Gβγ as a Therapeutic Target for Pain Modulation: Mechanisms of Opioid and Non-Opioid Receptor Potentiation

by

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Dedication

This dissertation is dedicated first to Dr. Emily Jutkiewicz. Throughout my dissertation I often found myself identifying with Emily more than my fellow trainees. Emily not only supported my career goals and family life – but understood them. This was no small feat given my multifaceted interests and objectives on the way to completing this dissertation. Emily and I shared the common language of candor that can be so difficult to cultivate with a mentor. I believe this way of communicating built a deep trust between us, allowing me to build confidence as a scientist and as a person. Emily's indelible legacy lives on through those of us fortunate enough to receive her mentorship and through countless more to whom she served in one of her many roles.

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Figure 3.1. Novel Gβγ modulators inhibit MOR-dependent PLC activation. HEK293 cells stably expressing MOR were co-transduced with BacMam viruses expressing M1 muscarinic receptor and a fluorescent DAG reporter and stimulated with either 300 nM carbachol (red line) or 300 nM carbachol plus 1 μM DAMGO. DAMGO alone gives no response in this assay. **A)** Compound 743 does not significantly inhibit DAMGO-dependent DAG production. **B)** Compound 744 concentration-dependently inhibits DAMGO-dependent DAG production. **C)** Compound 858 concentration-dependently inhibits DAMGO-dependent DAG production. 86

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Figure 3.5. Compound 895 has negligible activity *in vitro* and does not alter morphine-induced antinociception. A) Compound 895 does not alter Gβγ-GRK2 interactions at any concentration tested. **B)** Compound 895 does not alter morphine-induced antinociception compared to vehicle control. 2-way RM ANOVA with Sidak's post-test shows a main effect of treatment F (2, 34) = 6.089, P=0.0055 with compound 858 significantly different from vehicle at 60 min (P=0.0217) and compound 895 not different from vehicle at any time point (N=12 vehicle, N=13 compound 858, N=12 compound 895)..... 90

Figure 3.6. Gβγ modulators enhance morphine-induced antinociception in males, but not females. A) Males exhibit elevated tail withdrawal latencies following gallein pretreatment and morphine, while females do not (3-way RM ANOVA main effect of sex F (1, 20) = 10.14, P=0.0047) (N=6 vehicle females, N=6 vehicle males, N=6 gallein females, N=6 gallein males). **B)** Males exhibit elevated tail withdrawal latencies following compound 858 pretreatment and morphine, while females do not (3-way RM ANOVA main effect of sex F (1, 21) = 11.37, P=0.0029) (N=6 vehicle females, N=6 vehicle males, N=6 females compound 858, N=7 males compound 858). **C)** Tail withdrawal latencies are elevated in male mice treated with compound 743 and morphine, but not females (3-way RM ANOVA main effect of sex F (1, 21) = 11.37, P=0.0029) (N=6 vehicle females, N=6 vehicle males, N=6 compound 743 females, N=6 compound 743 males). **D)** Tail withdrawal latencies are elevated in male mice treated with compound 744 and morphine, but not females (3-way RM ANOVA main effect of sex F (1, 19) = 5.006, P=0.0374) (N=6 vehicle females, N=6 vehicle males, N=5 compound 744 females, N=6 compound 744 males). 91

Figure 3.7. Systemic 858 pretreatment does not significantly alter morphine-induced antinociception. A 30 min pretreatment of 50 mg/kg compound 858 i.p. did not alter tail withdrawal latencies compared to vehicle following morphine (2-way RM ANOVA no main effect of treatment F (1, 23) = 0.2361, P=0.6316) (N=12 vehicle, N=13 compound 858)..... 92

Figure 3.8. Dose comparisons of compound 858 and gallein. A) 30 nmol compound 858 does not enhance morphine-induced antinociception compared to vehicle (2-way RM ANOVA no main effect of treatment F (1, 22) = 2.659, P=0.1172) (N=12 vehicle, N=12 30 nmol compound 858). Mice pretreated with 50 nmol compound 858 display significantly elevated tail withdrawal latencies compared to vehicle pretreatment following morphine (2-way RM ANOVA main effect of treatment F (1, 23) = 6.147, P=0.0209) (N=12 vehicle, N=13 50 nmol compound 858). 100 nmol compound 858 did not enhance morphine-induced antinociception compared to vehicle (2-way RM ANOVA no main effect of treatment F (1, 22) = 2.737, P=0.1123) (N=12 vehicle, N=12 100 nmol compound 858). **B)** 50 nmol gallein does not alter tail withdrawal latencies compared to vehicle following morphine (2-way RM ANOVA no main effect of treatment F (1, 22) = 0.8335, P=0.3712) (N=12 vehicle, N=12 50 nmol gallein). Mice pretreated with 100 nmol gallein show significantly elevated tail withdrawal latencies compared to vehicle following morphine (2-way RM ANOVA main effect of treatment F(1, 22) = 8.997, P=0.0066) (N=12 vehicle, N=12 100 nmol gallein). 93

Abstract

The clinical utility of μ-opioid receptor (MOR) agonists is limited by side effects and tolerance. G protein βγ subunits (Gβγ) are critical mediators of MOR signaling that drive antinociception but also regulate phospholipase C and G protein-coupled receptor kinase pathways that feedback and inhibit MOR signaling. Modulation of Gβγ signaling with small molecules to preferentially inhibit feedback inhibition of MOR has been proposed as a strategy to enhance analgesia without exacerbating adverse effects. Overall, the experiments described in the current dissertation evaluated mechanisms by which Gβγ signal modulators, such as gallein, enhance the antinociceptive effects of analgesic drugs with a focus on opioids.

To assess whether enhancements in opioid-induced antinociception by gallein are dependent on MORs, we tested whether gallein could enhance the antinociceptive effects of opioids other than morphine and whether these effects were sensitive to naloxone pretreatment. We additionally sought to address whether gallein could enhance the antinociceptive effects of non-opioid analgesics. Gallein pretreatment significantly enhanced the antinociceptive effects of different MOR agonists and non-opioid analgesics that act through Gi -coupled GPCRs, with the strongest effects observed upon co-administration with morphine. Gallein's enhancement of morphine-induced antinociception was abolished by the opioid antagonist naloxone and was absent in MOR-knockout mice.

Given these data, we investigated whether these MOR-dependent effects of gallein were mediated centrally or peripherally. Peripherally administered MOR antagonist, route of gallein administration, and different nociceptive assays address this question. Systemic naloxone methiodide, which cannot cross the blood brain barrier, did not prevent gallein's enhancement of morphine-induced antinociception. Intracerebroventricular (i.c.v.), but not intrathecal (i.t.), administration of gallein enhanced morphine-induced antinociception. Gallein was also effective at enhancing morphine's action in a hot plate assay, implicating supraspinal MOR dependency.

Last, 10 S/T-A mice expressing phospho-site-deficient MORs lost responsiveness to gallein treatment, supporting a mechanism of action where gallein inhibits Gβγ-dependent inhibitory feedback phosphorylation of MOR in the CNS.

In another series of studies, we further validated the purported mechanism of gallein action to inhibit Gβγ-dependent inhibitory feedback on MORs by showing that a new series of completely novel and structurally distinct Gβγ modulators also enhanced morphine-induced antinociception. The underlying commonality between these molecules was their ability to inhibit Gβγ-mediated activation of PLCβ3 and GRK2. Given that these modulators enhanced morphine-induced antinociception, these data point to Gβγ as the molecular target *in vivo*. The most potent and efficacious of these novel modulators was compound 858. While compound 858 enhanced morphine-induced antinociception with greater potency than gallein when administered i.c.v., this was not the case for systemic administration where compound 858 had no effect. Pharmacokinetic data show that compound 858 did not reach brain tissue, highlighting the importance of central effects for these modulators.

The work presented in this dissertation furthers our understanding of *in vivo* mechanisms by which gallein enhances opioid-induced antinociception. This serves the underlying goal of widening the therapeutic index of opioid analgesics by advancing new avenues to improve safety and efficacy.

Chapter 1 Introduction

1.1 Pain

The International Association for the Study of Pain (IASP) defines pain as an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage (1). From this definition and from our lived experience it is apparent that pain plays the crucial physiological role of alerting an organism to the threat of tissue damage. This is particularly true for acute pain wherein a short-lived noxious stimulus (such as accidentally touching a hot stove) provides an incentive to cease the harmful behavior (2, 3). The importance of pain physiology is evidenced by observing those with congenital insensitivity to pain (CIP). These individuals are known to engage in self-harm or persist for long periods with untreated tissue damage. For these reasons, acute pain is often seen as an adaptive response of an organism to its environment (3).

In contrast to acute pain, some chronic pain states are considered maladaptive. Chronic pain is defined as pain that is experienced most days or every day for a period of three or more months (4). Importantly, certain chronic pain states may not be accompanied by actual tissue damage and there is often no way to distinguish this pain experience from that associated with tissue damage (5). This makes pain a highly subjective and affective experience. The IASP addresses this in their definition with the addition of the phrase "resembling that associated with." While it is valuable to address both acute and chronic pain conditions, we focus here on the neurophysiology of the acute pain response under normal physiological conditions and touch briefly on chronic pain.

1.2 Acute Pain

1.2.1 The Afferent Pain Pathway

Acute pain is largely described by the process of nociception, the communication between neurons transducing a peripheral noxious stimulus into electrochemical information processed by the central nervous system. Therefore, acute pain is often described as nociceptive pain. A noxious

stimulus is first detected by specialized primary afferent sensory neurons known as nociceptors which detect pain signals in the periphery through a variety of receptor-mediated signal transduction mechanisms (Figure 1.1). These neurons exhibit a distinctive pseudo unipolar morphology with cell bodies clustered in ganglia (dorsal root ganglia, DRG) alongside the spinal cord or within the skull in the case of the trigeminal ganglia which innervate cephalic tissues (2). Axonal projections extend outward toward the periphery where initial pain signals are received by transient receptor potential (TRP) channels sensitive to temperature changes and mechanical perturbation (6). TRP channel opening can ultimately lead to nociceptor depolarization and action potential generation. Shorter projections extend within different laminae of the spinal cord dorsal horn (7).

There are three broad categories of primary afferent neurons distinguished primarily by their axonal diameter and myelination: heavily myelinated Aβ fibers with wide diameter, lightly myelinated Aδ fibers with narrower diameter, and the narrower-still unmyelinated C fibers (2). These morphological differences translate into differences in functional conductance and the physiological roles these afferents play. With greater myelination and diameter, Aβ fibers exhibit rapid conduction velocity with low action potential thresholds. These fibers convey tactile sensations and light touch or hair movements. Higher threshold Aδ and C afferents conduct at lower velocities and are generally considered nociceptors and thermoreceptors. While mainly involved in detecting pain, C fibers are also responsible for pruritus. These morphological differences in primary afferents also translate into differences in termination within the laminae of the spinal cord. Higher threshold Aβ fibers terminate in deeper laminae (II-VI) as compared to Aδ and C afferents where termination is generally more superficial (laminae I-II) (7). C fibers themselves are split into two categories: peptidergic and non-peptidergic (8). Peptidergic C fibers contain neuropeptides such as substance P and innervate deep tissues (9-11) while non-peptidergic C fibers innervate the epidermis (12).

As previously mentioned, primary afferents synapse within various laminae of the dorsal horn. The vast majority of post-synaptic neurons in the dorsal horn are interneurons with local axonal projections and dendritic arborizations (7). These interneurons are either excitatory or inhibitory with glutamate or GABA as primary neurotransmitters, respectively. To communicate sensory inputs supraspinally, interneurons subsequently synapse onto projection neurons whose axons cross the midline of the spinal cord and travel to brain regions responsible for pain and

temperature perception (Figure 1.1). These regions include the caudal ventrolateral medulla, nucleus of the solitary tract, the lateral parabrachial area, the periaqueductal gray matter (PAG), and the thalamus (13). In this way, noxious sensory information travels from the periphery to the spinal cord – and ultimately to various regions of the brain for supraspinal processing.

1.2.2 Descending Modulation

Noxious stimuli are not only transmitted from the periphery up toward the brain but are also modulated by supraspinal inputs that terminate within the dorsal horn of the spinal cord – a phenomenon known as descending modulation. These descending inputs travel through key regions such as the PAG and the rostroventral medulla (RVM) (14) to alter neurotransmission at the level of the spine through two primary monoamine neurotransmitter systems: serotoninergic (originating in the raphe magnus) and noradrenergic (originating in the locus coeruleus and other pontine regions) (Figure 1.1). Interestingly, these inputs primarily modulate incoming afferent signals through bulk volume transmission as opposed to forming individual synapses (7). As such, these axons terminate diffusely throughout the dorsal horn. Most cells within the dorsal horn will hyperpolarize in response to serotonin or norepinephrine, reducing neurotransmission of incoming noxious stimuli at the level of the spine. However, long-term plasticity or acute signaling alterations in supraspinal circuits can lead to descending pain facilitation (14).

1.3 Chronic Pain

Chronic pain is defined as pain that is present most days or every day for a period of three or more months (4). The transition from adaptive pain processing to maladaptive chronic pain states is multifactorial and an ongoing area of investigation. Moreover, this process characterizes the change from normal pain processing to hyperalgesia (noxious stimuli become extremely noxious) or allodynia (benign stimuli such as touch become painful). Progression beyond these pain states is sometimes characterized as nociplastic pain where the sensation of pain persists in the absence of any apparent stimuli or tissue damage (15).

1.3.1 Maladaptive Pain and Central Sensitization

Perhaps unsurprisingly, one of the primary mechanisms underlying the transition from adaptive to maladaptive pain signaling is aberrant plasticity in dorsal horn neurons (2). In the context of pain, this plasticity is termed central sensitization. Central sensitization spans a continuum from rapid and reversible activity-dependent induction which serves to warn against

harms adjacent to the initial site of injury to longer-term changes in protein expression which may present as intractable pain (16). In the latter case, dorsal horn plasticity and resulting chronic pain may be maintained by lower threshold inputs (such as Aβ fibers) which would normally be insufficient to induce such adaptations.

Induction of central sensitization is activity-dependent and involves repeated dorsal horn stimulation via nociceptor inputs. Induction is rapid and lasts on the order of minutes to hours in the absence of further stimulus (16). Repeated input from primary nociceptor afferents results in several changes in the properties of postsynaptic neurons in the dorsal horn including increased spontaneous activity and lower action potential threshold. While these functional changes are mediated through a number of factors like NMDA and AMPA receptor trafficking, subunit composition, voltage-gated calcium channel (VGCC) activity, and signaling through various kinases, they all converge to increase intracellular calcium and cause hyperexcitability (16). This process is still considered largely adaptive by encouraging limited use of injured body parts.

Pathological central sensitization may be caused by persistent inflammation (16). Clinically, the transition to pathology might be marked by the autonomous maintenance of sensitization in the absence of a peripheral root cause. Thus, painful stimuli are not only perceived as more painful (hyperalgesia), but the physiological adaptations themselves may be maintained by less stimulation. For example, C-fiber stimulation insufficient to induce central sensitization has been shown to maintain central sensitization for periods on the order of days (17). This form of entrenched synaptic plasticity mediated by longer-term changes in protein expression and morphological changes at least partly describes the intractability of some chronic pain conditions including nociplastic pain.

1.3.2 Nociplastic Pain

The IASP defines nociplastic pain as “pain that arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing pain.” This definition does not necessarily imply that no historical noxious stimulus ever existed, but that it may not be the current driver of abnormal pain processing. The IASP definition maps relatively well onto the mechanistic description of later-stage (i.e. maladaptive) central sensitization described above. Clinically, individuals with nociplastic pain explain their symptoms as a diffuse, dull, and aching pain that is distributed widely throughout the body (15). Risk factors include

female sex, sleep disturbances, depression, anxiety, obesity, and physical inactivity among others. Although there are many correlational studies linking these risk factors to nociplastic pain, the directionality of causation is murky. Another hallmark of nociplastic pain is resistance to treatment with conventional analgesics.

Kaplan and colleagues propose two primary subtypes of nociplastic pain: top-down and bottom-up (15). As mentioned earlier, bottom-up nociplastic pain is relatively well described by activity-dependent central sensitization where removal of the noxious stimulus ultimately resolves aberrant pain processing as maladaptive plasticity reverts over time (16). On the other hand, top-down nociplastic pain can occur and be maintained in the absence of nociceptive input. This is perhaps more like later-stage entrenched central sensitization mediated by changes in protein expression. Clinical evidence supports a distinction between nociplastic pain subtypes. In an osteoarthritic population with pre-existing nociplastic pain, total knee or hip arthroplasty reduced pain outside of the surgical site for 2/3 of individuals while 1/3 showed no improvement (18). In other words, addressing the noxious stimulus did not resolve pain in 1/3 of patients. At present, the heterogenous nature of nociplastic pain makes it challenging to distinguish between these proposed subtypes prior to treatment.

1.3.3 Opioids in Chronic Pain

In chronic, neuropathic, and nociplastic pain, opioids are often contraindicated (15, 19-21). However, direct clinical evidence showing a lack of efficacy is actually quite sparse (22-25). One study showed that a non-opioid versus opioid treatment strategy in patients with chronic back, hip, or knee osteoarthritis led to lower pain intensity, no difference in pain-related function, fewer medication-related symptoms, and lower treatment discontinuation (26). However, given the study design, care should be taken not to interpret these findings as a blanket conclusion that opioids are not efficacious in chronic pain. Rather, this study suggests that tiered treatment beginning with low efficacy, low risk medications (like over the counter NSAIDs) and progressing to higher efficacy, higher risk medications only when necessary is advisable.

One meta-analysis examining the efficacy of two weeks or longer morphine treatment in neuropathic pain found a summary effect size in favor of morphine compared to placebo (24). Similar results were found for oxycodone (27). However, authors note that studies meeting inclusion criteria were few and were not of high quality (often due to sample size). The same group was only able to find single studies using similar inclusion criteria for fentanyl and hydromorphone

(28, 29). Overall, the group noted poor reporting of adverse events and high drop-outs for opioid groups. There were greater study withdrawals in the placebo group for the hydromorphone study, but this study used a pre-selection process to identify responders before continuing treatment (30). An older, but more comprehensive, meta-analysis evaluated all opioids for neuropathic pain with less stringent inclusion criteria (31). Both short (less than 6 weeks) and intermediate (greater than 6 weeks, but less than 12 weeks) term opioids were favored for pain relief compared to placebo. Opioids were non-significantly favored in the intermediate term when compared to active placebo (lorazepam or diphenhydramine). Adverse events were again more common in the opioid-treated groups. Looking at longer-term (6 months) opioid treatment in chronic non-cancer pain, adverse events were common in opioid treatment – leading to discontinuation of medication (32). However, there was modest pain relief for those who remained on opioid.

Therefore, it does appear that opioids are able to provide clinical analgesia in chronic pain states. The key problem is that sustained opioid therapy increases the risk of adverse events such that therapeutic benefit is outweighed. While many of the adverse events in the aforementioned studies were minor, other retrospective work shows significantly increased risk of overdose for those prescribed higher doses of opioid (33). Additionally, many of these studies showing benefits of opioids use imputation methods to account for high participant drop-out which may over-emphasize benefit and de-emphasize harm.

1.4 Animal Models of Pain

The measurement of pain – even in humans – is a controversial topic in the field. Here it is imperative to clarify the distinction between antinociception and analgesia. While analgesia refers to the entire phenomenology of pain relief including various affective and subjective components, antinociception implicates only the inhibition of neuronal transduction of noxious stimuli. Thus, in animals we do not measure analgesia, but only infer antinociception from behavioral responses. The animal cannot directly be asked to rate its level of pain (this is often the preferred method in human studies). Therefore, animal models of pain seek to measure some output of the subject's behavior (e.g. latency to a nocifensive behavior such as a withdrawal reflex). These models can be broadly classified into three main groups: 1) acute pain models measuring withdrawal reflex responses, 2) chronic pain or pre-existing pain state models, and 3) pain depressed behavior models.

1.4.1 Acute Pain Models

Acute pain models typically measure an animal's response latency (usually a withdrawal reflex) in response to some transient noxious stimulus. Importantly, these models do not require the experimenter to induce any sort of pre-existing pain state in the animal. Measuring the latency (time) until the animal exhibits a predefined behavior gives the experimenter an indirect measure of the degree of nociception within the animal. Using this paradigm, lower latencies indicate greater nociception. Or, subsequent to analgesic administration, higher latencies indicate greater antinociception. Acute pain models often use noxious thermal stimuli (heat or cold) but may also use chemical or mechanical stimuli.

Noxious thermal stimuli utilize extreme heat or cold. Common assays in this category include the tail flick, warm water tail withdrawal assay (WWTW), hot plate assay, Hargreaves assay, and cold plate assay. Because these assays measure latency until a response, a maximum cut-off time is generally used to avoid tissue damage to the animal. While tail flick and WWTW assay are similar in that they measure a spinally-mediated tail withdrawal reflex arc, the tail flick utilizes a radiant heat source focused on the animal's tail while WWTW immerses the animal's tail in a water bath (usually 50-55°C). In either case, longer latencies until a tail flick behavior in the presence of an analgesic drug suggest a greater antinociceptive effect. Like tail flick, the Hargreaves assay also uses a beam of radiant heat but instead applied the stimulus to the animal's hind paw. Hot plate also uses a 50-55°C temperature range, but responses in this assay are considered to be mediated supraspinally and typically include behaviors such as hind paw licking or jumping from the hot plate (escape behavior). As compared to WWTW or tail flick, hot plate requires relatively greater doses of analgesic drug to achieve a similar relative degree of antinociception. This is often phrased as a higher "efficacy requirement" of the assay. Cold plate is conceptually similar to the hot plate assay and measures similar responses, but plate temperature ranges from 5-15°C (with higher temperatures used more so in animals in pre-existing pain states).

An example of an acute pain model that utilizes a noxious chemical stimulus is the acetic acid stretch assay (AASA). In this assay the animal is treated with 0.6% acetic acid solution in sterile water subsequent to administration of an analgesic or vehicle. A 5 min latency period is typically allowed before beginning behavioral observation for 20 min. During this observation window, the amount of stretching behaviors exhibited by the animal is counted by the experimenter. Route of drug administration is an important consideration in this assay as the acetic

acid solution must be precisely administered into the intraperitoneal space (animals should exhibit roughly 20 stretches during the observation period). Therefore, it may be preferable to deliver an analgesic into a separate compartment (subcutaneously, for example). Compared to other acute pain models which are mediated by central pain circuits, the AASA is thought to involve primarily peripheral circuits as the effects of morphine are blocked by a peripherally restricted MOR antagonist in this assay (34). Last, on the spectrum of efficacy requirement, AASA is the lowest efficacy requiring assay among the acute pain models described here.

Acute mechanical assays typically use an algometer to measure the force, or pressure, applied to a specific region of the animal (usually the hind paw). The Randall Selitto assay is one such example. This assay requires acclimation of the animal to a restraint device before applying a gradually increasing pressure to the animal's paw. The maximum pressure observed before the animal exhibits a flinching/withdrawal behavior is recorded. Again, a maximum pressure is generally used to avoid tissue damage to the animal. Higher pressures in the presence of an analgesic indicate antinociception. More commonly, von Frey filaments are also used as a measure of mechanical pain. Filaments of increasing tensile strength are applied to the hind paw to measure withdrawal responses. Antinociception is indicated by the absence of hind paw withdrawal following application of higher tensile strength filaments.

It should be noted that most, if not all, of the aforementioned acute pain models can be and often are combined with manipulations to induce hyperalgesia or allodynia. However, these models do not require a pre-existing pain state and are frequently used on naïve animals. Therefore, they are included in this section as compared to models which are defined by the induction of hyperalgesia or allodynia.

1.4.2 Chronic Pain Models

Chronic or pre-existing pain state models seek to induce an allodynic or hyperalgesic effect in the animal. Depending on the model, this effect may be chronic (on the order of weeks or months) or simply a pre-existing condition present days or hours before nociceptive testing. In either case, these models are meant to replicate chronic pain and/or neuropathic conditions. The longest lasting of the chronic pain models are the spared nerve injury or sciatic nerve ligation models (among several others), while Complete Freund's Adjuvant (CFA) or carrageenan pretreatment and nitroglycerin-induced hyperalgesia models are common models with shorter durations.

Spared nerve injury involves the removal of a 2 mm section of the tibial and peroneal nerves, while the sural nerve is left unaltered. Sciatic nerve ligation constricts a portion of the sciatic nerve utilizing a suture. Both neuropathic pain models produce a chronic persistent hypersensitivity to noxious stimuli in the paw of the surgerized limb. Spared nerve injury may produce the most persistent hypersensitivity lasting as long as 8 months (35, 36). Such models enable experimenters to answer longitudinal questions.

In contrast to these chronic models involving surgically-induced neuropathic conditions, models which utilize CFA, carrageenan, or nitroglycerin-induced hyperalgesia seek to elicit an allodynic or hyperalgesic state shortly prior to testing. CFA or carrageenan pretreatment to the animal's hind paw are often used in conjunction with mechanical threshold measurements such as von Frey or Randall Selitto. Investigators look for a decrease in withdrawal threshold (the animal withdraws the hind paw at lower tensile strength filaments or applied pressure) subsequent to CFA/carrageenan pretreatment to identify the presence of allodynia. Importantly, peak inflammation induced by CFA can be observed 24 hrs later – or as soon as 3 hrs for carrageenan (37). Analgesics can then be tested to assess an increase in withdrawal threshold back to baseline. Nitroglycerin-induced hyperalgesia employs a similar pretreatment model to induce hypersensitivity to noxious stimuli. Here, animals typically receive a 10 mg/kg pretreatment of nitroglycerin and are allowed to incubate for 1-4 hrs. While nitroglycerin-induced hyperalgesia is typically thought to resemble a migraine phenotype, hyperalgesia appears generalizable, and animals exhibit significantly lower response latencies following pretreatment in assays such as WWTW (38).

1.4.3 Pain Depressed Behavior Models

Pain depressed behavior models seek to assess changes in normal animal behavior in response to painful stimuli. Crucially, these models measure antinociception as the restoration of behavior as opposed to previously discussed models which measure antinociception as absence of a behavior (e.g. withdrawal reflex). These models have been popularized relatively recently due to the idea that they more accurately represent the affective components of pain and may represent a more translationally relevant paradigm with greater face-validity (39). Behaviors of interest in these models include nesting, marble burying, voluntary locomotor activity, grooming, or operant behaviors like lever-pressing for food pellets. Some research has validated such methods by demonstrating animals that underwent chronic constriction injury to the sciatic nerve exhibited

fewer digging behaviors and total marbles buried (40). These reductions were rescued by the administration of various analgesic drugs including opioids. Likewise, Negus and colleagues found that reductions in nesting behavior following CFA were rescued by ketoprofen (an NSAID) and morphine (41). Another group found that various analgesics restored volitional wheel running following CFA-induced decreases in mice (42). Interestingly, animals returned to wheel running while allodynia measured via von Frey assay was still present, suggesting differences in sensitivity in these models. Last, operant models which measure orofacial responses in mice and rats have been well-characterized (43-45).

Proponents of these models claim that restoration of pain depressed behavior reduces the likelihood of false positives by avoiding behavioral disruption (39). For example, a sedative drug or muscle relaxant may be interpreted as analgesic through its ability to decrease withdrawal reflex responding. On the other hand, a sedative would not be expected to rescue pain-depressed behavior. Caution should be taken not to over-interpret these purported benefits. While true that these models may reduce the likelihood of false positives, they concomitantly create a false negative problem. A truly analgesic compound with some sedative effects may be thrown out by these models due to a failure to restore behavior. A compound thought to be analgesic in operant models may have altered the rewarding properties of a food pellet as opposed to providing analgesia.

As behavioral pharmacologists who cannot communicate directly with study subjects, we are always in a position to question whether we are really measuring what we think we're measuring – regardless of the model. This highlights the importance of rigorous science with adequate controls. Sedative effects in withdrawal reflex paradigms could be assessed through locomotor activity assays. Alteration of the rewarding properties of food could be evaluated by examining changes in operant responses from baseline following drug treatment alone. Pain depressed behavior models do provide additional ways to dissect components of the overall behavioral response following drug treatment and can provide valuable insight when performed rigorously. However, we do not yet have sufficient evidence to support the claim that these models, in and of themselves, provide a solution to a lack of predictive validity or translational relevance in preclinical models of pain.

1.5 Treatment and Management of Pain

The treatment of pain is characterized by alleviating or eliminating the noxious sensation, a phenomenon known as analgesia. Modern classification of pain treatments often broadly categorizes therapeutics into opioid and non-opioid analgesic categories. While opioid analgesics exert their effects through one or multiple of the opioid receptor subtypes, non-opioid analgesics function through various distinct mechanisms. Non-opioid analgesics include non-steroidal anti-inflammatory drugs (NSAIDs), acetaminophen, and adjuvant analgesics (e.g. benzodiazepines, α2-adrenergic agonists, antidepressants, and cannabinoid receptor agonists). Adjuvant analgesics are of particular interest here. While these drugs were originally developed for some purpose other than analgesia, they exert analgesic effects and often enhance the efficacy of opioids for pain without enhancing opioid-related side effects (a phenomenon known as "opioid-sparing"). The focus of this dissertation is opioid analgesics, but some attention will also subsequently be paid to opioid adjuvants – particularly α2-adrenergic agonists and cannabinoid receptor agonists.

1.6 History of Opioids in Pain Management

Opioids have a long history in use as analgesics. As early as 1680, laudanum (a mixture of opium and alcohol) was used for pain management by European physicians with opium itself being used long before this – dating back to ancient Sumerian culture (46, 47). Opiates continued as a standard of care for acute and chronic pain throughout the 19th century. Wilhelm Serturner extracted morphine from opium in 1804 (48) with industrial production commencing in 1820s Germany and followed by the United States in the 1830s (46). Still, use of industrially produced morphine in pain management did not become widespread until the advent of hypodermic needles and syringes in the 1850s (49). Almost 70 years after industrial morphine production in the US, diacetylated morphine (diamorphine) was introduced by the Bayer Company of Germany as Heroin – a cough medication. While acetylated salicylic acid (aspirin) came shortly thereafter, opioids remained crucial for severe pain conditions. Continued progress in the field led to the development of many new opioid analgesic drugs in the following years: dihydrocodeine (Paracodin) in 1911, oxycodone (Roxicodone, Eukodal) in 1915, hydrocodone (Dicodid) in 1921, hydromorphone (Dilaudid) in 1926, meperidine (Demerol, Dolantin) in 1939, and methadone marketed as Dolophine during WWII (3). Synthesis of the fentanyl series followed shortly thereafter in the 60s and 70s (50). Since this time, developments in opioid treatment for pain have largely focused on alternative delivery methods (transdermal fentanyl, for example) and

formulations including sustained release and local naloxone (a nonselective opioid receptor antagonist) release (3, 51).

1.7 Opioid Receptors

Opioids exert their effects by activating opioid receptors, which were first discovered in neural tissue by Pert and Snyder in 1973 (52). A few years later Yaksh and colleagues discovered that various opioids produced antinociception when injected into the spine of rats (53). Only months later, Yaksh also published work showing that the greatest antinociceptive effect of intracerebrally administered morphine was observed when drug was injected into the periaqueductal gray (PAG) (54). In both cases, the antinociceptive effects of morphine were reversed by naloxone. These findings suggested a role for opioid action at receptors spinally and supraspinally that contributed to analgesic effects. Around the same time it was proposed that opioid receptors were expressed in three distinct types which primarily responded to different ligands: μ-opioid receptors (MORs) activated by morphine, κ-opioid receptors (KORs) activated by ketocyclazocine, and δ-opioid receptors (DORs) activated by enkephalins (55, 56). Of these, MORs primarily mediate the analgesic effects of opioids, although the other types also mediate analgesia to a lesser extent.

Opioid receptors are found throughout both the afferent pain pathway and descending modulatory circuits. Opioid receptors are expressed at both peripheral and central terminals of primary afferent neurons, excitatory interneurons within the dorsal horn, and many of the aforementioned supraspinal brain stem regions (57, 58). At the level of the spine, the primary analgesic action of opioids is to produce hyperpolarization and reduce neurotransmission initiated by a noxious stimulus (59). Supraspinally, opioids act to inhibit GABAergic neurons in the PAG causing a net excitatory effect in the RVM (disinhibition) (60). Ultimately, this increases descending modulatory input to the spine to facilitate antinociception.

1.7.1 Intracellular Signaling of Opioid Receptors

Opioid receptors are G protein coupled receptors (GPCRs). GPCRs engage intracellular signaling pathways by activating constituent heterotrimeric G proteins subsequent activation by an orthosteric agonist (Figure 1.2) and this is the means by which opioids receptors achieve physiological effects at the cellular level. Agonist binding stabilizes an active form of the receptor which promotes exchange of GDP for GTP on the alpha subunit (Gα) of the G protein and

dissociation of Gα from the βγ subunit (Gβγ). GPCRs are primarily typified by the Gα subunit contained within the G protein heterotrimer that couple to the receptor, for example, Gαs, Gαi/o, Gαq, or Gα12/13. Coupling is thought to be more of a preference than absolute specificity. Structural studies implicate the α5 helix of Gα (61-64) and its interactions with receptor transmembrane domains 5 and 6 (65, 66) as a key driver of receptor coupling preference with G protein subtypes. However, the mechanisms responsible for GPCR G protein specificity are not fully understood (67). In any case, opioid receptors are widely considered Gαi/o-coupled GPCRs. Accordingly, G protein activation downstream of the agonist-receptor complex will produce Gαi/o-GTP subunits which inhibit adenylyl cyclase (in contrast to Gαs which activates adenylyl cyclase).

While Gα modulates adenylyl cyclase, it is Gβγ-mediated signaling that is largely responsible for the intracellular effects of opioid receptors which ultimately translate into analgesia. Gβγ engages with several functionally diverse proteins within the cell, including voltage gated calcium channels (VGCCs), inwardly rectifying potassium channels (GIRKs), soluble N-ethylmaleimide-sensitive factor attachment receptors (SNAREs), phospholipase-C β (PLCβ), and G protein-coupled receptor kinases (GRKs) (68). These effectors can be grouped into two general categories when thinking about their effects when engaged by Gβγ downstream of opioid receptor activation: 1) effectors which reduce neurotransmission and 2) effectors which negatively regulate the activated opioid receptor.

Gβγ interactions with VGCCs, GIRKs, and SNAREs reduce neurotransmission. These are the primary effects at the cellular level which produce opioid-induced analgesia. Gβγ inhibits VGCCs presynaptically (69, 70). Reduced presynaptic calcium influx ultimately reduces neurotransmitter release by decreasing calcium-dependent mechanisms responsible for vesicle fusion. For example, calcium binds directly to synaptotagmin to promote fusion of primed vesicles (71). Gβγ also inhibits vesicle fusion by directly interfering with SNARE binding to calcium-synaptotagmin (72). Postsynaptically, Gβγ activates GIRKs (73, 74), leading to increased potassium efflux and hyperpolarization of the neuron. Together, these effects of Gβγ reduce neurotransmission from primary afferents synapsing within the dorsal horn and reduce GABAergic tone on serotonergic and noradrenergic neurons supraspinally. This latter effect disinhibits descending modulation of pain. At the level of the whole organism, these changes in neuronal function produce analgesia.

Gβγ-mediated activation of GRKs (75) or PLCβ (76, 77) engage mechanisms which negatively regulate opioid receptor function. As the name suggests, GRKs phosphorylate the C-terminal tail of opioid receptors. This leads to recruitment of arrestins and subsequent G protein decoupling as well as receptor internalization. Similarly, activation of PLCβ3 by Gβγ also leads to downstream receptor phosphorylation – although the phosphorylation event is ultimately mediated by protein kinase C (PKC) in this case. In all, MORs have 11 serine/threonine sites on the C-terminal tail which can be phosphorylated by GRKs or PKC (78). These findings highlight the dual role of Gβγ to both regulate opioid receptor function as well as mediate their analgesic effects. Moreover, these Gβγ-mediated inhibitory effects on neurotransmission would be expected of synaptic Gαi-coupled GPCRs broadly.

1.8 α2-Adrenergic Agonists in Pain Management

α2-adrenergic agonists have been in clinical use for decades as antihypertensive agents. Like the opioid receptors, α2-adrenergic receptors (A2Rs) are also Gi/o-coupled GPCRs. These receptors are expressed throughout the peripheral (e.g. on vascular smooth muscle) (79) and central (e.g. dorsal horn neurons, dorsal root ganglia, brainstem and midbrain regions) (80-83). Antihypertensive effects of these drugs are generally thought to be due to action at presynaptic autoreceptors which reduces central sympathetic outflow via decreased epinephrine release. Similar to opioid receptors, activation of A2Rs generally causes a hyperpolarizing effect on dorsal horn neurons to suppress nociceptive input from primary afferents (84, 85). As analgesics, A2R agonists such as clonidine or tizanidine are often administered intrathecally and have shown some efficacy as monotherapies (86-88).

1.9 Cannabinoid Receptor Agonists in Pain Management

Cannabinoid receptors are categorized into CB1 and CB2 subtypes. CB1 receptors are primarily expressed on neurons in the central nervous system (89, 90), while CB2 receptors are found on various immune cell types in the periphery and CNS (91, 92). Both receptors are responsible for the analgesic effects of cannabinoid receptor agonists such as Δ9-tetrahydrocannabinol (Δ9-THC), although through differing mechanisms. Moreover, both receptors are expressed throughout the afferent pain pathway. As with opioid receptors and A2Rs, CB1 and 2 receptors are Gi/o-coupled GPCRs. Preclinical evidence has identified antinociceptive effects of the CB1 receptor in both acute and chronic pain models (93, 94). In accordance with

expression on immune cells, CB2 receptors have been shown to play a role in reducing neuropathic pain and inflammation (95, 96). Clinically, smoked cannabis provides analgesic benefit in chronic neuropathic pain (97).

1.10 Sex Differences in Pain and Pain Management

It is a common misconception that women are less sensitive to pain as compared to men. This view likely derives from a perceived evolutionary need to tolerate the pain of childbirth. However, the evidence points to greater pain sensitivity in women compared to men (98). While there is some debate as to whether this difference is based on sociocultural factors related to gender identity (e.g. lower healthcare utilization by men (99)) as opposed to real biological differences, the overwhelming majority of studies point to greater pain sensitivity in women (98, 100). Importantly, this relationship appears to hold irrespective of the applied noxious stimulus or the means of measurement. Despite these differences in humans, preclinical research has a history of bias against the inclusion of female subjects. Introduction of group variability due to estrous phases in female rodents has been a commonly argued reason for this. However, this apprehension is unfounded given the finding that it is male mice which exhibit greater variability when evaluated in large data sets (98, 101).

Differences in pain between sexes implicates mechanistic differences in underlying pain physiology and the efficacy of treatments to manage pain. One hallmark instance of such differences is the sex-dependent role of calcitonin gene-related peptide (CGRP) in pain (100). For example, intraplantar administration of CGRP into the hind paw produces mechanical (102), cold, and heat hypersensitivity in female, but not male rodents (103). Accordingly, FDA-approved migraine treatments designed to block the effects of CGRP show antihyperalgesic effects in female mice only (104, 105). These preclinical data are clinically validated (106).

Sex differences in pain management extend to opioids. When considering MOR agonists, antinociceptive effects are typically more pronounced in male as compared to female rodents (98, 107-109). However, this contrasts to the human case where women exhibit greater analgesic benefit (107). Preclinically, these differences are unlikely due strictly to peripheral pharmacokinetics (and subsequent CNS concentrations) as sex differences are still observed after central morphine administration (110-112). Evidence suggests that ligand binding and expression of opioid receptors and endogenous opioid peptides are modulated by gonadal steroid hormones (113-115). However, alterations in receptor-ligand binding by sex hormones *in vitro* may not

necessarily be reflected in antinociceptive responses *in vivo* (108). Interestingly, sex differences in opioid-induced antinociception could be related to agonist efficacy with differences being more pronounced for low efficacy agonists. This would suggest a lower receptor reserve in females either due to lower expression, or lower intrinsic efficacy of the receptor. One study showed greater antagonist mediated rightward shifts in dose-response curves for antinociception in female rats compared to males when lower efficacy agonists were used (116). Furthermore, pretreatment with irreversible antagonist produced greater rightward shifts in morphine-treated females than males, suggesting a difference in receptor reserve between sexes. However, this effect was absent when other MOR agonists were examined.

To make the matter even more complex, sex differences have also been observed for the antinociceptive effects of analgesic adjuvants: noradrenergic and cannabinoid receptor agonists. For instance, it was found that clonidine increased the propensity for male rats to undergo noxious heat in order to obtain a reward in an operant model of orofacial pain (117). This effect was also observed in ovariectomized females, but not when treated with estradiol. On the other hand, antinociception mediated by another α2-adrenergic receptor agonist, tizanidine, was demonstrated to be more robust in females as compared to males in a neuropathic pain model (118). In the case of the cannabinoids, Δ9-THC produces greater analgesic effects in female rats in both acute and chronic pain models (119, 120). In mice, it was found that females express CB1 receptors to a greater extent on GABAergic neurons within the ventrolateral periaqueductal gray (vl-PAG) (121). Activation of CB1Rs in coronal PAG slices elicited a greater reduction in spontaneous inhibitory postsynaptic currents in females compared to males, suggesting greater disinhibition of descending pain modulation. Interestingly, upon repeated administration of Δ9-THC either no sex difference or a greater benefit in males is observed (122, 123). This effect seems to be borne out in observational data from recreational cannabis users showing greater pain relief for men (124, 125).

Sex differences in pain and pain management can inform treatment strategies and hopefully improve outcomes. Treatment of pain in women with CGRP inhibitors or CGRP receptor antagonists present an example where data demonstrating underlying mechanistic differences was successfully implemented. Still, sex differences in response to pain treatments seems clearly dependent on the type or treatment and patient history.

1.11 Prior Attempts to Ameliorate Opioid-Related Side Effects

1.11.1 Drawbacks of Opioids as Analgesics

While opioids (specifically, MOR agonists) are often considered the gold standard class of analgesics in terms of potency and efficacy, their use is associated with multiple drawbacks, or side effects. These drawbacks include a relatively rapid development of tolerance to analgesic effects, risk of substance abuse, constipation, and respiratory depression. Widening the therapeutic index of opioid analgesics or altogether separating beneficial analgesic effects from unwanted side effects has been an ongoing objective in the field of opioid research. In 1929, Harvard pharmacologist Reid Hunt stated that, "A thorough study of the morphine molecule might show a possibility of separating the analgesic from the habit-forming property…" (126). This goal is particularly challenging given that both analgesic effects and side effects of opioids are mediated through target MORs. Prior work has focused on two primary strategies to improve the therapeutic index of MOR agonists: 1) opioid-sparing strategies using co-treatment with non-opioid analgesics and 2) the development of biased ligands.

1.11.1.1 Opioid Sparing Through Non-Opioid Co-Treatment

Opioid-sparing refers to the enhancement of therapeutic analgesic effects without the enhancement of common opioid-related side effects such as constipation and respiratory depression. In this way, the therapeutic index of MOR agonists can be improved. Clinicians may be able to use lower doses of MOR agonist to achieve the same analgesic effect under these circumstances. Opioid-sparing is often accomplished by co-administration of non-opioid analgesics alongside opioid treatment. Both α2-adrenergic receptor agonists and cannabinoid receptor agonists have been used for this purpose.

The antinociceptive interaction between opioid and α2-adrenergic receptors has been heavily studied (127). Synergy between clonidine and MOR agonists (morphine, fentanyl, and meperidine) was rigorously shown by Ossipov and colleagues in 1990 by isobolographic analysis (128), although some evidence precedes these studies (129). These data showed that synergy was most robust when clonidine was administered intrathecally, suggesting a spinal mechanism. One clinical study found that lower doses of alfentanil could be used during cholecystectomy in patients also receiving clonidine (130). Overall, though, meta-analysis of clinical studies examining

intrathecal clonidine co-administered with morphine shows only a small effect in reducing post-operative morphine with heterogeneous results (131).

The interaction between cannabinoid receptor agonists and opioids is complex and the apparent synergism between these classes of analgesics is an ongoing area of investigation. A somewhat recent meta-analysis of preclinical data demonstrated favorable effects of THC on opioid-sparing with moderate-to-large pooled effect estimate (132). However, the analyzed studies exhibited strong heterogeneity, and heterogeneity in clinical data precluded meta-analysis. In a separate preclinical study, isobolographic analysis found that pretreatment with cannabinoid receptor agonist WIN55212 was synergistic for morphine's antinociceptive effects, but only additive in terms of locomotor coordination deficits measured via rotarod (133). The interplay between cannabinoids and opioids may in part be evidenced by the fact that CB1 often colocalizes with opioid receptors. For example, both CB1 receptors and MORs are expressed on dorsal horn neurons receiving inputs from primary afferents (134). Furthermore, both CB1 and CB2 receptor agonists increase the release of endogenous opioids (135, 136).

One way to assess the interaction between the cannabinoid and opioid receptor systems for antinociceptive effects is to examine whether cannabinoid-induced antinociception is sensitive to opioid receptor antagonists. One study found that low doses of naloxone did not block the antinociceptive effects of cannabinoids, while a highly potent kappa-opioid receptor antagonist did (137). However, it is difficult to know whether the doses of naloxone and kappa-opioid receptor antagonist in this study are truly comparable. Another study demonstrated that 1 mg/kg of naloxone did not block the antinociceptive effects of 0.6 mg/kg of CB1/2 receptor agonist CP55940 (138). In contrast, it appears that antinociception mediated by a CB2-selective agonist was naloxone sensitive (135). Therefore, the dependency of CB1/2 receptor effects on opioid receptor function seems nuanced and may be specific to cannabinoid receptor subtype. Interpretation is also limited in single-dose studies.

Antidepressant medications (SSRIs, SNRIs) appear to provide benefit in chronic pain conditions (139). However, SSRIs can also limit opioid prodrug conversion, leading to reduced efficacy (140). Preclinical evidence suggests that tonic 5HT2A activity, may mitigate the beneficial analgesia associated with opioids (141). Ultimately, opioid-sparing through co-administration with non-opioid analgesics may be circumstantially relevant but seems to provide only inconsistent marginal benefits.

1.11.1.2 Biased Agonists

As an alternative approach to opioid-sparing through co-administration of non-opioid analgesics, investigators have long sought to develop opioid ligands with a signaling preference for analgesic benefits over unwanted side effects. Conceptually, the field thought this might be accomplished by producing an intracellular signaling bias in favor of pathways which result in analgesic effects. As such, these drugs have been called biased agonists. This idea hinges on the belief that the intracellular signaling pathways responsible for analgesia and for opioid-related side effects are dissociable from one another. Specifically, it was believed that the analgesic effects of opioid agonists were due to G protein signaling, while unwanted side effects were due to signaling through arrestins. This conception was, in part, due to work conducted by Bohn and colleagues wherein arrestin-3 knockout mice were shown to have enhanced opioid-mediated antinociception with reduced side effects (142, 143). Thus, developing an agonist which stabilized receptor conformations favoring G protein over arrestin signaling would be desirable. One such example is the biased agonist oliceridine, which showed a favored G protein profile *in vitro* (144) and exhibited a purportedly enhanced safety profile in clinical studies (145).

More recently, the dependence of opioid-related side effects on arrestin signaling has been called into question. Kliewer and colleagues found that both morphine and fentanyl did indeed induce enhanced respiratory depression in arrestin-3 knockout mice (146). Furthermore, the same group developed genetically modified knockin mice with serine/threonine to alanine mutations on the C-terminal tail of MORs (10 S/T-A mice) (78). These sites are phosphorylated by GRKs and PKC to recruit arrestins and initiate receptor downregulation. Therefore, arrestin signaling at MORs would be significantly abolished in these mice. Yet, these mice exhibit a worsened side effect phenotype in response to opioids. Gillis and colleagues demonstrated that the therapeutic window of opioid ligands is inversely correlated with intrinsic efficacy (147). Thus, the improved safety profile observed with biased ligands such as oliceridine may simply be due to partial agonism. Therefore, biased agonism may be an observable phenomenon *in vitro* but does not seem to mechanistically translate into an improved therapeutic window *in vivo* upon further scrutiny. The field continues to investigate possible mechanisms to dissociate opioid-related side effects from highly efficacious analgesic effects.

1.12 Gβγ Signal Bias as An Opioid-Sparing Strategy

Gβγ modulates the function of effectors which both aid and oppose MOR-mediated analgesia. These dual roles suggested to investigators that pharmacologically targeting Gβγ could be a means of producing signal bias downstream of opioid receptor activation. Evidence in support of this idea began when Ford and colleagues sought to understand the molecular basis for Gβγ interactions with its multiple effectors. This group performed a series of alanine scanning experiments on the switch-interface surface of Gβγ (where the β subunit interfaces with the switch II region of Gα) and found that mutations in this region caused non-overlapping changes in Gβγ function (148). In other words, altering a given amino acid residue might alter Gβγ-mediated activation of PLCβ3 while leaving other effectors unaltered, for example. This region of Gβγ was later called a protein-protein interaction (PPI) hot spot due to its role in accommodating structurally distinct effector proteins (149).

Considering these data, subsequent investigations worked to develop the notion that Gβγ signaling could be biased *in vitro*. Given that specific amino acids in the PPI region of Gβγ mediated binding and activation of certain effector subsets, this region could be targeted to alter the relative affinity of Gβγ for specific effectors (Figure 1.2). This would produce a biased signaling profile. Scott and colleagues tested this hypothesis using peptides derived from a phage-displayed library (149). Of these peptides, SIRKALNILGPDYD (SIRK) was found to inhibit Gβγ-mediated activation of PLCβ3 and PI3Kγ while leaving DAMGO-mediated inhibition of VGCCs unaltered. These initial findings supported the idea that Gβγ could be pharmacologically targeted to produce signal bias and led to subsequent experiments utilizing small molecules. Bonacci and colleagues found that small molecule M119 (Figure 1.3) inhibited Gβγ-mediated activation of PLCβ3 and PI3Kγ, whereas another small molecule that bound to a distinct subregion of the hot-spot (M201) enhanced it (150). Meanwhile, both molecules inhibited membrane association of GRK2. These data show that small molecules can alter Gβγ-mediated activation of specific effector subsets by interacting with different amino acids within the hot-spot region.

Based on prior data showing that PLCβ3 knockout mice exhibited an enhanced response to the antinociceptive effects of morphine (151), the effects of M119 to reduce Gβγ-mediated activation of PLCβ3 suggested that these small molecules could potentiate the analgesic effects of opioids. Indeed, Bonacci and colleagues tested this idea by co-administering M119 with morphine via intracerebroventricular (i.c.v.) injection in mice (150). In support of the potentiation

hypothesis, M119 co-administration produced a leftward shift in the dose-response curve for the antinociceptive effects of morphine as compared to morphine alone. Moreover, this effect was absent in PLCβ3 knockout mice. Further studies confirmed the enhancement of antinociceptive effects by M119 in response to both morphine and KOR agonist, but not DOR agonists (152). M119 also inhibited the development of acute tolerance to the antinociceptive effects of morphine and reduced jumps induced by naloxone-precipitated withdrawal. Further work showed that the structurally similar compound gallein (Figure 1.3) also acted as a Gβγ modulator in a model of neutrophil chemotaxis (153). This work was expanded by Hoot and colleagues showing that systemic gallein pretreatments enhanced morphine-induced antinociception (154). Importantly, these enhancements occurred in the absence of enhancements in opioid-related side effects such as respiratory depression and constipation. Furthermore, gallein pretreatments did not enhance the rewarding effects of morphine as measured by conditioned place preference (CPP). Recent electrophysiological findings support the notion that gallein does not alter the effects of Gβγ on effectors which contribute to analgesia (155).

More recent work from our group supports these findings by providing insights into properties and effects of gallein as a Gβγ signal modulator. For example, the effects of gallein to potentiate morphine-induced antinociception are dose-dependent with significant enhancements at 100 mg/kg of gallein, but not 50 mg/kg (156). When administered systemically, the effects of gallein were observable within 30 minutes, but were most robust 24 hrs later. Potentiated morphine responses did not completely diminish until sometime between 48 and 72 hrs, suggesting a long-lasting effect. This is curious given that the plasma half-life of gallein is approximately 2 hrs (unpublished data). While gallein binding to Gβγ is reversible, perhaps this effect could be explained by long off-rates (157) or sequestration within cells. Nevertheless, these studies also confirmed inhibition of morphine tolerance by gallein in a more chronic paradigm – in addition to enhancement of morphine-induced antinociception in a tolerant state (156). Moreover, gallein alone appears to enhance the antinociceptive effects of endogenous opioids in an ongoing pain state (38).

Effects of gallein may be attributable to inhibition of synergism between Gq- and Gi/o-mediated activation of PLCβ3 (Figure 1.4). Additional studies from our group indicate that Gq-mediated PLCβ3 activation is synergistically enhanced in the presence of Gβγ released downstream of Gi/o-coupled GPCRs (such as MORs) (158). Since PLCβ3 negatively regulated

MORs, inhibiting this synergism is likely an important component of gallein's effects. Interestingly, this hypothesis suggests a source of tonic Gq signaling within MOR expressing cells of pain circuits. Looking within the ventrolateral PAG would be a good starting point given high MOR expression, its importance in descending pain modulation, and given that bath-applied gallein enhanced inhibition of DAMGO-mediated evoked inhibitory post-synaptic currents (eIPSCs) (158). This effect was also observed in PLCβ3 knockout animals with no further enhancement upon gallein application. These data again link the effects of gallein with Gβγ-dependent PLCβ3 function.

Given the ubiquitous role of Gβγ throughout various organ systems and tissue types, the selectivity and safety of Gβγ inhibition as an opioid-sparing strategy might be called into question. In fact, beneficial effects of gallein have been demonstrated in other models unrelated to antinociception. For example, one group found that gallein was able to halt progression in a model of heart failure by inhibiting Gβγ-mediated recruitment of GRK2 (159-161). This restored β-adrenergic receptor density on myocardial membranes and normalized α2-adrenergic inhibitory feedback in response to catecholamines. Gallein treatment was also able to reduce kidney nephritis in lupus prone mice –by limiting PI3Kγ-induced lymphocyte migration downstream of Gβγ (162). In the latter study, mice were treated three times per week with 35 mg/kg of gallein for 20 weeks with no overt detrimental effects. One heart failure study treated animals daily with 30 mg/kg of gallein for 8 weeks with a similar lack of deleterious outcomes (160). A lack of nonspecific effects is also evidenced by the fact that gallein alone is only able to exert antinociception in an ongoing pain state where endogenous opioid concentrations are elevated (38, 156, 158). Furthermore, although these are not concrete safety data, long-term administration does not seem to be obviously harmful – indicating that non-therapeutic effects are probably not substantial (160, 162). These data suggest that gallein can interact with receptor systems other than opioid receptors, but exogenous agonist or heightened concentrations of endogenous agonist are likely required.

1.13 Objective of Current Experiments and Dissertation

The overall goal of this dissertation is to characterize and develop a therapeutic strategy that improves the safety and efficacy of opioid analgesics. The specific objectives of the experiments described in this thesis are 1) to elucidate mechanisms by which Gβγ inhibitors such as gallein potentiate the antinociceptive effects of morphine (and perhaps other analgesics) and 2) to screen novel Gβγ inhibitor compounds with distinct physicochemical properties beyond the

gallein/M119 scaffold that make them desirable candidates for drug development. These studies aim to address the current pitfalls of opioid treatments for pain (e.g. rapid development of tolerance to analgesic effects, respiratory depression, constipation, and abuse liability) by expanding the therapeutic window for these drugs.

Aim 1: To elucidate mechanisms by which Gβγ inhibitors such as gallein potentiate the antinociceptive effects of morphine (and perhaps other analgesics). The first studies of this thesis work addresses whether the effects of the Gβγ inhibitor, gallein, to enhance morphine-induced antinociception are actually due to the compound's action downstream of MOR activation and whether central or peripheral MORs are required for this effect. Furthermore, we sought to investigate whether gallein might enhance the antinociceptive effects of other analgesics. Finally, we wanted to validate gallein's purported mechanism of action using an *in vivo* mouse model where knockin MORs cannot undergo negative feedback phosphorylation. Based on previous work, I hypothesized that the effects of gallein to enhance morphine-induced antinociception would be dependent upon activation of supraspinal MORs within the central compartment, but not peripheral MORs. Additionally, I hypothesized that gallein would also enhance the antinociceptive effects of other analgesics that are agonists at GPCRs, but not non-GPCR agonists. Last, I hypothesized that gallein would have no effect in a 10 S/T-A mouse model where MORs cannot be phosphorylated by GRKs or PKC.

Aim 2: To screen novel Gβγ inhibitor compounds with physicochemical properties making them desirable candidates for drug development. These studies assess the efficacy of novel Gβγ inhibitor compounds to potentiate morphine-induced antinociception. Importantly, these modulators are structurally distinct with their underlying commonality being Gβγ signal modulation. It was hypothesized that novel compounds could be discovered with desirable physicochemical properties for drug development (as compared to gallein) would potentiate morphine-induced antinociception comparably to gallein with more favorable potency and validate Gβγ as the pharmacological target *in vivo*.

1.14 Figures

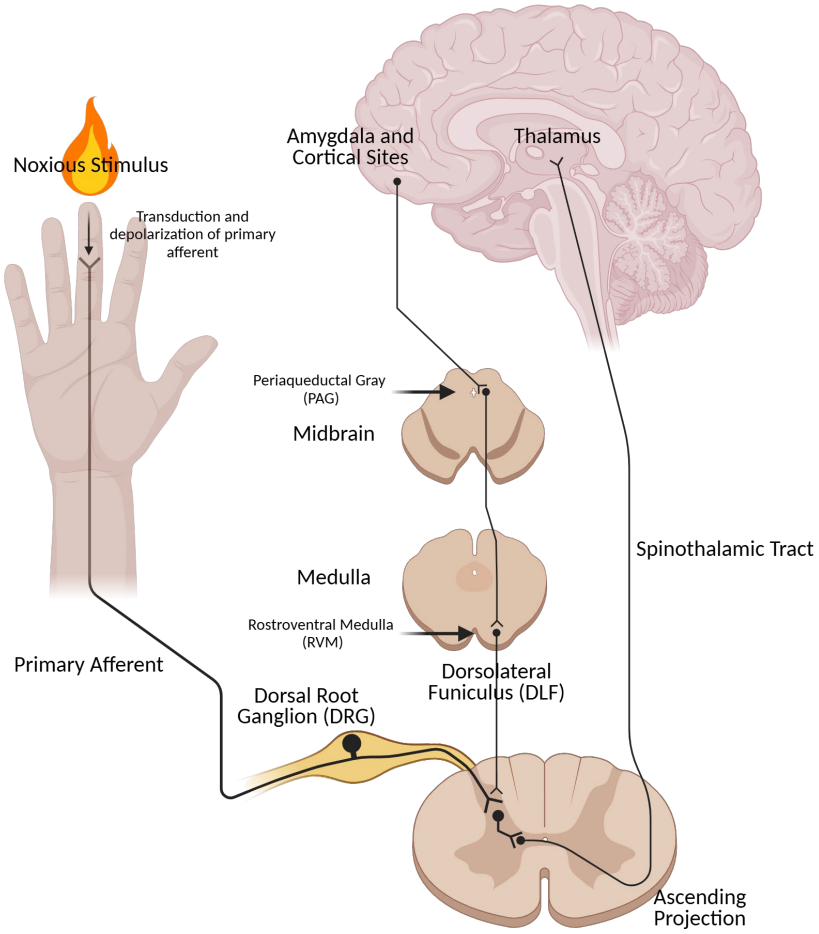


Figure 1.1. The ascending pain pathway and descending modulation of pain. Peripheral noxious stimuli are transduced into chemical signals by primary afferents. These signals are transmitted and integrated in the dorsal horn of the spinal cord before being sent to the brain via ascending projections. This information is received by the thalamus and relayed to the somatosensory cortex. The amygdala and cortical sites send information back down to the spine through the PAG and RVM to modulate neurotransmission in the dorsal horn.

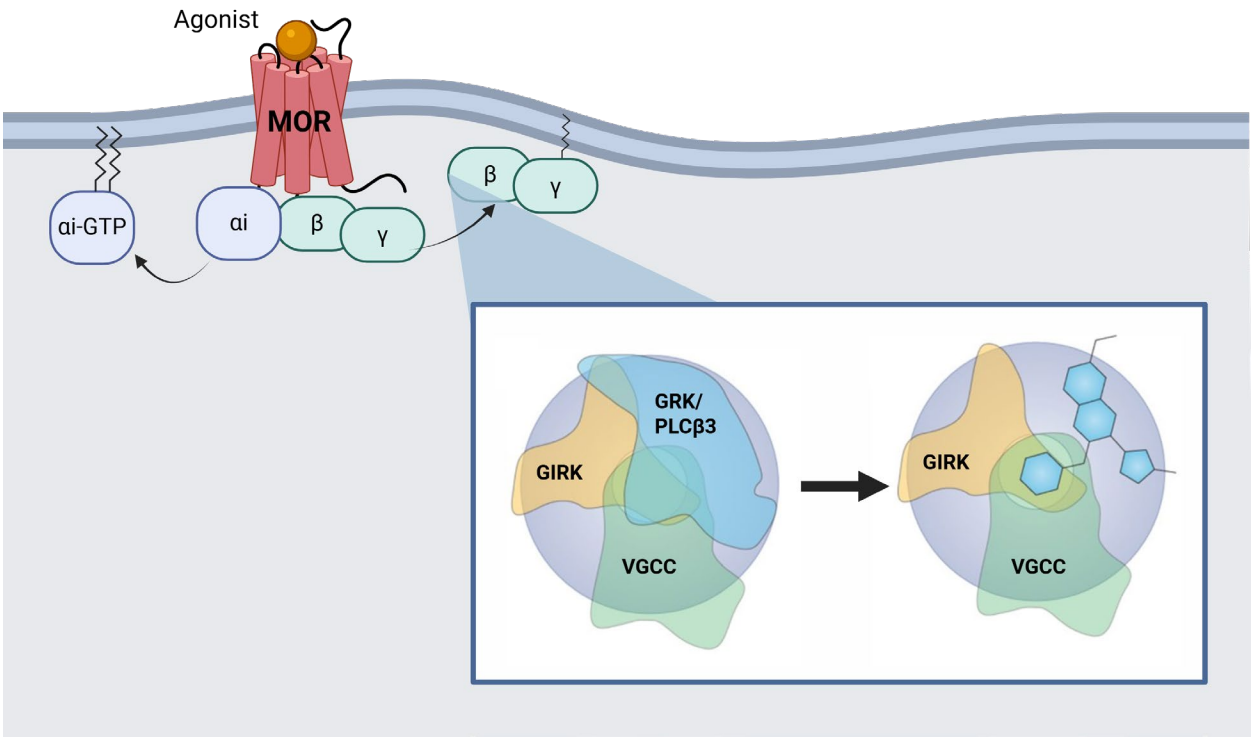


Figure 1.2. Small-molecule binding to the switch interface of the β subunit. Agonist-induced dissociation of G protein heterotrimers exposes the switch interface region of the β subunit. When exposed, residues within this region interact with downstream effectors. Small molecules that bind to this interface could alter the affinity of Gβγ for some effector subsets, but not others. Adapted from: (163).

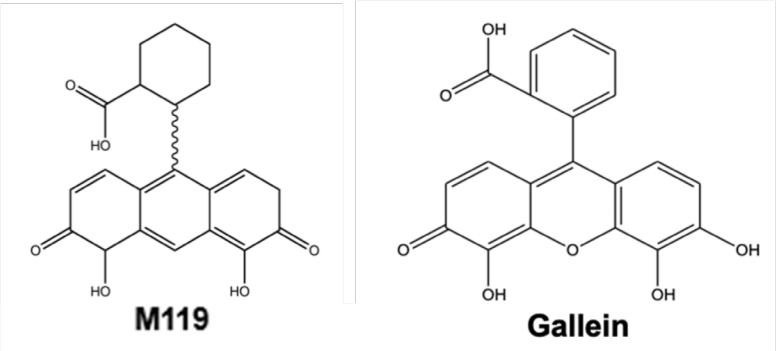


Figure 1.3. Structure of Gβγ Signal Modulators M119 and Gallein.

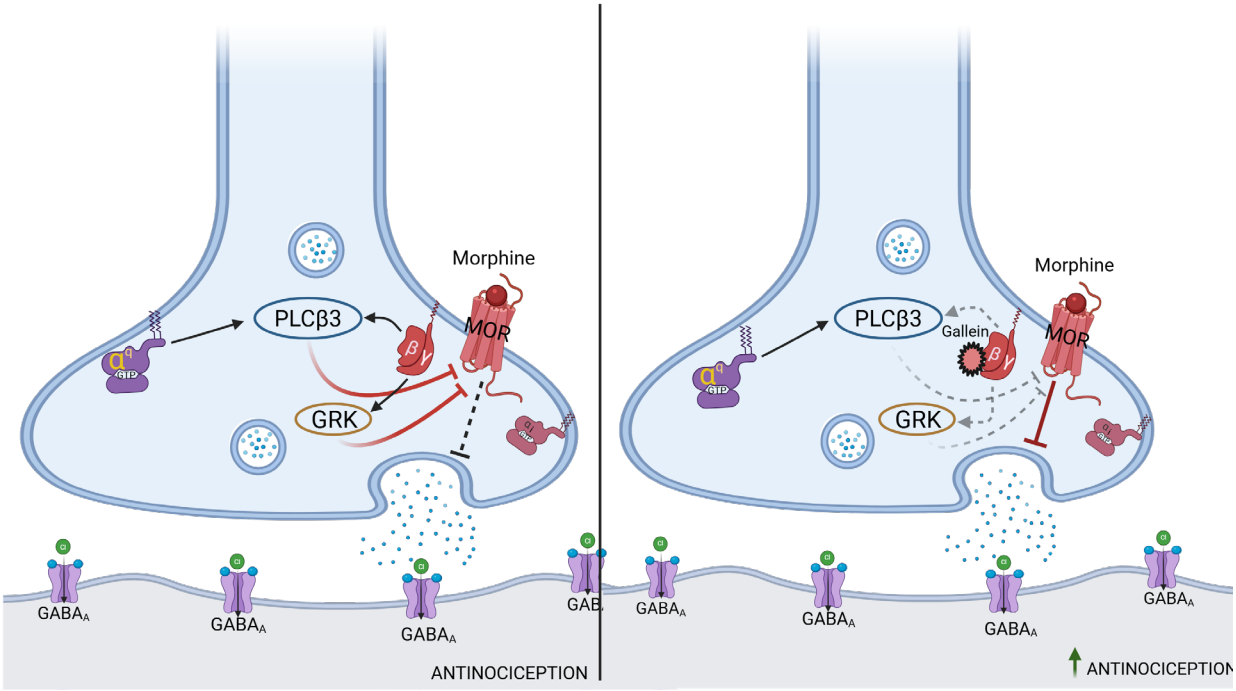


Figure 1.4. Mechanism of gallein action. MORs disinhibit descending inputs onto the dorsal horn to produce analgesia. Gβγ release downstream of MOR agonist-induced activation interacts with effectors such as PLCβ3 and GRKs. This causes negative feedback phosphorylation of the receptor, leading to G protein decoupling and arrestin recruitment. Gallein binds to Gβγ and reduces affinity for effectors which negatively regulate MORs, while leaving other signaling pathways unaltered. Consequent persistence of MOR signaling leads to enhancement in antinociception.

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Chapter 2 Mechanisms for Enhancement of μ-Opioid Receptor-Mediated Antinociception in the Central Nervous System Through Gallein-Dependent Modulation of Downstream βγ Subunit Signaling

2.1 Introduction

Considering both medical costs and lost economic productivity, a 2021 study estimated the economic costs of chronic pain in the United States at $722.8 billion (1). While opioids are indeed effective for pain management (2), they come with unwanted side effects such as respiratory depression and constipation, abuse liability, and tolerance development. Much work has been done in the field of opioid research and G protein-coupled receptor (GPCR) physiology to attempt to create new opioid analgesics which circumvent these issues. The fact that these efforts have been met with limited success highlights a critical need for the development of new treatment strategies to improve the safety and efficacy of opioid analgesics.

Opioids primarily mediate their analgesic effects through the μ-opioid receptor (MOR). MORs are GPCRs that couple to the Gi family of G proteins subunits. MORs regulate neuronal physiology largely via free G protein βγ subunits released upon activation and dissociation of the Gi α/βγ heterotrimer, and subsequent direct downstream effector regulation (3-5). Important Gβγ regulated effectors include inwardly rectifying potassium channels (GIRKs) (6, 7), N-type voltage-gated calcium channels (VGCCs) (8) and soluble N-ethylmaleimide-sensitive factor attachment receptors (SNAREs) (5, 9) which inhibit neuronal activity, mediating antinociception. Opposing MOR stimulation of antinociception are feedback inhibition pathways, at least two of which are stimulated by Gβγ. Gβγ subunits are required for activation of G protein-coupled receptor kinase 2/3(GRK 2/3) (10, 11) which phosphorylate MORs at the carboxyl terminus resulting in β-arrestin recruitment and desensitization (12, 13). Gβγ subunits also synergize with $G\alpha_q$ to activate PLCβ3 (14-17). Previous work demonstrated that the potency of morphine in antinociception is enhanced in PLCβ3$^{-/-}$ mice (18) supporting the idea that MOR antinociceptive signaling is opposed by

feedback inhibition by PLC activation, possibly through diacylglycerol dependent protein kinase C activation and MOR phosphorylation.

Two highly related small molecules, M119 and gallein, bind directly and reversibly to Gβγ *in vitro* (19-21). Binding of either of these molecules inhibits interactions between Gβγ and a subset of downstream targets including PLCβ and GRK2 (19, 22-26), while leaving ERK, GIRKs and VGCCs activation unaffected (19, 27, 28). This effector selectivity is thought to be driven by M119/gallein binding to a subsite on Gβγ that allows competition for only a subset of effectors because each effector engages Gβγ utilizing distinct protein contacts (19, 27-29).

Since M119/Gallein blocks two pathways involved in inhibition of MOR dependent antinociception, and since MOR signals largely through Gβγ subunits released from Gi, it was hypothesized that M119/Gallein might enhance MOR dependent antinociception. Indeed, it was shown that M119 administered i.c.v. enhanced the efficacy of morphine in a warm water tail withdrawal assay of antinociception (19, 22). This enhancement was blocked in PLCβ3$^{-/-}$ mice supporting the idea that M119-dependent blockade of Gβγ-PLCβ3 interactions mediate these effects(19). A subsequent study showed that systemic administration (i.p.) of gallein enhanced the antinociceptive effects of morphine without altering MOR-mediated side effects (i.e., constipation, reward, respiratory depression) (30). Multiple subsequent studies have confirmed and extended these initial findings (14, 31, 32). Measurements of enhanced postsynaptic currents (EPSCs) in mouse brain slices from the periaqueductal gray (PAG) demonstrated that gallein increases the potency of DAMGO-dependent inhibition of GABA release, and that this effect was eliminated in slices from PLCβ3$^{-/-}$ mice (14). These data provide *in vivo* and *ex vivo* support for the idea that these compounds block Gβγ interactions with effectors such as PLCβ3 and GRK2 preventing feedback inhibition of MOR signaling, increasing morphine antinociceptive potency.

Here we further investigate *in vivo* mechanisms for gallein action and provide further support for these proposed mechanisms and determine if systemic administration of gallein enhances MOR dependent antinociception through actions in the central nervous system or in the periphery. We find that enhancement of morphine-induced antinociception by systemically administered gallein is dependent on central MORs and these effects are generalizable across various MOR agonists, non-opioid Gi-coupled GPCR based analgesics, and various nociceptive stimuli. Gallein had no effect on antinociception mediated by epibatidine a non-GPCR dependent agonist. Importantly, gallein was unable to shift a dose-response curve for the antinociceptive

effects of morphine in mice lacking GRK and PKC phosphorylation sites in the C-terminus of MOR. Together these data provide further evidence for the proposed *in vivo* mechanism for gallein action where gallein-dependent blockade of signaling pathways that feedback inhibit of MORs via binding to Gβγ subunits released by Gi heterotrimers and that this requires engagement of MORs in the CNS. In addition, these data, in conjunction with previous reports, provide further evidence for a lack of generalized adverse effects of this approach, impacting primarily Gi mediated antinociceptive signaling.

2.2 Methods

2.2.1 Drugs and Reagents

Buprenorphine hydrochloride (Sigma-Aldrich, St. Louis, MO), CP55940 (Sigma-Aldrich, St. Louis, MO), clonidine (Cayman Chemical, Ann Arbor, MI), epibatidine (Cayman Chemical, Ann Arbor MI), fentanyl citrate (Sigma-Aldrich, St. Louis, MO), gallein (Cayman Chemical, Ann Arbor MI), morphine sulfate (Henry Schein, Melville NY), nalbuphine hydrochloride (Sigma-Aldrich, St. Louis, MO), naloxone hydrochloride (Sigma-Aldrich, St. Louis MO), naloxone methiodide (Sigma-Aldrich, St. Louis MO). All drugs were dissolved in 0.9% sterile saline except CP55940 and gallein. CP55940 was dissolved in 5% EtOH, 5% castor oil, 90% sterile saline vehicle and then further diluted in sterile saline for experiments. For intraperitoneal (i.p.) systemic administration and intrathecal (i.t.) administration, gallein was dissolved in a 5% DMSO, 10% castor oil, and 85% sterile water vehicle. Intracerebroventricular (i.c.v.) gallein treatments were dissolved in DMSO.

2.2.2 Animals

All animal procedures were conducted at the University of Michigan according to NIH *Health Guide for the Care and Use of Laboratory Animals* and with approval of the Institutional Animal Care and Use Committee (IACUC). Wild type C57BL/6 mice were purchased from Envigo (Indianapolis, IN) and bred in-house for these studies. MOR -/- mice were purchased from Jackson Laboratory and bred on a C57BL/6 background. 10 S/T-A mice were obtained from Stefan Schulz's laboratory and bred on C57BL/6 background. Mice were housed with a maximum of five animals per cage in clear polypropylene cages. Corn cob bedding and nestlets were provided for enrichment. Animals were housed in pathogen-free rooms maintained between 68°F and 72°F and 30% and 70% humidity. Rooms utilized a 12-hour light/dark cycle (lights on at 7 A.M. and lights

off at 7 P.M.) with free access to food. Experiments were conducted in the animals' housing room during the light cycle. All mice used in experiments were between 7 and 12 weeks of age and weighed 16-28 grams. A combination of male and female mice was used for all experiments. 8-16 animals per group were used for all experiments.

2.2.3 Pretreatment

Systemic gallein was administered i.p. 24 hrs prior to morphine. I.c.v. and i.t. gallein were administered 30 min prior to morphine. Systemic naloxone and naloxone methiodide were administered i.p. 15 min prior to morphine. I.c.v. naloxone methiodide was also administered 15 min prior to morphine.

2.2.4 Intracerebroventricular (i.c.v.) pretreatment injections

I.c.v. injections were performed 30 minutes after measurement of baseline withdrawal or response latencies and 30 minutes before morphine treatment. Hamilton syringes of 10 µL with a 26 G (catalog #7804-03; Point #4, 12° bevel) needles were used with a custom stopper that allowed 4 mm of the needle to enter the skull. Injections were free-handed utilizing the ears and eyes for orientation to target the lateral ventricles of the brain. Volume of injection was 3 µL for all experiments and the needle was held in place for 30 seconds after the total volume was injected to ensure perfusion. After 30 seconds, the needle was carefully removed and the mice placed into a separate recovery cage and observed for recovery from anesthesia (normal grooming and movement). Upon recovery, mice were placed back into their home cage.

To conduct the injection, mice were anesthetized using isoflurane until no longer responsive to tail pinch and breathing slowed to one inhalation per second. After completion of the experiment, mice were euthanized to confirm injection placement using methylene blue.

2.2.5 Intrathecal (i.t.) pretreatment injections

One hour prior to habituation measurement in warm water tail withdrawal, mice were briefly anesthetized in isoflurane and the thoracolumbar region shaved using handheld electric trimmers. Mice were then placed back into home cages for recovery. I.t. injections were performed 30 minutes after measurement of baseline tail withdrawal latency and 30 minutes before morphine treatment. BD 3/10 mL 5/16" x 31G Ultra-Fine Needle Insulin Syringes (Medline catalog #328438) were used to perform injections. Prior to injection, mice were again briefly anesthetized. Subsequently, mice were gripped by one hand at the pelvic girdle to aid visual confirmation of

spinous processes and sterile needle inserted between L4-L5 vertebrae (33, 34). Upon completion of injection, the needle was held in place for 30 seconds to prevent backflow. After 30 seconds, the needle was carefully removed and the mice placed into a separate recovery cage and observed for recovery from anesthesia (normal grooming and movement). Once recovered, mice were placed back into their home cage.

To confirm adequate hit-rate of i.t. injections prior to beginning experiments, separate cohorts of mice received intrathecal injections of 3 μg morphine and observed for Straub tail to confirm injection placement. Upon obtaining >90% accuracy, experiments were performed utilizing i.t. gallein.

2.2.6 Warm Water Tail Withdrawal

Mice were briefly placed into a cylindrical plexiglass restrainer and tails immersed approximately 3 cm into a 55°C water bath (50°C for experiments using nalbuphine). The latency to withdraw or rapidly flick the tail was recorded with a maximum of 15 seconds (20 seconds for experiments using 50°C water bath; nalbuphine experiments) to prevent tissue damage. Baseline tail withdrawal latencies between 2 and 5 seconds were consistent for each assay. Mice were briefly habituated to the plexiglass restrainer and tail dipped prior to injection with saline. After 30 min, baseline withdrawal latencies were recorded (BL, baseline withdrawal latency).

For i.c.v. and i.t. gallein pretreatment experiments, withdrawal latencies were recorded 30 min after i.c.v. injection (Pre, pretreatment withdrawal latency). Pretreatment withdrawal latencies were recorded 15 min after i.c.v. injection for i.c.v. naloxone methiodide pretreatment experiments. Mice received analgesic drug immediately following pretreatment measurement for gallein studies or immediately following baseline measurement for dose-response studies involving a single analgesic drug alone. For time course studies, withdrawal latencies were recorded every 15-30 min subsequent to MOR agonist, CP55940, or epibatidine treatment. For clonidine, latencies were recorded every 15 min for the first hour and every 30 min for the second hour. In dose-response curve studies, the inter-injection interval was 30 min for buprenorphine, nalbuphine, CP55940, and epibatidine. A 15 min inter-injection interval was used for fentanyl and clonidine dose-response curves. Doses of MOR agonists, CP55940, clonidine, and epibatidine utilized for time course studies were determined by selecting sub-effective doses from dose-response curves for each agonist alone. For morphine, a dose of 3.2 mg/kg was selected based on previous work illustrating enhancement of antinociceptive effects by gallein at this dose (14).

2.2.7 Hot Plate

To measure response latencies in the hot plate assay, mice were placed onto a Hot Plate Analgesia Meter (Columbus Instruments) set to 52°C surrounded by four plexiglass walls. Response latency in the hot plate assay was measured as time until licking of the hindpaws, flinching of the hindpaws, or escape/jumping behavior off the hot plate. The latency to responses was recorded with a maximum of 60 seconds to avoid tissue damage. Baseline latencies were consistently between 15 and 25 seconds. Mice were first habituated to the hot plate and injected with saline 30 min prior to baseline measurement (BL, baseline response latency).

For gallein experiments, response latency was recorded 30 min after i.c.v. injection (Pre, pretreatment response latency) and morphine administered immediately thereafter. Subsequent time course measurements were then taken every 30 min after morphine administration. Dose-response curve data of morphine on the hot plate assay utilized 30 min inter-injection interval.

2.2.8 Locomotor Activity

For locomotor activity studies, Opto-M3 Locomotor Activity Chambers (Columbus Instruments) were used. On Day 1 (habituation), animals habituated to locomotor activity chambers for 1 hr before receiving systemic gallein (i.p.) treatment and remained in the chamber for 3 hrs. At 24 hrs post gallein treatment (Day 2), mice were placed back into locomotor activity chambers and beam breaks (X,Y plane) recorded for 1 hr.

2.2.9 Statistics

For time course studies and dose response studies using gallein pretreatment, main effects and interactions were evaluated using repeated measures ANOVA in R with time or dose as a within-subjects factor and sex, genotype, and treatments as between subjects factors as appropriate to the specific experiment. Post-hoc analyses involved pairwise comparisons among groups at individual time points using the emmeans package with Tukey's HSD adjustment. Additionally, to control for the overall family-wise error rate across total comparisons, a global Bonferroni correction was applied.

Dose response data using a single MOR agonist and no pretreatment condition were analyzed using a repeated measures ANOVA with dose (baseline, dose1, dose 2, dose 3, dose 4, dose 5) as the within-subjects factor and sex as the between-subjects factor in R. Because no significant main effect of sex or sex by dose interaction were found, post-hoc comparisons were

subsequently performed using a Dunnett (treatment-vs-control) contrast computed from estimated marginal means obtained through the emmeans package in R. Baseline measurement served as the reference.

2.3 Results

2.3.1 Potentiation of morphine-induced antinociception by gallein requires activation of MORs

Prior data support a model where enhancement of morphine-induced antinociception by gallein depends upon morphine action at MORs, but this has not been formally demonstrated. To test this more directly, mice were systemically pretreated with gallein (100 mg/kg, i.p.) or equivalent volume of gallein vehicle (DMSO) 24 hrs prior to morphine. Previously published data demonstrated that gallein delivered i.p. is effective up to 48 hours after administration (32) and Figure 10 shows there are no effects on generalized locomotor activity by gallein after gallein administration in this paradigm. Naloxone (NLX) (3.2 mg/kg, i.p.) or saline (NLX vehicle) were then administered 15 minutes prior to administration of a subanalgesic dose of morphine (3.2 mg/kg, s.c.) (14, 32) (Figure 1A). Tail withdrawal latencies were measured in the 55°C warm water tail withdrawal (WWTW) assay. This dose of morphine alone had very little effect on tail withdrawal latencies compared to baseline. In accordance with prior findings (14, 30, 32), mice pretreated with gallein and saline (NLX vehicle) display significantly elevated morphine-dependent tail withdrawal latencies compared to morphine alone (gallein vehicle + NLX vehicle) – indicating potentiation of morphine-induced antinociception. However, when mice pretreated with gallein was followed by treatment with naloxone, gallein enhancement of morphine-induced antinociception was blocked indicating a requirement for morphine's action at opioid receptors for gallein efficacy.

Considering that neither morphine nor naloxone are highly selective for MOR relative to other opioid receptors, we investigated the effects of gallein on morphine-induced antinociception in MOR knockout (KO) mice and wildtype (WT) littermates (Figure 1B). MOR KO mice and WT littermates received a 30 min (i.c.v.) pretreatment with gallein (100 nmol) or vehicle prior to administration of morphine (3.2 mg/kg, s.c.) and assessment in the WWTW assay. This dose of i.c.v. gallein has previously been demonstrated to enhance morphine-induced antinociception (14) and also as shown previously, gallein alone does not alter tail withdrawal latencies over time in the absence of morphine (Figure 11). MOR KO mice administered gallein did not exhibit elevated

tail withdrawal latencies following morphine whereas WT littermates did. Together, these results confirm a requirement of morphine action at MOR for gallein to enhance antinociceptive effects of morphine.

2.3.2 Gallein potentiates the antinociceptive effects of other MOR agonists

To evaluate the effect of gallein on MOR agonist-mediated antinociception with agonists other than morphine, we first determined appropriate doses of MOR agonist for each study. Dose-response curves were generated in separate cohorts of mice treated in the absence of gallein administration (Figure 2 A, B, and C). Both fentanyl and buprenorphine produced dose-dependent increases in tail withdrawal latencies in the WWTW assay (Figure 2A and B), while nalbuphine only produced a significant increase in tail withdrawal latency at the highest dose tested (Figure 2C).

To test the effects of gallein on the responses to these MOR agonists a single sub-antinociceptive bolus dose of fentanyl, buprenorphine, or nalbuphine was used. Mice received a 30 min i.c.v. pretreatment of gallein (100 nmol, i.c.v.) or vehicle, prior to administration of agonist, followed by a time course in the WWTW assay. Gallein pretreatment significantly enhanced fentanyl-, buprenorphine-, and nalbuphine-induced antinociception as compared to vehicle pretreatment (Figure 2 D, E and F). Notably, there was a significant treatment by sex interaction in fentanyl experiments wherein gallein significantly enhanced morphine-induced antinociception in males, but not females (Figure 12).

2.3.3 Gallein enhances the effects of a cannabinoid receptor (CB1/2R) agonist and α2-adrenergic receptor agonist in a sex-dependent manner

Since Gβγ signaling arises primarily from Gi/o activation (10), gallein might be predicted modulate Gβγ signaling downstream of other antinociceptive Gi/o-coupled GPCRs to enhance their effects. We first evaluated cannabinoid receptors 1 and 2 (CB1/2Rs) which are both Gi/o-coupled. We utilized CP55940, a CB1/2R agonist with greater potency and efficacy than THC (35-37). A cumulative dose-response curve for CP55940 alone was used to determine a relatively equi-analgesic dose of drug compared to previous opioid experiments (Figure 3A). Animals were then pretreated with either 100 nmol i.c.v. gallein or vehicle prior to either cumulative dosing (Figure 3B) or a single bolus dose of CP55940 (Figure 3C). While there was no main effect of gallein in

either experiment, there was a significant effect of sex and gallein by sex interaction in the time course studies driven by higher response latencies in males pretreated with gallein (Figure 3D).

A similar set of experiments was conducted using the α2-adrenergic receptor (also Gi/o-coupled) agonist, clonidine. Although there was no effect of gallein treatment in dose-response studies (Figure 4B), a main effect was observed in the time course paradigm (Figure 4C) with a significant gallein by sex interaction (Figure 4D). While these effects do not appear as robust as those observed with MOR agonists, these data indicate that gallein potentiates antinociceptive signaling downstream of other Gi-coupled GPCRs.

2.3.4 Gallein does not enhance the antinociceptive effects of analgesics that do not rely on G protein signaling

Considering that gallein enhanced the antinociceptive effects of several distinct analgesics, it is important to further demonstrate that this is not a nonspecific effect on behavior. Prior research has shown that the nicotinic acetylcholine receptor agonist epibatidine potently elicits antinociception in rodents (38) and this effect was replicated in the WWTW assay here (Figure 5A). Similar to previous experiments, mice were pretreated i.c.v. with 100 nmol of gallein 30 minutes prior to a time course after epibatidine administration (Figure 5B). There was no effect of gallein pretreatment on tail withdrawal latencies demonstrating that gallein does not generally enhance antinociceptive responses.

2.3.5 Potentiation of morphine-induced antinociception by gallein requires activation of central, but not peripheral MORs

To investigate whether it is morphine action at central or peripheral MORs involved in potentiation of antinociceptive effects by gallein, we utilized naloxone methiodide (NLXM) (a blood-brain barrier impermeable analog of naloxone) (39). Gallein or gallein vehicle were administered i.p. 24 hours prior to morphine. 15 min prior to morphine, mice received either NLXM (10 mg/kg, i.p.) to block peripheral MORs, or saline (NLXM vehicle) (Figure 6A). As was demonstrated in Figure 1A, mice pretreated with gallein and saline exhibit significantly elevated tail withdrawal latencies as compared to mice pretreated with gallein vehicle and NLXM vehicle (this group of mice from Figure 1A is again depicted here in Figure 6A, open and closed circles). In contrast to naloxone pretreatment which blocked gallein's effects, pretreatment with peripherally restricted NLXM did not (Figure 6A, squares).

To support these data, we conducted a similar experiment in which mice were pretreated with systemic administration of gallein and central administration of NLXM(10 nmol, i.c.v.) (Figure 6B). In contrast to the elevated tail withdrawal latencies observed with systemic gallein and NLXM, centrally administered NLXM blocked the effects of systemically administered gallein and morphine.

2.3.6 Central gallein potentiates morphine-induced antinociception in the hot plate assay

To further isolate whether the effects of gallein to enhance morphine-induced antinociception primarily occur at spinal or supraspinal MORs within the central nervous system the effects of gallein were tested in the thermal antinociception hot plate assay. Responses observed in this assay are thought to be mediated supraspinally as compared to the spinal withdrawal reflex observed in WWTW. A dose response curve for s.c. morphine was generated in this assay to determine the appropriate subanalgesic dose (Figure 7A). Mice received i.c.v. gallein or vehicle pretreatment 30 min prior to morphine (5.6 mg/kg, s.c.) (Figure 7 B). Consistent with results observed in the WWTW assay, gallein pretreatment resulted in significantly elevated response latencies as compared to vehicle while gallein alone has no effect. These data indicate that gallein enhances the antinociceptive effects of morphine in nociceptive assays other than the WWTW and that its effects may be due to action downstream of supraspinal MORs within the central nervous system.

2.3.7 Intrathecal gallein pretreatment did not enhance morphine-induced antinociception in the WWTW assay as compared to i.c.v. gallein pretreatment

To complement data observed in the hot plate assay which suggest an effect of gallein at supraspinal MORs, studies were conducted in which mice were pretreated with gallein via the intrathecal route. These injections are expected to remain relatively local without substantial gallein concentrations in the brain (40-42). Mice pretreated with intrathecal gallein 30 minutes prior to morphine exhibited tail withdrawal latencies that were no different compared to vehicle pretreated mice (Figure 8A). In contrast, mice pretreated with i.c.v. gallein exhibit significantly elevated tail withdrawal latencies compared to vehicle pretreated mice (Figure 8B). These data corroborate hot plate findings which implicate enhancement of signaling downstream of supraspinal MORs for gallein to enhance morphine-induced antinociception.

2.3.8 Gallein does not potentiate morphine-induced antinociception in MOR 10S/T-A knock-in mice lacking MOR GRK and PKC phospho-sites

A central mechanistic hypothesis is that gallein exerts its effects on MOR by preventing Gβγ mediated activation of effectors such as GRK and PLCβ3 that inhibit MOR function. As a test of this *in vivo* we utilized knock-in mice where 10 critical GRK and PKC phosphorylation sites in MOR were mutated from serine/threonine to alanine (MOR 10S/T-A mice) (43). MORs in these animals cannot be phosphorylated at the C-terminal tail by GRK or PKC. If gallein acts by preventing activation of GRK and PKC-dependent phosphorylation of MORs, gallein would be expected to have minimal effect in these animals. Homozygous MOR 10S/T-A and wildtype littermates were pretreated with 100 nmol gallein, i.c.v. or vehicle prior to a dose response analysis of morphine in the WWTW assay (Figure 9 A). As previously shown, wildtype mice pretreated with gallein exhibited a significant leftward shift in the dose response curve for the antinociceptive effects of morphine compared to vehicle pretreated controls. Also as previously published, 10S/T-A mice also exhibit a significant leftward shift compared to WT in the absence of gallein treatment (43). No shift was observed in 10S/T-A littermates pretreated with gallein compared to vehicle. Surprisingly, gallein effects were also eliminated in heterozygous 10S/T-A mice (Figure 9 B). Overall, these data support the proposed model by indicating that gallein's action requires the ability to modulate the phosphorylation state of C terminal amino acids on MOR by PKC/GRK2.

2.4 Discussion

There is an ever-increasing body of evidence supporting pharmacological modulation of Gβγ subunit signaling with gallein downstream of Gi-coupled GPCRs as an efficacious therapeutic approach in preclinical models. Gallein efficacy has been demonstrated in multiple chronic disease models including mouse models of lupus, heart failure, cardiac fibrosis, and osteoarthritis (23-25, 44, 45) without overt side effects. There is also strong evidence that pharmacological manipulation of Gβγ signaling downstream of MOR activation with M119 or gallein enhances the antinociceptive effects of morphine (14, 19, 22, 30, 32). Importantly, these enhancements in morphine-induced antinociception occur in the absence of common opioid-related side effects such as respiratory depression and constipation (30). More recent investigation has demonstrated that gallein treatment inhibits the development of tolerance to the antinociceptive effects of morphine and enhances morphine-induced antinociception in a tolerant state (32). In this previous study it was found that gallein efficacy persisted up to 48 hours after administration. We do not understand

the mechanism for long duration of action which was confirmed in this current work and also noted in a previous study (44). We also recently demonstrated that gallein enhances the antinociceptive effects of endogenous opioids in a model of chronic pain (31).

Despite the accumulating evidence it remained possible that gallein's in vivo effects were unrelated to its ability to modulate MOR activity. Here we formally show that the ability of gallein to enhance the antinociceptive effects of morphine depends upon morphine action at MORs. Further, gallein enhances the antinociceptive effects of other opioid analgesics fentanyl, buprenorphine, and nalbuphine. That the effects of gallein are generalizable across different MOR agonists indicate that gallein's actions are not attributable to a specific idiosyncratic interaction of gallein and morphine (altering metabolism, for example). These enhancements in opioid-induced antinociception by gallein specifically depend upon morphine action at central, but not peripheral, MORs. This is evidenced by data showing naloxone methiodide only prevents the effects of gallein when administered centrally. Hot plate experiments (which measure supraspinally mediated behaviors) and intrathecal administration of gallein support this conclusion.

Gallein might be predicted to enhance the activity of any Gi/o-coupled GPCR. To test this idea, we studied the effects of gallein pretreatment on the antinociceptive effects of CP55940 and clonidine; agonists for two Gi/o coupled receptors (CB1/2 and α2-adrenergic receptors, respectively) (10, 46). Interestingly, we found that gallein's enhancement of CP55940- and clonidine-induced antinociception was only observed in males. While sex-dependent, these results support the notion that it is possible for gallein to interact with other, non-opioid, Gi/o coupled-receptor systems. Importantly, gallein did not enhance the antinociceptive effects of epibatidine, a nicotinic acetylcholine receptor agonist.

While sex differences were not an explicit area of investigation for these studies, there was a significant difference in the ability of gallein to potentiate fentanyl-, CP55940-, and clonidine-induced antinociception between males and females. Considering there were no sex differences observed in antinociceptive effects for mice administered fentanyl, CP55940, or clonidine alone, it is possible that there are sex-specific responses to the effects of Gβγ modulators such as gallein. This could be due to sex differences in expression and/or activity of various Gβγ effectors (47-49). Further work must be done to reach concrete conclusions regarding potential mechanisms for sex differences in the effects of gallein.

Finally, experiments utilizing 10 S/T-A mice which express MORs that cannot undergo C-terminal phosphorylation by GRK or PKC (43) strongly support the proposed mechanism of gallein action in an *in vivo* mouse model. Given that MORs cannot be directly regulated by GRKs or PKC in these mice, it would be expected, based on our model, that gallein would ineffective in these mice. Homozygous 10 S/T-A, and surprisingly heterozygous mice, did not respond to gallein in the WWTW assay. These data provide mechanistic *in vivo* evidence that gallein potentiates morphine-induced antinociception by abrogating Gβγ-mediated activation of GRK2 and PLCβ3 and subsequent MOR phosphorylation.

Together, these data support the proposed model where the "tool compound" gallein enhances opioid potency at neurons in the CNS by selectively inhibiting Gβγ interactions with enzymes involve in negative feedback regulation of MORs, while at the same time preserving Gβγ interactions with ion channels and/or SNAREs required for MORs antinociceptive actions. The preponderance of data suggest that this overall approach has therapeutic potential and incentivizes ongoing work to develop more "drug like" molecules that behave similarly to M119 and gallein.

2.5 Figures

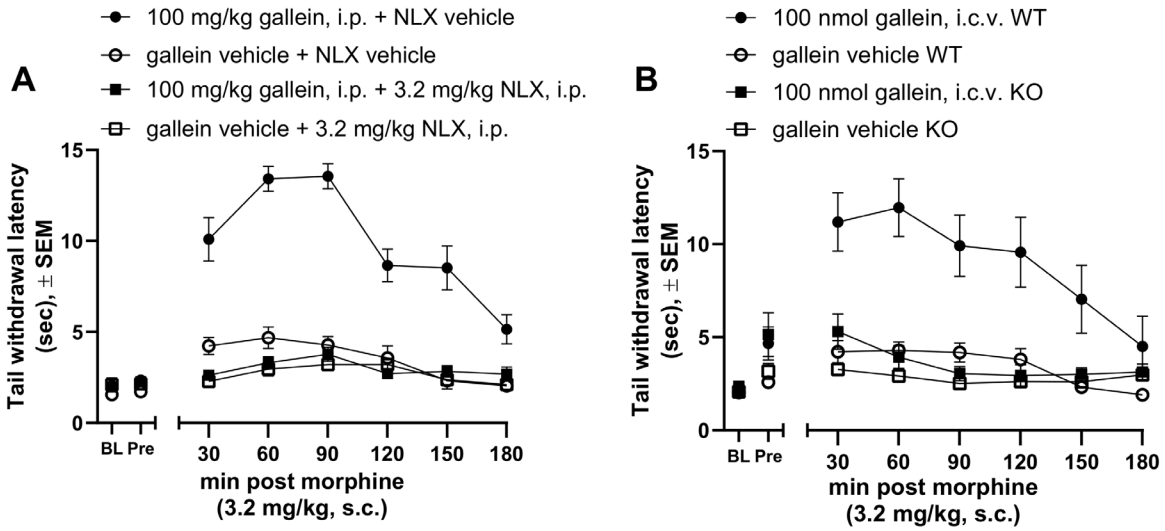


Figure 2.1. Enhancement of morphine-induced antinociception by gallein is dependent upon morphine action at MOR. A) There is a significant effect of 100 mg/kg gallein to enhance 3.2 mg/kg morphine-induced antinociception and this effect is blocked by 3.2 mg/kg naloxone pretreatment (Four-way RM ANOVA main effect of gallein pretreatment ($F(1, 41) = 52.33$, $P<0.0001$); main effect of naloxone pretreatment ($F(1, 41) = 62.85$, $P<0.0001$; gallein pretreatment by naloxone pretreatment interaction ($F(1, 41) = 42.60$, $P<0.0001$) (N=16 gallein + NLX vehicle, N=12 gallein + NLX, N=11 gallein vehicle + NLX, N=10 gallein vehicle + NLX vehicle). Refer to Table 1 for post-test comparisons. **B)** There is no significant effect of 100 nmol i.c.v. gallein to enhance 3.2 mg/kg morphine-induced antinociception in MOR knockout mice (Three-way RM ANOVA, main effect of gallein pretreatment ($F(1, 28) = 18.1428$, $P=0.0002088$; main effect of genotype ($F(1, 28) = 13.7496$, $P=0.000914$); gallein pretreatment by genotype interaction ($F(1, 28) = 9.3205$, $P=0.004926$) (N=8 vehicle WT, N=9 gallein WT, N=9 vehicle KO, N=10 gallein KO). KO mice treated with gallein did not significantly differ from KO or WT mice treated with vehicle at any time point (refer to Table 2 for post-test comparisons).

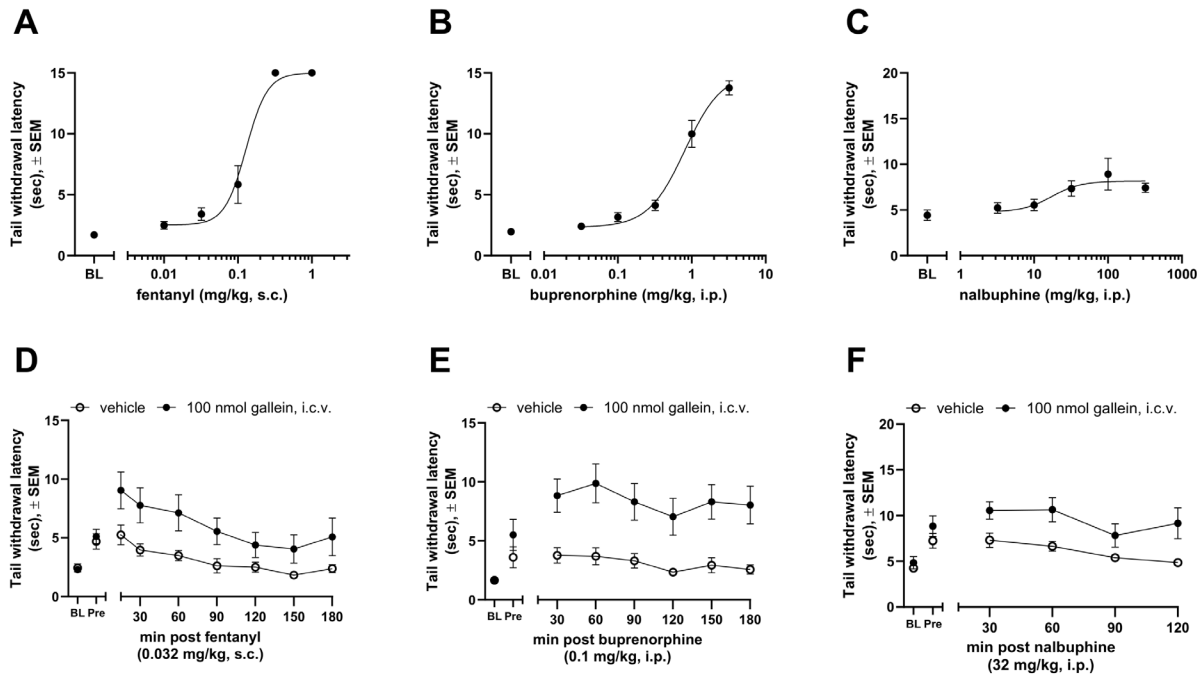


Figure 2.2. Gallein potentiates the antinociceptive effects of other MOR agonists in the WWTW assay. A) Fentanyl produces dose-dependent increases in tail withdrawal latency (Two-way RM ANOVA, main effect of dose $F(5, 30) = 108.1$, $P<0.0001$; no main effect of sex $F(1, 6) = 2.9861$, $P=0.1347$; no sex by dose interaction $F(5, 30) = 2.2826$, $P=0.0715$) (N=8). Doses 0.01 mg/kg ($P=0.043$), 0.032 mg/kg ($P=0.0347$), 0.32 mg/kg ($P<0.0001$), and 1 mg/kg ($P<0.0001$) are significantly different from baseline (Dunnett's post-test). **B)** Buprenorphine produces dose-dependent increases in tail withdrawal latency (2-way RM ANOVA, main effect of dose $F(5, 45) = 90.1$, $P<0.0001$; no main effect of sex $F(1, 9) = 0.6431$, $P=0.4432$; no sex by dose interaction $F(5, 45) = 0.1957$, $P=0.9626$) (N=11). Doses 0.32 mg/kg ($P=0.0054$), 1 mg/kg ($P=0.0004$), and 3.2 mg/kg ($P<0.0001$) are significantly different from baseline (Dunnett's post-test). **C)** Nalbuphine produces antinociception in the WWTW assay at 50°C (2-way RM ANOVA, main effect of dose $F (5, 30) = 4.38$, $P=0.0407$; no main effect of sex $F(1, 6) = 0.0485$, $P=0.8330$; no sex by time interaction $F(5, 30) = 1.4674$, $P=0.2297$) (N=8). Only the largest 320 mg/kg ($P=0.0018$) dose is significantly different from baseline (Dunnett's post-test). **D)** 100 nmol i.c.v. gallein pretreatment significantly enhances 0.032 mg/kg fentanyl-induced antinociception (Three-way ANOVA, main effect of gallein pretreatment $F(1, 20) = 12.46$, $P=0.0021$); main effect of sex $F(1, 20) = 13.09$, $P=0.0017$; gallein pretreatment by sex interaction $F(1, 20) = 8.117$, $P=0.0099$) (N=12 vehicle, N=12 gallein). See Figure 12 for effects of sex. **E)** 100 nmol i.c.v. gallein pretreatment significantly enhances 0.1 mg/kg buprenorphine-induced antinociception (Three-way RM ANOVA, main effect of gallein pretreatment $F(1, 22) = 19.97$, $P=0.0002$; main effect of sex $F(1, 22) = 6.269$, $P=0.0202$); no gallein pretreatment by sex interaction $F(1, 22) = 1.3467$, $P=0.2583$) (N=14 vehicle, N=12 gallein). Tail withdrawal latencies in gallein pretreated mice were significantly higher than vehicle at every time point post buprenorphine treatment (Three-way RM ANOVA Tukey post-test with Bonferroni correction, 30 min $P=0.023$, 60 min $P=0.01$, 90 min $P=0.032$, 120 min $P=0.014$, 150 min $P=0.0095$, 180 min $P=0.005$) **F)** 100 nmol i.c.v. gallein pretreatment significantly enhances 32 mg/kg nalbuphine-induced antinociception (Three-way RM ANOVA, main effect of gallein pretreatment $F(1, 21) = 8.87$, $P=0.0072$) (N=14 vehicle, N=11 gallein). The gallein pretreated group had significantly elevated tail withdrawal latencies (Three-way RM ANOVA Tukey post-test with Bonferroni correction) compared to vehicle at 60 min ($P=0.0467$).

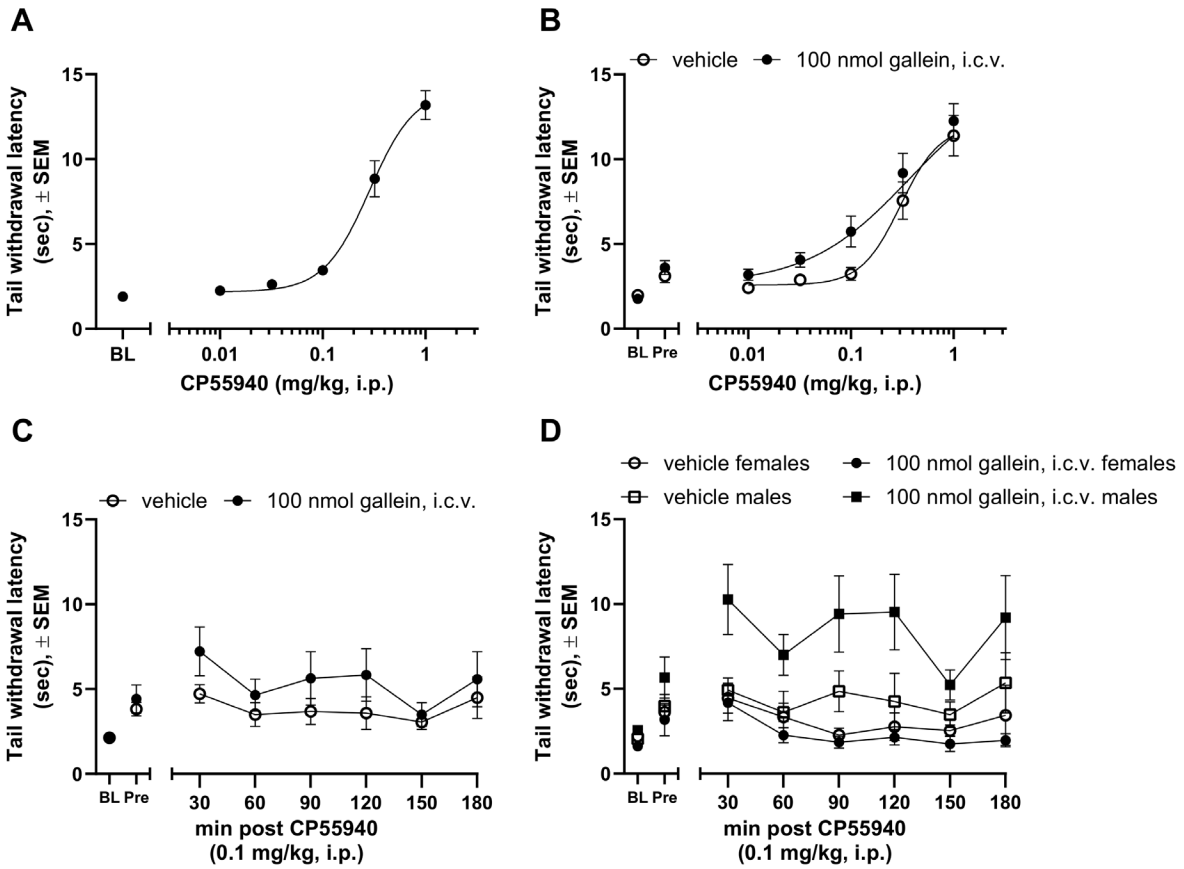


Figure 2.3. Gallein enhances antinociception mediated by a bolus dose of a cannabinoid receptor agonist, CP55940, in male mice. A) CP55940 produces dose-dependent increases in tail withdrawal latencies (Two-way RM ANOVA, main effect of dose $F(5,70) = 66.3341$, $P<0.0001$; no main effect of sex $F(1, 14) = 0.7246$, $P=0.409$ or sex by dose interaction $F(5, 70) = 0.4733$, $P=0.795$) (N=16). All doses 0.032 mg/kg or greater produced latencies significantly greater than baseline (Dunnett's post-test). **B)** 100 nmol i.c.v. gallein does not significantly shift a dose-response curve of CP55940 in the WWTW assay (Three-way RM ANOVA, main effect of dose $F(6, 150) = 82.8825$, $P<0.0001$; no main effect of gallein pretreatment $F(1, 25) = 1.6988$, $P=0.2043$); no main effect of sex $F(1, 25) = 1.2045$, $P=0.2829$; no sex by gallein interaction $F(1, 25) = 0.4941$, $P=0.4886$) (N=14 vehicle, N=14 gallein). **C)** There is no main effect across sexes of 100 nmol i.c.v. gallein to enhance 0.1 mg/kg CP55940-induced antinociception (Three-way RM ANOVA, no main effect of gallein pretreatment $F(1, 19) = 2.2962$, $P=0.146153$) (N=11 vehicle, N=12 gallein). **D)** 100 nmol i.c.v. gallein enhances 0.1 mg/kg CP55940-induced antinociception in males, but not females (Three-way RM ANOVA main effect of sex $F(1, 19) = 12.308$, $P=0.002351$; gallein pretreatment by sex interaction $F(1, 19) = 5.5386$, $P=0.029522$) (N=5 vehicle females, N=6 vehicle males, N=6 gallein females, N=6 gallein males).

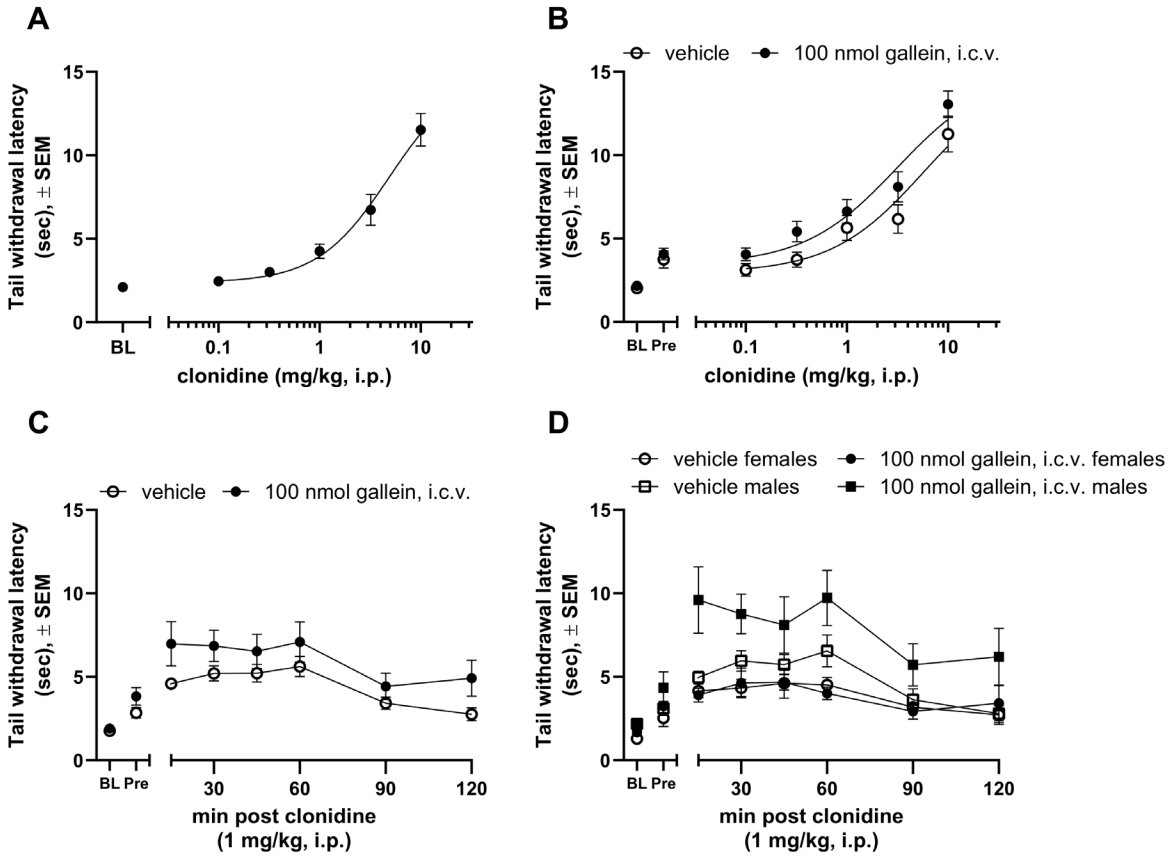


Figure 2.4. Gallein enhances antinociception mediated by a bolus dose of clonidine in males. A) Clonidine produces a dose-dependent increase in tail-withdrawal latency (Two-way RM ANOVA, main effect of dose $F(5, 70)=47.5651$, $P<0.0001$; no main effect of sex $F(1, 14)=1.0123$, $P=0.3314$; no sex by dose interaction $F(5, 70) = 0.5781$, $P=0.7165$) with doses 0.32 mg/kg or greater all significantly different from baseline (Dunnett post-test) (N=16). **B)** 100 nmol i.c.v. gallein did not significantly alter a dose-response curve of clonidine (Three-way RM ANOVA, main effect of gallein $F(1,24) = 3.6064$, $P=0.06964$; main effect of dose $F(6,144) = 71.6202$, $P<0.0001$) (N=14 vehicle, N=14 gallein). **C)** 100 nmol i.c.v. gallein enhanced the antinociceptive effects of a bolus dose of 1 mg/kg clonidine (Three-way RM ANOVA, main effect of gallein $F(1, 21) = 7.8237$, $P=0.0108$) (N=13 vehicle, N=13 gallein). **D)** Enhancements in clonidine-induced antinociception by gallein are driven by elevated tail withdrawal latencies in males (Three-way RM ANOVA, main effect of sex $F(1, 21) = 17.7986$, $P=0.000385$; gallein pretreatment by sex interaction $F(1, 21) = 6.4958$, $P=0.018697$) (N=6 vehicle females, N=7 vehicle males, N=6 gallein females, N=7 gallein males).

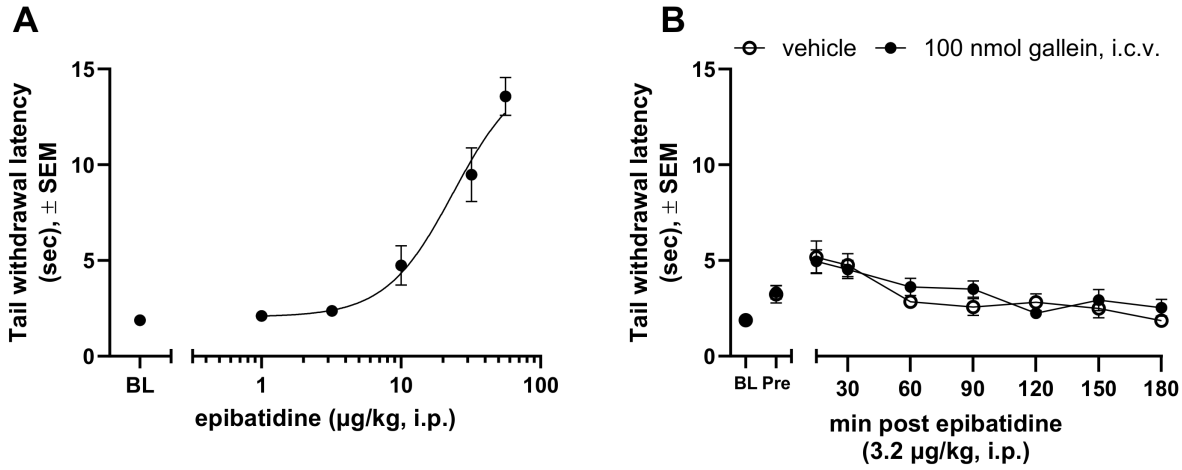


Figure 2.5. Gallein does not enhance epibatidine-induced antinociception. A) Epibatidine produces dose-dependent increases in tail withdrawal latency (Two-way RM ANOVA, main effect of dose $F(5, 60) = 40.6934$, $P<0.0001$; no main effect of sex $F(1, 12) = 0.2091$, $P=0.6556$) (N=14). 3.2, 32, and 56 µg/kg doses were significantly different from baseline (Dunnett's post-test). **B)** 100 nmol i.c.v. gallein did not enhance 3.2 µg/kg epibatidine-induced antinociception (Three-way RM ANOVA, no main effect of gallein $F(1, 20) = 0.4234$, $P=0.5227$) (N=12 vehicle, N=12 gallein).

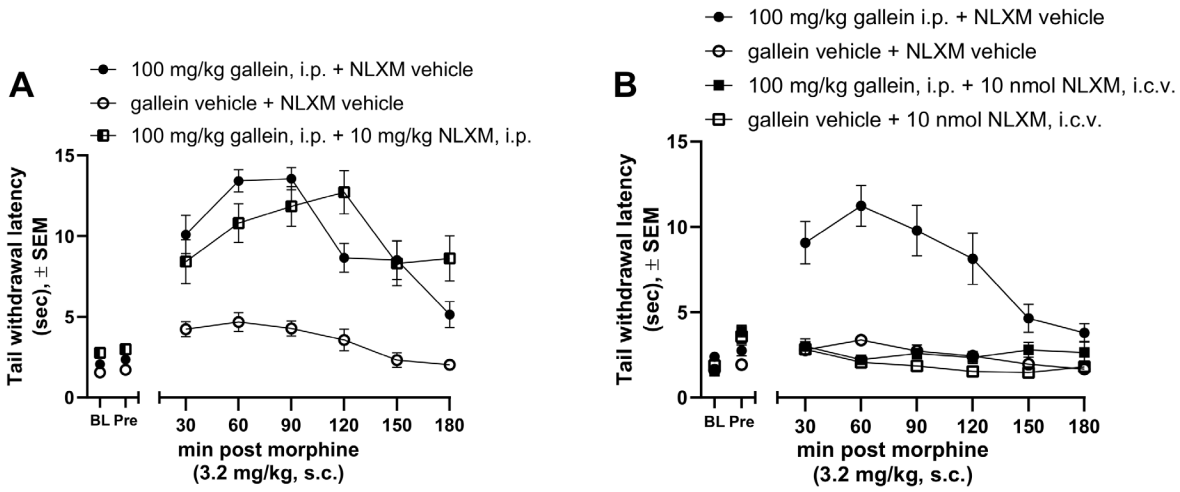


Figure 2.6. Enhancement of morphine-induced antinociception by gallein is dependent upon morphine action at central MORs. A) Enhancement of 3.2 mg/kg morphine-induced antinociception by 100 mg/kg i.p. gallein is not blocked by 10 mg/kg systemic naloxone methiodide (Three-way RM ANOVA, main effect of pretreatment condition ($F(2, 32) = 28.13$, $P<0.0001$). Post-test shows that mice treated with gallein and NLXM had elevated tail withdrawal latencies compared to mice treated with gallein vehicle while not being significantly different from mice treated with gallein and NLXM vehicle (See Table 3) (N=16 gallein + NLXM vehicle; N=12 gallein + NLXM; N=10 gallein vehicle + NLX vehicle). **B)** When administered centrally, 10 nmol NLXM blocks enhancement of 3.2 mg/kg morphine-induced antinociception by 100 mg/kg gallein, i.p. (Four-way ANOVA, main effect of gallein pretreatment ($F(1, 40) = 43.52$, $P<0.0001$); i.c.v. NLXM pretreatment ($F(1, 40) = 32.58$, $P<0.0001$; gallein pretreatment by i.c.v. NLXM pretreatment interaction ($F(1, 40) = 26.87$, $P<0.0001$). (N=12 gallein + vehicle; N=12 gallein + NLXM; N=12 gallein vehicle + vehicle; N=12 gallein vehicle + NLXM). See Table 4 for post-test comparisons.

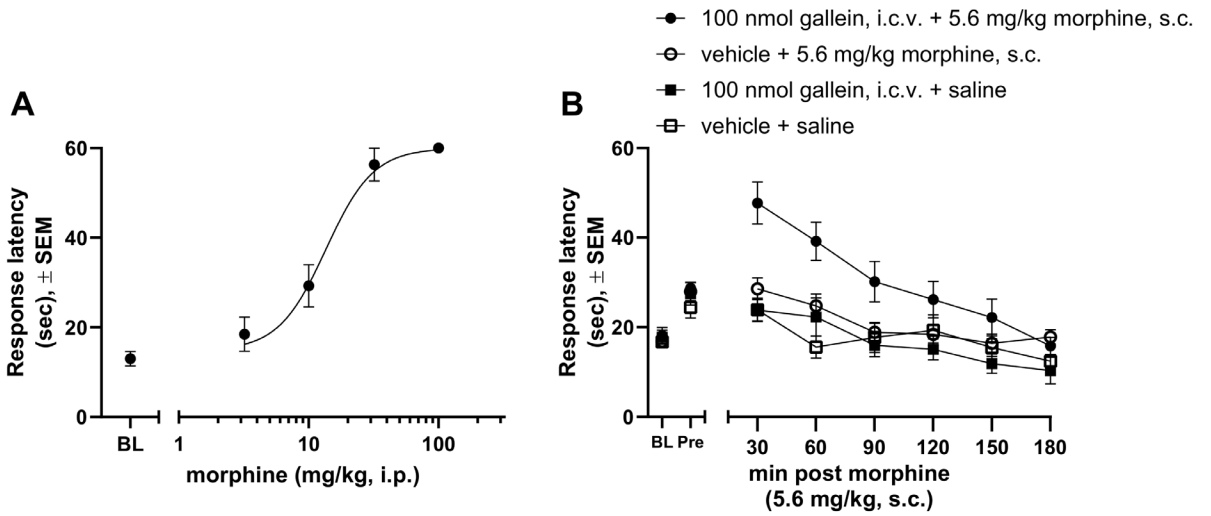


Figure 2.7. **Gallein potentiates morphine-induced antinociception in the hot plate assay. A)** Morphine produces dose-dependent increases in tail withdrawal latency in the hot plate assay (Two-way RM ANOVA, main effect of dose $F(4, 24) = 43.036$, $P<0.0001$). All doses greater than 3.2 mg/kg produce response latencies significantly above baseline (Dunnett's post-test) (N=8). **B)** 100 nmol i.c.v. gallein significantly enhances morphine-induced antinociception in the hot plate assay (Four-way RM ANOVA, gallein pretreatment by morphine interaction ($F (1, 40) = 4.26$, $P=0.0455$; main effect of morphine ($F (1,40) = 13.96$, $P=0.0006$). See Table 5 for post-test comparisons. (N=12 gallein vehicle + morphine; N=12 gallein + morphine; N=12 gallein vehicle + saline; N=12 gallein + saline).

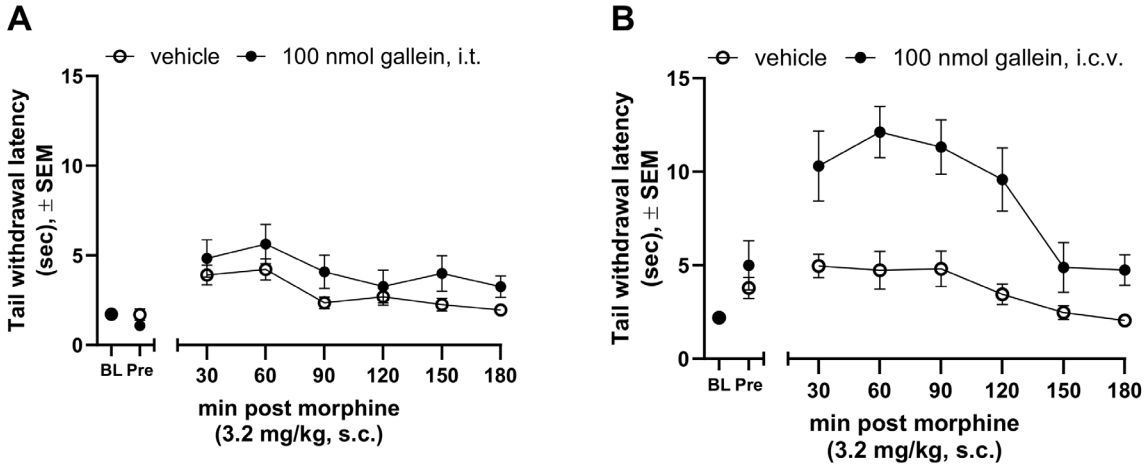


Figure 2.8. Intrathecal (i.t.) gallein pretreatment does not enhance morphine-induced antinociception, but i.c.v. gallein pretreatment does. A) The antinociceptive effects of morphine were not enhanced by 100 nmol i.t. gallein (Two-way RM ANOVA, no main effect of gallein pretreatment $F(1, 25) = 1.2892$, $P=0.267$) (N=13 vehicle; N=16 gallein). **B)** Mice pretreated with 100 nmol i.c.v. gallein exhibit significantly elevated tail withdrawal latencies compared to vehicle pretreated mice (Two-way RM ANOVA, main effect of gallein pretreatment $F(1, 20) = 22.0584$, $P=0.0001385$; no main effect of sex $F(1, 20) = 1.2268$, $P=0.2812$; no sex by gallein pretreatment interaction $F(1, 20) = 0.0013$, $P=0.9716$) (N=8 vehicle; N=9 gallein). See Table 6 for post-test comparisons.

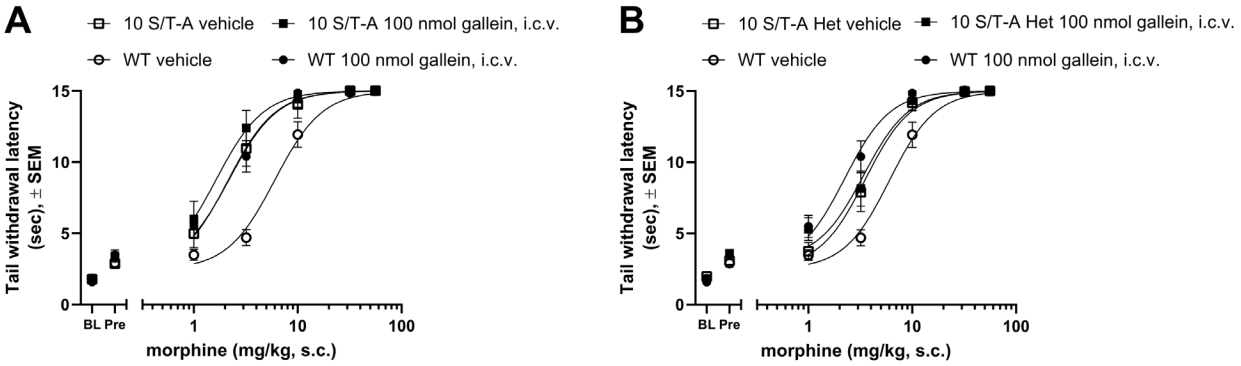


Figure 2.9. Gallein does not potentiate morphine-induced antinociception in mice expressing MOR lacking phosphorylation in the carboxyl terminus, 10 S/T-A mice. A) 100 nmol i.c.v. gallein produced a significant leftward shift in the dose-response curve for morphine and the effect of gallein on the dose-response relationship of morphine was significantly altered in homozygous 10 S/T-A knock-in mice (Three-way RM ANOVA main effect of gallein $F(1, 52) = 9.8159$, $P=0.00284$, main effect of genotype $F(1, 52) = 7.1717$, $P=0.00989$, gallein by dose interaction $F(6, 312)=6.1542$, $P<0.0001$, gallein by dose by genotype interaction $F(6, 312)=2.5217$, $P=0.0213$) (N=16 WT vehicle, N=16 WT gallein, N=12 10 S/T-A vehicle, N=12 10 S/T-A gallein). At 3.2 mg/kg morphine, WT animals treated with gallein ($P=0.00485$), 10 S/T-A mice treated with vehicle ($P=0.00392$), and 10 S/T-A mice treated with gallein ($P=0.000147$) all had significantly elevated tail withdrawal latencies compared to WT animals treated with vehicle but did not differ from each other. **B)** 100 nmol i.c.v. gallein did not alter a dose-response curve of morphine in heterozygous 10 S/T-A mice (Two-way RM ANOVA no main effect of gallein pretreatment $F(1, 30)=0.517$, $P=0.478$; no gallein pretreatment by dose interaction $F(6, 180)=0.505$, $P=0.804$) (N=16 10 S/T-A Het vehicle, N=16 10 S/T-A Het gallein).

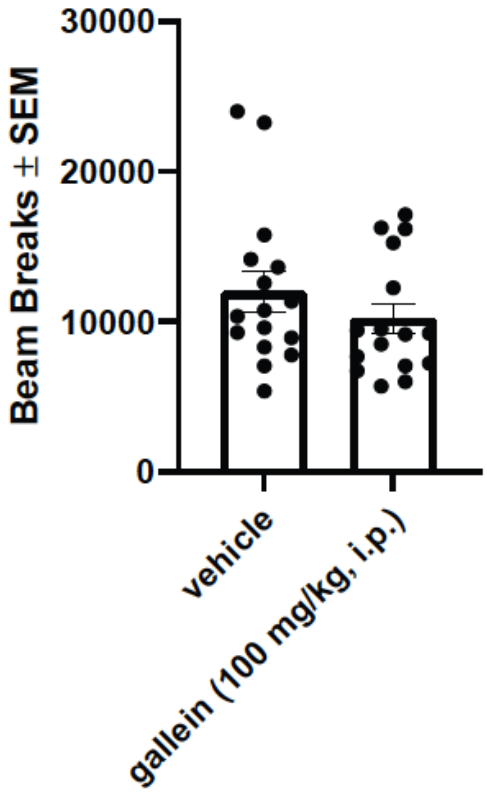


Figure 2.10. Systemic gallein does not alter locomotor activity 24 hrs post administration. Total beam breaks over the course of 1 hr are not different between gallein and vehicle pretreated groups 24 hrs post administration (Unpaired t-test T (30) = 1.098, P=0.2807) (N=16 vehicle, N=16 gallein).

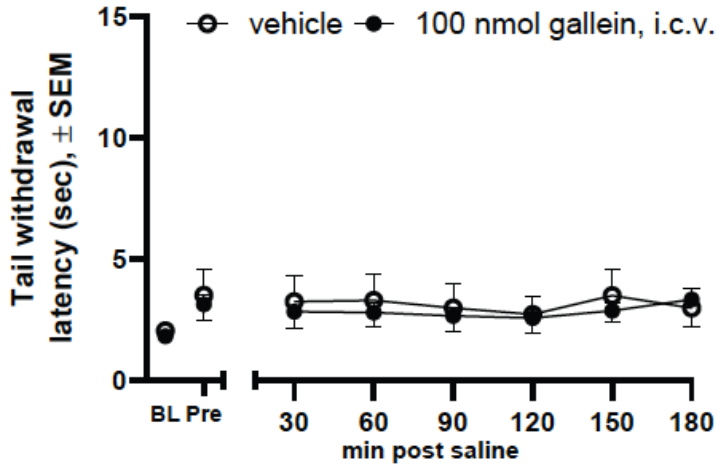


Figure 2.11. Gallein has no effect on antinociception in the absence of morphine. Tail withdrawal latencies do not differ in mice pretreated with gallein or vehicle prior to a time course of saline in the WWTW assay (Two-way RM ANOVA, no main effect of gallein pretreatment ($F(1, 19) = 0.1401$, $P=0.712$) (N=12 vehicle, N=11 gallein).

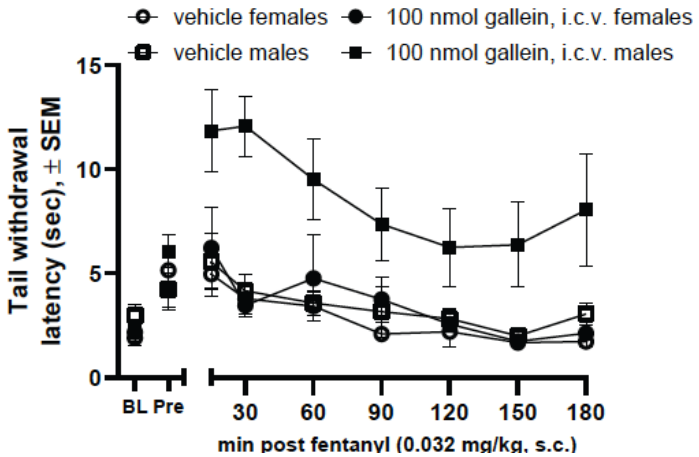


Figure 2.12. Sex differences in potentiation of antinociceptive effects of fentanyl by gallein. Fentanyl-induced antinociception is enhanced by gallein in males, but not females (Three-way RM ANOVA, main effect of sex ($F(1, 20) = 13.09$, $P=0.0017$); gallein pretreatment by sex interaction ($F(1, 20) = 8.117$, $P=0.0099$) (N=6 vehicle females, N=6 vehicle males, N=6 gallein females, N=6 gallein males). At the 30-minute time point males treated with gallein exhibited significantly elevated tail withdrawal latencies (Three-way RM ANOVA Tukey post-test with Bonferroni correction) compared to females treated with gallein ($P=0.0008$) and compared to males treated with vehicle ($P=0.0025$).

2.6 Tables

Comparison	Time (min)	p-value
gallein + NLX vehicle vs gallein + NLX	30	<0.0001
gallein + NLX vehicle vs gallein vehicle + NLX vehicle	30	0.0036
gallein + NLX vehicle vs gallein vehicle + NLX	30	<0.0001
gallein + NLX vehicle vs gallein + NLX	60	<0.0001
gallein + NLX vehicle vs gallein vehicle + NLX vehicle	60	<0.0001
gallein + NLX vehicle vs gallein vehicle + NLX	60	<0.0001
gallein + NLX vehicle vs gallein + NLX	90	<0.0001
gallein + NLX vehicle vs gallein vehicle + NLX vehicle	90	<0.0001
gallein + NLX vehicle vs gallein vehicle + NLX	90	<0.0001
gallein + NLX vehicle vs gallein + NLX	120	<0.0001
gallein + NLX vehicle vs gallein vehicle + NLX vehicle	120	0.000637
gallein + NLX vehicle vs gallein vehicle + NLX	120	0.000124
gallein + NLX vehicle vs gallein + NLX	150	0.00417
gallein + NLX vehicle vs gallein vehicle + NLX vehicle	150	0.00404
gallein + NLX vehicle vs gallein vehicle + NLX	150	0.00171

Table 2.1. Figure 1A Post-test Comparisons.

Comparison	Time (min)	p-value
gallein WT vs vehicle WT	30	0.00250
gallein WT vs vehicle KO	30	0.000257
gallein WT vs gallein KO	30	0.00538
gallein WT vs vehicle WT	60	<0.0001
gallein WT vs vehicle KO	60	<0.0001
gallein WT vs gallein KO	60	<0.0001
gallein WT vs vehicle WT	90	0.014
gallein WT vs vehicle KO	90	0.000311
gallein WT vs gallein KO	90	0.000555
gallein WT vs vehicle KO	120	0.00543
gallein WT vs gallein KO	120	0.00744

Table 2.2. Figure 1B Post-test Comparisons.

Comparison	Time (min)	p-value
gallein + NLXM vs gallein vehicle + NLXM vehicle	0	0.009
gallein + NLXM vs gallein vehicle + NLXM vehicle	60	0.016
gallein + NLXM vehicle vs gallein vehicle + NLXM vehicle	60	<0.0001
gallein + NLXM vs gallein vehicle + NLXM vehicle	90	0.003
gallein + NLXM vehicle vs gallein vehicle + NLXM vehicle	90	<0.0001
gallein + NLXM vs gallein vehicle + NLXM vehicle	120	<0.0001
gallein + NLXM vs gallein vehicle + NLXM vehicle	180	0.011

Table 2.3. Figure 6A Post-test Comparisons.

Comparison	Time (min)	p-value
gallein + NLXM vehicle vs gallein vehicle + NLXM vehicle	30	<0.0001
gallein + NLXM vehicle vs gallein vehicle + NLXM	30	<0.0001
gallein + NLXM vehicle vs gallein + NLXM	30	0.00012
gallein + NLXM vehicle vs gallein vehicle + NLXM vehicle	60	<0.0001
gallein + NLXM vehicle vs gallein vehicle + NLXM	60	<0.0001
gallein + NLXM vehicle vs gallein + NLXM	60	<0.0001
gallein + NLXM vehicle vs gallein vehicle + NLXM vehicle	90	<0.0001
gallein + NLXM vehicle vs gallein vehicle + NLXM	90	<0.0001
gallein + NLXM vehicle vs gallein + NLXM	90	<0.0001
gallein + NLXM vehicle vs gallein vehicle + NLXM vehicle	120	0.0032
gallein + NLXM vehicle vs gallein vehicle + NLXM	120	0.00026
gallein + NLXM vehicle vs gallein + NLXM	120	0.0025
gallein + NLXM vehicle vs gallein vehicle + NLXM	150	0.0102

Table 2.4. Figure 6B Post-test Comparisons.

Comparison	Time (min)	p-value
gallein + morphine vs gallein vehicle + morphine	30	0.046
gallein + morphine vs gallein + saline	30	0.002
gallein + morphine vs gallein vehicle + saline	30	0.002
gallein + morphine vs gallein vehicle + saline	60	0.009

Table 2.5. Figure 7B Post-test Comparisons.

Comparison	Time (min)	p-value
gallein vs vehicle	30	0.0339
gallein vs vehicle	60	0.0006
gallein vs vehicle	90	0.0054
gallein vs vehicle	120	0.0234

Table 2.6. Figure 8B Post-test Comparisons.

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Chapter 3 Novel Gβγ Inhibitors Enhance Morphine-Induced Antinociception

3.1 Introduction

On-target side effects of opioids such as constipation, respiratory depression, rapid tolerance development, and abuse liability have been a consistent impediment to their repeated use in pain conditions. As such, there have been numerous attempts to dissociate these side effects from the beneficial analgesia conferred by MOR agonists and effectively broaden the therapeutic window for these drugs. To date, these attempts, such as biased agonism, have not been successful (1). Thus, there is a critical need to improve the safety and efficacy of opioid analgesics – especially in patients with chronic pain conditions where repeated opioid administration increases prevalence of adverse effects (2). A novel strategy to address this issue is Gβγ signal modulation.

Downstream of MOR agonist-induced activation, Gβγ engages several structurally distinct effectors which both facilitate and oppose the analgesic effects of opioids. For example, Gβγ inhibits voltage-gated calcium channels (VGCCs) (3, 4) and soluble N-ethylmaleimide-sensitive factor attachment receptors (SNAREs) (5, 6) to reduce presynaptic GABA release in the RVM and disinhibit descending pain modulation. Conversely, Gβγ negatively regulates MORs by activating GRKs (7) and PLCβ3 (8, 9). Because Gβγ performs these dual roles for intracellular MOR signaling, investigators hypothesized that Gβγ signal bias could be a viable pharmacological strategy to enhance the analgesic effects of MOR agonists. Indeed, *in vitro* studies showed that both peptides and small molecules could differentially inhibit certain Gβγ effector subsets while leaving other signaling pathways unaltered (10-12).

Of particular interest were two structurally similar compounds, M119 and gallein, which selectively inhibited Gβγ-mediated activation of GRK2 and PLCβ3. *In vivo* experiments found that pretreatments with these Gβγ modulators potentiated the antinociceptive effects of morphine (10, 11, 13). More recent work demonstrated that gallein pretreatments abrogated the development of tolerance to morphine-induced antinociception and even potentiated antinociception in a tolerant state (14). Gallein alone appears to enhance the effects of endogenous opioids in a pre-

existing pain state (15). The work presented within this dissertation has furthered our understanding of Gβγ modulation *in vivo* by illustrating that it is a receptor-mediated phenomenon.

While these findings are promising, they utilize the tool compound gallein. Gallein is a dye-like compound on a fused planar ring scaffold. Moreover, gallein exhibits undesirable physicochemical properties leading to curious pharmacokinetics and poor solubility. For these reasons, gallein is not a suitable candidate for drug development. The translational relevance of Gβγ signal modulation as a clinical strategy is ultimately dependent upon viable drug candidates. Therefore, we sought to characterize the efficacy of three novel Gβγ signal modulators: compound 743, 744, and 858 (Table 1) for their ability to modulate Gβγ signaling *in vitro* and potentiate morphine-induced antinociception. As a negative control, we also tested whether compound 895 could alter morphine-induced antinociception. While compound 895 is designed on the same scaffold as compound 858, it exhibited weak activity *in vitro*.

We found that central administration of compounds 743 and 858 significantly enhanced morphine-induced antinociception in the warm water tail withdrawal assay. These data confirmed observations that these compounds significantly alter Gβγ signaling *in vitro*. Compound 895, which is structurally similar to 858, but with limited *in vitro* activity, had no effect. Because compound 858 exhibited the greatest potency when administered centrally, we examined whether systemic administration was viable. There was no effect of compound 858 to alter the antinociceptive effects of morphine when administered systemically. Based on pharmacokinetic analysis, this is likely due to poor central penetration. Still, these data support the overall approach of Gβγ signal bias and show that novel compounds exhibit effects comparable to gallein.

3.2 Methods

3.2.1 Drugs and Reagents

Gallein (Cayman Chemical, Ann Arbor MI) and morphine sulfate (Henry Schein, Melville NY). Morphine was dissolved in 0.9% sterile saline. Centrally administered gallein and compounds 743, 744, and 858 were dissolved in DMSO. For intraperitoneal (i.p.) systemic administration compound 858 was dissolved in a 10% DMSO, 10% castor oil, and 80% sterile water vehicle.

3.2.2 Animals

All animal procedures were conducted at the University of Michigan according to NIH *Health Guide for the Care and Use of Laboratory Animals* and with approval of the Institutional Animal Care and Use Committee (IACUC). Wild type C57BL/6 mice were purchased from Envigo (Indianapolis, IN) and bred in-house for these studies. Mice were housed with a maximum of five animals per cage in clear polypropylene cages. Corn cob bedding and nestlets were provided for enrichment. Animals were housed in pathogen-free rooms maintained between 68°F and 72°F and 30% and 70% humidity. Rooms utilized a 12-hour light/dark cycle (lights on at 7 A.M. and lights off at 7 P.M.) with free access to food. Experiments were conducted in the animals' housing room during the light cycle. All mice used in experiments were between 7 and 12 weeks of age and weighed 16-28 grams. A combination of male and female mice was used for all experiments. 8-12 animals per group were used for all experiments.

3.2.3 Transduction of diacylglycerol (DAG) fluorescent biosensor and M1 muscarinic acetylcholine receptor in HEK293 cells

HEK293 cells were used for these studies. Green-fluorescent up DAG assay kit (#U0300G) and CAAX-Green downward DAG kit (#D0331G) were purchased through Montana Molecular (Bozeman, MT). HEK293 cells were incubated (8-24 hrs) with a viral transduction reaction including DAG Sensor BacMam, sodium butyrate, and M1 muscarinic acetylcholine BacMam (receptor control was not added for endogenous Gq-coupled receptor experiments). A 96-well black plate with transparent bottom (Corning, Corning NY) was used (50 µL of 500,000 cells/mL per well) with BacMam transduction reaction (100 µL per well).

3.2.4 Activation of DAG sensor and collection of data

Assays were conducted with a Hamamatsu µCell FDSS plate reader. Agonists were loaded into a 96-well plastic conical bottom source plate (Thermo Fisher Scientific, Rochester NY) prior to transfer by the instrument. Before placing the cells in the plate reader, transduction media was exchanged with 120 µL of warmed Gibco Dulbecco's Phosphate Buffered Saline (DPBS) with Ca2+ and Mg2+ (Life Technologies Co, Grand Island, NY). Cells at 80% confluency were kept in the dark inside the plate reader and incubated in DPBS for 10 min before agonist was added. Baseline fluorescence at 540 nm was measured each second for 30 sec followed by 15 µL of agonist added simultaneously to each well of the plate. Fluorescence intensity measurements were acquired every second for 230 sec. Data are normalized to baseline fluorescence ($\Delta F/Fo=1$) in each

well and the change in fluorescence in each well relative to baseline is monitored over time. Each condition was tested in 3-4 wells in at least 3 different sets of experiments.

3.2.5 Transient transfection of MOR in HEK293 cells

HEK293 cells were transiently transfected with flag-MOR cDNA using Lipofectamine 2000 in a 10 cm plate at 70% confluency one day before BacMam transduction. After 24 h cells were transferred to a 96-well plate at 80% confluency and incubated for 24 hrs before the assay.

3.2.6 Pretreatment

All Gβγ modulators were administered 30 min prior to morphine, including systemic 858.

3.2.7 Intracerebroventricular (i.c.v.) pretreatment injections

I.c.v. injections were performed 30 minutes after measurement of baseline withdrawal or response latencies and 30 minutes before morphine treatment. Hamilton syringes of 10 µL with 26 G (catalog #7804-03; Point #4, 12° bevel) needles were used with a custom stopper that allowed 4 mm of the needle to enter the skull. Injections were free-handed utilizing the ears and eyes for orientation to target the lateral ventricles of the brain. Volume of injection was 3 µL for all experiments and the needle was held in place for 30 seconds after the total volume was injected to ensure perfusion. After 30 seconds, the needle was carefully removed and the mice placed into a separate recovery cage and observed for recovery from anesthesia (normal grooming and movement). Upon recovery, mice were placed back into their home cage.

To conduct the injection, mice were anesthetized using isoflurane until no longer responsive to tail pinch and breathing slowed to one inhalation per second. After completion of the experiment, mice were euthanized to confirm injection placement using methylene blue.

3.2.8 Warm Water Tail Withdrawal

Mice were briefly placed into a cylindrical plexiglass restrainer and tails immersed approximately 3 cm into a 55°C water bath. The latency to withdraw or rapidly flick the tail was recorded with a maximum of 15 seconds (20 seconds for experiments using 50°C water bath; nalbuphine experiments) to prevent tissue damage. Baseline tail withdrawal latencies between 2 and 5 seconds were consistent for each assay. Mice were briefly habituated to the plexiglass restrainer and tail dipped prior to injection with saline. After 30 min, baseline withdrawal latencies were recorded (BL, baseline withdrawal latency).

For pretreatments, withdrawal latencies were recorded 30 min after i.c.v. injection (Pre, pretreatment withdrawal latency). Mice received analgesic immediately following pretreatment measurement for all studies. Morphine dose of 3.2 mg/kg was selected based on previous work illustrating enhancement of antinociceptive effects by gallein at this dose (16).

3.2.9 Pharmacokinetics

Performed by Pharmaron in CD1 mice were treated with 10 mg/kg compound 858, i.p. in 40% DMSO, 40% PEG400, 20% water. Plasma and brain samples were taken at 15 min, 30 min, 1 hr, and 3 hrs with 3 mice at each time point. Samples were processed using protein precipitation extraction with acetonitrile and the resulting extracts were used for LC-MS/MS quantification. Hepatocyte microsome stability studies were also performed by Pharmaron.

3.2.10 Statistics

Time course studies and dose response studies using gallein pretreatment, main effects and interactions were evaluated using repeated measures ANOVA in Graphpad Prism with time as a within-subjects factor and sex and treatment as between subjects factors as appropriate to the specific experiment. Sidak's post-test was used to compare groups at individual timepoints.

3.3 Results

3.3.1 In vitro Screening and Precursor Compound Selection

83,000 compounds from the DART Neurosciences collection were screened for their ability to reduce Gβγ-PLCβ interactions using a Time-resolved FRET based assay. 50 positive "Hits" were tested in triplicate in dose response curves. Compounds with IC50's <30 μM with Hill coefficients between -2 and 2 were selected for low molecular weight, desirable predicted ADME properties, and removal of pan-assay interference compounds (PAINs). This led to a subset of 10 compounds from which two were selected as scaffolds for medicinal chemistry optimization (Table 1). Compound 342472 served as a precursor for compounds 743 and 744, while compound 338800 served as a precursor for compounds 858 and 895.

3.3.2 Novel Gβγ Modulators Inhibit MOR-Dependent PLC Activation

As shown previously, concomitant Gq activation with carbachol and DAMGO-mediated Gβγ release downstream of MORs leads to synergistic PLC activation as measured by fluorescent DAG reporter (17). Compound 744 concentration-dependently decreased this synergistic

activation (Figure 1B), while compound 743 did not (Figure 1A). Compound 858 exhibited effects similar to 30 μM 744 although at a lower concentration of 10 μM (Figure 1C).

3.3.3 Inhibition of Arrestin Recruitment

PathHunter arrestin recruitment assay demonstrated concentration-dependent inhibition of DAMGO-dependent luminescence by gallein (positive control) and compound 743 but not compound 744 (Figure 2A). Compound 858 inhibited arrestin recruitment more potently than gallein (Figure 2B) without altering Gβγ interactions with Gα in the Tru-Path assay (Figure 2C).

3.3.4 Novel Gβγ Modulators Enhance Morphine-Induced Antinociception

To examine whether novel Gβγ modulators could potentiate the antinociceptive effects of morphine, 100 nmol of compounds 743 and 744 were centrally administered 30 min prior to morphine in the WWTW assay. Compound 743 significantly enhanced 3.2 mg/kg, s.c. morphine-induced antinociception (Figure 3A) while compound 744 only trended to do so (P=0.0541) compared to vehicle (Figure 3B). Importantly, this dose of morphine has been shown previously to produce no significant antinociception on its own (13, 14, 17).

3.3.5 Compound 858 Enhances Morphine-Induced Antinociception at a Lower Dose than Gallein

Due to the relative efficacy and potency of compound 858 *in vitro*, we sought to evaluate the ability of compound 858 to potentiate 3.2 mg/kg of morphine at three doses: 30 nmol, 50 nmol, and 100 nmol (Figure 8A). Interestingly, compound 858 produced significant increases in tail withdrawal latencies compared to vehicle at 50 nmol (Figure 4A). These effects were comparable to 100 nmol gallein pretreatment (Figure 4B). However, neither 30 nmol or 100 nmol of compound 858 produced significant increases in tail withdrawal latencies compared to vehicle (Figure 8A). Considering that 50 nmol gallein did not significantly potentiate morphine-induced antinociception (Figure 8B), these data indicate greater potency for compound 858 compared to gallein.

3.3.6 Compound 895 Does not Enhance Morphine-Induced Antinociception

Compound 858 is designed on a pyrazolopyridine scaffold. To test an additional negative control condition and to validate the translation of *in vitro* mechanisms to *in vivo* results, we tested whether compound 895 potentiated morphine's antinociceptive effects in WWTW. Compound

895 is also designed on the pyrazolopyridine scaffold but does not potently inhibit arrestin recruitment (Figure 5A). Therefore, we hypothesized that compound 895 should not affect morphine-induced antinociception. Indeed, compared to vehicle, compound 895 had no effect on tail withdrawal latencies following morphine treatment (Figure 5B).

3.3.7 Gβγ Modulators Sex-Dependently Enhance Morphine-Induced Antinociception

To investigate sex differences in response to the effects of Gβγ modulation on morphine-induced antinociception, all studies included both female and male mice. Analysis for sex differences revealed that, while there were no sex by treatment interactions in any study, there was a significant effect of sex for 100 nmol gallein treatment (Figure 6A), 50 nmol compound 858 (Figure 6B), and 100 nmol compound 744 (Figure 6D) (trending for 100 nmol compound 743 P=0.057). Male mice treated with Gβγ modulator dependably exhibit elevated tail withdrawal latencies compared to all other groups. The consistency of this effect across all Gβγ modulators used suggests that there is a sexually dimorphic response to this pharmacologic intervention.

3.3.8 Systemic Compound 858 Does not Alter Morphine-Induced Antinociception

Because compound 858 exhibited the greatest efficacy and potency across novel Gβγ modulators, we sought to evaluate whether systemic administration would exhibit similar effects. However, systemic administration of 50 mg/kg compound 858 30 min prior to morphine had no effect on morphine-induced antinociception (Figure 7). Pharmacokinetic testing showed that, while stable *in vitro* (half-life of 143 min in mouse liver microsomes), systemic 858 displays an exceptionally short half-life in plasma and does not appear in brain homogenates, suggesting that it is rapidly cleared (Table 2 and 3). Given that prior i.c.v. studies show significant effects, these data support conclusions in Chapter 2 showing that central action is required for these compounds to influence morphine-induced antinociception.

3.4 Discussion

Evidence validating Gβγ signal modulation as a pharmacologic strategy to improve the safety and efficacy of MOR agonists continues to grow. Initial *in vivo* studies showing a leftward shift in the dose response curve for antinociceptive effects of morphine with Gβγ modulator co-administration were followed by data showing no enhancement of MOR agonist-related side effects (11, 13). These data supported a widening of the therapeutic window for MOR agonists. Our lab continues to bolster these findings by uncovering mechanisms of Gβγ signaling modulators

in this paradigm. For example, it was demonstrated that repeated gallein pretreatments could prevent the development of tolerance to morphine's antinociceptive effects (14). Furthermore, gallein treatment in already-tolerant mice still potentiated morphine-induced antinociception. Additional work implies that gallein alone enhances the antinociceptive effects of endogenous opioids (15). To further strengthen the use of Gβγ modulation as a potential therapeutic strategy, we assessed the efficacy of novel Gβγ modulators to potentiate morphine-induced antinociception. Our data show that novel Gβγ modulators designed on chemical scaffolds entirely distinct from gallein can enhance morphine-induced antinociception, although these effects appear largely sexually dimorphic. Compound 858 was the most potent modulator tested *in vitro* and in i.c.v. administration experiments but exhibited poor pharmacokinetics when administered systemically. Inadequate brain penetration is likely responsible for the lack of activity observed following systemic 858 treatment.

Here we showed that novel Gβγ modulators can inhibit DAMGO-dependent synergistic PLC activation and arrestin recruitment *in vitro*. These data translated to *in vivo* models of pain where modulator pretreatment enhanced morphine-induced antinociception when administered centrally. Both compound 743 and 858 pretreatment prior to morphine produced significant increases in tail withdrawal latencies compared to vehicle. The effects of compound 744 trended closely to significance ($p=0.0541$). Moreover, compound 895, which exhibited limited *in vitro* activity, had no effect on morphine-induced antinociception. It should also be noted that these compounds are structurally distinct from each other and from gallein. This suggests that there are several design strategies available to produce Gβγ signal bias and strengthens the notion that Gβγ is the mechanistic target *in vivo* (since Gβγ inhibition is the primary commonality between compounds).

Interestingly, all compounds including gallein exhibited sex-dependent effects. While there were not strict sex by treatment interactions, all compounds except 743 showed main effects of sex. Furthermore, we believe it is meaningful that this effect is observationally consistent (if not statistically significant) across all Gβγ modulators tested. Males treated with Gβγ modulator tend to have higher average withdrawal latencies compared to all other groups. This finding is especially intriguing because of the structural dissimilarity between Gβγ modulators. It is therefore unlikely that the sex differences observed in these studies are a result of a specific response to a

certain molecular structure. Rather, these data suggest a difference in responses to Gβγ modulation broadly as a result of underlying discrepancies in physiology between sexes.

Some evidence indicates sexual dimorphism in Gβγ-GRK2 interactions downstream of opioid receptor activation (18). These data implicated sex hormones as a potential factor for this effect. Ovariectomized females treated with estradiol had greater Gβγ-GRK2 associations in striatal tissue than ovariectomized females alone. Increases interactions between Gβγ and GRK2 implies greater Gβγ-mediated activation of GRK2 and consequent negative regulation of the receptor. This would insinuate that a larger dose of Gβγ modulator is necessary to compete for binding and observe significant efficacy in females (i.e. that Gβγ modulation is less potent in females). However, we did not see that a larger dose of compound 858 potentiated morphine-induced antinociception in females. Regardless of sex, 100 nmol 858 had no effect on morphine-induced antinociception. Potentially this higher dose interferes with Gα-Gβγ heterotrimer assembly and therefore prevents appropriate G protein receptor coupling.

An alternative explanation is that there is an underlying sex difference in the response to morphine. While there is evidence that opioids are more potent in male as compared to female rodents (19), there were no significant sex differences for this dose of morphine (3.2 mg/kg) alone (granted, this may be difficult to observe at single dose with low efficacy). Moreover, our previous data showed no sex-dependent shift in dose-response curves for fentanyl, clonidine, or buprenorphine alone even though there were sex-dependent effects for potentiation of these analgesics in response to gallein. Ultimately, more work must be done to investigate the potential mechanisms causing these sex differences. Future work examining the full dose-response relationship for these modulators could provide key insights as to whether Gβγ modulation is a viable therapeutic strategy in females.

Our data support conclusions from Chapter 2 that central action of these Gβγ modulators is crucial for their efficacy. Systemic pretreatment of compound 858 did not enhance morphine-induced antinociception, while central administration did. Subsequent pharmacokinetic data showed that intraperitoneal 858 is cleared rapidly with no brain penetration. Findings from Chapter 2 showed that central, but not peripheral, MOR activation was required to observe enhancements in morphine-induced antinociception by gallein in the WWTW assay. Given this, it makes sense that these compounds must access the CNS to act downstream of central MORs.

Overall, these data support the idea that novel Gβγ modulators designed on scaffolds entirely distinct from gallein can potentiate morphine-induced antinociception with efficacy comparable to gallein. Results from *in vitro* testing translated to *in vivo* mouse models of pain. This strengthens the hypothesis that Gβγ signal modulation is the mechanism of action *in vivo*. Moreover, our findings highlight the importance of CNS penetration for this therapeutic strategy. Sex differences with respect to Gβγ modulation should be further investigated to determine a potential mechanism underlying robust effects in males, but not females.

3.5 Acknowledgments

In vitro experiments in Figure 1, Figure 2, and Figure 5A were conducted by Michael Burroughs in the Smrcka Laboratory.

3.6 Figures

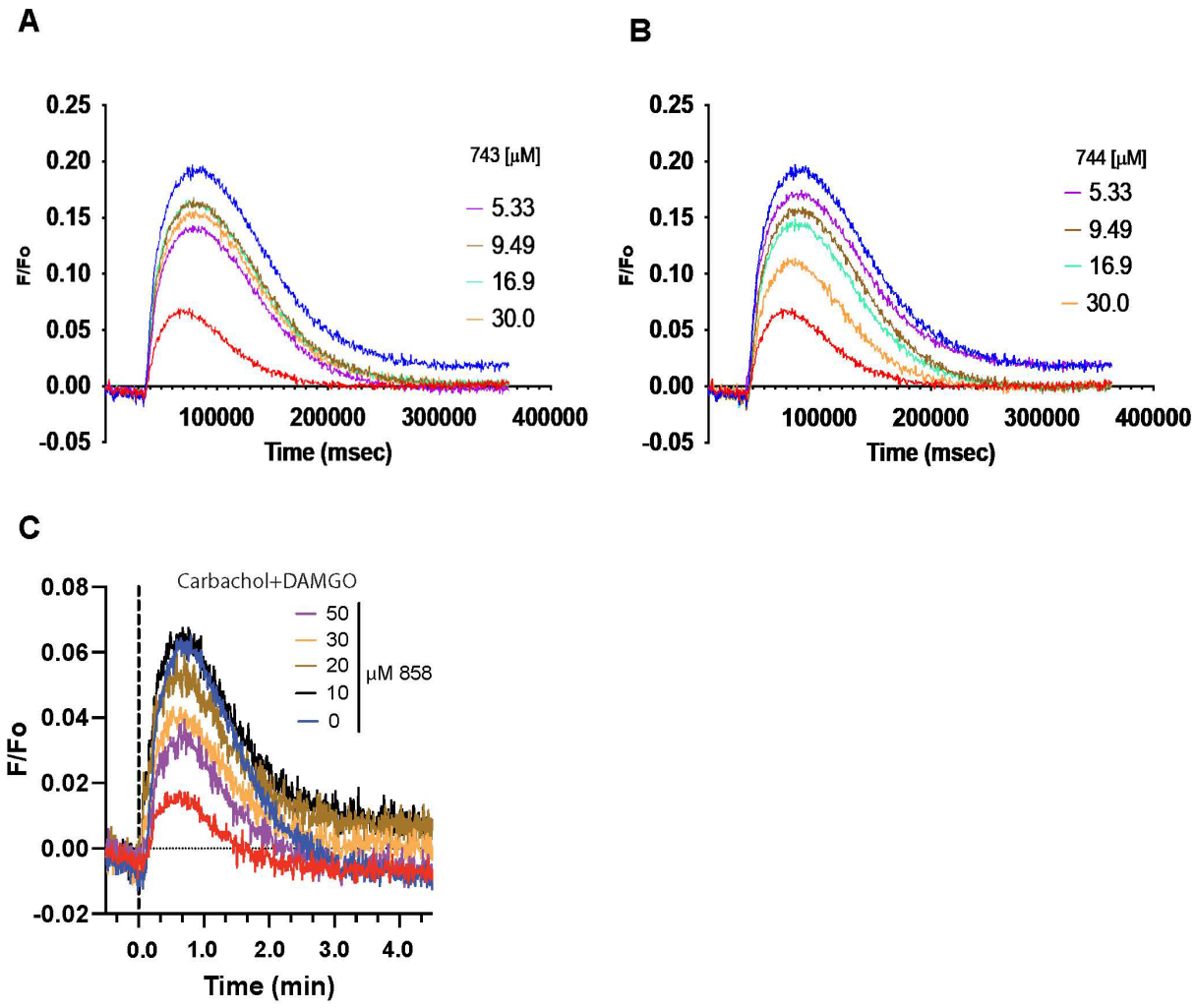


Figure 3.1. Novel Gβγ modulators inhibit MOR-dependent PLC activation. HEK293 cells stably expressing MOR were co-transduced with BacMam viruses expressing M1 muscarinic receptor and a fluorescent DAG reporter and stimulated with either 300 nM carbachol (red line) or 300 nM carbachol plus 1 µM DAMGO. DAMGO alone gives no response in this assay. **A)** Compound 743 does not significantly inhibit DAMGO-dependent DAG production. **B)** Compound 744 concentration-dependently inhibits DAMGO-dependent DAG production. **C)** Compound 858 concentration-dependently inhibits DAMGO-dependent DAG production.

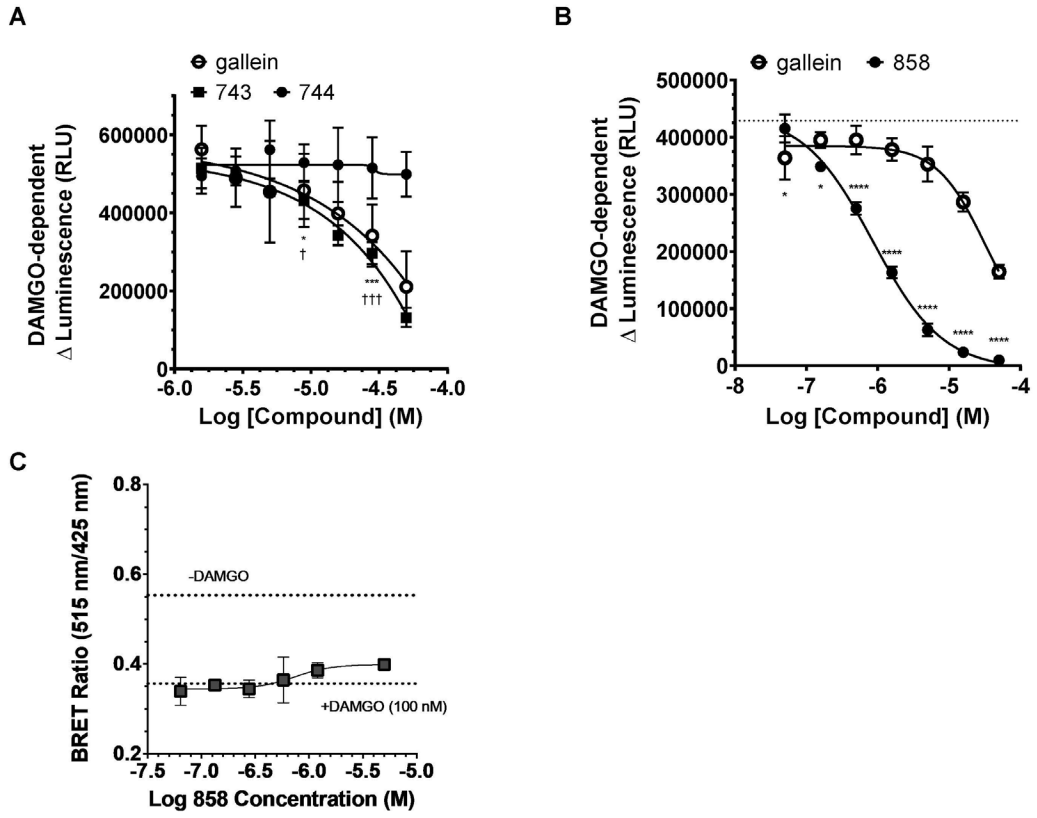


Figure 3.2. Novel Gβγ modulators inhibit DAMGO-dependent arrestin recruitment. A) Compound 743 inhibits DAMGO-dependent (100 nM) arrestin recruitment similarly to gallein, while compound 744 does not (2-way ANOVA main effect of treatment $F(2, 21) = 16.3$, $P<0.001$, main effect of concentration $F(6, 21) = 9.608$, $P<0.001$, and treatment by concentration interaction $F(12, 21) = 2.324$, $P=0.0437$). Dunnett's post-test shows that gallein (†) and 743 (*) significantly differ from baseline, while 744 does not. **B)** Compound 858 inhibits DAMGO-dependent arrestin recruitment more potently than gallein (2-way ANOVA main effect of treatment $F(1, 28) = 632.4$, $P<0.0001$, main effect of concentration $F(6, 28) = 215.6$, $P<0.0001$, and treatment by concentration interaction $F(6, 28) = 60.75$, $P<0.0001$). Sidak's post-test shows 858 significantly different from gallein at each concentration tested. **C)** Compound 858 does not alter Gβγ interactions with Gα.

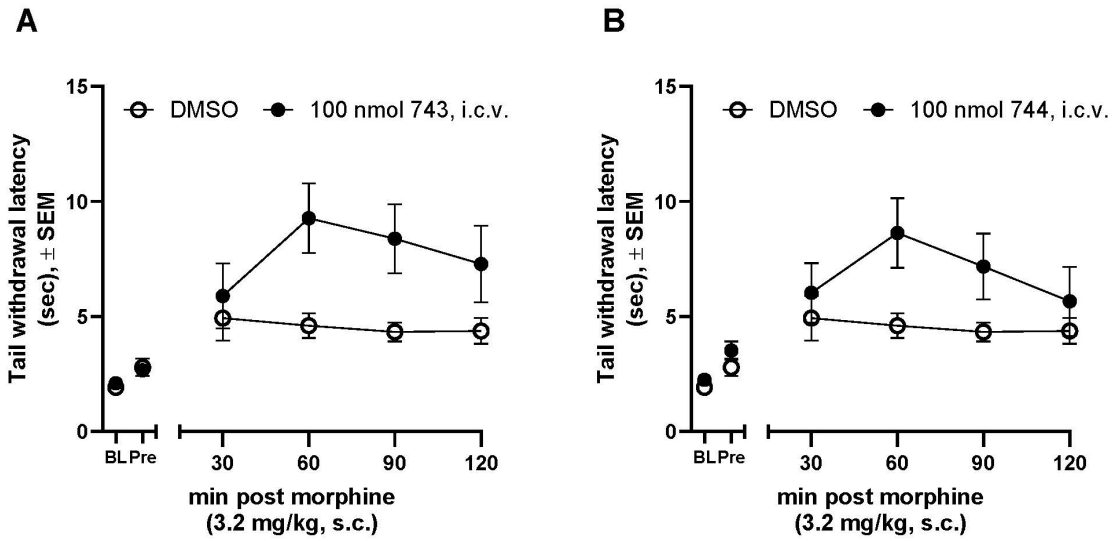


Figure 3.3. A novel Gβγ modulator enhances morphine-induced antinociception. **A)** Mice pretreated with 100 nmol compound 743 exhibit significantly elevated tail withdrawal latencies following morphine administration compared to vehicle (2-way RM ANOVA main effect of treatment F (1, 22) = 6.589, P=0.0176) (N=12 vehicle, N=12 compound 743). **B)** Tail withdrawal latencies were not significantly elevated following pretreatment with 100 nmol compound 744 compared to vehicle (2-way RM ANOVA no main effect of treatment F (1, 21) = 4.164, P=0.0541) (N=12 vehicle, N=11 compound 744).

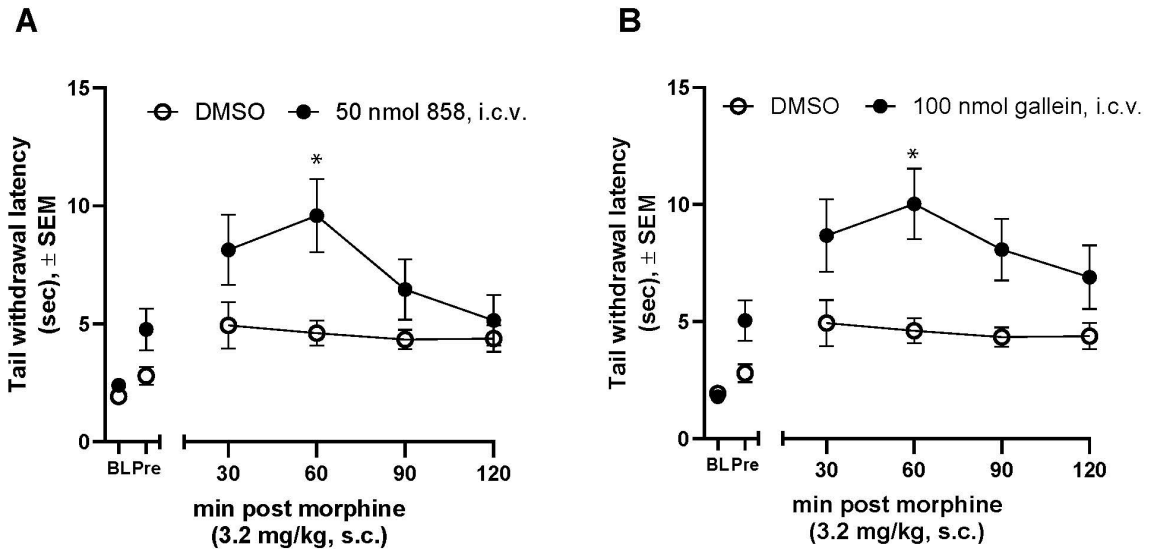


Figure 3.4. Compound 858 produces comparable enhancements in morphine-induced antinociception at a lower dose than gallein. A) Mice pretreated with 50 nmol compound 858 display significantly elevated tail withdrawal latencies compared to vehicle pretreatment following morphine (2-way RM ANOVA main effect of treatment $F(1, 23) = 6.147$, $P=0.0209$) (N=12 vehicle, N=13 compound 858). The compound 858 group was significantly different from vehicle at 60 min (Sidak's post-test $P=0.0496$). **B)** Mice pretreated with 100 nmol gallein show significantly elevated tail withdrawal latencies compared to vehicle following morphine (2-way RM ANOVA main effect of treatment $F(1, 22) = 8.997$, $P=0.0066$) (N=12 vehicle, N=12 gallein). The gallein treated group was significantly different from vehicle at 60 min (Sidak's post-test $P=0.0271$).

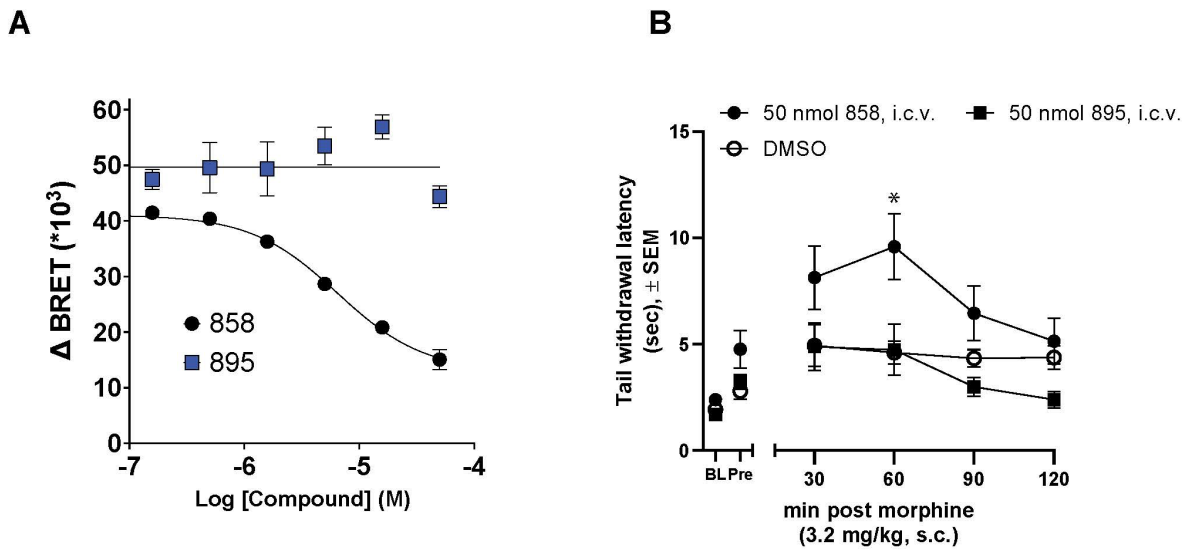


Figure 3.5. Compound 895 has negligible activity *in vitro* and does not alter morphine-induced antinociception. A) Compound 895 does not alter Gβγ-GRK2 interactions at any concentration tested. **B)** Compound 895 does not alter morphine-induced antinociception compared to vehicle control. 2-way RM ANOVA with Sidak's post-test shows a main effect of treatment F (2, 34) = 6.089, P=0.0055 with compound 858 significantly different from vehicle at 60 min (P=0.0217) and compound 895 not different from vehicle at any time point (N=12 vehicle, N=13 compound 858, N=12 compound 895).

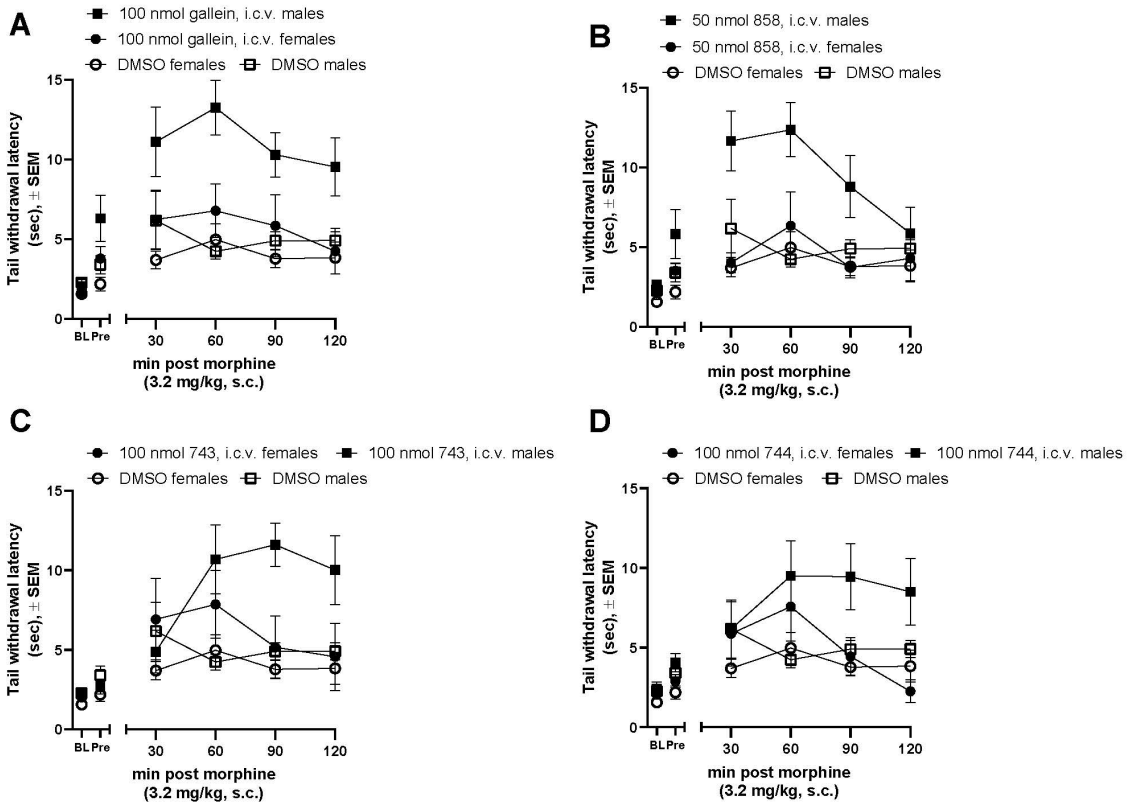


Figure 3.6. Gβγ modulators enhance morphine-induced antinociception in males, but not females. A) Males exhibit elevated tail withdrawal latencies following gallein pretreatment and morphine, while females do not (3-way RM ANOVA main effect of sex F (1, 20) = 10.14, P=0.0047) (N=6 vehicle females, N=6 vehicle males, N=6 gallein females, N=6 gallein males). **B)** Males exhibit elevated tail withdrawal latencies following compound 858 pretreatment and morphine, while females do not (3-way RM ANOVA main effect of sex F (1, 21) = 11.37, P=0.0029) (N=6 vehicle females, N=6 vehicle males, N=6 females compound 858, N=7 males compound 858). **C)** Tail withdrawal latencies are elevated in male mice treated with compound 743 and morphine, but not females (3-way RM ANOVA main effect of sex F (1, 21) = 11.37, P=0.0029) (N=6 vehicle females, N=6 vehicle males, N=6 compound 743 females, N=6 compound 743 males). **D)** Tail withdrawal latencies are elevated in male mice treated with compound 744 and morphine, but not females (3-way RM ANOVA main effect of sex F (1, 19) = 5.006, P=0.0374) (N=6 vehicle females, N=6 vehicle males, N=5 compound 744 females, N=6 compound 744 males).

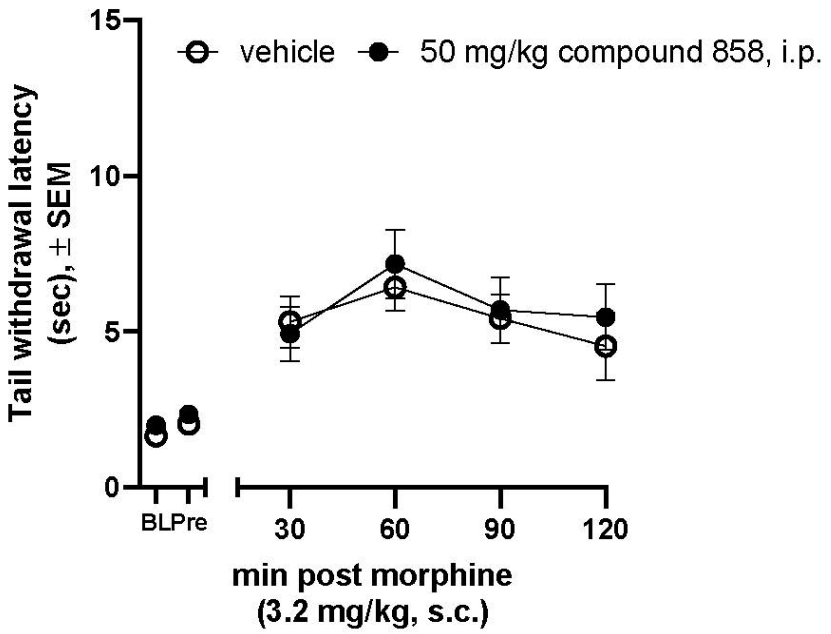


Figure 3.7. Systemic 858 pretreatment does not significantly alter morphine-induced antinociception. A 30 min pretreatment of 50 mg/kg compound 858 i.p. did not alter tail withdrawal latencies compared to vehicle following morphine (2-way RM ANOVA no main effect of treatment F (1, 23) = 0.2361, P=0.6316) (N=12 vehicle, N=13 compound 858).

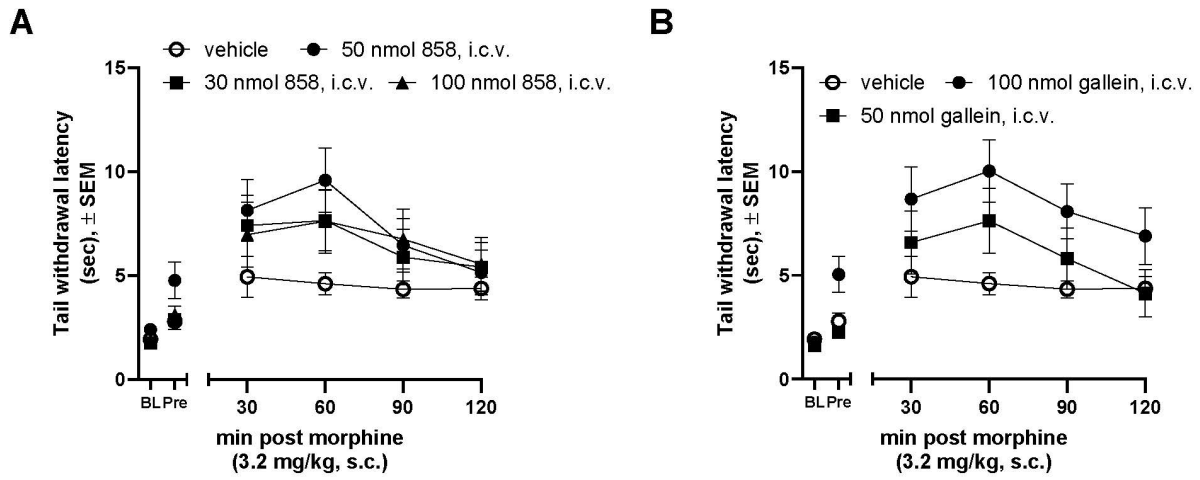


Figure 3.8. Dose comparisons of compound 858 and gallein. A) 30 nmol compound 858 does not enhance morphine-induced antinociception compared to vehicle (2-way RM ANOVA no main effect of treatment F (1, 22) = 2.659, P=0.1172) (N=12 vehicle, N=12 30 nmol compound 858). Mice pretreated with 50 nmol compound 858 display significantly elevated tail withdrawal latencies compared to vehicle pretreatment following morphine (2-way RM ANOVA main effect of treatment F (1, 23) = 6.147, P=0.0209) (N=12 vehicle, N=13 50 nmol compound 858). 100 nmol compound 858 did not enhance morphine-induced antinociception compared to vehicle (2-way RM ANOVA no main effect of treatment F (1, 22) = 2.737, P=0.1123) (N=12 vehicle, N=12 100 nmol compound 858). **B)** 50 nmol gallein does not alter tail withdrawal latencies compared to vehicle following morphine (2-way RM ANOVA no main effect of treatment F (1, 22) = 0.8335, P=0.3712) (N=12 vehicle, N=12 50 nmol gallein). Mice pretreated with 100 nmol gallein show significantly elevated tail withdrawal latencies compared to vehicle following morphine (2-way RM ANOVA main effect of treatment F(1, 22) = 8.997, P=0.0066) (N=12 vehicle, N=12 100 nmol gallein).

3.7 Tables

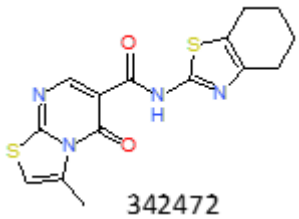
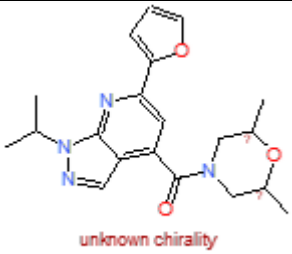
Compound ID	342472	338800
Scaffold for:	Compounds 743, 744	Compounds 858, 895
Structure		 338800

Table 3.1. Lead scaffolds for medicinal chemistry optimization.

Summary of MCTI-858IP pharmacokinetic parameters

IP Dose 10 mg/kg

PK parameters	Unit	Mean
$T_{1/2}$	h	0.285
T_{max}	h	0.250
C_{max}	ng/mL	1124

Table 3.2. Plasma PK for 10 mg/kg systemic compound 858.

Summary of MCTI-858IP pharmacokinetic parameters

IP Dose 10 mg/kg

PK parameters	Unit	Mean
$T_{1/2}$	h	ND
T_{max}	h	ND
C_{max}	ng/mL	ND

Table 3.3. Brain PK for 10 mg/kg systemic compound 858. ND: not detected.

3.8 References

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Chapter 4 Discussion

4.1 Significance

The experiments described within this dissertation sought to characterize mechanisms by which Gβγ signal modulation alters the antinociceptive effects of analgesic drugs. While most of this work focused on the effects of gallein on opioid-induced antinociception, we also examined the effects of gallein on non-opioid analgesics and the ability of novel Gβγ modulators to enhance morphine-induced antinociception. This work led to several key findings: 1) the ability of gallein to potentiate morphine-induced antinociception is dependent upon activation of MORs, 2) enhancement of morphine-induced antinociception by gallein in the warm water tail withdrawal (WWTW) assay is dependent upon activation of central, but not peripheral, MORs, 3) gallein enhances the antinociceptive effects of non-opioid analgesics that are agonists at GPCRs, 4) enhancement of morphine-induced antinociception by gallein requires feedback phosphorylation at the C-terminal tail of MORs, and 5) novel Gβγ modulators enhance morphine-induced antinociception when centrally administered. Together these studies provide mechanistic insights on Gβγ modulation as a strategy to improve the safety and efficacy of opioid analgesics.

MOR agonists have long been recognized as efficacious treatments for pain. However, their efficacy is limited by several on-target drawbacks such as rapid development of tolerance, constipation, respiratory depression, and abuse liability. For many years the field has sought to dissociate the therapeutic benefits of opioids from deleterious side effects. One example of this was the concept of biased agonism – MOR agonists preferentially activating pathways which instantiate therapeutic effects as opposed to side effects. Efforts to produce biased agonists with a clinically improved therapeutic window has been challenging and the effects of the single approved “biased” MOR agonist, oliceridine, may simply be explained by partial agonism (1).

An alternative approach to producing a biased signaling profile at the receptor-ligand complex is to produce signal bias downstream of MOR activation. In examining this strategy, Gβγ has been of particular interest due to its ability to interact with various functionally distinct effectors. Importantly, Gβγ engages effectors which both negatively regulate MORs, and others

which are responsible for the analgesic effects of opioids. For example, Gβγ inhibits VGCCs (2, 3) to reduce transmission of primary afferents in the spine but also activates GRK2 (4) which phosphorylates the C-terminal tail of MORs resulting in receptor internalization. Therefore, if Gβγ signaling could be biased away from effectors negatively regulating the receptor while leaving other pathways unaltered, enhancements in the analgesic effects of MOR agonists would be expected. This idea was verified *in vivo* using M119 (5, 6) and expanded using gallein pretreatment which potentiated morphine-induced antinociception without enhancing constipation or respiratory depression (7).

Still, at the outset of this dissertation work there were many open questions regarding the use of Gβγ signal modulators to improve the safety and efficacy of opioids. There was no direct *in vivo* evidence showing that the ability of gallein to enhance morphine-induced antinociception was actually dependent upon activation of MORs – or which MOR populations are required for this effect. Although there was some evidence that gallein could interact with other receptor systems, it was not clear whether this would be the case for analgesics and whether gallein may nonspecifically enhance the antinociceptive effects of drugs which are not agonists at GPCRs. For these reasons, we sought to characterize mechanisms by which gallein enhances antinociception using *in vivo* mouse models of pain and determine whether novel Gβγ modulators developed on different chemical scaffolds could also enhance morphine-induced antinociception.

4.2 Characterizing the Mechanisms of Gallein *In Vivo*

4.2.1 Dependence on MORs

Our studies confirm that the effects of gallein to enhance morphine-induced antinociception are dependent upon the ability of morphine to activate MORs. This is based on our observations that potentiation of morphine's antinociceptive effects were blocked by naloxone pretreatment and were absent in MOR knockout (KO) mice. While naloxone itself is non-selective among opioid receptor subtypes, the absence of an effect in MOR KO mice demonstrates a requirement of morphine action at MORs specifically. Prior to these studies it remained possible, although unlikely, that the effects of gallein were mediated through some other non-receptor-dependent effect. Here we crucially support the purported mechanism of gallein action downstream of receptor activation *in vivo*.

To further support these findings, we found that gallein enhanced the antinociceptive effects of other MOR agonists: fentanyl, buprenorphine, and nalbuphine. Therefore, regardless of the agonist used to activate the receptor, gallein can exert its effects at Gβγ to enhance antinociception. This further points to a receptor-mediated effect as opposed to some idiosyncratic effect of gallein with morphine, such as altering metabolism. While morphine, buprenorphine, and nalbuphine are all morphinans, fentanyl has a distinct structural scaffold. Morphine (and nalbuphine) primarily undergoes glucuronidation in the liver via UDP-glucuronosyltransferases to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) (8, 9), while fentanyl (and buprenorphine) is primarily N-dealkylated by CYP3A4 to norfentanyl (and norbuprenorphine) (10, 11). Given these distinct metabolic pathways, it is unlikely that gallein exerts its effects by inhibiting metabolism (competing for CYP enzymes, for example).

4.2.2 Probe-Dependency and Effects on Partial Agonists

As with some allosteric modulators, gallein appears to have probe-dependent effects at MORs. In other words, the ability of gallein to enhance antinociception is dependent upon the agonist used. While the effect of gallein on buprenorphine and fentanyl was comparable to morphine, there was only a minor effect on nalbuphine-induced antinociception. This is likely because nalbuphine is not particularly efficacious in the WWTW assay – even at less noxious temperatures. Although gallein can enhance low doses of efficacious opioid analgesics, it does not appear to convert inefficacious analgesics to high efficacy. This makes sense if the ligand-receptor complex insufficiently drives GRK or PLC activation which gallein then inhibits. It is also possible that the dose of nalbuphine used in time course experiments was not equivalent in efficacy compared to MOR agonists used in other experiments. Considering that the dose-response curve for nalbuphine in the WWTW assay is relatively flat with only the highest dose of 320 mg/kg significantly different from baseline, perhaps a larger dose would exhibit a more robust effect when paired with gallein. A full understanding of potentially unique dose-response relationships between different MOR agonists paired with gallein calls for further studies utilizing a dose-response paradigm.

Regarding efficacy, it is somewhat curious that gallein robustly enhanced the antinociceptive effects of buprenorphine – a partial agonist relative to most other MOR agonists examined here. Buprenorphine does not potently recruit arrestins *in vitro* and this could indicate that gallein would have a reduced effect since there is less Gβγ-mediated negative regulation of

the receptor available to disinhibit. However, this idea conflates negative regulation independently mediated through GRKs with subsequent arrestin recruitment and internalization. GRKs can independently produce receptor decoupling from G proteins in the absence of arrestins (12). GRKs and PLCβ may also sequester Gβγ from other effectors (13-15). For a partial agonist liberating less overall Gβγ, a greater fractional amount may be diverted toward negative regulatory mechanisms. By using gallein to "free-up" this fraction, a robust MOR signaling effect could be expected. Thus, gallein may shift the Gβγ effector stoichiometry in a way that is more meaningful for partial agonists.

The robust effects of gallein on buprenorphine-induced antinociception may also be explained by a receptor reserve effect where limits on efficacy due to the requirement for high receptor occupancy are alleviated by gallein-induced increases in signal transduction efficacy. Last, these effects might be explained by an assay-specific effect. The 55°C WWTW assay is particularly noxious and likely increases the Hill slope of dose-response curves for analgesic drugs (effectively creating a threshold "on or off" effect rather than a smooth curve). The addition of gallein in the presence of buprenorphine may have been sufficient to push antinociception over the threshold required to observe a significant effect.

Last, it is important to recall that while buprenorphine may not potently recruit arrestins *in vitro*, MOR-mediated PLCβ3 activation is synergistic alongside Gq signaling (16). Therefore, a relatively low intrinsic efficacy ligand-receptor complex may contribute to outsized negative regulation of MORs in cells and circuits with tonic Gq activity. Gallein could still be expected to exert robust effects on MOR-mediated antinociception in this context.

4.2.3 Dependence on Central MORs

We showed that enhancements in morphine-induced antinociception by gallein in the WWTW assay are dependent upon central MORs because systemic pretreatment with blood-brain barrier impermeable naloxone methiodide (NLXM) did not block gallein's effects. Yet, when NLXM was injected directly into the brain, it did block potentiation of morphine's antinociceptive effects by gallein. These central NLXM studies indicate that systemic gallein does access the CNS and confirm that NLXM does effectively antagonize morphine action at opioid receptors. To support these findings and examine where in the CNS gallein acts, we tested the effects of central gallein on morphine-induced antinociception in the hot plate assay where nociceptive responses are thought to be mediated supraspinally. We also investigated whether i.t. gallein pretreatments

could enhance morphine-induced antinociception. Given that gallein was efficacious in the hot plate assay, but not when injected i.t., gallein likely acts primarily downstream of supraspinal MORs to enhance the antinociceptive effects of morphine. This aligns with prior data showing bath-applied gallein increases DAMGO-mediated inhibition of eIPSCs in vl-PAG slices (16). As a whole, these data point to a primary mechanism of gallein to potentiate MOR-mediated disinhibition of descending modulatory pain circuits.

4.2.4 Presynaptic MORs

As disinhibition is primarily due to reductions in presynaptic GABA release, these data highlight the importance of gallein action downstream of presynaptic MORs. Given that presynaptic MORs are thought to be resistant to acute desensitization (17), these findings seem paradoxical. If gallein prevents negative regulation of MORs, why would enhancement of presynaptic MOR signaling be important if these populations are already resistant to negative regulation? There are some potential (non-mutually exclusive) explanations for this: 1) the presynaptic effects of gallein are chiefly a result of shifting the available free Gβγ pool toward effector interactions which contribute to analgesia, 2) gallein reduces presynaptic MOR cycling because they are responsive to GRKs, and 3) the time course of this effect does not match our observed effects *in vivo*. These ideas are discussed in detail below.

As previously discussed, gallein produces Gβγ signal bias by reducing affinity for certain effectors (GRK2, PLCβ3) which negatively regulate MORs while leaving other signaling pathways unaltered. Even if presynaptic MORs were less responsive to negative feedback machinery such as GRKs, PKC, and arrestins, the effects of gallein are not completely dependent on inhibiting these mechanisms. By altering the affinity of Gβγ for GRK2/3 and PLCβ3, the available fraction of free Gβγ could have an increased probability to interact with presynaptic SNAREs or VGCCs. Inhibition of these effectors contributes to the analgesic effects of opioids by reducing GABA release, disinhibiting descending pain modulation. This mechanism would be largely independent of direct GRK or PKC action at MORs.

Secondly, the apparent resistance of presynaptic MORs to desensitization is measured functionally. MOR agonist-mediated reductions in postsynaptic eIPSCs do not appear to decrease during acute exposure (minutes) to agonist (17). This does not indicate that presynaptic MORs are unresponsive to canonical mechanisms of desensitization and/or internalization. In fact, Jullié and colleagues demonstrated that presynaptic MORs do internalize in a GRK-dependent manner (18).

There appears to be a lack of desensitization from a functional standpoint because internalized MORs are rapidly replaced via lateral diffusion from extrasynaptic or axonal pools. In turn, the internalized pool can be inserted extrasynaptically to replace those MORs which undergo diffusion or reinserted into the synapse. Thus, total MOR surface expression appears relatively unchanged. Although this process is rapid, inhibition of this cycling process with gallein could very plausibly contribute to a greater duration of MORs in synaptic microdomains important for inhibiting vesicle fusion.

Last, the time course of this presynaptic resistance to desensitization (minutes) does not match our observed effects *in vivo* (hours). This resistance of presynaptic MORs to desensitization is a short-lived effect (17). Extended exposure to MOR agonist *in vivo* (morphine is not cleared within minutes) is unlike bath-applied agonist wash-on in electrophysiology experiments. Therefore, presynaptic resistance of MORs to functional desensitization likely has only limited relevance in our animal models where morphine action persists beyond a few minutes.

4.2.5 Gallein Enhances Antinociceptive Effects of Non-Opioid Analgesics

Our studies show that gallein enhances the antinociceptive effects of CP55940 and clonidine: a CB1/2 agonist and α2-adrenergic agonist, respectively. Both receptors are Gi/o-coupled GPCRs like MORs. Since Gβγ effects are largely pertussis toxin sensitive, staying within the same family of G protein heterotrimers seemed relevant for examining the ability of gallein to interact with these systems. Crucially, gallein did not enhance the antinociceptive effects of epibatidine, a nicotinic acetylcholine receptor agonist. Together, these findings support the notion that the effects of gallein require GPCR-dependent release of free Gβγ as opposed to exerting a nonspecific action unrelated to G protein activation.

These data align with previous findings which show that gallein interacts with Gβγ downstream of adrenergic receptor activation at presynaptic nerve terminals and within immune cells downstream of chemokine receptors (19-22). Studies from the Blaxall laboratory examined the effects of gallein in models of heart failure. One hallmark of heart failure is the presence of dysfunctional adrenergic receptor signaling resulting from unusually elevated concentrations of circulating catecholamines and overexpression of GRK2 (23, 24). This group hypothesized that Gβγ modulator compounds may ameliorate heart failure progression by normalizing GRK2 activity and adrenergic receptor signaling. Indeed, long-term gallein treatment halted heart failure progression and reduced GRK2 expression in a transgenic mouse model of heart failure (19). The

same group found that 8 weeks of daily gallein treatment (10 mg/kg per day) in a transverse aortic constriction heart failure model normalized plasma catecholamines, rescued β-adrenergic receptor density in myocardial membranes, and attenuates cardiac remodeling leading to improved survival (20). Three weeks of gallein (2.5 mg/kg titrated up to 10 mg/kg per day) also attenuated fibrotic remodeling in an ischemia-reperfusion injury model (21). In a chronic inflammatory disease model of lupus, 20 weeks of gallein injections 3 times per week (20 mg/kg or 35 mg/kg each treatment) ameliorated kidney damage in lupus-prone mice with elevated and dysregulated chemokines (22). Here, gallein inhibited PI3Kγ-mediated immune cell migration downstream of chemokine receptors.

Combined with our findings, these prior findings provide some additional insights regarding Gβγ signal bias as a therapeutic strategy. For one, long-term treatments of gallein appear to be well-tolerated. The studies discussed above all incorporate a relatively long-term frequent treatment paradigm without notable adverse effects. This is important to consider since one concern of Gβγ signal modulation is potentially deleterious effects due to ubiquitous cellular expression of the target. It does not appear that gallein produces the sort of systemic disruption that might be anticipated from such an intervention. This highlights the second crucial point – gallein seems to require elevated levels of agonist (endogenous or exogenous) to exert its effects. The current dissertation work along with recently published findings reinforces this idea. Here we have shown no effect of gallein treatment alone on antinociception, but recent work demonstrates gallein-induced antinociception alone in an ongoing pain state with presumably elevated concentrations of endogenous opioids (25). Thus, although gallein may bind to free Gβγ downstream of normal physiological signaling, robust increases in receptor mediated Gβγ activity are likely necessary to observe meaningful effects of gallein.

4.2.6 Sex-Dependent Effects

An interesting observation was the sex difference in the ability of gallein to enhance fentanyl-induced antinociception. While gallein pretreatment significantly potentiated the antinociceptive effects of fentanyl in males, there was no effect in female mice. Notably, there was no significant difference in the effects of fentanyl alone between males and females (although there was a trend, $p=0.07$) and there was no sex difference observed for other MOR agonists. The fact that effects were greater in males is consistent with literature showing greater antinociceptive effects of opioids in male mice, but this would be expected to show up in the dose-response curve

for fentanyl alone. These results suggest a difference in the response to gallein itself rather than the underlying analgesic. Some evidence suggests that there may be differences in important effectors downstream of Gβγ. These differences could plausibly alter the efficacy of gallein. For example, one study found that treatment of estradiol in ovariectomized female mice increased associations of Gβγ with GRK2 in striatal tissue homogenates (26). Importantly, GRK2 mRNA was equivalent between the sexes. Greater engagement of GRK2 by Gβγ in females indicates that larger doses of gallein might be required in females to compete for binding.

Another study found that PKCε, a downstream target of PLCβ known to regulate MORs (27), produced sex-dependent hyperalgesic priming following agonist treatment (28). Priming was observed only in male rats and reversed by estrogen treatment. These data may indicate an overall lower responsiveness of PKCε in females and less inhibitory feedback at MORs through this signaling pathway. Gβγ signal modulation to alter activity of this signaling cascade and reduce negative regulation of MORs would thus be expected to exhibit a reduced response in females and could explain sex differences observed in this dissertation. Crucially, sex differences for two key effectors in our proposed mechanism of Gβγ modulation appear in prior work.

Potentiation of clonidine- and CP55940-induced antinociception by gallein was also sex-specific. As observed for fentanyl, only gallein-treated males exhibited significantly elevated tail withdrawal latencies compared to vehicle pretreated groups. While there are reported sex differences for the antinociceptive effects of noradrenergic and cannabinoid receptor agonists, we did not observe statistically significant differences in our dose-response curves for the agonists alone. As with opioids, α2-adrenergic receptor agonists show greater antinociceptive efficacy in males (29, 30), which may at least partially account for differences observed after gallein treatment. Interestingly though, female rats exhibit greater sensitivity to cannabinoid-induced antinociception in some studies (31-33). However, with repeated administration this sex difference seems to be lost or even reversed (34, 35). This is consistent with work in humans which shows that, among recreational cannabis users, men exhibit greater analgesic benefit (36, 37). One hypothesis is that gallein produces a signaling profile which facilitates this sex-switching. Investigating sex differences in signaling and/or allosteric modulators will be challenging since studies will need to dissect effects of underlying orthosteric agonists from modulator compounds. Presently this work is sparse and focuses primarily on GABAA positive allosteric modulators (38, 39).

Finally, gallein did not enhance the effects of an analgesic which is not an agonist at a GPCR – epibatidine. Although epibatidine has been shown to produce antinociceptive effects (40) and demonstrated dose-dependent antinociception in our hands, its action was not potentiated by gallein. This supports the mechanism of gallein action producing signal bias specifically at Gβγ downstream of GPCR activation.

4.3 Novel Gβγ Modulators Enhance Morphine-Induced Antinociception

We found that novel Gβγ modulator compounds potentiated the antinociceptive effects of morphine in the WWTW assay. These findings are crucial to the translatability of Gβγ signal bias as a therapeutic strategy because gallein's poor physicochemical properties make it unsuitable for drug development. Therefore, we examined the ability of three structurally distinct compounds to enhance the antinociceptive effects of morphine: 743, 744, and 858. These compounds were selected for their potency and efficacy to inhibit Gβγ-PLCβ3 and Gβγ-GRK2 interaction *in vitro*. Additionally, compound 895 built on the same scaffold as 858, but which did not bind to Gβγ was evaluated as a negative control.

Both compounds 743 and 858 significantly potentiated morphine-induced antinociception, while 744 only trended ($p=0.0541$). Importantly, 895 negative control did not enhance morphine-induced antinociception. These data demonstrate that novel Gβγ inhibitors are efficacious in animal models of pain and illustrate predictive validity for *in vitro* screens of these compounds. Furthermore, the data reify Gβγ as the primary mechanistic target *in vivo*, since the commonality between these structurally distinct compounds is that they bind to Gβγ *in vitro*. Interestingly, 50 nmol compound 858 produced potentiation that was comparable to 100 nmol of gallein. In contrast, 50 nmol of gallein exhibited a reduced effect compared to 100 nmol. This suggests that compound 858 is more potent than gallein in potentiating morphine-induced antinociception.

Because compound 858 was the most promising novel Gβγ modulator tested with potentially greater potency and comparable efficacy to gallein, we sought to examine whether systemic treatment of compound 858 could enhance morphine-induced antinociception. Compared to DMSO vehicle, systemic 858 treatment had no effect on tail withdrawal latencies following morphine treatment. Subsequently, we performed pharmacokinetic analysis on compound 858 and found that it is rapidly cleared with little ever appearing in the plasma and none in the brain. While these results are not ideal from a drug development perspective, they reinforce the importance of CNS penetration for these compounds to exert their effects. Centrally-administered novel Gβγ

modulators enhanced morphine-induced antinociception, while systemic treatment did not because they did not reach the brain. These findings strengthen our previous data showing that central, but not peripheral, MOR activation is required for gallein to exert its effects.

All novel Gβγ signal modulators used in these studies produced sex-dependent effects on morphine-induced antinociception. While these effects were not all strictly statistically significant (sex by treatment interaction p-value less than 0.05), the fact that they are observable across all compounds tested suggests that it is a real and meaningful phenomenon. In every case, males treated with Gβγ modulator exhibit greater mean tail withdrawal latencies than other groups. This was also the case for gallein. Here it is important to reiterate that these compounds are structurally distinct with 858 developed on a different scaffold than 743 or 744. In addition to previous studies which utilized analgesics which are agonists are different GPCRs, these findings support the idea that there is a sex difference with respect to Gβγ modulation rather than orthosteric agonist. Furthermore, structural differences between these modulators and gallein indicate that the cause of these sex differences may not be intrinsic to the modulator itself, but a result of underlying physiology. This would be consistent with increased Gβγ-GRK2 interactions and a potential lack of PKCε responsivity in females as previously discussed (26, 28). While this would suggest greater doses of Gβγ modulator are necessary in females, 100 nmol of compound 858 did not produce enhancements in morphine-induced antinociception in female mice. A critical next step in this body of work is to determine the precise mechanism by which these sex differences manifest and whether they might be overcome by dosing or whether this therapeutic strategy might not be viable in females.

4.4 Therapeutic Considerations

With respect to Gβγ signal modulation as a potential therapeutic strategy, there are two important points to consider: 1) the efficacy of Gβγ modulation as compared to other potentially similar strategies and 2) the benefits and limitations of such a strategy in practice. While methods for enhancing opioid-induced antinociception have been discussed previously, another investigational strategy, direct GRK2 inhibition, merits attention. This method is especially relevant since this is one mechanism by which Gβγ modulators exert their effects (though PLCβ inhibition may be more relevant and there are no current strategies which address this).

The FDA-approved SSRI, paroxetine, has been shown to bind directly to GRK2 and inhibit its kinase activity (41). Furthermore, Bouley and colleagues found that both paroxetine and

derivative compounds rescued DAMGO-mediated MOR internalization (42). More recently, this work has largely shifted toward cardiac therapies. In multiple animal models of heart failure, a paroxetine derivative exhibited therapeutic efficacy by improving left ventricular contractility (43). This effect seems mediated through restoration of β-adrenergic receptor function by preventing desensitization (41). Interestingly, this is similar to the effects of gallein in heart failure (19, 20). These data are especially promising given that paroxetine is already FDA-approved, which may allow for a drug repurposing strategy. Despite these encouraging findings, paroxetine did not outperform placebo to reduce adverse left ventricular remodeling in patients with acute anterior ST-segment-elevation myocardial infarction (STEMI) (44).

While paroxetine as a GRK2 inhibitor could be a promising therapeutic avenue to potentiate opioid-induced antinociception, this strategy may not widen the therapeutic window. We know that MORs which are unresponsive to GRK or PKC phosphorylation (10 S/T-A mouse model) enhance both antinociceptive effects and side effects of MOR agonists (45). However, gallein enhanced antinociception without altering side effects (7). This is curious considering we found no leftward shift in morphine-induced antinociception in homozygous 10 S/T-A mice following gallein treatment (suggesting that the effects of gallein on morphine-induced antinociception are wholly mediated through its ability to prevent GRK2 or PKC receptor phosphorylation). It may be the case that synergistic PLCβ activation plays an outsized role in specific circuits related to opioid-mediated antinociception as compared to those mediating side effects. Therefore, Gβγ modulation could be expected to have a greater impact on certain receptor-mediated effects. On the other hand, global receptor knockin or GRK2 inhibition might not exhibit circuit-specific effects. Regardless, further investigation is needed for either strategy – alongside studies of allosteric modulation – to reach a clinically relevant therapeutic strategy.

The current body of evidence suggests that gallein exerts effects across multiple organ and receptor systems (19-22, 46). This is in addition to the data shown here which demonstrates the ability of gallein to enhance both α2-adrenergic and cannabinoid receptor mediated antinociception. This has both positive and negative implications for Gβγ signal modulation as a therapeutic strategy. On the positive end, Gβγ signal modulation could be expected to confer therapeutic benefit in a number of conditions where the underlying etiology involves Gαi-coupled GPCR dysfunction. This is evidenced by the diverse set of preclinical models discussed throughout this dissertation where gallein exhibits beneficial effects. Thus, a commercially available and safe

Gβγ modulator could present tremendous clinical value in contrast to most traditional medicines which focus on a single disease state.

On the other hand, Gβγ signal modulation may also be challenging to implement in practice for this reason. Patients are often prescribed multiple medications for diverse disease states which may influence concentrations of several orthosteric ligands for various GPCRs. In these patient populations Gβγ signal modulation could very well have unpredictable and unintended effects. Therefore, polypharmacy could present a contraindication for this therapeutic strategy – or a need for careful monitoring. Further studies evaluating the effects of Gβγ signal modulation on drug-drug interactions and animal tolerability with administration of multiple Gαi-coupled GPCR agonists would provide valuable insights to this question.

4.5 Conclusion

While opioids continue to be a highly efficacious treatment for pain conditions, their repeated use has always been hampered by significant clinical drawbacks including constipation, respiratory depression, rapid tolerance development, and abuse liability. Because these drawbacks are all considered on-target at MORs, efforts to dissociate therapeutically beneficial effects from side effects have been an ongoing challenge in the field of pain and analgesic research. Strategies to address this challenge include "biased" agonists and co-administration with analgesic adjuvants like clonidine or cannabinoids. These strategies have not provided a substantial solution to the problem. Rather than producing signal bias at the receptor-ligand complex like biased agonists, another strategy involves directly interacting with Gβγ to produce signal bias downstream of receptor activation. Gβγ engages effectors downstream of MOR activation which both facilitate and negatively regulate MOR-mediated analgesia. Small molecules reducing the affinity of Gβγ for effectors opposing opioid-induced analgesia while leaving other effector interactions unaltered could effectively enhance the therapeutic window of MOR agonists.

Early *in vitro* work showed that both peptides and small molecules could produce a biased signaling profile with respect to Gβγ. These data were validated by subsequent *in vivo* work illustrating enhanced morphine-induced antinociception following gallein pretreatment. This dissertation built upon this work by further elucidating mechanisms by which gallein enhances analgesic effects *in vivo*. Activation of central (and perhaps supraspinal) MORs is required for gallein to enhance the antinociceptive effects of morphine in the WWTW assay. Gallein also enhanced the antinociceptive effects of non-opioid analgesics in a sex-dependent fashion. Sex-

dependent effects were also observed in experiments evaluating novel Gβγ inhibitors. Ultimately, the data presented in this thesis suggest that Gβγ signal modulation is a promising avenue for improving the safety and efficacy of MOR agonists. Continued work should focus on sex differences in order to determine whether this is a viable strategy in both sexes, mechanisms for gallein's observed specificity for therapeutic effects, and on further development of novel translatable compounds.

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