12-2020

The Role of the Recently Deorphanized G-Protein Coupled Receptor, GPR171, in Morphine Tolerance and Withdrawal

Leela Afrose
Utah State University

THE ROLE OF THE RECENTLY DEORPHANIZED G-PROTEIN COUPLED RECEPTOR, GPR171, IN MORPHINE TOLERANCE AND WITHDRAWAL

by

Leela Afrose

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biology

Approved:

Erin Bobeck, Ph.D. Major Professor	Karen Kapheim, Ph.D. Committee Member
Amy Odum, Ph.D. Committee Member	D. Richard Cutler, Ph.D. Interim Vice Provost of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2020

ABSTRACT

The Role of the Recently Deorphanized G-protein Coupled Receptor, GPR171, in Morphine Tolerance and Withdrawal

by

Leela Afrose, Master of Science

Utah State University, 2020

Major Professor: Erin Bobeck, Ph.D.
Department: Biology

Mu-opioid receptor (MOPr) agonists, such as morphine and its derivatives, are the most effective and commonly used analgesics to treat acute pain. However, the utility of morphine for the treatment of chronic pain is restricted due to the development of tolerance to its analgesic efficacy over time and the development of withdrawal upon cessation of the treatment. Morphine works by activating the MOPr within the descending pain modulatory pathway, which includes the periaqueductal gray (PAG), rostroventromedial medulla (RVM), and the dorsal horn (DH) of the spinal cord to change how the body responds to pain. The PAG has been found to be especially important in the development of morphine tolerance. Our recent research has shown that a newly characterized G protein-coupled receptor, GPR171, was highly expressed in the PAG and modulated morphine antinociception and opioid signaling. The main focus of this research was to explore the functional interaction of GPR171 in the effects of morphine induced tolerance and withdrawal to evaluate this receptor's function during chronic morphine treatment. Our results demonstrate that activation of GPR171 with an agonist attenuates morphine tolerance in female mice. Most importantly, GPR171 agonist in combination with morphine does not exacerbate morphine induced tolerance and withdrawal during long term morphine treatment. Taken together,

the results of this study suggest that this drug combination could possibly be used as a potential pain therapeutic for chronic pain, especially for females.

(62 pages)

PUBLIC ABSTRACT

The Role of the Recently Deorphanized G-protein Coupled Receptor, GPR171, in Morphine Tolerance and Withdrawal

Leela Afrose

Opioid analgesics, such as morphine, represent the gold standard pain killer and the most frequently used drugs for the treatment of moderate to severe pain. Despite being a potent analgesic, morphine has unwanted and dangerous side effects with repeated use, such as tolerance and withdrawal. Tolerance is a state when a person no longer responds to a drug and a higher dose is required to achieve the same initial pain relief. Withdrawal is a set of undesirable psychological and physiological symptoms that occur after someone stops taking a drug or reduces the dose. Morphine tolerance and withdrawal play a vital role in the development of opioid addiction. One of the crucial goals to reduce opioid addiction is to develop pain therapeutics for chronic pain with high efficacy and reduced side effects. Despite centuries of extensive research, the existing treatments for chronic pain have met with limited success and developing better and alternative therapies are urgently needed. A novel G-protein coupled receptor, GPR171, is found to be highly expressed throughout the pain modulating regions of the brain. Our previous study found that activating this receptor with an agonist, enhances morphine's pain relieving property in combination therapy during acute treatment in mice. In this study, we investigated the effects of activating this receptor during long-term morphine treatment to evaluate tolerance and withdrawal. Our results demonstrate that, activating this receptor reduces morphine induced tolerance in female mice (but not males) on a thermal pain test and it does not have any additional adverse effects on morphine tolerance and withdrawal syndrome. These results suggest the potentiality of GPR171 as a novel pain therapeutics in combination with morphine with

enhanced efficacy and reduced tolerance and dependence for the treatment of chronic pain, especially for females.

To my parents....

ACKNOWLEDGMENTS

I would like to extend my sincere gratitude to my advisor Dr. Erin Bobeck for her directions, insightful discussions, tolerance and encouragement that led to the success of this project. I have been fortunate enough to have her personal and professional guidance and mentorship throughout my graduate study. Thank you, Erin for choosing me as your first graduate student. I am grateful to Dr. Amy Odum and Dr. Karen Kapheim for serving as my committee members and for their professional guidance and valuable comments to enrich this research work.

Thanks to all my colleagues at USU Biology Department for their wonderful collaboration. I strongly thank Max McDermott and Akila Ram for their continuous support throughout my stay in the research group. I am grateful to the Bobeck Lab of Utah State University for the academic and financial support which made the completion of my Masters program possible.

Finally, I would like to thank my parents and my siblings for their unconditional love and support throughout my graduate school journey. A special thanks to my husband, who believes in me more than I believe in myself.

Leela Afrose

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACRONYMS

cAMP	Cyclic Adenosine Monophosphate
DH	Dorsal Horn
DMSO	Dimethyl Sulfoxide
DOPr	Delta Opioid Receptor
ED50	Median Effective Dose
ERK1/2	Extracellular-Signal Regulated Kinase 1/2
FDA	Food and Drug Administration
GPCR	G-Protein Coupled Receptor
GPR171	G-Protein Coupled Receptor 171
IASP	International Association for the Study of Pain
MOPr	Mu Opioid Receptor
PAG	Periaqueductal Gray
PBS	Phosphate Buffered Saline
RVM	Rostroventromedial Medulla
TNFα	Tumor Necrosis Factor alpha

CHAPTER 1

INTRODUCTION

1.1 Chronic Pain

Chronic pain costs approximately $635 billion annually which is greater than the combined annual costs of heart disease, cancer, and diabetes in the United States (Gaskin & Richard, 2012). Approximately 11% of the U.S. population suffer from daily pain (Nahin, 2015). In addition, pain is more prevalent in vulnerable subpopulations, including the women, elderly, and socioeconomically challenged population (Green, Baker, Smith, & Sato, 2003). Chronic pain is associated not only with the complex web of heterogeneous illnesses and injuries, but also with the treatment of disease. For example, most patients with chronic pain related to cancer have pain related to chemotherapy, radiation therapy, and surgery. Besides physical suffering, chronic pain patients suffer psychologically, emotionally, and socially because of poor quality of life, and lost productivity. Management of chronic pain is considered as one of the pivotal issues in public healthcare with currently available analgesics because of its unclear etiology (Cherubino, Sarzi-Puttini, Zuccaro, & Labianca, 2012). Despite opioids being commonly prescribed, unwanted side effects, poor analgesic efficacy over time and the growing opioid epidemic limit the effectiveness of opioid analgesics specially for chronic pain. Our current health care system struggles with uncertainties when treating a chronic pain patient in the face of opioid epidemic as well as the morbidity and mortality associated with opioid overdose. Creating safer and better alternative pain therapeutics than opioids is a dire need.

1.2 G Protein-Coupled Receptors as Pain Therapeutics

Around 35% of all FDA approved drugs act by targeting the G protein-coupled receptors (GPCRs) (Insel et al., 2019). GPCRs are considered to be therapeutic targets due to

their high number of cell surface expression and their ability to modulate many pathophysiological processes, including pain. Their critical roles in processing physiological functions makes them an attractive target for developing pain medication. Studies have shown that there are at least 40 members of the GPCR family that are considered to be potential therapeutic targets for the regulation of pain (Stone & Molliver, 2009). The agonists of Mu-opioid receptor (MOPr), which is a G protein-coupled receptor, have been used for decades as an effective pain killer. However, side-effects associated with their use limit the clinical benefits of these drugs. Some of the most advanced efforts to develop safer pain treatments have been focused on alternative GPCR targets, for example cannabinoids. Orphan receptors, a class of G protein-coupled receptors with unknown endogenous ligands, are an unexplored avenue for developing new therapeutics (Nourbakhsh, Atabaki, & Roohbakhsh, 2018). Recently it has been identified that ProSAAS derived peptide, BigLEN, is the endogenous ligand for the previously orphan G protein-coupled receptor, GPR171 (Gomes et al., 2013). Various smaller peptides including little SAAS, PEN and BigLEN are derived from ProSAAS (Mzhavia et al., 2002). ProSAAS is one of the most abundant pro-peptides in the brain (Fricker, 2010) and has been implicated in a wide range of functions (Wei et al., 2004; D. J. Morgan et al., 2010; Hoshino et al., 2014). Recently, ProSAAS was found to be upregulated in cerebrospinal fluid in fibromyalgia patients (Khoonsari et al., 2019) indicating that it may be involved in chronic pain. Little is known about the BigLEN-GPR171 neuropeptide receptor system's function except for its role in anxiety (Bobeck et al., 2017) and feeding behaviors (Wardman et al., 2016). In addition, our recent study found that GPR171 modulates opioid induced signaling and antinociception (McDermott et al., 2019). The goal of this current study was to evaluate the effect of GPR171 activation on morphine tolerance and withdrawal to determine the potentiality of this receptor for developing new therapeutics to treat pain.

1.3 Descending Pain Pathway and Opioid Function

The descending pain modulatory pathway includes the periaqueductal grey (PAG), the rostral ventromedial medulla (RVM), and the dorsal horn (DH) of the spinal cord (Lueptow,

Fakira, & Bobeck, 2018). These structures together take part in a mechanism through which cortical and subcortical sites modulate nociception. Majority of the analgesic drugs, including opioids target the descending pain modulatory system to relief pain (Ossipov, Dussor, & Porreca, 2010). The PAG integrates information received from higher centers of the brain and is capable of activating a powerful analgesic effect. The RVM, on the other hand, facilitates or inhibits nociceptive inputs and acts as the final relay station in the control of descending pain modulation (Ossipov, Morimura, & Porreca, 2014). Opioid analgesics, such as morphine, produce their antinociceptive effects in part by inhibition of neurons projecting from the PAG to the RVM, which in turn inhibits incoming pain signals at the dorsal horn of the spinal cord (Heinricher, Tavares, Leith, & Lumb, 2009; Lau & Vaughan, 2014). Many studies have highlighted the importance of the PAG in opioid antinociception, tolerance, and withdrawal (Behbehani, 1995; Hao et al., 2011; Bobeck, Haseman, Hong, Ingram, & Morgan, 2012). Chronic morphine produces downstream effects and neuroadaptations along the descending pain pathway (Vanderah et al., 2001). The Allen Brain Atlas shows high mRNA expression of both the BigLEN peptide and GPR171 in the descending pain modulatory pathway including the PAG. In addition, we found that GPR171 is expressed in the PAG using immunohistochemistry (McDermott et al., 2019) The ubiquitous expression of this neuropeptide and receptor in the descending pain pathway make it likely for it to regulate morphine antinociception, tolerance and withdrawal.

1.4 Opioid Tolerance and Withdrawal

Despite being one of the most potent analgesics to date, morphine produces tolerance and withdrawal syndromes when used repeatedly for a long period of time. Tolerance is a state when a person stops responding at the current dose after repeated exposures and a higher dose of the drug is needed to produce the same initial effects, resulting in dose escalation (Chu, Clark, & Angst, 2006). Preclinical studies have established that chronic administration of morphine produce a progressive increase in the ED50 over time (Way, Loh, & Shen, 1969). In clinical settings, more than 10-fold dose escalations of opioid dose are reported to treat chronic pain patients (Buntin-Mushock, Phillip, Moriyama, &

Palmer, 2005). Opioids should be administered in an amount sufficient to achieve analgesia, however, greater adverse effects are associated with a higher dose which poses a challenge for the clinicians. One theory of morphine tolerance is linked to regulations of MOPr desensitization, internalization, and downregulation (Al-Hasani & Bruchas, 2011; Williams et al., 2013; Allouche, Noble, & Marie, 2014). It is well established that chronic morphine treatment upregulates the cAMP-PKA signaling and leads to adaptations in many signaling proteins within the descending pain modulatory pathway (Sharma, Klee, & Nirenberg, 1975; Guitart & Nestler, 1989; Gintzler, Chakrabarti, & Dray, 2004). Despite significant amount of research, the cellular and molecular mechanisms mediating the development of opioid tolerance remain unclear and controversial.

Prolonged use of morphine in higher doses develops physical and psychological dependence. Once the users stop taking morphine, they go through a severe withdrawal and want to keep using morphine to feel normal. This is one of the reason people misuse morphine and become addicted (Burma, Kwok, & Trang, 2017). The severity of opioid dependence and withdrawal syndromes are considered as one of the major contributors to the addictive potential of opioid narcotics. Once someone become dependent to morphine, withdrawal syndrome is observed spontaneously upon the cessation of morphine after chronic use or precipitated through the administration of an opiate antagonist both in human and rodents. The symptoms develop within 24 hours of the cessation of morphine and persist for a week or longer in human. The signs and symptoms of withdrawal in humans include diarrhea, increased heart rate and blood pressure, insomnia, anxiety, irritability and stomach and muscle cramps (Kosten & George, 2002) whereas in rodents jumping, diarrhea, weight loss, paw tremors, wet-dog shakes, hyper-reactivity, teeth chatter, abrupt weight loss, ptosis, and lacrimation behaviors are observed (Laschka, Teschemacher, Mehraein, & Herz, 1976). To assess the ability of different pharmacological manipulations to alleviate the presence or intensity of these signs and symptoms are used as a measure of their potential in the treatment of opiate dependence.

1.5 Morphine Tolerance and Withdrawal in the PAG

Several previous studies have demonstrated that after chronic exposure to morphine, tolerance and dependance develop as a result of multiple signaling proteins and neural system adaptation. For review see (Lueptow et al., 2018). Morphine induced tolerance is largely linked to changes in the GABAergic neurons within the PAG (M. M. Morgan, Clayton, & Lane, 2003). Repeated microinjection of morphine into the PAG develops tolerance (M. M. Morgan, Clayton, & Boyer-Quick, 2005) and blocking of mu opioid receptors within the ventrolateral PAG inhibit the development of morphine tolerance (Lane, Patel, & Morgan, 2005). Another study observed that repeated microinjection of morphine within the ventrolateral PAG caused a significant increase in Extracellular-Signal Regulated Kinase 1/2 (ERK1/2) and enhanced the development of morphine tolerance (Macey et al., 2009). Inhibition of adenylyl cyclase within the ventrolateral PAG leads to a reduction in morphine tolerance (Bobeck, Chen, Morgan, & Ingram, 2014). These findings suggest that the PAG is particularly important in the regulation of morphine tolerance.

In rodent models, several brain regions are implicated to the physical signs of opiate withdrawal including the PAG, locus coeruleus, nucleus accumbens, ventral tegmental area, hypothalamus, amygdala and the spinal cord (Couceyro & Douglass, 1995; McPhie & Barr, 2009). Among the brain regions implicated in opiate dependence and withdrawal, the role of the ventrolateral PAG appears to be one of the critical areas in the expression of many withdrawal signs evident by biochemical and in vivo studies (Maldonado, Stinus, Gold, & Koob, 1992; Stornetta, Norton, & Guyenet, 1993; Chieng, Keay, & Christie, 1995). A number of studies show alterations in c-fos immunoreactivity (Chieng et al., 1995) and changes in intracellular recordings into the ventrolateral PAG after opioid withdrawal (Chieng & Christie, 1996). The Tumor Necrosis Factor alpha (TNFα) and glial activation within the PAG also take part in modulating withdrawal responses as has been reported by others (Hao et al., 2011). Taken together, these data suggest that the PAG performs a key role in the development of morphine tolerance and withdrawal.

1.6 Sex Differences in Pain, Opioid Analgesia, Tolerance and Withdrawal

There is increasing evidence that chronic pain is more prevalent among female compared to male patients due to biological, psychological and sociocultural factors (Fillingim, King, Ribeiro-Dasilva, Rahim-Williams, & Riley, 2009; Bartley & Fillingim, 2013). A number of studies have demonstrated that females have greater pain sensitivity and lower response of analgesic drugs compared to males in both human and rodents (Cicero, Nock, & Meyer, 1996; Bartok & Craft, 1997; Wiesenfeld-Hallin, 2005). Several rodent studies have shown that male rodents produce greater stress-induced analgesia and tend to have a higher response to MOPr agonists, such as morphine, compared to female rodents (Cicero et al., 1996; Zubieta et al., 2002). Male rats show higher levels of MOPr expression and binding than female rats within the PAG (Loyd, Wang, & Murphy, 2008). Another article reports that females have greater morphine potency but slower onset and offset of analgesic effect compared to males (Sarton et al., 2000). In addition, emerging evidence suggests that sex hormones have numerous influences in pain sensitivity. Sex hormones and hormonal levels are implicated in pain sensitivity and opioid tolerance with different stages of the menstrual cycle (Kepler, Kest, Kiefel, Cooper, & Bodnar, 1989). A number of preclinical studies have shown that males develop greater tolerance to morphine than females on the hot plate (Badillo-martinez, Kirchgessner, Butler, & Bodnar, 1984), and tail flick (Mousavi, Shafaghi, Kobarfard, & Jorjani, 2007). In addition, males have higher precipitated withdrawal scores than females, indicating greater morphine dependence in males than females (Kasson & George, 1984; Craft, Stratmann, Bartok, Walpole, & King, 1999). Research on sex differences in morphine tolerance and withdrawal is still very limited and further study is necessary to explain sex differences in morphine tolerance and dependence. Additional exploration of these sex specific observation is required to determine these disparities in gender biased pain mechanisms.

1.7 Purpose of Study

The goal of this research was to investigate the interactions of GPR171 and the opioid system by using GPR171 small molecule ligands that were characterized previously using

homology modeling and virtual screening (Gomes et al., 2013; Wardman et al., 2016; Bobeck et al., 2017). Our previous data shows that GPR171 agonist, MS15203, and antagonist, MS21570, do not have any antinociceptive effects on their own in male mice. In addition, the GPR171 antagonist, MS21570, in combination with morphine causes a reduction in antinociception and the agonist, MS15203, enhances morphine antinociception on the hot plate and tail flick tests compared to morphine alone (McDermott et al., 2019). In this study, we sought to investigate the cellular distribution of GPR171 within the PAG using immunohistochemistry and the functional interaction of the GPR171 in morphine induced tolerance and withdrawal in vivo. This study addressed three distinct levels of characterization: first, we sought to determine the colocalization of this receptor within different neuronal subtypes in the PAG and our results show that the GPR171 is primarily found in GABAergic neurons. Second, we evaluated the behavioral effects of this receptor agonist in morphine tolerance by using the hot plate and tail flick behavioral assays in vivo and found that this GPR171 agonist attenuates morphine tolerance in female mice on the tail flick test and has no effect in male mice. Third, we set out to consider the role of GPR171 on naloxone-precipitated withdrawal and our data reveals that this receptor has no influence on morphine dependence and withdrawal. Taken together, these data suggest the possibility of the GPR171 agonist to be safely combined with morphine treatment to reduce the dose of morphine, therefore, making it a new novel area of study towards the development of pain therapeutics with reduced side effects and abuse liability. The outcome of this study is clearly an important step towards decoding this receptor's interaction in opioid function and the development of better and safer pain therapeutics and pain management modalities for long term pain treatment[1].

[1]Part of this thesis has been published in (McDermott et al., 2019)

CHAPTER 2

MATERIALS AND METHODS

2.1 Subjects

Male and female C57BL/6 mice ($n = 236$) (Charles River Laboratories, CA) were used. Mice were 6-13 weeks old and weighing 13-27g at the beginning of the experiment. Mice were housed (4–5 per cage) in a humidity and temperature-controlled room with a 12:12 hour light/dark cycle (on 0700–1900) hours. Mice were handled for three days prior to testing. All procedures were conducted in accordance with the guidelines by International Association for the Study of Pain (IASP) and approved by the Utah State University Institutional Care and Use Committee (Protocol #2775).

2.2 Distribution of GPR171 in the PAG

2.2.1 Fluorescence Immunohistochemistry

Male C57BL/6 mice ($n = 5$) were used. Immunohistochemistry was performed as described previously (Wardman et al., 2016; Bobeck et al., 2017). Briefly, mice were deeply anesthetized with Isoflurane and perfused transcardially through the ascending aorta with 4% paraformaldehyde. Collected brain tissues were postfixed for 1 hour and then stored in 1X PBS. Immunohistochemistry was performed on free-floating coronal cut brain tissues (50 μm) containing PAG. Sections were incubated in 1% sodium borohydride in 1X PBS for 30 min followed by blocking buffer (5% normal goat serum and 0.3% Triton X-100 in 1X PBS) at room temperature for 1 hour. Tissues were incubated overnight at 4°C in primary antibodies against GPR171 (GeneTex, Irvine, CA) (rabbit, 1:400), glutamatergic neurons (contain vglut2 immunoreactivity) (Millipore, Temecula, CA) (guinea pig, 1:500), GABAergic neurons (contain GAD67 immunoreactivity) (Millipore, Temecula, CA) (mouse, 1:500), 1% Bovine Serum Albumin (Sigma-Aldrich) and 0.1% Triton X-100 (Sigma-Aldrich).

Primary antibodies were visualized with goat anti-rabbit 594, goat anti rabbit 488, goat anti-mouse 488 and goat anti-guinea pig 647 (Invitrogen, 1:1000) followed by 5 min of incubation with the nuclear stain DAPI (100 ng/ml). Sections were then mounted with ProLong Diamond Antifade (Invitrogen) (McDermott et al., 2019).

2.2.2 Microscopy and Image Analysis

Antibodies were detected by confocal microscopy at the Utah State University Microscopy CORE using Zeiss 710 Confocal Microscope by an experimenter blinded to the experimental conditions. Images were processed using ImageJ (NIH), Excel (Microsoft) and Prism software (version 7.0; GraphPad Software).

2.3 Role of GPR171 in Acute Morphine Administration

2.3.1 Drug Treatments

Male and female C57BL/6 mice (n = 119) were used. GPR171 agonist, MS15203 (10mg/kg, i.p., ChemBridge Co, and a gift from Sanjai Pathak, Queens College, NY), GPR171 antagonist, MS21570 (5 mg/kg, i.p.; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), were diluted in 10% DMSO (Sigma- Aldrich, St. Louis, MO) in Saline. Morphine Sulfate (2 or 5mg/kg s.c., Hikma, London, UK) was suspended in 0.9% Saline. All drugs were administered at a volume of 10 ml/kg. These doses were chosen based on previous studies (Wardman et al., 2016; Bobeck et al., 2017). Mice were randomly divided into 8 groups: Vehicle + Morphine (5mg), Vehicle + Morphine (2mg), Vehicle + Saline, MS15203 + Saline, MS15203 + Morphine (5mg), MS15203 + Morphine (2mg), MS21570+Morphine (5 mg), MS21570 + Saline group. A power analysis was conducted to determine a sample size of $n = 8$ based on preliminary studies (McDermott et al., 2019).

2.3.2 Time Course Paradigm

Mice were injected with MS15203 (10 mg/kg, i.p.), MS21570 (5 mg/kg, i.p.), or 10% DMSO (10 ml/kg, i.p.) 10 minutes prior to injections of morphine (2 or 5 mg/kg, s.c.) or

saline (10 ml/kg, s.c.). All animals were tested on hot plate and tail flick tests prior to any drug administration to assess baseline scores, and then at 15, 30, 60, and 120 minutes after the second drug injection of morphine or saline (McDermott et al., 2019).

2.4 Role of GPR171 in Chronic Morphine Administration

2.4.1 Drug Treatments

Male and female C57BL/6 mice ($n = 59$) were used. GPR171 agonist, MS15203 (10mg/kg, i.p.), GPR171 antagonist, MS21570 (5 mg/kg, i.p.) were diluted in 10% DMSO in Saline. Morphine sulfate (5mg/kg s.c.) was suspended in 0.9% saline. Mice were randomly divided into 6 groups: DMSO + Morphine, DMSO + Saline, MS15203 + Saline, MS15203 + Morphine, MS21570 + Morphine, MS21570 + Saline group. A power analysis was conducted to determine a sample size of $n = 8$ based on preliminary tolerance studies.

2.4.2 Tolerance Induction and Dose Response Paradigm

On day 1, mice were injected with MS15203 (10 mg/kg, i.p.), MS21570 (5 mg/kg, i.p.), or 10% DMSO (10 mL/kg, i.p.) 10 min prior to injections of morphine (5mg/kg, s.c.) or equal volume of saline (10 mL/kg, s.c.). On Days 1-4, mice were injected with their specific drug combination twice daily (10 AM and 4 PM) to induce morphine tolerance. On Day 5, Cumulative quarter log doses of morphine (to obtain final doses of 1, 1.8, 3.2, 5.6, 10, and 18 mg/kg, s.c.) were administered at 30 min intervals, and mice were tested on the hotplate and tail flick test 15 min after morphine/saline injection. These doses and injection times were modified from known procedures that produce dose-dependent antinociception in rats (Bobeck, McNeal, & Morgan, 2009; Bobeck et al., 2012, 2014) and in mice during our preliminary studies.

2.5 Role of GPR171 in Morphine Withdrawal

2.5.1 Drug Treatments

Male C57BL/6 mice ($n = 53$) were used. GPR171 agonist, MS15203 (10mg/kg, i.p.) and GPR171 antagonist MS21570 (5 mg/kg, i.p.), was diluted in 10% DMSO in Saline. Morphine sulfate (5 mg/kg, s.c.) was suspended in 0.9% saline. Mice were randomly divided into 6 groups: DMSO + Morphine, DMSO + Saline, MS15203 + Saline, MS15203 + Morphine, MS21570 + Morphine, MS21570 + Saline group.

2.5.2 Withdrawal Paradigm

On day 1, mice were injected with MS15203 (10 mg/kg, i.p.), MS21570 (5 mg/kg, i.p.) or 10% DMSO (10 mL/kg, i.p.), 10 minutes prior to injections of morphine (5 mg/kg, s.c.) or equal volume of saline (10 mL/kg, s.c.). Mice were injected twice daily (10 AM and 4 PM) with their designated drug combination for 4 days to induce morphine dependance. On day 5, each mouse received their designated drug combination in morning only. Two hours later they were given Naloxone Hydrochloride (2 mg/kg, i.p.; Tocris Bioscience) and monitored and video recorded for counting the number of jumping for 30 min. Number of jumps were observed and counted as withdrawal behavior. Jumping is considered as a well-documented withdrawal behavior in mice (Kest et al., 2002).

2.6 Behavioral Tests

2.6.1 Hot Plate Test

Nociception was assessed using the hot plate (Harvard Apparatus, Holliston, MA). The hot plate test involves higher brain function and is considered to be a supraspinally organized response. We measured the latency for the mice to lick the hind paw when placed on a 50°C hot plate. To avoid tissue damage, mice were removed from the hot plate if no response occurs within 60s.

2.6.2 Tail Flick Test

Thermal nociception was assessed with tail flick, warm water bath (Thermo Fisher

Scientific) test. Tail flick test was conducted to observe spinal reflex. We measured the tail-withdrawal latency of the mice using a 52°C water bath. Cut off time was 20s to avoid tissue damage.

2.7 Data and Statistical Analyses

Statistical analyses of data were conducted by using Microsoft Excel and Prism software (version 7.0; GraphPad Software). Data were generated by one-way or two-way ANOVA (repeated measures), when appropriate using Prism software. Dunnett's or Tukey's honestly significant difference post hoc tests were conducted to make pairwise comparisons.

CHAPTER 3

RESULTS

3.1 Distribution of GPR171 in the PAG

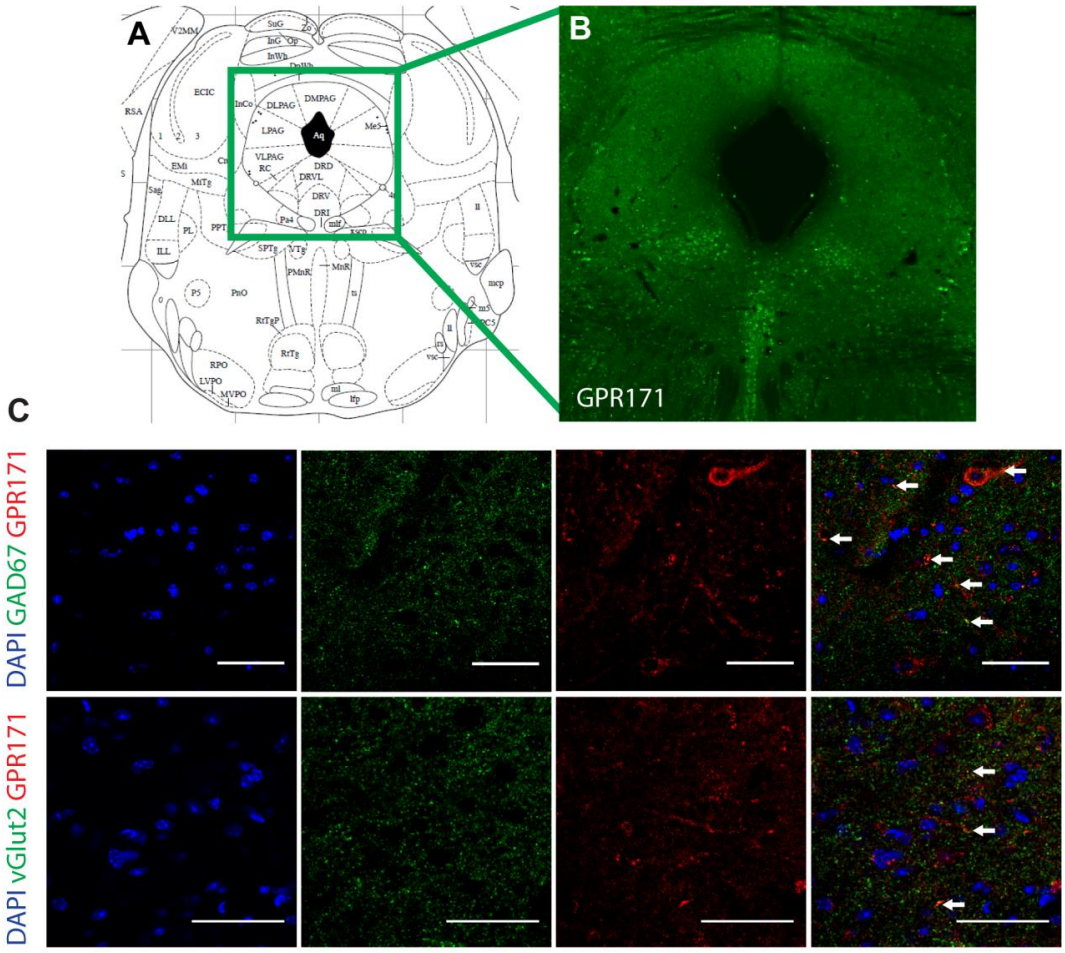

Fig. 3.1: GPR171 expression in different neuronal populations. (A) Image from mouse brain atlas indicating location of PAG adapted from (Paxinos & Franklin, 2001). (B) Immunohistochemistry shows high expression of GPR171 in the PAG. (C) Immunohistochemistry results show that GPR171 is colocalized with GAD67 (i.e., GABA neurons). There are fewer cells that show colocalization of GPR171 and vGlut2. Data are representative of two sections from four mice. Arrows indicate colocalization. Scale bars, 50 mm (McDermott et al., 2019).

We performed free floating fluorescence immunohistochemistry to investigate the expression of GPR171 within the PAG. We found that GPR171 is highly expressed in all areas of PAG, especially the ventrolateral PAG, which has been implicated in pain modulation (Fig. 3.1A and B). Fig. 3.1A is adapted from (Paxinos & Franklin, 2001). Our data shows that GPR171 was primarily colocalized with GAD67 within the ventrolateral PAG. In addition, there was some colocalization of this receptor with vGLUT2 (Fig. 3.1C) (McDermott et al., 2019). This data indicates that GPR171 is predominately expressed within the GABAergic neurons within the ventrolateral PAG, it is also expressed in a subset of glutamatergic neurons.

3.2 GPR171 Ligands Do Not Produce Antinociception

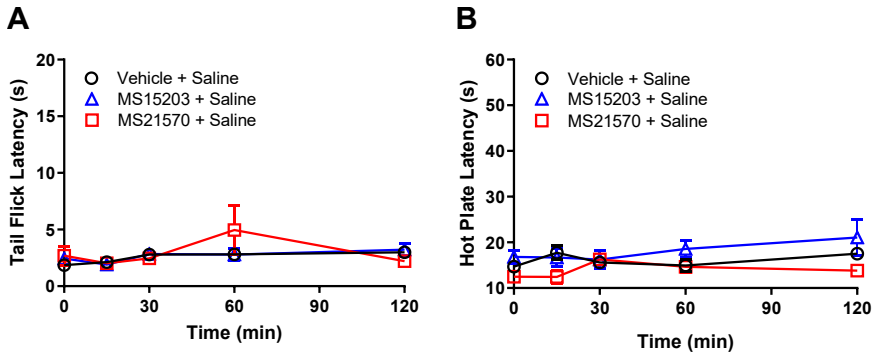

Fig. 3.2: Small molecule ligands targeting GPR171, alone do not produce antinociception in males. GPR171 agonist (MS15203; 10 mg/kg, i.p.), GPR171 antagonist (MS21570; 5 mg/kg, i.p.), or vehicle (10% DMSO in saline, i.p.) were administered to mice and then tested on the warm water (52°C) tail flick assay (A) or hot plate (50°C) assay (B) at 15, 30, 60, or 120 minutes. The data reveal no change in nociception on either test. Data are the means ± S.E. of 8–12 animals/group (McDermott et al., 2019).

There was no statistically significant difference in the baseline tail flick [$F(7, 79) = 1.16, P = 0.338$] or hot plate [$F(7, 79) = 0.877, P = 0.528$] latencies between the groups prior to starting the experiment. Administration of MS15203 + Saline or MS21570 + Saline were not significantly different from the Vehicle + Saline group [$F(2, 25) = 0.309, P = 0.737$] at any time point [$F(4, 100) = 2.91, P = 0.253$] on the tail flick test (Fig. 3.2A). Simi-

larly, MS15203 and MS21570 groups were not different from the control group [$F(2, 25) = 2.98, P = 0.069$] at any time point [$F(4, 100) = 1.55, P = 0.194$] on the hot plate test (Fig. 3.2B). These data suggest that GPR171 agonist or antagonist alone does not produce antinociception (McDermott et al., 2019).

3.3 GPR171 Ligands Alter Morphine-Induced Antinociception

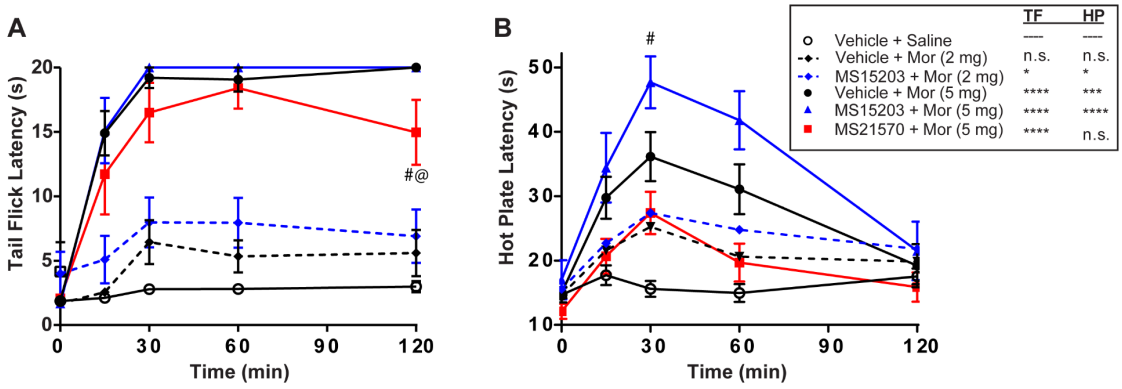

Fig. 3.3: GPR171 ligands alter morphine antinociception in male mice. GPR171 agonist (MS15203; 10 mg/kg, i.p.), GPR171 antagonist (MS21570; 5 mg/kg, i.p), or vehicle (10% DMSO in saline, i.p.) were administered 10 minutes prior to saline (10 ml/kg) or morphine (Mor; 2 or 5 mg/kg, s.c.). Antinociception was evaluated at 15, 30, 60, or 120 minutes on the warm water (52°C) tail flick assay (A) or hot plate (50°C) assay (B). GPR171 agonist, MS15203, does not alter morphine (5 mg)-induced antinociception while GPR171 antagonist decreased antinociception as measured on the tail flick assay. MS15203 increased antinociception following the lower dose of morphine (2 mg). (B) On the hot plate test, MS15203 increased 5 mg morphine-induced antinociception at 15, 30, and 60 minutes, while MS21570+Mor (5 mg) does not induce antinociception greater than saline controls. MS15203+Mor (2 mg) produced significant antinociception at 30 minutes, whereas Mor (2 mg) did not. Inset: Dunnett's post hoc to evaluate main effect compared with saline. *P< 0.05; ***P< 0.001; ****P< 0.0001, compared with saline; #P< 0.05, compared with morphine; @$P < 0.05$, compared with morphine + agonist. n.s., Not significant; HP, hot plate; TF, tail flick. Data are the means ± S.E. of 8–15 animals/group (McDermott et al., 2019).

On the tail flick test in male mice, there was no statistically significant difference in the baseline tail flick latencies (Tukey's, $P > 0.05$) between any group before starting the

experiment. All morphine treated groups showed increased latencies [$F(5, 65) = 29.12, P < 0.0001$] across all time points [$F(4, 260) = 122.5, P < 0.0001$] compared to the saline treated groups (Fig. 3.3A). There was an overall significant main effect between Vehicle + Morphine (5 mg), MS15203 + Morphine (5 mg), MS15203 + Morphine (2 mg), and MS21570 + Morphine (5 mg) compared with Vehicle + Saline treated mice (Dunnett's, $P < 0.05$). The agonist, MS15203 + Morphine (5 mg) group was not significantly different than the Vehicle + Morphine (5 mg) group on the tail flick test at any time point (Tukey's, $P > 0.05$). Given that the 5 mg morphine dose produced a maximal antinociceptive effect on the tail flick test, another group of animals received 2 mg morphine in combination with MS15203. MS15203 + Morphine (2 mg) group showed an increase in antinociception on the tail flick test compared with saline group (Dunnett's, $P < 0.05$), whereas Vehicle + Morphine (2 mg), did not produce significant anticonception compared with saline group (Dunnet's, $P > 0.05$). On the other hand, the antagonist, MS21570 + Morphine (5 mg) caused a significant reduction in morphine antinociception compared with Vehicle + Morphine (5 mg) group at the 120-minute time point (Tukey's, $P < 0.05$). (Fig. 3.3A).

A similar finding is seen in male mice on the hot plate test. There was no statistically significant difference in the baseline hot plate latencies (Tukey's, $P > 0.05$) between any group before starting the experiment. Morphine treated groups showed increased hot plate latencies [$F(5, 65) = 8.94, P < 0.0001$] over time [$F(4, 260) = 35.51, P < 0.0001$] (Fig. 3.3B). There was an overall significant main effect between Vehicle + Morphine (5 mg), MS15203 + Morphine (5 mg) and MS15203 + Morphine (2 mg) compared to Vehicle + Saline group (Dunnett's, $P < 0.05$). Vehicle + Morphine (5 mg) and MS15203 + Morphine (5 mg) groups showed a significant increase in hot plate latencies compared to Vehicle + Saline treated mice at the 15, 30, and 60 minute time points (Tukey's, $P < 0.05$). Importantly, MS15203 + Morphine (5 mg) treatment produced significantly greater antinociception than Vehicle + Morphine (5 mg) at 30 min time point (Tukey's, $P < 0.05$). However, MS21570 + Morphine (5 mg) was not significantly different from the Vehicle + Saline group at any time point (Tukey's, $P > 0.05$), but was significantly different from MS15203 + Morphine

(5 mg) at 15, 30, and 60 minutes (Tukey's, $P < 0.05$). Similar to the tail flick result, 2 mg morphine did not produce antinociception compared to saline controls (Tukey's, $P > 0.05$). However, MS15203 + Morphine (2 mg) produced a significant increase in hot plate latencies compared to saline controls at the 30 minute time point (Tukey's , $P < 0.05$) (Fig. 3.3B). These results indicate that GPR171 agonist increases morphine induced antinociception on the hot plate test, conversely, the antagonist reduces morphine antinociception on the tail flick and hot plate tests (McDermott et al., 2019).

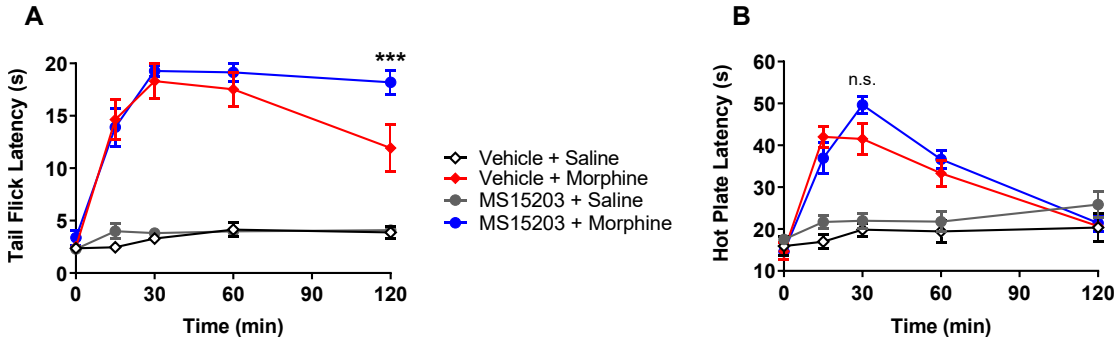

Fig. 3.4: GPR171 agonist increased morphine antinociception in female mice on the tail flick test. GPR171 agonist (MS15203; 10 mg/kg, i.p.) or vehicle (10% DMSO in saline, i.p.) were administered 10 minutes prior to morphine (5 mg/kg, s.c.) or saline (10 ml/kg, s.c.) injection. Antinociception was assessed at 15, 30, 60, or 120 minutes on the (A) warm water (52°C) tail flick assay or (B) hot plate (50°C) assay. (A) On the tail flick assay, MS15203 enhanced morphine induced antinociception at 120 min time point. (B) On the hot plate thermal assay, MS15203 induces greater, but not significant antinociception compared to morphine treated mice. ***P< 0.001, n.s., Not significant. 7-9 animals/group.

Similar results were observed in female mice. There was no statistically significant difference in the baseline tail flick and hot plate latencies (Tukey's, $P > 0.05$) between any group before starting the experiment. All morphine-treated groups showed increased latencies on the tail flick test $[F(3, 28) = 104.2, P < 0.0001]$ across time $[F(4, 112) = 47.34, P < 0.0001]$ comparing to the saline treated groups. There was an overall significant main effect between Vehicle + Morphine and MS15203 + Morphine compared with Vehicle + Saline treated mice (Dunnett's, $P < 0.05$). The agonist, MS15203 + Morphine group was significantly different from the Vehicle + Morphine group at 120 min time point

(Tukey's, $P < 0.05$). But at the 15, 30, and 60 min time point it was not significant (Tukey's, $P > 0.05$) (Fig. 3.4A) indicating that MS15203 enhanced the duration of morphine antinociception.

On the hot plate test, morphine-treated groups showed increased hot plate latencies $[F(3, 28) = 20.48, P < 0.0001]$ over time $[F(4, 112) = 40.80, P < 0.0001]$ compared to the saline treated groups. There was an overall significant main effect between Vehicle + Morphine and MS15203 + Morphine compared with Vehicle + Saline treated group (Dunnett's, $P < 0.05$). MS15203 + Morphine group showed increased, but not statistically significant antinociception at any time point (Tukey's, $P > 0.05$) compared to the Vehicle + Morphine group (Fig. 3.4B). These data suggest that GPR171 agonist enhances morphine antinociception on the tail flick but not on the hot plate test in female mice.

3.4 GPR171 Agonist Decreases Morphine Tolerance in Female on the Tail Flick Test

In order to assess the role of MS15203 on the development of morphine tolerance and to ascertain differences between groups a dose response on the tail flick and hotplate were run concurrently. Morphine produced a dose-dependent increase in tail flick latencies for all groups (Fig. 3.5A). Mice treated with twice-daily injections of morphine for 4 days were tolerant as seen by a rightward shift in the dose response curve compared to saline treated mice on the tail-flick test in both female $[F(3, 172) = 6.392, P < 0.0001]$ (Fig. 3.5A, Table 3.1). and male mice $[F(3, 166) = 38.40, P < 0.0001]$ (Fig. 3.5B, Table 3.1). Pretreatment with MS15203 + Morphine significantly reduced ED50 values compared to DMSO + Morphine group in female mice on the tail flick test which reveals a reduction in the development of tolerance (Fig. 3.5A; see Table 3.1 to compare ED50 values). Conversely, pretreatment with MS15203 + Morphine did not alter tolerance to morphine compared to DMSO + Morphine group in male mice on the tail flick test (Table 3.1). MS15203 + Saline and DMSO + Saline groups were not different in female mice. Notably, MS15203 + Saline group caused a rightward shift in the morphine dose response curve compared to DMSO + Saline group in male mice (Fig. 3.5B), suggesting that repeated injection of MS15203 alone reduces morphine

antinociception in male but not in female mice. ED50s are presented in Table 3.1.

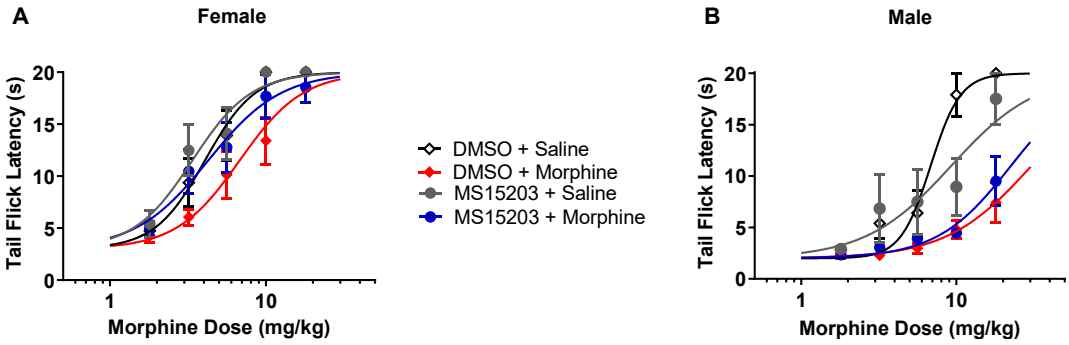

Fig. 3.5: GPR171 agonist attenuated morphine tolerance in female but not in male mice on the tail flick test. Tolerance was induced by twice-daily injections of morphine for 4 days as seen by a rightward shift in the dose response curve compared to saline treated mice. Dose response curves were performed on Day 5 using a cumulative quarter log dose procedure of morphine (1, 1.8, 3.2, 5.6,10, and 18 mg/kg, s.c.). Repeated injections of MS15203+Morphine significantly increased tail flick latency compared to morphine alone, thus reducing morphine tolerance in female mice (A), while there was no difference in tolerance in male mice (B) after repeated injection of MS15203+Morphine. Interestingly, repeated injection of MS15203 alone did not produce any effect in female mice (A) whereas, MS15203 decreased morphine analgesia compared to saline treated group in male mice (B). 5-8 animals/group.

Table 3.1: Comparison of ED50 Values
for Tail Flick Test

Treatment	**Females**	**Males**
DMSO+Saline	$4.057 \pm 0.3651^{*}$	$6.819 \pm 0.4904^{*}$
DMSO+Morphine	$6.770 \pm 0.6899^{@}$	$30.46 \pm 8.494^{@}$
MS15203+Saline	$3.367 \pm 0.3710^{*}$	$9.264 \pm 1.926^{@*}$
MS15203+Morphine	$4.256 \pm 0.5710^{*}$	$22.46 \pm 3.649^{@}$

$@p < 0.05$ from saline treated mice; $^{*}p < 0.05$ from morphine treated mice.

3.5 GPR171 Agonist Does Not Alter Morphine Tolerance in Either Male or Female on the Hot Plate Test

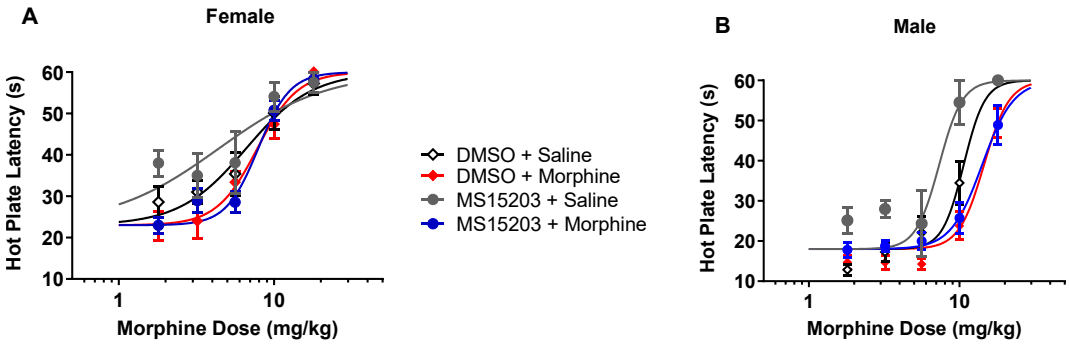

Fig. 3.6: GPR171 agonist did not alter morphine tolerance in female or male mice on the hot plate test. Tolerance was induced by twice-daily injections of morphine for 4 days as seen by a rightward shift in the dose response curve for DMSO+Morphine group compared to DMSO+Saline group in male (B), but not in female mice (A). Dose response curves were performed on Day 5 using a cumulative quarter log dose procedure of morphine (1, 1.8, 3.2, 5.6, 10, and 18 mg/kg, s.c.). The data reveals that pretreatment with MS15203+Morphine produced a rightward shift similar to DMSO+Morphine, suggesting that morphine tolerance is not affected by repeated administration of MS15203 in female (A) and male (B) mice during hot plate thermal pain assay. Interestingly, repeated administration of MS15203 alone on Day 1-4 caused an increase in morphine induced antinocieption on Day 5 in both female (A) and male (B) mice. 5-9 animals/group.

Table 3.2: Comparison of ED50 Values for Hot Plate Test

Treatment	Females	Males
DMSO+Saline	6.584 ± 0.8422	$10.65 \pm 0.6109^{*}$
DMSO+Morphine	7.781 ± 0.6273	$14.55 \pm 0.7941^{@}$
MS15203+Saline	$4.387 \pm 0.9597^{@*}$	$7.321 \pm 0.7588^{@*}$
MS15203+Morphine	7.886 ± 0.4549	$14.13 \pm 0.9023^{@}$

$@p < 0.05$ from saline treated mice; $^{*}p < 0.05$ from morphine treated mice.

Mice treated with twice daily injections of morphine for 4 days were tolerant on the hot plate test as seen by the rightward shift in the dose response curve compared to saline treated male mice $[F(3, 178) = 16.43, P < 0.0001]$ (Fig. 3.6B; Table 3.2). Female mice

treated with 4 days morphine injection did not show morphine tolerance on the hot plate test [$F(3, 178)4.025, P = 0.0084$] (Fig. 3.6A; Table 3.2). There was a significant dose-dependent increase in hot plate latencies for all female groups comparing to the saline treated mice. Pretreatment with MS15203 + Morphine did not alter ED50 values compared to DMSO + Morphine group suggesting that morphine induced tolerance was not affected by repeated administration of the GPR171 agonist, MS15203, on the hot plate test in either male or female mice (Fig. 3.6). MS15203 + Saline group showed a reduction in ED50 values compared to DMSO + Saline group in both sexes (Table 3.2) suggesting that repeated injection of MS15203 alone enhances morphine antinociception. ED50s are presented in Table 3.2.

3.6 GPR171 Agonist Does Not Alter Morphine Withdrawal in Male

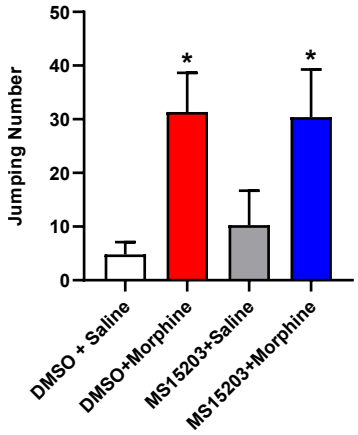

Fig. 3.7: Pretreatment with GPR171 agonist did not alter withdrawal behaviors in male mice. Animals were treated with GPR171 agonist, MS15203 (10 mg/kg, i.p.) or vehicle (10% DMSO in saline, i.p.) 10 minutes before morphine (5 mg/kg, s.c.) or saline (10 ml/kg, s.c.) injection twice daily for 4 days to induce morphine dependence. On day 5, mice were treated with their designated drug combination. Two hours later Naloxone Hydrochloride (2 mg/kg, i.p.) was administered to induce precipitated withdrawal. Mice were video recorded immediately for 30 minutes to observe withdrawal behavior- jumping. Repeated injection of DMSO + Morphine caused an increase in jumping number compared to the DMSO + Saline treated mice indicating that those mice developed morphine dependence. Administration of MS15203 + Morphine did not alter jumping number comparing to the morphine treated group. Administration of MS15203 + Saline increased the jumping number compared with the saline treated mice, although the increase is not significant. $^*P < 0.05$, compared with saline. Data are the means ±SEM. 8–10 animals/group.

Mice treated with twice-daily injections of morphine for 4 days showed morphine dependence by significant increase in jumping compared to the DMSO + Saline treated mice (Tukey's, $P < 0.05$). Administration of MS15203 + Morphine did not alter jumping compared to the DMSO + Morphine treated group (Tukey's, $P > 0.05$), suggesting that GPR171 agonist, MS15203, does not have any effect on morphine withdrawal (Fig. 3.7). Repeated administration of MS15203 + Saline did not produce a significant change in the jumping number compared to the DMSO + Saline group (Tukey's, $P > 0.05$).

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Discussion

In this present study, we investigated the expression of GPR171 within the PAG and its ability to modulate morphine antinociception, tolerance, and withdrawal in male and female mice. We found that GPR171 is primarily expressed in GABAergic neurons in the PAG. Consistent with our previous findings in male mice, we found that MS15203 enhances morphine antinociception in female mice (McDermott et al., 2019). Our data also demonstrate that pretreatment with GPR171 agonist, MS15203, attenuates morphine tolerance on the tail flick test in female mice while having no effects in male mice. Interestingly, repeated injection of MS15203 alone enhanced morphine antinociception on the hot plate test in both sexes. In contrast, MS15203 did not alter morphine tolerance on the hot plate test in either sex. Furthermore, female mice did not show any morphine tolerance on the hot plate test. The result of our withdrawal study suggests that GPR171 agonist, MS15203, treatment does not alter morphine dependence or withdrawal.

4.1.1 GPR171 is Found in GABAergic Neurons

Given that the neurons in the ventrolateral PAG primarily contribute to morphine antinociception and tolerance, we performed immunohistochemistry to observe the colocalization of GPR171 with the neuronal subtypes in this region. We found that GPR171 is mainly found within GABAergic neurons. It is also found in a smaller number of glutamatergic neurons. Several previous studies suggest that under normal conditions, the GABAergic interneurons in the PAG have tonic activity, thereby causing a steady release of GABA and inhibition of PAG output neurons. However, upon binding of opioids to the MOPr within the PAG, the activity of GABAergic neurons are decreased, resulting in an

increase in activity of the PAG output neurons to the RVM (Vaughan, Ingram, Connor, & Christie, 1997; Bobeck et al., 2014). This increased activity from the PAG to the RVM is considered as the main contributor to the opioid-induced antinociception, by blocking incoming pain signals in the spinal cord. In addition, both in vitro (Chieng & Christie, 1994; Bobeck et al., 2014) and in vivo (M. M. Morgan & Liebeskind, 1987; Tortorici, Robbins, & Morgan, 1999) studies have linked alterations within these PAG GABAergic neurons in morphine tolerance. It has been proposed that blocking the MOPr within the PAG, inhibits the development of tolerance following systemic morphine injection and inactivation of the RVM by a GABA agonist along with microinjection of morphine into the PAG, still leads to the development of tolerance in rats (Lane et al., 2005). These data suggest that morphine tolerance is primarily associated with a decrease in opioid mediated inhibition of GABA release within the ventrolateral PAG. It is possible that the GPR171 located on these GABAergic neurons regulate morphine antinociception and tolerance. Although the underlying mechanism warrants further research.

4.1.2 GPR171 Agonist Increases Morphine Antinociception

Our recent study has demonstrated that GPR171 agonist, MS15203, enhances morphine antinociception, whereas antagonism of this receptor decreases morphine antinociception during acute morphine treatment (McDermott et al., 2019). Given that we tested only male mice in our previous study, here in this study, we performed a time course to observe the effects of MS15203 on acute morphine antinociception in female mice. We found that MS15203 enhances morphine antinociception both on the tail flick and hot plate test in female mice, which is consistent with our previous findings in male mice. Although the increase in hot plate latencies is not statistically significant compared to the morphine treated group. Overall, these data suggest that GPR171 agonist, MS15203, enhances morphine-induced antinociception on spinal and supraspinal measures in both sexes.

On the hot plate assay, repeated administration of MS15203 alone led to increased latencies in both sexes. Our previous study reports that acute administration of MS15203 does not have any analgesic property on its own (McDermott et al., 2019). However,

we did not look at the analgesic effects of repeated injection of MS15203. It should be noted that the morphine dose-response study was performed at least 12 hours after the last injection, which suggests that MS15203 was likely not active in the bloodstream. A similar effect of MS15203 administration is also observed on the tail flick test in female mice although not significant comparing to saline treated groups. However, an opposite result is observed in male mice meaning that repeated injection of MS15203 decreased morphine antinociception. It is unclear why this effect of repeated MS15203 treatment would be different on the two tests. The pharmacokinetics and pharmacodynamics of MS15203 are unknown, which could contribute to the differences in its effects in the brain versus spinal cord. However, it has been shown that MS15203 crosses the blood brain barrier and activates neurons in the hypothalamus (Wardman et al., 2016). Our hypothesis is that repeated administration of MS15203 leads to enhanced downstream signaling which in turn causes enhanced morphine antinociception. Our previous study shows that blockade of GPR171 decreases MOPr mediated G protein activity in vitro (McDermott et al., 2019). In this present study, we only investigated the behavioral aspects of the function of MS15203. Further studies are needed to interpret the cellular changes of underlying mechanisms.

4.1.3 Development of Morphine Tolerance is Reduced in Female Mice on the Hot Plate Test

In this study we found that female mice developed less morphine tolerance compared to the male mice on the hot plate assay. Previous literature on sex differences in morphine tolerance are controversial and have reported mixed findings. A number of studies have found that females are less tolerant to morphine than males (Badillo-martinez et al., 1984; Craft et al., 1999; South, Wright, Lau, Mather, & Smith, 2001), where other studies show no sex difference in tolerance in rats (Thornton & Smith, 1997; Holtman, Sloan, & Wala, 2004). It has been shown that chronic administration of systemic morphine induces greater tolerance in male than in female rats (Loyd, Morgan, & Murphy, 2008). The greater sensitivity of males to acute morphine analgesia could account for the greater tolerance observed in male rats. Likewise, female rats develop tolerance against morphine to a lesser

extent and slower than male rats (Barrett, Cook, Terner, Craft, & Picker, 2001). It should be noted that female rats differ in the degree of morphine tolerance in particular phase of the estrous cycle (Shekunova & Bespalov, 2004). However, we did not attempt to examine the estrous cycle phase of the female mice in this current experiment. One possible mechanism for the dissimilar tolerance development observed in female mice on the tail flick and hot plate test may be explained by the supraspinal versus spinal nature of the two tests. The hot plate test is a supraspinal pain assay while tail-flick measures a spinal reflex. These two methods predominantly reflect nociception and tolerance at different levels of the central nervous system. In addition, repeated testing of mice using the hot plate has a learning component which the mice learn to exhibit certain behaviors so that they can be quickly removed from the hotplate apparatus (Chapman et al., 1985; Le Bars D, Gozariu M, 2001). This learning phenomenon can confound the results on the hot plate test. It should be noted that female mice developed morphine tolerance to a lesser extent both on the hot plate and tail flick test compared to the male mice in this current study. On the hot plate test, male mice showed 2-fold shift in the ED50, whereas female mice showed no difference comparing to the saline treated group. On the tail flick test, male and female mice showed 5-fold and 2-fold shift in the ED50 values respectively compared to the saline group. Considering all these above-mentioned factors, it can be said that this female specific lack of tolerance development might be a result of many associated factors.

4.1.4 Combined Treatment of GPR171 Agonist and Morphine Decreases Tolerance in Female Mice

Our data shows that activation of GPR171 with the agonist, MS15203, reduced morphine induced tolerance in female mice on the tail flick test. A similar trend is observed in male mice showing that the ED50 is in between saline and morphine treated groups, but not significant from morphine. These results are consistent with our previous data where injection of MS15203 in combination with morphine enhanced analgesia during acute morphine treatment in both male (McDermott et al., 2019) and female mice in the current study. Given that MS15203 increases morphine's pain-relieving property during acute treatment,

MS15203 leads to a corresponding progressive increase in morphine analgesia during repeated administration of MS15203 + Morphine, resulting in enhanced morphine function or decreased morphine tolerance. Our immunohistochemistry data shows that GPR171 is expressed on the GABAergic neurons of PAG. It is evident that MOPr are also located on the GABAergic neurons of PAG (Stiller, Bergquist, Beck, Ekman, & Brodin, 1996; Lueptow et al., 2018). Due to the co-expression of these two receptors in the GABAergic neurons of the PAG, activation of these two receptors together inhibit the GABA release to a greater extent than morphine alone. Thereby, increasing morphine antinociception during acute treatment and in parallel, decreasing morphine tolerance during chronic treatment are observed. Interestingly, our results show that MS15203 does not alter morphine tolerance in male mice on the hot plate test. One plausible explanation is that GPR171 is more prevalent in the periphery and modulating morphine tolerance at the peripheral level that contributes primarily to the spinal sites to the development of tolerance rather than the supraspinal sites. Taken together, these results indicate that combined treatment of GPR171 agonist, MS15203 and morphine does not have any adverse effect on morphine tolerance. This combination therapy has the potential to reduce morphine dose, thereby attenuating tolerance and dependence to morphine during long-term treatment. Most importantly, this combination therapy is likely to reduce morphine tolerance in female.

4.1.5 GPR171 Agonist Has No Effect on Morphine Withdrawal

Our withdrawal experiment result shows that MS15203 does not alter morphine withdrawal induced jumping. We only explored the effects of the GPR171 agonist, MS15203, on morphine withdrawal in male mice. Given that MS15203 has no effect on morphine tolerance, it is not surprising that it does not regulate morphine withdrawal in male mice. Multiple brain regions are found to be involved in morphine withdrawal including the PAG, locus coeruleus, nucleus accumbens, ventral tegmental area, hypothalamus, frontal cortex, amygdala and the spinal cord (McPhie & Barr, 2009). Although the PAG is thought to perform a key role in morphine withdrawal (Ouyang et al., 2012), other studies have highlighted the role of locus coeruleus, ventral tegmental area (McClung, Nestler, & Zachariou, 2005)

and frontal cortex (Ammon, Mayer, Riechert, Tischmeyer, & Höllt, 2003) as the primary contributor of morphine withdrawal symptoms. By immunohistological analysis, we only explored the expression of GPR171 within the PAG, while the expression of this receptor in other areas is still unknown. A likely explanation to our observation is that morphine withdrawal is mediated largely by the areas other than PAG and that is why MS15203 has no influence on this phenomenon. Additionally, It has been reported that the glutamatergic neurons are involved in naloxone-precipitated withdrawal symptoms (Zhang et al., 2020). Our immunohistochemistry data shows that the GPR171 is primarily colocalized with the GABAergic neurons and, to a lesser extent, in glutamatergic neurons of PAG. Given that this receptor is not highly expressed on the glutamatergic neurons, activation of this receptor might not have any effect on morphine withdrawal. It is evident that although Mu opioid receptor agonist, morphine inhibits adenylyl cyclase activity during acute treatment (Fedynyshyn & Lee, 1989), there is a compensatory increase in adenylyl cyclase signaling during chronic treatment with morphine causing an increased neuronal adenylyl cyclase activity, thereby cAMP is upregulated during withdrawal (Terwilliger, Beitner-Johnson, Sevarino, Crain, & Nestler, 1991). The GPR171 agonist, MS15203, works in a similar fashion as morphine meaning that it also reduces cAMP production. It is possible that the reduction in the adenylyl cyclase signaling by GPR171 is not potent enough to attenuate morphine tolerance or withdrawal. In this study, we used relatively lower doses of morphine (5mg/kg) and naloxone (2mg/kg), where other researchers have used higher or escalating doses of morphine and a wide range of naloxone doses (Kasanga et al., 2017). As a sign of withdrawal behavior, only jumping number was observed in this study. Although jumping is a well-documented withdrawal behavior in mice (Kest et al., 2002), other studies measured individual withdrawal signs and an overall opiate withdrawal score was calculated (Zachariou et al., 2003; Kasanga et al., 2017) which may have prevented us from observing differences in this current study. Previous studies have used different routes of administration of drugs, length of drug administration and different mouse strains (Cichewicz & Welch, 2003; Rzasa Lynn & Galinkin, 2018). It is worth mentioning that we did not in-

clude female mice in this withdrawal study. Given that MS15203 showed greater difference during morphine tolerance in female mice, it is possible that the agonist might show an effect on morphine withdrawal if tested in female mice. Future studies with different doses and testing paradigms are needed to rule out that GPR171 is not involved in morphine withdrawal. Overall, these data suggest that GPR171 agonist, MS15203, can be safely used as a combination therapy with morphine without worsening morphine withdrawal.

4.1.6 Interactions Between GPR171 and MOPr Systems

All these results support the idea that these two receptors systems work together. Our previous study reported that antagonism or knockdown of GPR171 reduces MOPr mediated G protein signaling (McDermott et al., 2019), meaning that GPR171 may be a regulator of MOPr signaling. This could happen if these two receptors interact by heterodimer formation. A number of studies have postulated that several GPCRs form both homo and heterodimers (Terrillon & Bouvier, 2004; Alfaras-Melainis, 2009). Notably, both the MOPr and GPR171 have been found to functionally interact with other receptors and form heterodimers (Fujita, Gomes, & Devi, 2015; Gomes et al., 2016; Margolis, Fujita, Devi, & Fields, 2017). These receptor–receptor interactions modulate ligand binding and the signaling properties of the individual receptors (Al-Hasani & Bruchas, 2011; Stockton & Devi, 2012). It has been previously reported that MOPrs can be modulated by other receptors. Antinociceptive effects of MOPrs is regulated by the DOPr (Porreca, Mosberg, Hurst, Hruby, & Burks, 1984; Abul-Husn, Sutak, Milne, & Jhamandas, 2007), cannabinoid receptors (Cichewicz & McCarthy, 2003), glutamate receptors (Nishiyama, Yaksh, & Weber, 1998; M. M. Morgan, Bobeck, & Ingram, 2009), orexin receptors (Azhdari-Zarmehri, Esmaeili, Sofiabadi, & Haghdoost-Yazdi, 2013; Emam et al., 2016) and alpha-2 adrenergic receptor (Drasner & Fields, 1988). We know that GPR171 can interact with another GPCR, GPR83 (Gomes et al., 2016), but it is unknown whether it dimerizes with the MOPr as well. Our immunohistochemistry data reports the expression of GPR171 on the GABA neurons of PAG while it is well known that MOPr are also highly expressed on the GABA neurons (Bobeck et al., 2014). Activation of these two receptors together will likely inhibit

GABA release to a greater extent thereby exciting the projection neurons from the PAG to the RVM (Terwilliger et al., 1991; Lau & Vaughan, 2014; Lueptow et al., 2018). It is also noteworthy that the GPR171 and MOPr both are inhibitory GPCRs meaning that they are coupled to Gai/o proteins and inhibit cAMP production (Gomes et al., 2013). When these two receptors are activated together, there is crosstalk between signaling pathways which could produce enhanced downstream signaling and greater morphine antinociception. To our knowledge, this current study is the first to look at this receptor's role in opioid tolerance and withdrawal. Additionally, we are not aware of any previous studies about GPR171 that included both sexes. Our primary focus in this current study was to investigate this receptor's function in vivo. Future studies are needed to determine the underlying cellular changes of these behavioral observations.

4.1.7 Limitations of the Study

Although this research has revealed some exciting findings, there are some unavoidable limitations that should be noted. One of the limitations of this study is the cumulative dosing procedure of morphine. In order to complete testing within the antinociceptive time course of the opioid, only 5 doses of morphine were administered. Previous studies have shown that using this cumulative dosing paradigm causes higher ED50 values than a paradigm where dose-response curves are created by testing animals once with only a particular dose (Kumar et al., 2008; Sirohi, Dighe, Madia, & Yoburn, 2009). However, with those studies hundreds more animals must be used to get a large enough sample size at each dose. Furthermore, we investigated only one dose of the agonist, MS15203 (Wardman et al., 2016). We also used just one specific injection and testing time that were adapted from our previous studies (Bobeck et al., 2014). Because GPR171 is relatively a new area of research, there is not enough data available to know the optimal dose and timing of MS15203, thus a thorough dose-responses and time course of the effectiveness of MS15203 still needs further exploration. The other problem with this study is that there is an arbitrary tail flick and hotplate cutoff latency. In receptor binding studies, calculation of EC50s are more precise, because there is not a given cutoff value (Tallarida, 2000). In the current experiment a cutoff

value must be used in order to prevent tissue damage of the mouse tail and paw. Another limitation of this present study is that we performed immunohistochemistry only in male mice to see the expression of GPR171 in the PAG. Additionally, we have not examined the phase of estrous cycle of the female mice. It is evident that female shows variability in morphine tolerance development at different stages of estrous cycle (Shekunova & Bespalov, 2004).

4.1.8 Implications and Future Directions

The findings of this study are an important step for the development of a novel pain therapeutic particularly for chronic pain with reduced tolerance and dependance than opioid treatment alone. This study shows that GPR171 agonist, MS15203, does not have any adverse effects during long term morphine use indicating that MS15203 can be safely used with morphine for better synergistic analgesic effects. This combination therapy will allow for a reduction in the dose of morphine thereby reducing tolerance and dependence to morphine during long-term treatment. Further studies are needed to expand the results of this study. Our results demonstrate several sex differences in the function of MS15203. This is the first study that has been conducted on GPR171 that includes female mice. Given that MS15203 shows female specific attenuation of morphine tolerance, it has the potential to be used in gender specific drug tailoring. Future studies are needed to interpret the possible mechanisms underlying sexual dimorphism in the role of GPR171 in regulating the analgesic effects of morphine. The gender specific population and distribution of GPR171 in the brain and spinal cord, pharmacokinetics, and neuronal circuitry need to be investigated. In this present study, we only used a specific dose of MS15203, further studies are warranted with different does of MS15203 to determine the optimal dose. It is also recommended to study the effects of chronic MS15203 administration in combination with acute and chronic morphine treatment to evaluate if there is any cross tolerance between these two drugs. We investigated the role of GPR171 only in morphine function, the other MOPr agonists should be evaluated in future. Another important future experiment is to evaluate the function of GPR171 in other pain models such as inflammatory and neuropathic pain.

4.2 Conclusion

Collectively, these findings lay the groundwork for future studies aimed at GPR171 function, therefore, making it a novel area of study towards the development of pain therapeutics. Here, we presented that the GPR171 agonist, MS15203, decreases morphine tolerance in female mice and notably, it does not aggravate morphine induced tolerance and withdrawal during chronic morphine treatment. Clinically, this would be a beneficial outcome in reducing opioid dosage, because patients will require smaller doses of the opioid agonist to produce the same amount of antinociception. The outcome of this study helps to elucidate the sex differences in GPR171 function and will be an important step to support sex specific tailoring of pain therapeutics in future.

References

Abul-Husn, N. S., Sutak, M., Milne, B., & Jhamandas, K. (2007). Augmentation of spinal morphine analgesia and inhibition of tolerance by low doses of μ- And δ-opioid receptor antagonists. *British Journal of Pharmacology*. doi: 10.1038/sj.bjp.0707277

Alfaras-Melainis, K. (2009). Modulation of opioid receptor function by protein-protein interactions. *Frontiers in Bioscience*. doi: 10.2741/3474

Al-Hasani, R., & Bruchas, M. R. (2011). *Molecular mechanisms of opioid receptor-dependent signaling and behavior.* doi: 10.1097/ALN.0b013e318238bba6

Allouche, S., Noble, F., & Marie, N. (2014). *Opioid receptor desensitization: Mechanisms and its link to tolerance.* doi: 10.3389/fphar.2014.00280

Ammon, S., Mayer, P., Riechert, U., Tischmeyer, H., & Höllt, V. (2003). Microarray analysis of genes expressed in the frontal cortex of rats chronically treated with morphine and after naloxone precipitated withdrawal. *Molecular Brain Research*. doi: 10.1016/S0169-328X(03)00057-3

Azhdari-Zarmehri, H., Esmaeili, M. H., Sofiabadi, M., & Haghdoost-Yazdi, H. (2013). Orexin receptor type-1 antagonist SB-334867 decreases morphine-induced antinociceptive effect in formalin test. *Pharmacology Biochemistry and Behavior*. doi: 10.1016/j.pbb.2013.09.018

Badillo-martinez, D., Kirchgessner, A. L., Butler, P. D., & Bodnar, R. J. (1984). Monosodium glutamate and analgesia induced by morphine. Test-specific effects. *Neuropharmacology*. doi: 10.1016/0028-3908(84)90231-4

Barrett, A. C., Cook, C. D., Terner, J. M., Craft, R. M., & Picker, M. J. (2001). Importance of sex and relative efficacy at the μ opioid receptor in the development of tolerance and cross-tolerance to the antinociceptive effects of opioids. *Psychopharmacology*. doi: 10.1007/s002130100821

Bartley, E. J., & Fillingim, R. B. (2013). Sex differences in pain: A brief review of clinical and experimental findings. *British Journal of Anaesthesia*. doi: 10.1093/bja/aet127

Bartok, R. E., & Craft, R. M. (1997). Sex differences in opioid antinociception. *Journal of Pharmacology and Experimental Therapeutics*.

Behbehani, M. M. (1995). Functional characteristics of the midbrain periaqueductal gray. *Progress in Neurobiology*, *46*(6), 575–605. doi: 10.1016/0301-0082(95)00009-K

Bobeck, E. N., Chen, Q., Morgan, M. M., & Ingram, S. L. (2014). Contribution of adenylyl cyclase modulation of pre- and postsynaptic GABA neurotransmission to morphine antinociception and tolerance. *Neuropsychopharmacology*. doi: 10.1038/npp.2014.62

Bobeck, E. N., Gomes, I., Pena, D., Cummings, K. A., Clem, R. L., Mezei, M., & Devi, L. A. (2017). The BigLEN-GPR171 peptide receptor system within the basolateral amygdala regulates anxiety-like behavior and contextual fear conditioning. *Neuropsychopharmacology*, *42*(13), 2527–2536. doi: 10.1038/npp.2017.79

Bobeck, E. N., Haseman, R. A., Hong, D., Ingram, S. L., & Morgan, M. M. (2012). Differential development of antinociceptive tolerance to morphine and fentanyl is not linked to efficacy in the ventrolateral periaqueductal gray of the rat. *Journal of Pain*, *13*(8), 799–807. doi: 10.1016/j.jpain.2012.05.005

Bobeck, E. N., McNeal, A. L., & Morgan, M. M. (2009). Drug dependent sex-differences in periaqueducatal gray mediated antinociception in the rat. *Pain*, *147*(1-3), 210–216. doi: 10.1016/j.pain.2009.09.008

Buntin-Mushock, C., Phillip, L., Moriyama, K., & Palmer, P. P. (2005). *Age-dependent opioid escalation in chronic pain patients.* doi: 10.1213/01.ANE.0000152191.29311.9B

Burma, N. E., Kwok, C. H., & Trang, T. (2017). Therapies and mechanisms of opioid withdrawal. *Pain management*. doi: 10.2217/pmt-2017-0028

Chapman, C. R., Casey, K. L., Dubner, R., Foley, K. M., Gracely, R. H., & Reading, A. E. (1985). Pain measurement: an overview. *Pain*. doi: 10.1016/0304-3959(85)90145-9

Cherubino, P., Sarzi-Puttini, P., Zuccaro, S. M., & Labianca, R. (2012). *The management of chronic pain in important patient subgroups.* doi: 10.2165/11630060-000000000-00000

Chieng, B., & Christie, M. J. (1994). Inhibition by opioids acting on μ-receptors of

GABAergic and glutamatergic postsynaptic potentials in single rat periaqueductal gray neurones in vitro. *British Journal of Pharmacology*. doi: 10.1111/j.1476-5381.1994.tb16209.x

Chieng, B., & Christie, M. J. (1996). Local opioid withdrawal in rat single periaqueductal gray neurons in vitro. *Journal of Neuroscience*. doi: 10.1523/jneurosci.16-22-07128.1996

Chieng, B., Keay, K. A., & Christie, M. J. (1995). Increased fos-like immunoreactivity in the periaqueductal gray of anaesthetised rats during opiate withdrawal. *Neuroscience Letters*. doi: 10.1016/0304-3940(94)11119-4

Chu, L. F., Clark, D. J., & Angst, M. S. (2006). Opioid tolerance and hyperalgesia in chronic pain patients after one month of oral morphine therapy: A preliminary prospective study. *Journal of Pain*. doi: 10.1016/j.jpain.2005.08.001

Cicero, T. J., Nock, B., & Meyer, E. R. (1996). Gender-related differences in the antinociceptive properties of morphine. *Journal of Pharmacology and Experimental Therapeutics*.

Cichewicz, D. L., & McCarthy, E. A. (2003). Antinociceptive synergy between Δ9-tetrahydrocannabinol and opioids after oral administration. *Journal of Pharmacology and Experimental Therapeutics*. doi: 10.1124/jpet.102.045575

Cichewicz, D. L., & Welch, S. P. (2003). Modulation of oral morphine antinociceptive tolerance and naloxone-precipitated withdrawal signs by oral δ9-tetrahydrocannabinol. *Journal of Pharmacology and Experimental Therapeutics*. doi: 10.1124/jpet.102.046870

Couceyro, P., & Douglass, J. (1995). Precipitated morphine withdrawal stimulates multiple activator protein-1 signaling pathways in rat brain. *Molecular Pharmacology*.

Craft, R. M., Stratmann, J. A., Bartok, R. E., Walpole, T. I., & King, S. J. (1999). Sex differences in development of morphine tolerance and dependence in the rat. *Psychopharmacology*. doi: 10.1007/s002130050911

Drasner, K., & Fields, H. L. (1988). Synergy between the antinociceptive effects of intrathecal clonidine and systemic morphine in the rat. *Pain*. doi: 10.1016/0304-3959(88)90042-5

Emam, A. H., Hajesfandiari, N., Shahidi, S., Komaki, A., Ganji, M., & Sarihi, A. (2016). Modulation of nociception by medial pre-optic area orexin a receptors and its relation with morphine in male rats. *Brain Research Bulletin*. doi: 10.1016/j.brainresbull.2016.09.009

Fedynyshyn, J. P., & Lee, N. M. (1989). μ type opioid receptors in rat periaqueductal gray-enriched P2 membrane are coupled to G-protein-mediated inhibition of adenylyl cyclase. *FEBS Letters*. doi: 10.1016/0014-5793(89)80921-4

Fillingim, R. B., King, C. D., Ribeiro-Dasilva, M. C., Rahim-Williams, B., & Riley, J. L. (2009, may). Sex, Gender, and Pain: A Review of Recent Clinical and Experimental Findings. *The Journal of Pain*, *10*(5), 447–485. doi: 10.1016/J.JPAIN.2008.12.001

Fricker, L. D. (2010). *Analysis of mouse brain peptides using mass spectrometry-based peptidomics: Implications for novel functions ranging from non-classical neuropeptides to microproteins.* doi: 10.1039/c003317k

Fujita, W., Gomes, I., & Devi, L. A. (2015). *Heteromers of μ-δ Opioid receptors: New pharmacology and novel therapeutic possibilities.* doi: 10.1111/bph.12663

Gaskin, D. J., & Richard, P. (2012). The economic costs of pain in the United States. *Journal of Pain*. doi: 10.1016/j.jpain.2012.03.009

Gintzler, A. R., Chakrabarti, S., & Dray. (2004). *Chronic morphine-induced plasticity among signalling molecules.* doi: 10.1002/0470869127.ch13

Gomes, I., Aryal, D. K., Wardman, J. H., Gupta, A., Gagnidze, K., Rodriguiz, R. M., ... Devi, L. A. (2013). GPR171 is a hypothalamic G protein-coupled receptor for BigLEN, a neuropeptide involved in feeding. *Proceedings of the National Academy of Sciences*, *110*(40), 16211–16216. Retrieved from `http://www.pnas.org/cgi/doi/10.1073/pnas.1312938110` doi: 10.1073/pnas.1312938110

Gomes, I., Bobeck, E. N., Margolis, E. B., Gupta, A., Sierra, S., Fakira, A. K., ... Devi,

L. A. (2016). Identification of GPR83 as the receptor for the neuroendocrine peptide PEN. *Science Signaling*. doi: 10.1126/scisignal.aad0694

Green, C. R., Baker, T. A., Smith, E. M., & Sato, Y. (2003). The effect of race in older adults presenting for chronic pain management: A comparative study of black and white Americans. *Journal of Pain*. doi: 10.1054/jpai.2003.8

Guitart, X., & Nestler, E. J. (1989). Identification of morphine- and cyclic AMP-regulated phosphoproteins (MARPPs) in the locus coeruleus and other regions of rat brain: Regulation by acute and chronic morphine. *Journal of Neuroscience*. doi: 10.1523/jneurosci.09-12-04371.1989

Hao, S., Liu, S., Zheng, X., Zheng, W., Ouyang, H., Mata, M., & Fink, D. J. (2011). The role of TNFα in the periaqueductal gray during naloxone-precipitated morphine withdrawal in rats. *Neuropsychopharmacology*. doi: 10.1038/npp.2010.197

Heinricher, M. M., Tavares, I., Leith, J. L., & Lumb, B. M. (2009). *Descending control of nociception: Specificity, recruitment and plasticity.* doi: 10.1016/j.brainresrev.2008.12.009

Holtman, J. R., Sloan, J. W., & Wala, E. P. (2004). Morphine tolerance in male and female rats. *Pharmacology Biochemistry and Behavior*. doi: 10.1016/j.pbb.2003.12.020

Hoshino, A., Helwig, M., Rezaei, S., Berridge, C., Eriksen, J. L., & Lindberg, I. (2014, feb). A novel function for proSAAS as an amyloid anti-aggregant in Alzheimer's disease. *Journal of neurochemistry*, *128*(3), 419–430. doi: 10.1111/jnc.12454

Insel, P. A., Sriram, K., Gorr, M. W., Wiley, S. Z., Michkov, A., Salmerón, C., & Chinn, A. M. (2019). *GPCRomics: An Approach to Discover GPCR Drug Targets.* doi: 10.1016/j.tips.2019.04.001

Kasanga, E. A., Boakye-Gyasi, E., Biney, R. P., Kyekyeku, J. O., Agyare, C., & Woode, E. (2017). Geraniin attenuates naloxoneprecipitated morphine withdrawal and morphine-induced tolerance in mice. *Journal of Intercultural Ethnopharmacology*. doi: 10.5455/jice.20161229015413

Kasson, B. G., & George, R. (1984). Endocrine influences on the actions of morphine: IV.

effects of sex and strain. *Life Sciences*. doi: 10.1016/0024-3205(84)90633-7

Kepler, K. L., Kest, B., Kiefel, J. M., Cooper, M. L., & Bodnar, R. J. (1989). Roles of gender, gonadectomy and estrous phase in the analgesic effects of intracerebroventricular morphine in rats. *Pharmacology, Biochemistry and Behavior*. doi: 10.1016/0091-3057(89)90363-8

Kest, B., Palmese, C. A., Hopkins, E., Adler, M., Juni, A., & Mogil, J. S. (2002). Naloxone-precipitated withdrawal jumping in 11 inbred mouse strains: Evidence for common genetic mechanisms in acute and chronic morphine physical dependence. *Neuroscience*. doi: 10.1016/S0306-4522(02)00458-X

Khoonsari, P. E., Musunri, S., Herman, S., Svensson, C. I., Tanum, L., Gordh, T., & Kultima, K. (2019). Systematic analysis of the cerebrospinal fluid proteome of fibromyalgia patients. *Journal of Proteomics*. doi: 10.1016/j.jprot.2018.04.014

Kosten, T. R., & George, T. P. (2002). *The neurobiology of opioid dependence: implications for treatment.* doi: 10.1151/spp021113

Kumar, P., Sunkaraneni, S., Sirohi, S., Dighe, S. V., Walker, E. A., & Yoburn, B. C. (2008). Hydromorphone efficacy and treatment protocol impact on tolerance and μ-opioid receptor regulation. *European Journal of Pharmacology*. doi: 10.1016/j.ejphar.2008.08.025

Lane, D. A., Patel, P. A., & Morgan, M. M. (2005). Evidence for an intrinsic mechanism of antinociceptive tolerance within the ventrolateral periaqueductal gray of rats. *Neuroscience*. doi: 10.1016/j.neuroscience.2005.06.014

Laschka, E., Teschemacher, H., Mehraein, P., & Herz, A. (1976). Sites of action of morphine involved in the development of physical dependence in rats - II. Morphine withdrawal precipitated by application of morphine antagonists into restricted parts of the ventricular system and by microinjection into various brain a. *Psychopharmacologia*. doi: 10.1007/BF00421383

Lau, B. K., & Vaughan, C. W. (2014). *Descending modulation of pain: The GABA disinhibition hypothesis of analgesia.* doi: 10.1016/j.conb.2014.07.010

Le Bars D, Gozariu M, C. S. (2001). Animal models of nociception. - PubMed - NCBI. *Pharmacol Rev*.

Loyd, D. R., Morgan, M. M., & Murphy, A. Z. (2008). Sexually dimorphic activation of the periaqueductal gray-rostral ventromedial medullary circuit during the development of tolerance to morphine in the rat. *European Journal of Neuroscience*, *27*(6), 1517–1524. doi: 10.1111/j.1460-9568.2008.06100.x

Loyd, D. R., Wang, X., & Murphy, A. Z. (2008). Sex differences in μ-opioid receptor expression in the rat midbrain periaqueductal gray are essential for eliciting sex differences in morphine analgesia. *Journal of Neuroscience*. doi: 10.1523/JNEUROSCI.4123-08.2008

Lueptow, L. M., Fakira, A. K., & Bobeck, E. N. (2018). *The contribution of the descending pain modulatory pathway in opioid tolerance.* doi: 10.3389/fnins.2018.00886

Macey, T. A., Bobeck, E. N., Hegarty, D. M., Aicher, S. A., Ingram, S. L., & Morgan, M. M. (2009). Extracellular signal-regulated kinase 1/2 activation counteracts morphine tolerance in the periaqueductal gray of the rat. *Journal of Pharmacology and Experimental Therapeutics*. doi: 10.1124/jpet.109.152157

Maldonado, R., Stinus, L., Gold, L. H., & Koob, G. F. (1992). Role of different brain structures in the expression of the physical morphine withdrawal syndrome. *Journal of Pharmacology and Experimental Therapeutics*.

Margolis, E. B., Fujita, W., Devi, L. A., & Fields, H. L. (2017). Two delta opioid receptor subtypes are functional in single ventral tegmental area neurons, and can interact with the mu opioid receptor. *Neuropharmacology*. doi: 10.1016/j.neuropharm.2017.06.019

McClung, C. A., Nestler, E. J., & Zachariou, V. (2005). Regulation of gene expression by chronic morphine and morphine withdrawal in the locus ceruleus and ventral tegmental area. *Journal of Neuroscience*. doi: 10.1523/JNEUROSCI.0062-05.2005

McDermott, M. V., Afrose, L., Gomes, I., Devi, L. A., & Bobeck, E. N. (2019). Opioid-induced signaling and antinociception are modulated by the recently deorphanized receptor, GPR171. *Journal of Pharmacology and Experimental Therapeutics*. doi:

10.1124/jpet.119.259242

McPhie, A. A., & Barr, G. A. (2009). Regional Fos expression induced by morphine withdrawal in the 7-day-old rat. *Developmental Psychobiology*. doi: 10.1002/dev.20392

Morgan, D. J., Wei, S., Gomes, I., Czyzyk, T., Mzhavia, N., Pan, H., ... Pintar, J. E. (2010). The propeptide precursor proSAAS is involved in fetal neuropeptide processing and body weight regulation. *Journal of Neurochemistry*, *113*(5), 1275–1284. doi: 10.1111/j.1471-4159.2010.06706.x

Morgan, M. M., Bobeck, E. N., & Ingram, S. L. (2009). Glutamate modulation of antinociception, but not tolerance, produced by morphine microinjection into the periaqueductal gray of the rat. *Brain Research*. doi: 10.1016/j.brainres.2009.07.100

Morgan, M. M., Clayton, C. C., & Boyer-Quick, J. S. (2005). Differential susceptibility of the PAG and RVM to tolerance to the antinociceptive effect of morphine in the rat. *Pain*. doi: 10.1016/j.pain.2004.09.039

Morgan, M. M., Clayton, C. C., & Lane, D. A. (2003). Behavioral evidence linking opioid-sensitive GABAergic neurons in the ventrolateral periaqueductal gray to morphine tolerance. *Neuroscience*. doi: 10.1016/S0306-4522(02)00822-9

Morgan, M. M., & Liebeskind, J. C. (1987). Site specificity in the development of tolerance to stimulation-produced analgesia from the periaqueductal gray matter of the rat. *Brain Research*. doi: 10.1016/0006-8993(87)90519-1

Mousavi, Z., Shafaghi, B., Kobarfard, F., & Jorjani, M. (2007). Sex differences and role of gonadal hormones on glutamate level in the nucleus accumbens in morphine tolerant rats: A microdialysis study. *European Journal of Pharmacology*. doi: 10.1016/j.ejphar.2006.10.010

Mzhavia, N., Qian, Y., Feng, Y., Che, F.-y., DEVI, L., & FRICKER, L. (2002). Processing of proSAAS in neuroendocrine cell lines. *Biochem. J.* doi: 10.1042/0264-6021:3610067

Nahin, R. L. (2015). Estimates of Pain Prevalence and Severity in Adults: United States, 2012. *Journal of Pain*. doi: 10.1016/j.jpain.2015.05.002

Nishiyama, T., Yaksh, T. L., & Weber, E. (1998). Effects of intrathecal NMDA and non-NMDA antagonists on acute thermal nociception and their interaction with morphine. *Anesthesiology*. doi: 10.1097/00000542-199809000-00023

Nourbakhsh, F., Atabaki, R., & Roohbakhsh, A. (2018). *The role of orphan G protein-coupled receptors in the modulation of pain: A review* (Vol. 212). doi: 10.1016/j.lfs.2018.09.028

Ossipov, M. H., Dussor, G. O., & Porreca, F. (2010). *Central modulation of pain.* doi: 10.1172/JCI43766

Ossipov, M. H., Morimura, K., & Porreca, F. (2014). *Descending pain modulation and chronification of pain.* doi: 10.1097/SPC.0000000000000055

Ouyang, H., Liu, S., Zeng, W., Levitt, R. C., Candiotti, K. A., & Hao, S. (2012). *An emerging new paradigm in opioid withdrawal: A critical role for glia-neuron signaling in the periaqueductal gray.* doi: 10.1100/2012/940613

Paxinos, G., & Franklin, K. (2001). *The Mouse Brain in Stereotaxic Coordinates, 2nd edition.*

Porreca, F., Mosberg, H. I., Hurst, R., Hruby, V. J., & Burks, T. F. (1984). Roles of Mu, delta and kappa opioid receptors in spinal and supraspinal mediation of gastrointestinal transit effects and hot-plate analgesia in the mouse. *Journal of Pharmacology and Experimental Therapeutics*.

Rzasa Lynn, R., & Galinkin, J. L. (2018). *Naloxone dosage for opioid reversal: current evidence and clinical implications.* doi: 10.1177/2042098617744161

Sarton, E., Olofsen, E., Romberg, R., Den Hartigh, J., Kest, B., Nieuwenhuijs, D., ... Dahan, A. (2000). Sex differences in morphine analgesia: An experimental study in healthy volunteers. *Anesthesiology*. doi: 10.1097/00000542-200011000-00018

Sharma, S. K., Klee, W. A., & Nirenberg, M. (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.72.8.3092

Shekunova, E. V., & Bespalov, A. Y. (2004). Estrous cycle stage-dependent expression of

acute tolerance to morphine analgesia in rats. *European Journal of Pharmacology*. doi: 10.1016/j.ejphar.2004.01.012

Sirohi, S., Dighe, S. V., Madia, P. A., & Yoburn, B. C. (2009). The relative potency of inverse opioid agonists and a neutral opioid antagonist in precipitated withdrawal and antagonism of analgesia and toxicity. *Journal of Pharmacology and Experimental Therapeutics*. doi: 10.1124/jpet.109.152678

South, S. M., Wright, A. W., Lau, M., Mather, L. E., & Smith, M. T. (2001). Sex-related differences in antinociception and tolerance development following chronic intravenous infusion of morphine in the rat: Modulatory role of testosterone via morphine clearance. *Journal of Pharmacology and Experimental Therapeutics*.

Stiller, C. O., Bergquist, J., Beck, O., Ekman, R., & Brodin, E. (1996). Local administration of morphine decreases the extracellular level of GABA in the periaqueductal gray matter of freely moving rats. *Neuroscience Letters*. doi: 10.1016/0304-3940(96)12638-0

Stockton, S. D., & Devi, L. A. (2012). *Functional relevance of μ-δ opioid receptor heteromerization: A Role in novel signaling and implications for the treatment of addiction disorders: From a symposium on new concepts in mu-opioid pharmacology.* doi: 10.1016/j.drugalcdep.2011.10.025

Stone, L. S., & Molliver, D. C. (2009). *In search of analgesia: Emerging roles of GPCRS in pain.* doi: 10.1124/mi.9.5.7

Stornetta, R. L., Norton, F. E., & Guyenet, P. G. (1993). Autonomic areas of rat brain exhibit increased Fos-like immunoreactivity during opiate withdrawal in rats. *Brain Research*. doi: 10.1016/0006-8993(93)90055-R

Tallarida, R. J. (2000). *Drug synergism and dose-effect data analysis.*

Terrillon, S., & Bouvier, M. (2004). Roles of G-protein-coupled receptor dimerization. *EMBO Reports*. doi: 10.1038/sj.embor.7400052

Terwilliger, R. Z., Beitner-Johnson, D., Sevarino, K. A., Crain, S. M., & Nestler, E. J. (1991). A general role for adaptations in G-proteins and the cyclic AMP system in

mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Research*. doi: 10.1016/0006-8993(91)91111-D

Thornton, S. R., & Smith, F. L. (1997). Characterization of neonatal rat fentanyl tolerance and dependence. *Journal of Pharmacology and Experimental Therapeutics*.

Tortorici, V., Robbins, C. S., & Morgan, M. M. (1999). Tolerance to the antinociceptive effect of morphine microinjections into the ventral but not lateral-dorsal periaqueductal gray of the rat. *Behavioral Neuroscience*, *113*(4), 833–839. doi: 10.1037/0735-7044.113.4.833

Vanderah, T. W., Suenaga, N. M., Ossipov, M. H., Malan, J., Lai, J., & Porreca, F. (2001). Tonic descending facilitation from the rostral ventromedial medulla mediates opioid-induced abnormal pain and antinociceptive tolerance. *Journal of Neuroscience*. doi: 10.1523/jneurosci.21-01-00279.2001

Vaughan, C. W., Ingram, S. L., Connor, M. A., & Christie, M. J. (1997). How opioids inhibit GABA-mediated neurotransmission. *Nature*. doi: 10.1038/37610

Wardman, J. H., Gomes, I., Bobeck, E. N., Stockert, J. A., Kapoor, A., Bisignano, P., ... Devi, L. A. (2016). Identification of a small-molecule ligand that activates the neuropeptide receptor GPR171 and increases food intake. *Science Signaling*, *9*(430). doi: 10.1126/scisignal.aac8035

Way, E. L., Loh, H. H., & Shen, F. H. (1969). Simultaneous quantitative assessment of morphine tolerance and physical dependence. *Journal of Pharmacology and Experimental Therapeutics*.

Wei, S., Feng, Y., Che, F.-Y., Pan, H., Mzhavia, N., Devi, L. A., ... Fricker, L. D. (2004, mar). Obesity and diabetes in transgenic mice expressing proSAAS. *The Journal of endocrinology*, *180*(3), 357–368.

Wiesenfeld-Hallin, Z. (2005). *Sex differences in pain perception.* doi: 10.1016/S1550-8579(05)80042-7

Williams, J. T., Ingram, S. L., Henderson, G., Chavkin, C., von Zastrow, M., Schulz, S., ... Christie, M. J. (2013). *Regulation of μ-opioid receptors: Desensitization,*

phosphorylation, internalization, and tolerance. doi: 10.1124/pr.112.005942

Zachariou, V., Brunzell, D. H., Hawes, J., Stedman, D. R., Bartfai, T., Steiner, R. A., ... Picciotto, M. R. (2003). The neuropeptide galanin modulates behavioral and neurochemical signs of opiate withdrawal. *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1533224100

Zhang, X. Y., Li, Q., Dong, Y., Yan, W., Song, K., Lin, Y. Q., & Sun, Y. G. (2020). Mu-Opioid Receptors Expressed in Glutamatergic Neurons are Essential for Morphine Withdrawal. *Neuroscience Bulletin*. doi: 10.1007/s12264-020-00515-5

Zubieta, J. K., Smith, Y. R., Bueller, J. A., Xu, Y., Kilbourn, M. R., Jewett, D. M., ... Stohler, C. S. (2002). μ-opioid Receptor-Mediated Antinociceptive Responses Differ in Men and Women. *Journal of Neuroscience*. doi: 10.1523/jneurosci.22-12-05100.2002

APPENDICES

APPENDIX A

GPR171 Antagonist Does Not Alter Morphine Tolerance in Male

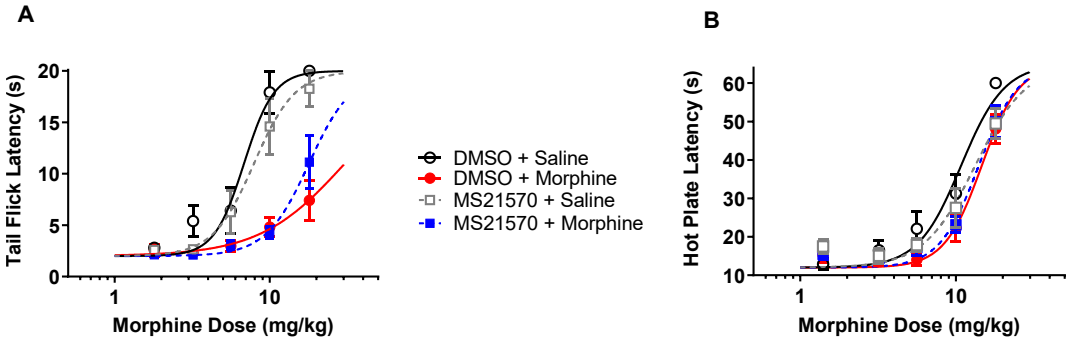

Fig. A.1: GPR171 antagonist does not alter morphine tolerance in male mice on the tail flick and hot plate test. Tolerance was induced by twice-daily injections of morphine for 4 days as seen by a rightward shift in the dose response curve for DMSO + Morphine group compared to DMSO + Saline group in tail flick (A), but not in the hot plate (B) test. Dose response curves were performed on Day 5 using a cumulative quarter log dose procedure of morphine (1, 1.8, 3.2, 5.6, 10, and 18 mg/kg, s.c.). Repeated administration of MS21570 + Morphine on Day 1-4 did not affect morphine tolerance on the tail flick (A) and hot plate (B) test. The data reveals that morphine tolerance is not affected by GPR171 antagonist in male mice. 5-9 animals/group.

APPENDIX B

GPR171 Antagonist Does Not Alter Morphine Withdrawal in Male

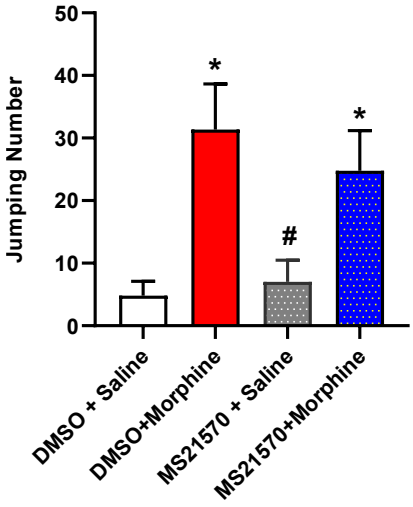

Fig. B.1: GPR171 antagonist does not alter morphine withdrawal. Animals were treated with GPR171 antagonist, MS21570 (5 mg/kg, i.p.) or vehicle (10% DMSO in saline, i.p.) 10 minutes before morphine (5 mg/kg, s.c.) or saline (10 ml/kg, s.c.) injection twice daily for 4 days to induce morphine dependence. On day 5, mice were treated with their designated drug combination. Two hours later Naloxone Hydrochloride (2 mg/kg, i.p.) was administered to induce precipitated withdrawal. Mice were video recorded immediately for 30 minutes to observe withdrawal behavior- jumping. Repeated injection of DMSO + Morphine caused an increase in jumping number compared to the DMSO + Saline treated mice indicating that those mice developed morphine dependence. Pretreatment with MS21570 + Morphine reduced jumping number compared to the DMSO + Morphine group, although the reduction is not significant. Repeated injection of MS21570 + Saline increased the jumping number compared to the DMSO + Saline group, although not significant. $^*P < 0.05$, compared with saline; $\#P < 0.05$, compared with morphine. Data are the means ±SEM. 9–10 animals/group.