrations in the BBB would decrease the amount of morphing reaching the CNS, ultimately shifting the morphine dose effect curve and giving the appearance of tolerance

development but might not be associated with opioid receptor desensitization or downregulation. These data suggest that upregulation of P-gp may reduce the central antinociceptive effects of morphine via an increase of morphine efflux from CNS[39]. Another peripherally mediated proposed mechanism for opioid tolerance is the involvement of the microbiome. Recent studies demonstrate altered gut microbiota following chronic opioid administration resulting in the compromise of the gut barrier[40,41]. Interestingly, both in germ-free mice or with the administration of probiotics, mice chronically administered morphine did not produce tolerance to the antinociceptive effects of morphine[42]. It is possible that NLX-M pretreatment inhibited morphine-induced compromisation of the gut barrier, leading to tolerance attenuation. However, studies suggest TLR4 receptors may also play a role in this mechanism, and no activity of NLX-M on TLR4 receptors has been demonstrated[43]. These data offer significant implications of the microbiome on opioid-mediated tolerance development.

This study demonstrated that morphine-induced tolerance was attenuated through antagonism of peripheral opioid receptors, similar to previous reports[25]. Additionally, we demonstrated opioid-induced tolerance through activation of peripheral opioid receptors. Previous work has shown antiallodynic properties of loperamide, which were attenuated with NLX-M suggesting a peripherally-mediated mechanism[26]. In the same study, chronic loperamide induced tolerance to its antiallodynic effects, and NTX pretreatments attenuated this effect. The study, however, did not assess how NLX-M might impact the tolerance development, although the study suggests the tolerance was induced via activation of peripheral MORs residing in the dorsal root ganglion, since the antinociception observed was mediated through peripheral opioid receptors[26]. This provides further evidence that peripheral opioid receptors play a role in tolerance development. The intriguing component of these results in this manuscript is that tolerance

development occurred to CNS-mediated antinociception through peripheral opioid receptor manipulation. This suggests that the manipulation of peripheral opioid receptors may potentially alter opioid-receptor function in the CNS. There are many proposed mechanisms involved in morphine-induced tolerance, either following repeated administration *in vivo* or prolonged exposure *in vitro*. Opioid receptor desensitization is a common phenomenon that occurs following opioid administration and has been thought of as a cause for opioid tolerance[44]. Down-regulation is the reduction of opioid receptor number, which may result in the degradation of the receptor. Further, down-regulation could also be due to a decrease in receptor synthesis and both could contribute to tolerance development. Mice administered chronic subcutaneous etorphine infusions produced significant antinociceptive tolerance and these effects were correlated with a significant down-regulation of MORs in mouse brain[20]. Conversely, morphine-tolerant mice who received either morphine pellet implantation or chronic subcutaneous infusion did not display decreases in MOR number[20]. These data suggest tolerance development can be produced through differing mechanisms depending on the agonist, further suggesting multiple mechanisms contributing to opioid tolerance. Multiple signaling pathways, including ACase inhibition, activation of MAP kinases, inhibition of voltage-gated calcium channels, and activation of GIRK channels, are utilized to measure opioid receptor desensitization[45] and are instrumental measurements for future studies to elucidate an intracellular mechanism responsible for the effects seen in this manuscript. While the mechanism behind our results in this manuscript is unclear, it suggests that activation of peripheral opioid receptors is sufficient for inducing tolerance to centrally acting opioids. Concern for centrally active opioids for the use of chronic pain has risen due to adverse effects, and usage of peripherally restricted opioid receptor agonists has gained attention. However, it is imperative to elucidate the involvement of peripheral opioid receptors in developing opioid-

induced adverse effects that hinder chronic opioid therapy's effectiveness. Future studies should illuminate the mechanism contributing to the tolerance demonstrated in this manuscript and to understand if the mechanisms are similar to those following CNS administration of opioids.

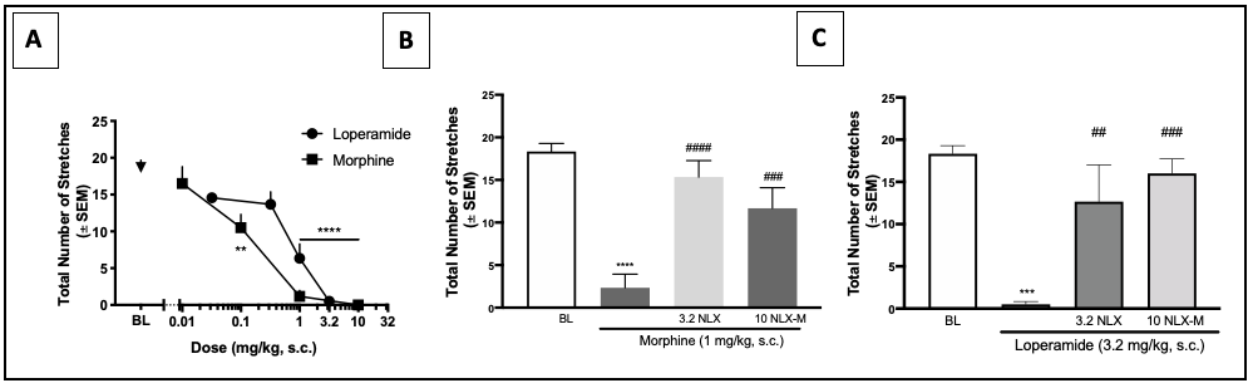

Figure 4.1 Antinociceptive Effects of Morphine and Loperamide in the AASA

Figure 4.1: (A) Dose effect curve of systemically administered morphine or loperamide in the AASA. (B) Pharmacological inhibition of morphine-induced antinociception with naloxone, or naloxone-methiodide. (C) Pharmacological inhibition of loperamide-induced antinociception with naloxone, or naloxone-methiodide (N=6 for each group) **** = P<0.0001, *** = P<0.001, ** = P<0.01, versus vehicle control. ### = P<0.001, ## = P<0.01, versus 1mg/kg morphine, or 3.2mg/kg loperamide.

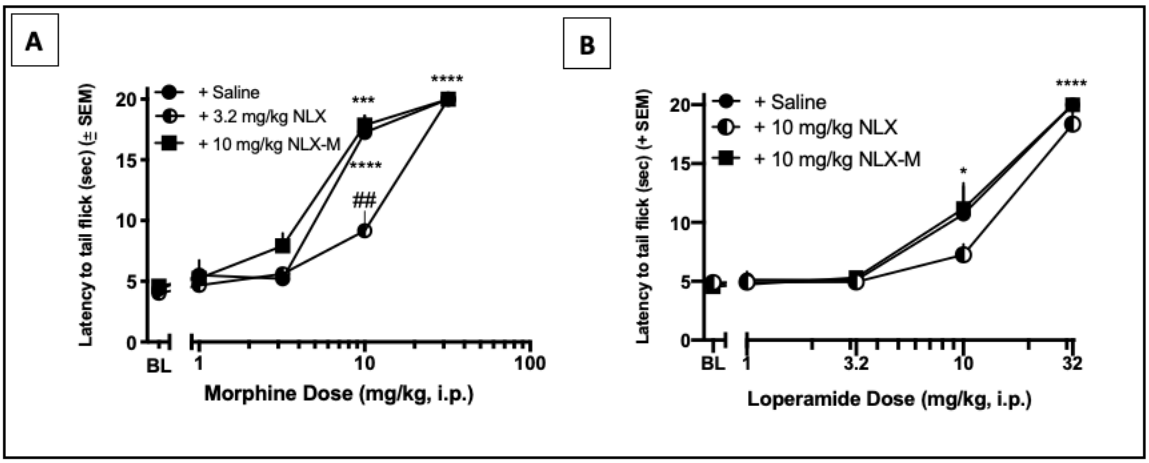

Figure 4.2 Antinociceptive Effects of Morphine and Loperamide in the WWTW

Figure 4.2: (A) Cumulative dose effect curves for morphine in the presence of naloxone, or naloxone methiodide measuring thermal nociception. Y-axis plotted at tail-flick latencies. (B) Cumulative dose effect curves for loperamide in the presence of naloxone, or naloxone methiodide measuring thermal nociception. Y-axis plotted at tail-flick latencies (N=6 for each group) **** = P<0.0001, *** = P<0.001, * = P<0.05, versus BL control.

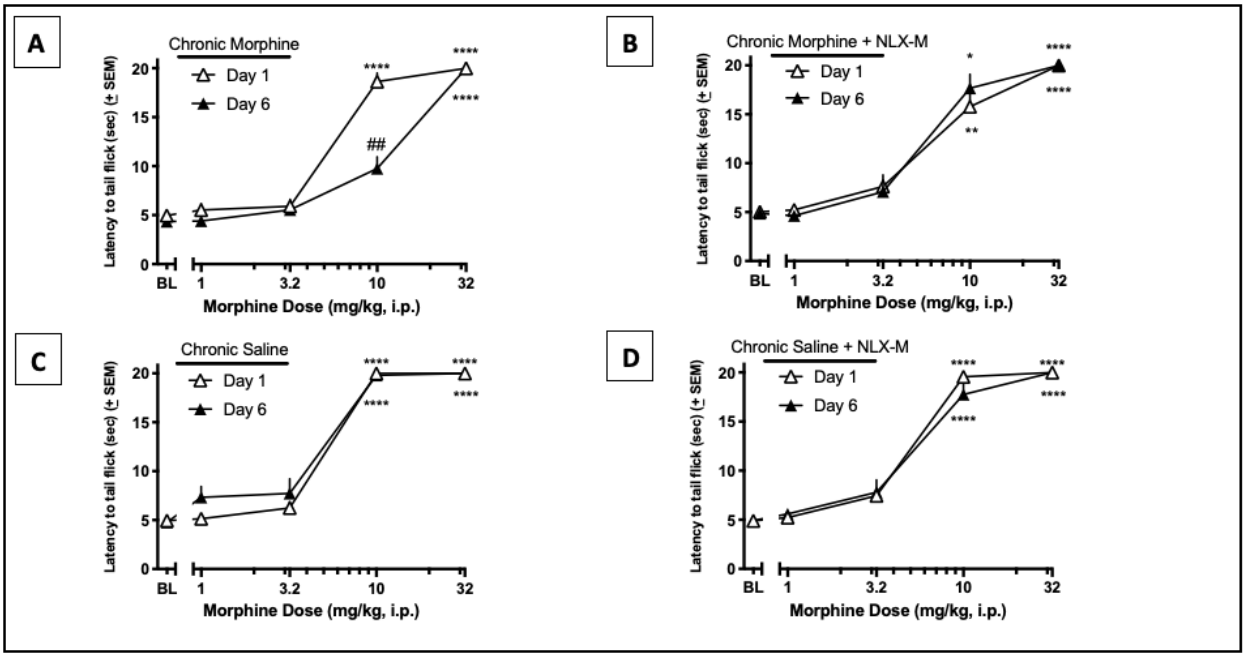

Figure 4.3 Effects of Naloxone-Methiodide on the Development of Tolerance Produced by Repeated Administration of Morphine in the WWTW

Figure 4.3: Morphine-induced tolerance in the presence and absence of the peripherally restricted opioid receptor agonist, naloxone-methiodide. (A) Tolerance development to the antinociceptive effects of morphine following five days of repeated administration. (B) Morphine dose effect curve before and after pretreatments of naloxone-methiodide to repeated administration of morphine. (C) Acute antinociceptive effects of morphine following chronic saline treatment. (D) Acute antinociceptive effects of morphine following chronic naloxone-methiodide treatment (N=6 for each group) **** = P<0.0001, ** = P<0.01, * = P<0.05, versus BL control. ## = P<0.01 versus same dose Day 1.

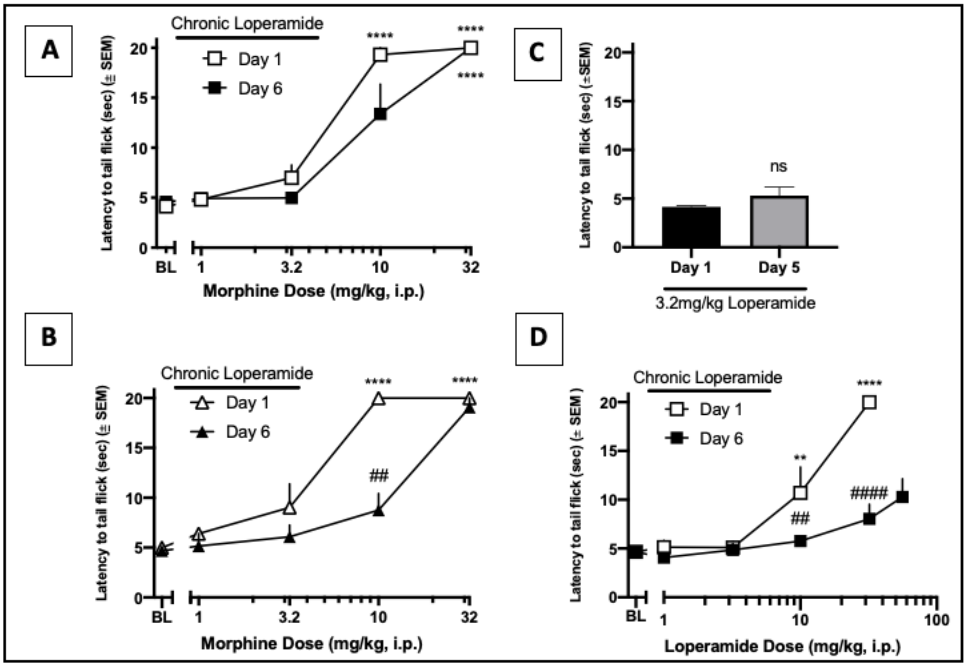

Figure 4.4 The Antinociceptive Effects of Morphine or Loperamide Following Repeated Administration of 3.2mg/kg Loperamide

Figure 4.4: Loperamide-induced tolerance and cross tolerance to the thermal antinociceptive effects of morphine. (A) Cumulative dose effects curves of loperamide, Day 1 vs Day 6, following 5 days of 3x daily repeated administration of 3.2mg/kg loperamide. (B) Cumulative dose effect curves of morphine, Day 1 vs Day 6 tail flick latencies following 5 days of 3x daily repeated administration of loperamide. (C) Cumulative dose effect curves of morphine, Day 1 vs Day 5 tail flick latencies following 5 days of 4x daily repeated administration of loperamide. (D) Baseline tail-flick latencies following 3.2mg/kg loperamide day 1, and day 5 of chronic loperamide treatment. (N=6 for each group) **** = P<0.0001, *** = P<0.001, ** = P<0.01, * = P<0.05 versus BL control, ns = not significant.

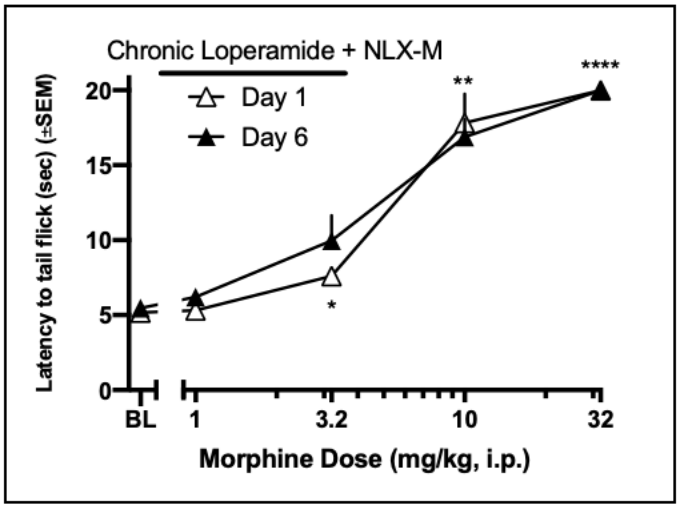

Figure 4.5 Effects of Chronic Naloxone-Methiodide on Loperamide-Induced Tolerance to the Antinociceptive Effects of Morphine

Figure 4.5: NLX-M attenuates loperamide-induced cross tolerance in the WWTW. (A) Naloxone methiodide pretreatments to repeated administration of morphine attenuate morphine induced tolerance. (N=6 for each group) **** = P<0.0001, ** = P<0.01, * = P<0.05 versus vehicle control.

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Chapter V: General Discussion

The experiments described in this thesis sought to examine the receptor-mediated mechanisms responsible for the acute and chronic effects of known selective MOR agonists and novel, mixed-efficacy opioid ligands. While the majority of this work focused on measuring antinociceptive effects of opioid ligands, we also evaluated physical dependence, constipation, and abuse liability. The data presented here demonstrate two major findings: 1) mixed-efficacy opioid ligands produce antinociceptive effects in number of animal models and seem to produce less tolerance, physical dependence, and abuse liability as compared with selective MOR agonists, and 2) peripheral opioid receptors play a role in the development of tolerance to the antinociceptive effects of centrally acting μ-opioid receptor (MOR) agonists. Together, the studies reported in this thesis identify potential opioid receptor targets and mechanisms that may be used to create safer, opioid-based treatments for pain management.

It is well established that MOR agonists are highly effective treatments for attenuating pain. Unfortunately, MOR agonist-induced adverse effects plague clinical utility for long-term, chronic pain patients. It was hoped that the mechanisms contributing to opioid-induced analgesia were distinct from the adverse effects. Nearly two centuries of work have contributed to improving opioid-based therapy by solely targeting the MOR. These investigations yielded higher affinity, more potent, and more efficacious MOR agonists, but failed to generate a safer

opioid analgesic. Elucidating how morphine and other opioids produce adverse effects is essential in the development of safer opioid analgesics.

Antinociceptive effects and the undesired adverse effects of opioid use are primarily mediated through activation of the MOR, as shown by the absence of antinociceptive effects, rewarding effects, and withdrawal-like effects in mice lacking the MOR[1]. However, DOR activity can attenuate some of the undesired effects of MOR agonists without disrupting the acute antinociceptive properties. Antagonism of the DOR attenuates MOR-mediated tolerance development[2], which was further recapitulated with ligands exhibiting MOR agonist/DOR antagonist profiles, such as UMB425 and VRP26[3,4]. Our lab recently published on AAH8, a MOR agonist/DOR antagonist mixed-efficacy opioid ligand that produced acute antinociceptive effects without producing tolerance or physical dependence[5].

Interestingly, DOR agonists have been shown to increase the potency of MOR agonists to produce antinociceptive effects and increase the efficacy of MOR agonists[2,6-8]. SRI-22141, a dual mixed-efficacy MOR/DOR agonist exhibited enhanced efficacy in pre-clinical neuropathic pain models, including reduced tolerance and physical dependence development, providing an addition approach to mixed-efficacy opioid ligands[9]. These data suggest combining DOR agonist activity with a conventional MOR agonist may decrease the dose of the MOR agonist necessary to produce significant antinociception.

Through a screening process of many mixed-efficacy opioid ligands, we found AMB67, a dual MOR/DOR agonist, that displayed acute antinociceptive effects in the acetic acid stretch assay (AASA). Therefore, we chose to conduct an *in vivo* characterization of AMB67, and further evaluate a compound, AAH8, that our lab previously reported in the literature[5]. Considering the decreased tolerance observed with these peptidomimetic compounds, we sought to further explore

mechanisms of tolerance that may explain the improved profile of activity observed with multifunctional opioid ligands.

In Vivo Characterization of The Dual MOR/DOR Agonist, AMB67: Acute and Chronic Antinociception, Physical Dependence, and Abuse Potential

Numerous studies have reported that the therapeutic profile of MOR agonists can be improved with simultaneous modulation of the DOR[2,10,11]. These data resulted in many groups probing bivalent ligands[12,13] and mixed-efficacy MOR-agonist/DOR-antagonist ligands. Generally, these compounds exhibit acute antinociceptive effects with limited tolerance and physical dependence development[14-17]. Interestingly, dual MOR/DOR agonists, such as MMP2200, have also been shown to display similar characterization as MOR agonist/DOR antagonist ligands. MMP2200 is a dual MOR/DOR agonist and produces substantial antinociceptive effects with reduced tolerance, physical dependence, locomotor activation, and self-administration[18-20]. Other dual MOR/DOR agonists, SRI-22141 and RV-Jim-C3, display acute antinociceptive effects in the tail flick and inflammatory models of pain[9,21]. SRI-22141 was effective at attenuating neuropathic pain-like states in a mouse neuropathic pain model. *In vitro*, cells exposed to prolonged SRI-22141, produced robust cAMP overshoot, an *in vitro* marker for dependence liability[22]. However, repeated administration of 10 mg/kg SRI-22141, 2x each day for 4 days, produced significantly less tolerance compared to morphine, and the mice did not develop physical dependence[9]. Discrepancies between *in vivo* and *in vitro* data suggest the improved *in vivo* profiles of mixed-efficacy opioids ligands may not be selective targeting of receptors in a single cell, but instead the involvement of more complex neuronal circuits. *In vitro*, MMP2200 also produced cAMP overshoot, however it failed to induce physical dependence *in vivo*[20]. However, MMP2200 is the only dual MOR/DOR agonist to have been studied for abuse potential

and failed to induce conditioned place preference[20]. To further elucidate the potential advantages of dual MOR/DOR agonists, Chapter II of this thesis characterizes the antinociceptive effects, as well as adverse effects, including abuse potential, of the dual MOR/DOR agonist, AMB67.

Competitive binding studies using radiolabeled diprenorphine show morphine exhibited low nanomolar affinity at the MOR[23], while AMB67 exhibited sub nanomolar affinity at the MOR. In rat MOR expressing C6 cells, morphine produced 48.2 ± 6.5% of DAMGO-induced [^{35}S]GTPγS stimulation[23], suggesting partial agonist activity. These data correlated with our results where morphine produced 21.8 ± 1.9% [^{35}S]GTPγS stimulation at rat MOR expressing C6 cells. Also, in rat MOR expressing C6 cells, AMB67 produced 81 ± 4.3% [^{35}S]GTPγS stimulation as compared to DAMGO, nearly 4-fold more efficacious at the MOR than morphine, displaying full agonist activity. AMB67 is nearly 100-fold more potent at the MOR than morphine. At the DOR, AMB67 had 21-fold higher affinity than morphine. Both morphine and AMB67 exhibited similar efficacy at DOR, with 56% and 34% [^{35}S]GTPγS stimulation, respectively, and AMB67 was nearly 16-fold more potent at DOR than morphine. Together, these data suggest that AMB67 is nearly a full agonist at MORs and a low efficacy agonist at DORs.

In vivo, AMB67 displayed antinociceptive effects in assays of chemical, thermal, and inflammatory nociception in C57BL/6N mice with EC_{50}s of approximately 0.3mg/kg, 8.6 mg/kg, and 3.3 mg/kg, respectively. To assess drug-induced chemical antinociception, we utilized the AASA, where diluted acetic acid was used to produce a writhing or stretching response indicative of a pain-like behavior. In the AASA, naloxone, naltrindole, and βFNA, attenuated AMB67-induced antinociception, suggesting this effect is mediated through actions at both the MOR and the DOR, consistent with the *in vitro* profile of AMB67. In contrast, morphine-induced antinociception was attenuated by naloxone, but not naltrindole. Even though both compounds

exhibit modest agonist effects at the DOR *in vitro*, the antinociceptive effects of morphine in the AASA assay are likely MOR-mediated. These data suggest either potency differences between morphine and AMB67 measuring [^{35}S]GTPγS stimulation may explain the lack of DOR-mediated effects of morphine, or that G-protein activation in this cell line does not adequately predict the *in vivo* activity of an opioid ligands.

In the WWTW, morphine exhibited potent thermal antinociceptive effects, and studies show this effect is mediated through the MOR, as MOR KO mice fail to elicit morphine-induced antinociception[24]. Similarly, AMB67-induced thermal antinociception was shifted to the right in MOR heterozygous mice, and abolished in mice lacking the MOR, demonstrating MOR-mediated effects. These effects were a little surprising considering the antinociceptive effects of AMB67 in the AASA were at least partially mediated by DORs. However, prototypical DOR agonists, such as SNC80, fail to elicit increases in tail-flick latencies, suggesting that DOR activation alone cannot produce antinociceptive effects in thermal, spinally-mediated nociceptive assays. Convulsive effects of DOR agonists are common and may impact the antinociceptive effects of DOR agonists. It is important to note that AMB67 failed to induce convulsive behavior on its own, as shown by no increases in the Racine score in mice administered AMB67, up to doses of 32mg/kg. AMB67 did slightly enhance PTZ-induced convulsions in a DOR-mediated behavior (data not shown). Future studies should assess AMB67 in DOR KO mice in the WWTW to compare and contrast DOR-mediated antinociceptive effects across different nociceptive measures.

In mice administered CFA to induced mechanical hypersensitivity, both morphine and AMB67 exhibited significant antinociceptive effects. Morphine was more efficacious than AMB67; however, we were restricted in using larger morphine doses because stimulatory effects

impeded reliable measurements of mechanical nociception. The EC_{50} of both morphine and AMB67 in mice pretreated with naloxone was shifted to the right, suggesting opioid-receptor mediated effects. It was surprising AMB67 was not more effective in mice treated with CFA, given the DOR agonist activity of AMB67 in the AASA. It was previously shown that CFA induced an up-regulation of DORs in the dorsal spinal cord, suggesting a role for the DOR in nociceptive mechanisms of inflammation[25]. There is, however, contradictory data in the literature with regards to the effects of DOR agonists on CFA-induced mechanical allodynia and thermal hypersensitivity, as some studies show DOR agonists are effective in one but not the other, and vice versa[25,26]. Our studies were conducted 24h post CFA injection. It is possible that upregulation of the DOR was not significant at this timepoint, and if AMB67 was assessed 48h or 72h post CFA injection, AMB67 might have been more effective. Studies assessing the antinociceptive effects of AMB67 in a model of inflammation in MOR KO mice would assess if the DOR is at all involved in the effects shown here. It would be possible that if the DOR component of AMB67 was involved, that mice lacking the MOR would still display modest antinociceptive effects.

The three pain models discussed above are models of pain elicited behavior, whereas an antinociceptive effect is demonstrated by decreasing a behavior induced by a noxious stimulus. Thus, we also assessed morphine and AMB67 in a model of pain depressed behavior. We utilized a pain depressed behavior where animals were conditioned to run on a running wheel and CFA was administered bilaterally into the hind paws to decrease the wheel-running behavior. Morphine significantly restored wheel running behavior but did not completely restore CFA-induced suppression of wheel running (See Appendix, Figure 5.1A). Conversely, AMB67 failed to restore wheel-running behavior in CFA-treated mice (See Appendix Figure 5.1B). The ability of morphine to improve the affective components of pain contributes to its antinociceptive effects[27]. CFA-

induced pain depressed wheel running might incorporate affective pain to a greater extent than the nociceptive models utilized within this manuscript. Since AMB67 produced significantly fewer rewarding properties compared with morphine, this may explain the ineffectiveness of AMB67 in CFA-induced suppression of wheel running. Unfortunately, since AMB67 robustly suppressed wheel running at larger doses in the absence of pain, I was unable to evaluate larger doses. Future studies should assess AMB67 in a self-administration model to gain further insight into the abuse potential of AMB67 and if it produces less abuse potential compared to current opioid therapeutics.

Overall, the acute antinociceptive studies demonstrated in this thesis show AMB67 produces robust chemical, mechanical, and thermal antinociceptive effects in an opioid mediated manner, but was ineffective at attenuating CFA-induced suppression of wheel running. We were interested in assessing the antinociceptive effects of AMB67 and morphine following repeated administration. Repeated administration of morphine produced a rightward shift in the EC_{50} in all three pain models, recapitulating previous studies[28,29]. Conversely, AMB67 failed to induce tolerance in all models utilizing the same dosing paradigm as morphine. Based on previous literature, the DOR activity of AMB67 may contribute to the lack of tolerance. However, directly assessing the impact the DOR component of AMB67 has on tolerance development is difficult. Since mice lacking the DOR exhibit attenuated morphine-induced tolerance[10], this limits our ability to use DOR KO mice to assess the contribution of the DOR activity of AMB67. Similarly, naltrindole attenuates morphine-induced tolerance development and therefore provides further limitations to our assessment. It is possible that if the dosing regimen was more strenuous, either increasing the number of injections per day or increasing the dose per injection, that tolerance development may have occurred. However, we used the same dosing and injection frequency paradigm for both AMB67 and morphine, suggesting AMB67 would be beneficial in a patient

population, and therefore demonstrate an improvement in pain therapy, compared to morphine. However, even though AMB67 failed to elicit tolerance development when repeatedly administered, assessing the abuse potential of AMB67 is imperative, as euphoric effects of opioids have been demonstrated time and time again to induce drug abuse behavior.

We assessed the rewarding and abuse potential of AMB67 through two different paradigms and two different species. In mice, AMB67 was assessed in the conditioned place preference, which involves the association of one environment with a drug of abuse, followed by the association of a different environment with the absence of the drug, or the drug's vehicle. Both compounds produced conditioned place preference, but AMB67 was nearly 10-fold less potent than morphine. Similarly, in a drug discrimination paradigm assessing interoceptive effects of morphine, AMB67 substitutions produced full generalization to the subjective effects of morphine; however, AMB67 was approximately 5.5-fold less potent than morphine. These data suggest that AMB67 may produce less rewarding effects or abuse potential than morphine. An interesting component of these data is the comparison of potency in rewarding effects compared to antinociceptive effects. While morphine and AMB67 exhibited equipotent antinociceptive effects across a variety of pain assays, AMB67 was consistently less potent in rewarding effects. One concern in the drug discrimination study is morphine was administered SC and AMB67 was administered IP. Drugs administered IP tend to undergo more extensive first pass metabolism, so it is possible less AMB67 reached the CNS compared to morphine and therefore a decrease in potency was shown. However, further investigation demonstrated morphine, either administered SC or IP, produced the same EC_{50}, suggesting administration route does not explain the differences (See Appendix, Figure 5.2). However, future studies should assess CNS concentrations of AMB67 following various routes of administration.

Lastly, physical dependence was assessed. Repeated administration of morphine elicited withdrawal-like behaviors, indicative of physical dependence development. However, animals repeatedly administered AMB67 displayed minimal withdrawal-like behaviors, although this effect was not significant from control. One concern with these studies is the relative affinities between AMB67 and naltrexone (the antagonist used to elicit the withdrawal-like behaviors). AMB67 exhibited a roughly 2-fold greater affinity for the MOR than naltrexone and was administered at 5x the dose. It is possible that naltrexone was unable to displace enough of AMB67 from the MOR to induce a significant withdrawal-like effect. Future studies should address this concern by potentially using a ligand with a higher affinity, such as diprenorphine. Diprenorphine may be sufficient in outcompeting with AMB67 for the MOR and, therefore, eliciting a significant withdrawal-like response. Physical dependence is a major driver for continued opioid use because patients will begin using drug to minimize the effects of withdrawal. Having an opioid that does not cause drug dependency, as determined by withdrawal symptoms in the absence of drug, is a major advancement in opioid-based therapy. The combination of robust antinociceptive effects, no tolerance development, diminished physical dependence, and lessened abuse potential makes AMB67 a potential candidate for treating patients' long term.

Continued Evaluation of The Mixed Efficacy MOR Agonist/DOR Antagonist, AAH8: Pharmacokinetics May Limit the Centrally Mediated Antinociceptive Effects of AAH8

Previous research demonstrated the mixed-efficacy opioid, AAH8, produced equipotent antinociceptive effects to morphine, no tolerance development following repeated administration, and no abuse potential[5]. These data strengthen the rationale for assessing mixed-efficacy opioid ligands as a safer alternative to current opioid therapeutics. In this section, the assessment of AAH8-induced antinociception was initially attempted in a chronic model of inflammatory pain.

However, acute AAH8 administration failed to attenuate CFA-induced mechanical allodynia, up to 32mg/kg, and was only modestly effective at 32mg/kg in the WWTW, contradictory to recently published work. Therefore, this section first assessed potential rationales for why AAH8 lacks antinociceptive effects in assays requiring CNS receptor activation.

In the AASA, AAH8 induced nearly equipotent antinociceptive effects as morphine, with an EC_{50} of approximately 0.5mg/kg. Antinociceptive effects in the AASA is predominately mediated through peripheral opioid receptors, as naloxone methiodide (NLX-M), a peripherally restricted opioids receptor antagonist, attenuated the effects of morphine. However, NLX-M failed to attenuate the antinociceptive effects. These data suggest that AAH8 induces antinociception via a central mechanism. However, systemically administered AAH8 did not produce antinociceptive effects in the WWTW or VF assays, suggesting that it does not reach the CNS. When administered ICV, AAH8 produced dose-dependent antinociceptive effects in the WWTW. These data suggest that when AAH8 is given in a manner that allows it to reach central MORs, it produces robust antinociceptive effects. A potential rationale for why NLX-M failed to attenuate the antinociceptive effects of AAH8 in the AASA is that AAH8 has a 3000-fold greater affinity for the MOR than NLX-M[30,31]. Therefore, at equal doses, NLX-M may not be able to compete with AAH8 for receptor occupancy and cannot block the antinociceptive effects of AAH8. Higher doses of NLX-M cannot be used as NLX-M can access the CNS if at a high enough dose. Peripherally acting MOR antagonist (PAMORA) usage has risen due to the effectiveness in reversing opioid-induced constipation[32]. Synthesizing a PAMORA with greater affinity than those already in use would be an additional approach to assess how AAH8 is inducing antinociceptive effects in the AASA. Loperamide, a MOR selective agonist, is well characterized as a useful drug in patients with IBD, as it is restricted to the GI tract and induces constipation effects. The restrictiveness of

loperamide is due to it being a major substrate for p-glycoproteins (PGP), a protein involved in drug detoxification and tissue protection from administered drugs[33,34]. It is unknown if AAH8 is a substrate for PGP, but it would be interesting to assess, via microanalysis, if CNS concentrations of AAH8 differ between WT and PGP null mice.

Understanding that AAH8 induced opioid receptor-mediated antinociceptive effects in a peripherally mediated model of pain, we were interested in assessing the constipation effects of AAH8 following different routes of administration. AAH8 (10mg/kg) was shown to induce constipation in WT mice[5]. We chose to assess an AAH8 dose-response curve compared to morphine and interestingly, AAH8 was approximately 20-fold more potent than morphine at inducing constipation effects. These results were unexpected, given the equipotent antinociceptive effects in the AASA. However, these data are consistent with the *in vitro* evaluation of AAH8, where AAH8 is approximately 100-fold more potent at activating the MOR than morphine. When administered IP, AAH8 was more potent than AAH8 (SC) at producing constipation effects, but why this was the case was puzzling. AAH8 includes a THQ core within the structure that has been shown to exhibit significant CYP3A4 metabolism[35], an enzyme predominately found in the liver, with small traces in the small intestines. Drugs administered IP are subject to extensive first-pass metabolism within the liver via absorption through the hepatic portal system[36], whereas SC administration bypasses first-pass metabolism. This would suggest AAH8 would undergo more significant metabolism following IP than SC; however, this contradicts the constipation data collected here. Another rationale is potential absorption issues from the injection site. Following SC administration, less drug reaches circulation, including the site of action in the GI tract; therefore, larger doses of AAH8 would be needed to produce constipation following SC administration. Future studies should evaluate systemic AAH8 concentrations following IP and

SC administration to assess if the metabolism of AAH8 differs. Additionally, studies should evaluate the metabolism and pharmacokinetics of AAH8 between different routes of administration to improve metabolic stability of future ligands.

Elucidation of Additional Mechanisms Involved in Opioid-Induce Tolerance

Much research has exposed mechanisms involved in opioid tolerance[37-39]. *In vitro* models have been instrumental in elucidating receptor effects that contribute to tolerance development. Cells exposed to prolonged morphine show morphine-induced receptor desensitization, and these results have been recapitulated in *ex vivo* studies looking at various brain regions[40,41,42,43]. Opioid receptor downregulation has been shown following chronic opioid exposure and is proposed to be involved in tolerance. *Ex-vivo* studies using beta-arrestin-2 KO mice showed a disruption in morphine-induced tolerance and resensitization[44]. These effects were recapitulated in the same strain of mice where tolerance development following repeated administration of morphine was diminished in beta-arresting-2 KO mice compared to WT controls[45]. Further, the use of *in vivo* models has also aided in elucidating tolerance mechanisms. Beta-arrestin-2 proteins are crucial for regulating agonist-mediated G-protein coupled receptor (GPCR) signaling; both by mediating receptor desensitization and receptor resensitization[34], which are both thought to be critical for tolerance development. Additionally, MOR residing in the DRG exhibited attenuated morphine-induced decreases in voltage-gated calcium currents following repeated administration[46], but it is unknown if this effect leads to any physiologically relevant behavioral effect such as tolerance development to the antinociceptive effects of MOR agonists.

The presence of opioid receptors on peripheral nerve terminals of primary afferent neurons, and the DRG, sparked interest in using opioid agonists that lack CNS effects for treating pain[47]. If applicable, the adverse effect profile should be improved as many of the undesired effects of

opioids, such as abuse liability and respiratory depressant effects, are predominately mediated in the CNS. Although pre-clinical studies indicate peripheral opioid receptor-mediated antinociception in inflammatory models[48-52], clinical results of peripheral opioid treatment have been modest at best[53-56], and appear to be most efficacious when employed for knee pain following knee surgery, or local infiltration following dental surgery[57]. Although relatively effective in some pain cases, peripheral opioid use may not be sustainable when chronically used because tolerance development may be inevitable.

A recent study suggested antagonism of peripheral opioid receptors on primary afferent neurons may be sufficient for attenuating morphine-induced tolerance[58], in the absence of pain and in a model of chronic neuropathic pain. Therefore, we continued this evaluation by assessing peripheral opioid receptor involvement in the development and attenuation of tolerance to the centrally-mediated antinociceptive effects of MOR agonists. I show, in this thesis, as have many others, that tolerance to the antinociceptive effects of morphine occur following repeated administration. 10 mg/kg morphine was administered 3 time daily, for five days and the morphine dose-effect curve was assessed before and after repeated administration. In both thermal and inflammatory pain models, the non-selective peripherally restricted opioid antagonist, naloxone-methiodide, fully attenuated morphine-induced tolerance. These results align with those shown by Corder et al., 2017, and therefore we chose to assess if repeated activation of opioid receptors in the PNS was sufficient for inducing tolerance. To assess this, initial morphine dose-effect curves were established on day 1, then 3.2 mg/kg loperamide was administered 4 times daily, for 5 days. On day 6, we assessed the morphine dose-effect curve again in the same group of animals. Repeated administration of low dose loperamide (3.2mg/kg) induced tolerance and cross-tolerance to the antinociceptive effects of morphine. These results are the first to show that centrally-

mediated antinociception can be disrupted through chronic activation of opioid receptors in the PNS. The underlying mechanism causing the rightward-shift in the morphine dose effect curve is unknown; however, it may be that chronic activation of opioid receptors in the PNS induce alterations in opioid receptor function and signaling in the CNS. For example, following systemically administered chronic morphine in mice, MORs in the PAG and in the DRG display decreased morphine-induced GIRK inhibition compared to vehicle control mice, a canonical mechanism involved in opioid tolerance. It would be interesting to assess morphine-induced GIRK inhibition and cAMP inhibition in PAG neurons vs. DRG neurons of mice chronically administered loperamide. This would begin to elucidate the downstream effects of chronic loperamide. Nonetheless, to further confirm our results are due to activation of peripheral opioid receptors, we demonstrated that naloxone-methiodide blocks loperamide-induced cross-tolerance to morphine. However, which opioid receptor is mediating this effect is still unknown. It would be interesting to synthesize peripherally restricted antagonists at DOR or KOR to assess the involvement of each receptor subtype in tolerance development to the centrally mediated antinociceptive effects of morphine following repeated, peripheral opioid receptor activation. Understanding which opioid receptors, and which population of opioid receptors promote tolerance development will improve our knowledge on tolerance mechanisms and potentially alter the way in which opioid-based treatment is conducted.

As mentioned previously, it is imperative to elucidate all mechanisms that contribute to opioid tolerance to improve opioid-based therapy. These studies offer evidence that usage of peripheral opioid agonists may not be the best nor safest approach and that alternative methods are required for treating pain.

Conclusion:

MOR agonists produce significant analgesic effects, but the clinical utility is hindered due to unwanted effects such as constipation, respiratory depression, abuse potential, physical dependence, and tolerance development. Many decades of research have focused on synthesizing novel, selective ligands targeting the MOR for treating clinical pain. However, with no improvements in adverse effect profiles, new approaches are required to improve opioid-based therapy. Therefore, the current studies explored the contribution of other opioid receptor types and populations of opioid receptors contributing to the acute and chronic effects of MOR agonists. Literature on mixed-efficacy MOR/DOR ligands continue to show improvements in adverse effect profiles compared to current clinically used opioids. Further, understanding that the production and attenuation of tolerance development can be disrupted through actions at opioid receptors in the periphery, these data may shed light on potential mechanisms involved in the improved adverse effect profiles of mixed-efficacy opioid ligands. For example, it would be interesting if either the DOR or the KOR in the PNS mediate tolerance to MOR agonists, as this would evolve clinical approaches for pain and minimize tolerance in the human population. Implications of targeting the DOR in the periphery may provide a more distinct explanation as to why mixed-efficacy opioid ligands display a lessened adverse effect profile. Overall, the work presented in this thesis should benefit the generation of safer analgesics and further our understanding of opioid tolerance. The possibility of attenuating the crippling adverse effects associated with opioid use would greatly benefit opioid-based treatment, fight the opioid epidemic, and potentially save the lives of those who require opioids for normal, everyday function.

Appendix

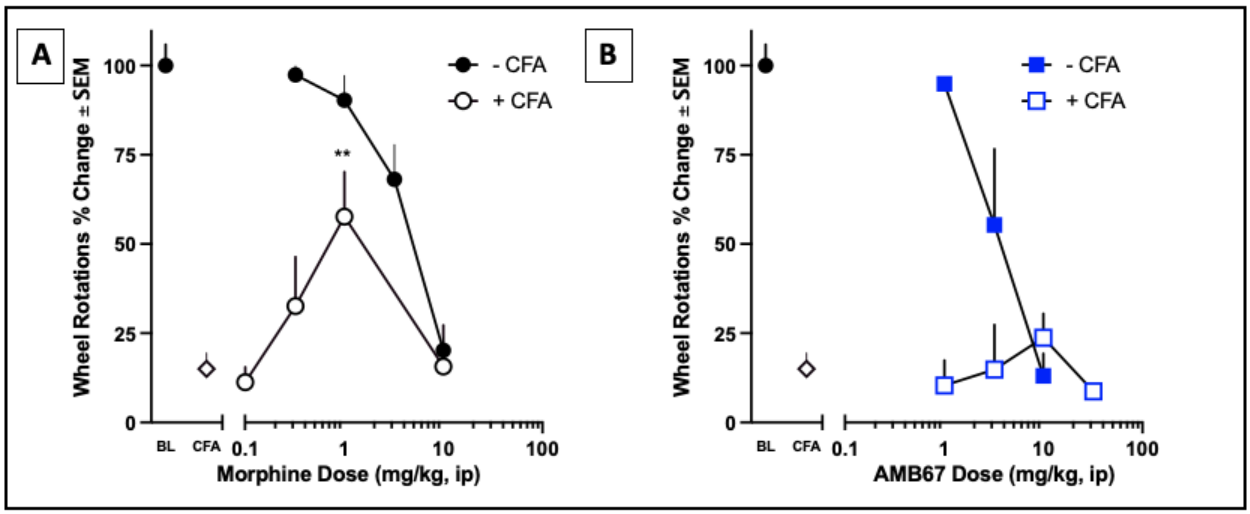

Figure 5.1 Acute Antinociceptive of AMB67 and Morphine in Pain-Depressed Wheel Running

Figure 5.1: Acute Antinociceptive of AMB67 and Morphine in Pain-Depressed Wheel Running. (A) Effects of different doses of morphine on wheel running behavior in the absence or presence of hindpaw CFA injections. (B) Effects of different doses of AMB67 on wheel running behavior in the absence or presence of hindpaw CFA injections. ** $p < 0.01$ relative to CFA.

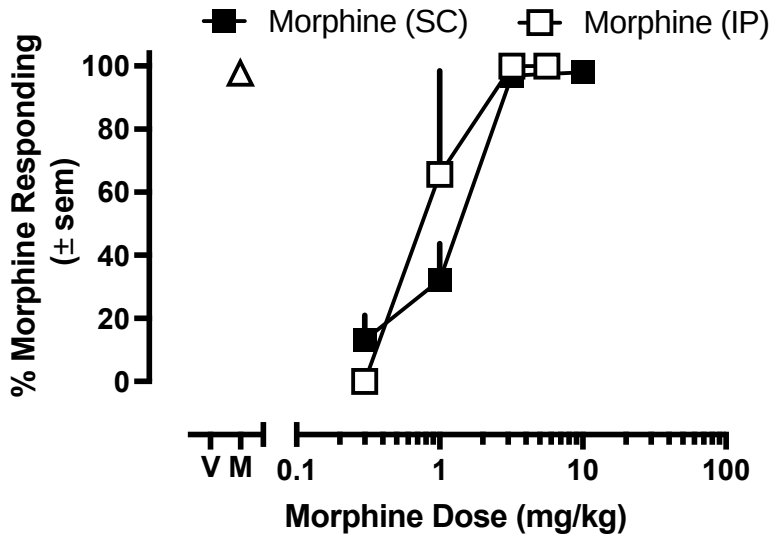

Figure 5.2 Drug Discriminative Properties of Morphine

Figure 5.2: Characterization of the Drug Discriminative Properties of Morphine Following SC or IP administration. The % morphine responding in rats trained to discriminate the subjective effects of morphine (IP) from saline (IP) was assessed following either IP or SC morphine administration.

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