Biasing of Gβγ-signaling with Small Molecules as a Novel Therapeutic Approach to Improve Opioid-mediated Antinociception.

by

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Dedication

This dissertation is dedicated first and foremost to my family –my inspiration for what I do. To José de Jesús Sanchez Jiménez, Cynthia Carmen Aguayo Navarro, Brian Sanchez Aguayo and Bradley A. Sanchez Aguayo. Todos mis esfuerzos y logros están inspirados en ustedes, los amo. This work is dedicated to what I believe represents to be Mexican American in STEM. My work, my passion and my determination are gifts that are rooted in my ancestors and the values that my family have taught me. During graduate school, I had to navigate spaces in which I felt I had to dampen my Latinidad. But it is in these types of spaces where my identity as a Latina has made me strong and has inspired me to take the space that I deserve. To all my Latinas in STEM, own your identity and occupy the space you deserve. I dedicate this accomplishment to Tuxpan Jalisco, a magical pueblo that nurtured my childhood and lives within me to keep me grounded. To all my friends who have supported me throughout this experience – too many to name. You made Michigan home for me, and I truly believe that without your support I would've not been as successful. Juan, Loyda, Caroline, and Liz, thank you for your friendship since day one. Lastly, I dedicate this accomplishment to the personal growth I achieved during this period of my life with the help of J.C. The work I dedicated to myself made the Doctor I am soon to become.

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Abstract

Opioid analgesics are widely used as a treatment option for pain management and relief. However, the misuse of opioid analgesics has contributed to the current opioid epidemic in the United States. The National Institute of Drug Abuse (NIDA) reports that in 2021, more than 106,000 deaths were attributed to opioid overdoses, and an estimated 16,000 involved a prescription opioid. Prescribed opioids such as morphine, codeine, oxycodone, and fentanyl are primarily used in the clinic to treat pain or during medical procedures. Nevertheless, the rewarding and reinforcing effects of opioids have led to patient misuse of prescribed opioids and increased their illicit use and distribution. Patients who take prescribed opioids report adverse effects like constipation, nausea, dizziness, itchiness, respiratory depression, and development of tolerance to the analgesic effects. Scientists in the drug discovery field aim to identify molecular targets that could separate the therapeutic effects of opioid analgesics from the detrimental side effects and improve pharmacological strategies to relieve pain.

This thesis explores the application of targeting G protein βγ subunit signaling as a novel therapeutic approach to increase opioid-induced analgesia and decreases the development of opioid tolerance. To bias Gβγ-signaling, we used gallein, a small molecule that binds to the Gβγ subunit downstream of opioid receptors. We proposed that when gallein is bound, Gβγ promotes pro-analgesic signaling but cannot interact with signaling pathways that oppose the analgesic response.

First, we investigated activation of phospholipase Cβ (PLCβ) signaling downstream of the μ-opioid receptor (MOR). We hypothesized that PLCβ signaling opposes the opioid analgesic response and that activation of PLCβ requires Gβγ signaling downstream of MOR and coincident $G\alpha_q$ signaling. We assessed this model in cellular, ex vivo, and in vivo assays. Using a fluorescent biosensor, we tested the coactivation of PLCβ by MOR and $G\alpha_q$-coupled receptors in HEK-293 cells. Then, MOR-dependent inhibition of neurotransmission was tested in GABA-ergic synapses in the mouse periaqueductal grey (PAG) in presence of a Gβγ-inhibitor (gallein) or $G\alpha_q$-inhibitor. And lastly, we evaluated the effects of gallein and $G\alpha_q$ inhibitor treatment on morphine-dependent antinociception. Our results show that coincident activation of MOR and $G\alpha_q$-coupled receptors produces synergistic activation of PLCβ in HEK-293 cells. In ex vivo and in vivo experiments, treatment with either gallein or a $G\alpha_q$-inhibitor increased DAMGO-mediated inhibition of GABA-release and increased morphine-mediated antinociception.

We continued exploring the therapeutic potential of biasing Gβγ-signaling and its application to chronic morphine treatment in vivo. We hypothesized that biasing Gβγ-signaling with gallein could prevent activation of regulatory signaling pathways that result in opioid tolerance. We tested gallein using two paradigms for treatment: administration during the development of opioid tolerance and administration after tolerance is developed. Our results showed that gallein cotreatment during repeated administration of morphine decreased opioid tolerance development, and gallein treatment in an opioid-tolerant state enhanced the potency of morphine. Additionally, our data showed that PLCβ is necessary for gallein's potentiating effects in an opioid-tolerant state but not in preventing the development of tolerance.

Overall, we propose that biasing Gβγ-signaling could translate into a novel therapeutic approach that improves the analgesic effects of prescription opioids and aids in preventing opioid

tolerance. These studies demonstrate that small molecules that target Gβγ-signaling could reduce the need for large opioid doses to treat pain and, therefore, benefit the opioid epidemic.

Chapter 1 Introduction

1.1 Opioid-induced antinociception from protein to neuron, to behavioral pharmacology.

1.1.1 Opioid receptors and G-protein signaling.

There are three canonical types of opioid receptors in mammals: the μ-opioid receptor (MOR), κ-opioid receptor (KOR), and δ-opioid receptor (DOR) [1, 2]. Activation of opioid receptors promotes analgesia, but also produces unwanted physiological effects that each receptor facilitates differently. MOR activation produces physiological responses such as analgesia, respiratory depression, euphoria, sedation, constipation, and physical dependence [1]. KOR activation stimulates spinal analgesia, sedation, dyspnea, and dysphoria [1]. DOR activation causes analgesia, convolutions, and anxiolysis [2]. Activation of all opioid receptors promotes analgesia, but MOR agonists are the most effective for treating moderate to severe pain compared with KOR and DOR agonists.

Opioid receptors are members of the G-protein coupled receptors (GPCRs) family. GPCRS are membrane proteins composed of 7 transmembrane domains that use heterotrimeric G-proteins for receptor signaling transduction. GPCRs are the most drug-targeted class of receptors since their activation regulates many physiological responses. Activation of a GPCR by either an endogenous or exogenous agonist initiates a signaling cascade based on the G-protein coupled to this receptor [3]. Each G-protein in its inactive heterotrimeric form is bound to guanosine diphosphate (GDP). Once the receptor is activated, the heterotrimer is recruited to the receptor, and the α-subunit releases GDP resulting in guanosine triphosphate (GTP) binding. The

binding of GTP causes the dissociation of the active Gα-GTP subunit from the Gβγ subunit and the receptor allowing each subunit to interact with their respective effectors [3]. The cellular response upon activation by a GPCR depends on the Gα subunit coupled to it, of which there are 4 types: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$ [3, 4].

Opioid receptors are coupled to the $G\alpha_{i/o}$ subfamily of G-proteins. When an agonist activates MOR, it inhibits adenylyl cyclase via the $G\alpha_{i/o}$ subunit, reducing the cyclic adenosine monophosphate (cAMP) levels [1, 2]. The Gβγ subunit plays an important role in $G_{i/o}$-coupled receptor signaling since it interacts with a wide list of effectors, including ion channels, enzymes, and kinases that are unique to Gβγ subunits that dissociate from $G\alpha_{i/o}$ proteins [5]. Therefore, MOR-mediated activation of Gβγ promotes signaling cascades that result in opioid-mediated antinociception.

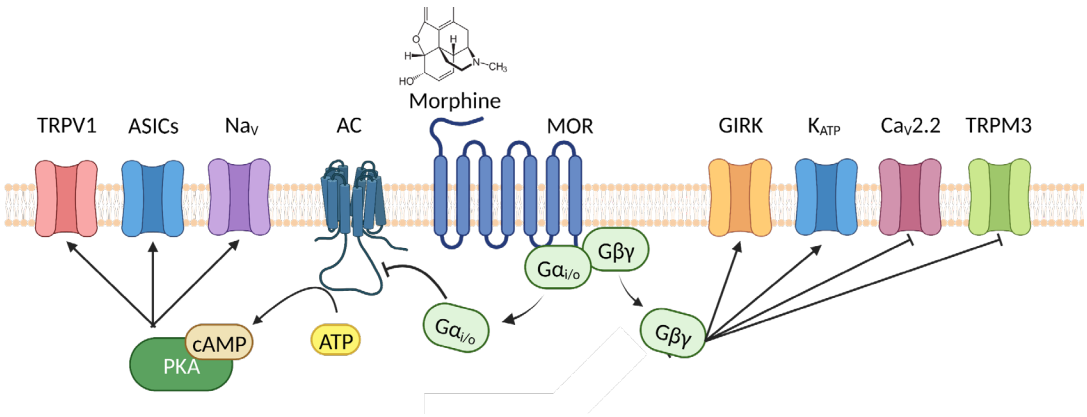

Figure 1. G-protein signaling transduction upon MOR activation.

Activation of MOR by an agonist such as morphine induces G-protein dissociation and activation of different signaling pathways. The $G\alpha_i$-GTP subunit inhibits adenylyl cyclase, whereas the Gβγ subunit interacts with other effectors such as ion channels to mediate neuronal activation.

1.1.2 Gβγ-signaling in neuronal pain-modulatory circuitry.

MOR-mediated antinociception occurs by Gβγ-dependent activation of effectors that modulate neuronal activity and neurotransmitter release. A model for understanding opioid-induced antinociception is the disinhibition hypothesis of the descending modulatory pain pathway [6, 7]. The descending modulatory pain pathway is a neuronal circuit in the brainstem that causes antinociception by modulating the ascending nociceptive inputs from the peripheral nervous system (PNS) [7]. The disinhibition hypothesis proposes that neurons in the descending pain pathway are tonically inhibited by the neurotransmitter release of gamma-aminobutyric acid (GABA). To cause opioid antinociception, MOR activation decreases GABA release (disinhibition) to produce pain modulation from the central nervous system (CNS) to the periphery [6, 7]. This process is well known and studied in opioid synapses occurring in the periaqueductal grey (PAG), which belong to the brain areas in the descending modulatory pain pathway [6, 8]. The PAG is located strategically in this pathway since it is the point of interception from the forebrain and midbrain inputs to the brainstem. It also has a rich expression of MORs and neurons secreting endogenous opioid peptides [6]. The descending modulatory pain system relies on projection from the ventral PAG to the rostral ventromedial medulla (RVM), modulating the spinal cord's nociceptive afferent signals [6].

At the neuronal terminal axons, activation of presynaptic MOR decreases GABA-neurotransmitter release and it does so by Gβγ-signaling. MOR-Gβγ signaling promotes inhibition of voltage-gated calcium channels (VGCC) which reduces the inflow of extracellular calcium [9]. Reduction of intracellular calcium and Gβγ-dependent inhibition of synaptotagmin proteins disrupt the formation of soluble N-ethylmaleimide-sensitive factor attachment protein

receptor (SNARE) complex, which is important for the attachment of neurotransmitter vesicles to the plasma membrane [10, 11]. Decreasing the release of GABA from presynaptic cells removes the inhibitory inputs to postsynaptic cells in the descending pain modulatory pathway to promote supraspinal antinociception [8].

Another signaling mechanism in which Gβγ regulates opioid-mediated antinociception by MOR stimulation is the activation of G-protein-gated inwardly rectifying potassium channels (GIRK channels) in the spinal cord [12]. Direct activation of GIRK channels by Gβγ in dorsal root ganglion (DRG) neurons is an important mechanism for spinal opioid-mediated antinociception [13]. Activation of GIRK channels and K^+ currents cause cell hyperpolarization in DRG cell bodies that connect peripheral sensory information to central ascending pathways. Hyperpolarization of DRG cell bodies decreases action potential firing and blocks peripheral sensory information from reaching the brain cortex [14].

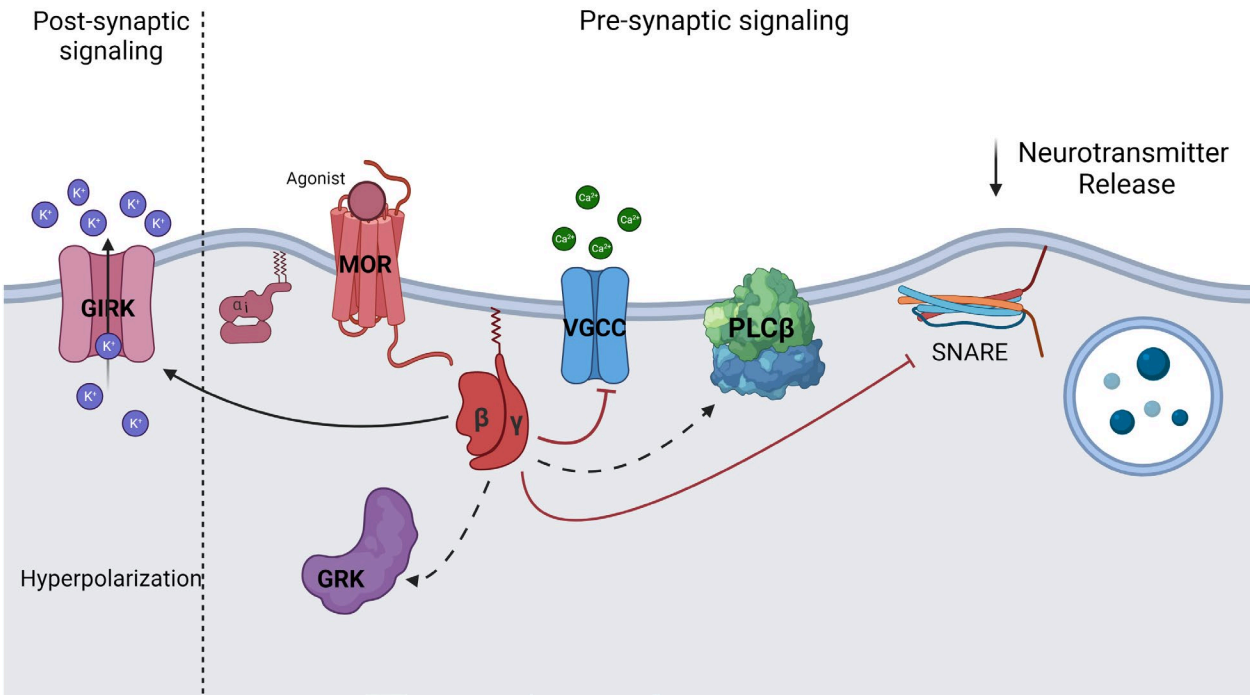

Figure 2. Gβγ-effectors that modulate opioid-mediated antinociception.

Examples of Gβγ-effectors that promote analgesia downstream of MOR activation are in solid lines and include post and presynaptic ion channels and proteins in the SNARE complex. However, Gβγ-signaling also activates enzymes— in dashed lines— like PLCβ and GRK that lead to receptor desensitization. PLCβ and GRK are ubiquitously expressed.

1.1.3 In vivo opioid pharmacology and acute behavioral assessment of pain-associated response in rodents.

A general way to understand pain is to describe it as the physiological response to protect the body from further harm triggered by a noxious stimulus. Specifically, in vivo studies using rodent behavior, acute pain is the processing of a noxious stimulus that leads to withdrawal from it, referred to as nociception. However, activation of the opioid system leads to a decrease in the sensation of pain (antinociception). MOR activation alters rodents' behavioral response to noxious stimuli by reducing this behavioral output [15]. Thermal nociception uses exposure to noxious temperatures, such as hot water or surfaces, to elicit a withdrawal response [15]. Experimental assays such as warm-water tail withdrawal (WWTW) and hot plate the

experimenter measures the latency to react to the thermal-noxious stimulus—either cold or hot. WWTW is the in vivo experimental model utilized to investigate morphine-induced antinociception for all data collected in this thesis. This methodology consists of immersing a rodent's tail in water temperatures ranging from 46°C to 55°C and measuring the latency to tail withdrawal or flick. The tail withdrawal response relies on a spinal reflex arc that acts on motor response. Activation of MOR—and other opioid receptors—modulates the tail withdrawal response by acting on the descending and ascending modulatory pain systems.

Other acute pain models use mechanical stimulation and inflammation to measure hypersensitivity to sensory stimuli, and the use of opioid treatment is utilized to reverse it. There are different treatments to induce hypersensitive state in rodents such as Freund's complete adjuvant (CFA), nitroglycerin (NTG), or collagen. A hypersensitive state is measured by changing mechanical thresholds such as Von Frey, Randell stiletto, or changes in typical rodent behavior. For example, CFA injection in an animal paw induces a hypersensitive state in which a mildly noxious stimulus becomes very nociceptive [15]. The Von Frey test measures this hypersensitive state by determining how much pressure on the injured paw it takes to elicit a withdrawal response by pressing thin filaments that do not exert a withdrawal response on the non-injured paw. [15]. Another experimental assay that assess opioid-induced antinociception in a state of hyperalgesia is an injection of nitroglycerin that produces a headache-like state [16]. The study of nociception using acute thermal-nociceptive assay contributes to the pharmacological exploration of the mechanism of action of small molecules that bias Gβγ-signaling using central and spinal mediated behaviors to measure opioid-mediated antinociception.

1.2 Phospholipase-Cβ (PLCβ) signaling and its importance in opioid-mediated antinociception.

1.2.1 G-protein activation of Phospholipase-Cβ (PLCβ)

Activation of $G\alpha_q$-coupled receptors regulates physiological functions such as smooth muscle tone, platelet activation, pituitary hormone release, and regulation of cell synapses [3]. The canonical effector of activated $G\alpha_q$-GTP is the enzyme phospholipase-C (PLC). PLC catalyzes the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-biphosphote (PIP_2), leading to the production of two second messengers: diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG stimulates the activity of protein kinase C (PKC), and IP_3 opens IP_3-gated-calcium channels in the endoplasmic reticulum, leading to increased intracellular calcium. Activation of PLC regulates different downstream signaling cascades, including MAPK activation, Rho-mediated signaling, and transcription factors. The specific cellular responses by PLC activation depend on tissue-specific cell type and subtype of PLC involved [17, 18].

There are 6 different PLC isozymes – β, γ, δ, ε, ζ, and η- that have structurally conserved domains: a TIM barrel, four EF domains, and a C2 domain [19]. This thesis focuses on the β isozyme, which is expressed ubiquitously and regulates neuronal activity. PLCβ is divided into 4 different isoforms: PLCβ1, PLCβ2, PLCβ3, and PLCβ4. These 4 different isoforms are classified depending on which cell type it is being expressed and activation by G-proteins [18, 19]. The canonical G-protein activator of PLCβ is $G\alpha_q$-GTP, but the Gβγ subunit can also activate some PLC isoforms such as PLCβ3 [18, 20]. The isoform PLCβ3 is interesting because it can be activated by $G\alpha_q$-GTP and Gβγ individually but also in a synergistic fashion when these two G-proteins are present [21]. PLCβ3 has been previously investigated in opioid-antinociception and is thought to negatively regulate opioid signaling [22, 23]. In the next

sections, we discuss the different mechanisms by which PLCβ signaling could regulate nociception in more detail.

1.2.2 $G\alpha_q$-PLCβ signaling in opioid antinociception.

Opioid-mediated antinociception depends on Gβγ-signaling by activation of opioid receptors; however, evidence suggests that activation of other GPCRs and G-protein signaling cascades can indirectly alter opioid-induced antinociception. The opioid system relies on $G\alpha_{i/o}$ signaling transduction since all opioid receptors preferentially couple to this family of G-proteins. Although activation of $G\alpha_q$ signaling pathways is not directly associated with the opioid system, these two pathways converge and share certain effectors downstream of receptor activation. Some studies have explored how the activation or antagonism of $G\alpha_q$-coupled receptors directly or indirectly affect opioid-antinociception; some examples are muscarinic acetylcholine receptors, serotonin 5-HT(2A) receptors, and even orphan GPCRs.

Muscarinic acetylcholine receptors M1, M3, and M5 are $G\alpha_q$-coupled receptors, in contrast to M2 and M4, which are coupled to $G\alpha_i$. Muscarinic antagonist treatment does not produce antinociceptive effects alone, but there are conflicting results in the outcome of opioid antinociception. Some reports say that muscarinic antagonism mostly potentiates opioid-induced antinociception, but few others claim that muscarinic antagonism attenuates opioid antinociception or has no significant effect [24-30]. The discrepancies seem to be related to the specificity of the antagonist, route of administration, and mouse strain. However, a study by Carrigan and Dykstra (2007) used an M1 muscarinic KO model and M1 antagonism and showed that deletion or antagonism of antinociception of M1 increased morphine-mediated antinociception [24]. This study shows the influence of $G\alpha_q$ activation on opioid-induced

antinociception using in vivo and pharmacological approaches to dissect the selectivity of the M1 receptor in opioid signaling.

GPR139, an orphan $G\alpha_q$-coupled receptor, also has been shown to oppose the opioid system [31]. When GPR139 was knocked-out in mice, it promoted higher sensitivity to morphine in different opioid behavioral assessments, including nociception. Behavioral assays showed that when GPR139 was activated, it dampened morphine-induced antinociception [32]. The proposed molecular mechanism for opioid-signaling attenuation by GPR139 is to disrupt GIRK channel function by PLCβ-dependent depletion of PIP_2 in the plasma membrane, which negatively affects these channels [31, 33]. This study proposes a model of PLCβ–dependent attenuation of opioid signaling on postsynaptic MOR activation of GIRK but does not account for presynaptic regulation of PLCβ on presynaptic MOR signaling.

In the past few years, there has been a boom in psychedelics research and their potential therapeutic influence in different disease models, such as their use for chronic pain. Psychedelics effects are attributed to the activation of serotonin 5-HT(2A) receptors, a $G\alpha_q$-coupled receptor. Although there is evidence that psychedelics such as psilocybin promote analgesia, the mechanism in which 5-HT(2A)R activation leads to antinociception remains unknown. Some suggest that 5-HT(2A)R activation indirectly affects nociceptive modulatory pathways, or it is attributed to the ability of psychedelics to promote synaptic plasticity [34]. However, a study explored the crosstalk between MOR and 5-HT(2A)R activation in a cellular model and showed that coactivation of these receptors enhances MOR desensitization and receptor internalization [35]. This study also showed that MOR and 5-HT(2A)R are expressed in the PAG [35].

1.2.3 Gβγ-PLCβ signaling, and synergistic activation in opioid antinociception.

Activation of PLCβ can be modulated differently depending on its interaction with G-proteins and other effectors. Biochemical experiments using purified proteins showed that Gβγ produces a low efficacy activation of PLCβ3, $G\alpha_q$ robustly activates it, but coactivation produces a highly elevated PLCβ enzymatic response that is synergistically higher than Gβγ and $G\alpha_q$ stimulation alone [21]. This synergistic model is also observed within the cellular context of G-protein signaling. Coactivation of a $G\alpha_q$-coupled receptor (Histamine 1 receptor) and a $G\alpha_i$-coupled receptor (GPR17) in HEK-293 cells produces a synergistic response of intracellular Ca^{2+} release by PLCβ3 activation [36]. It showed that Gβγ could not elicit a Ca^{2+} response independently in a cellular context but needs Gαq signaling coactivation [36]. Another study utilized a fluorescent biosensor in HEK-293 that measured PLCβ-dependent PKC activation and showed that Gβγ activation by morphine alone did not elicit a PKC response compared with direct PKC activators indicating that MOR activation alone does not produce PLCβ response [37]. Structural studies show that Gβγ and $G\alpha_q$ occupy distinct binding sites on PLCβ3, and this could contribute to its synergistic enzymatic response [38-40].

Subsequently, PLCβ3 has been shown to be involved in MOR activation and antinociceptive response [23]. Transgenic mice lacking PLCβ3 were tested to determine if PLCβ3 alters the antinociceptive response of morphine using WWTW. Genetic deletion of PLCβ3 potentiated the antinociceptive effects of morphine in a dose-dependent manner compared with control [23]. In addition, electrophysiological studies of [D-Ala2-MePhe4-Gly-ol] enkephalin (DAMGO)-induced reduction of VGCC-calcium currents in PLCβ3 KO

determined that the lack of PLCβ3 also improved opioid-dependent reduction of Ca^{2+} currents in DRG neurons [23].

In summary, biochemical and physiological studies suggest that the activation of PLCβ3 modulates antinociceptive opioid response by opposing MOR signaling. Determination of how synergistic G-protein activation of PLCβ3 modulates MOR signaling could reveal molecular pathways that could be explored to increase the antinociceptive effects of opioid analgesics and improve the efficiency and safety of opioid treatment for pain.

1.3 MOR phosphorylation, receptor desensitization, and tolerance.

1.3.1 MOR phosphorylation as a negative feedback mechanism.

After a GPCR has been activated and G-proteins are dissociated, the receptor undergoes post-activation modifications that result in receptor desensitization. Desensitization of the receptor causes a reduction in receptor response after acute or repeated stimuli [41]. When an agonist has acutely activated MOR, it leads to a rapid desensitization that begins with the phosphorylation of the receptor and ends in receptor internalization[41]. MOR receptors can go through surface resensitization, and internalized receptors could be recycled back to the surface or degraded by lysosomes [41]. However, prolonged activation of MOR results in a constant toggle of equilibrium between desensitization and resensitization, leading to lasting signaling modifications. These post-activation modifications greatly influence the dose-dependent response of an agonist by decreasing its potency and efficacy; this physiological adaptation is referred to as tolerance. A proposed molecular mechanism for chronic exposure to MOR agonist such as morphine and the development of tolerance is caused by an impaired balance in receptor desensitization [41]. Chronic exposure to MOR agonist causes changes in MOR's constitutive activity [42, 43], accelerated receptor desensitization [44, 45], impaired recovery from

desensitization [44, 46], and impaired receptor recycling [47, 48]. These imbalances in receptor homeostasis could be attributed to kinase signaling.

It is hypothesized that receptor phosphorylation plays an important role in the physiological changes that result in tolerance. Studies have reported that MOR is basally phosphorylated in brain tissue [49], but agonist-induced phosphorylation of MOR is higher and dependent on phosphosites on the c-tail [49, 50]. An increase in receptor-phosphorylation correlates to increased receptor trafficking and internalization. Phosphorylation of the receptor is mediated by serine/threonine kinases such as G-coupled receptor kinases (GRK) and protein kinase C (PKC). Gβγ signaling is known to influence these two kinase pathways: PKC—downstream of Gβγ-PLCβ activation—and GRK recruitment to the plasma membrane by Gβγ. Mutations in MOR's c-tail have been used to study the implications of phosphorylation in MOR cellular dynamics and signaling in cellular models. But more recently, a mutant mouse line was created containing MOR that cannot be phosphorylated by kinases [51]. The mutant MOR 10S/T-A contains 10 amino acid mutations serine/threonine to alanine out of the 11 phosphorylatable sites of the c-tail. Knock-in mice expressing MOR with the 10S/T-A showed blunted opioid tolerance development to pain stimulus in the hot plate assay after exposure to chronic opioid treatment using a subcutaneous osmotic pump [51]. Kinase phosphorylation of the receptor could work as a homologous or heterologous negative feedback mechanism since it depends on MOR-G-protein activation. Post-translational modification by receptor phosphorylation after prolonged activation of MOR could be a significant signaling pathway to investigate the development of opioid tolerance.

1.3.2 Protein Kinase C (PKC) in opioid signaling.

Downstream of PLCβ signaling activation of PKC by the second messengers (DAG and IP_3-dependent Ca^{2+} release) results in phosphorylation of the c-tail of GPCRs [52]. PKC has been a target of investigation in opioid signaling because it induces MOR desensitization. MOR-phosphorylation by PKC has been investigated using techniques and tools that target phospho-site-specific antibodies and use kinase inhibitors and siRNA knockdown screening [30]. One study determined that morphine agonism –compared to DAMGO — produced PKC-mediated rapid desensitization of MOR in rat locus coeruleus, suggesting agonist specificity for this pathway [53]. PKC activators phorbol 12,13-dibutyrate (PDBu) and phorbol 12-myristate 13-acetate (PMA) have been shown to induce opioid desensitization as measured by reduced hyperpolarization induced by or caused by morphine and [Met]5enkephalin in rat brain slices [21]. Activation of $G\alpha_q$ signaling by M3 muscarinic receptors enhanced morphine desensitization by PKC and highlights the importance of $G\alpha_q$ -PLCβ downstream signaling pathway and how it modulates opioid signaling [53]. In vivo studies used oligonucleotides to reduce the expression of PKC in the spinal cord, which led to attenuated morphine tolerance to thermal nociception [54]. Another study supporting the idea that PKC has a role in the induction of opioid tolerance used kinase inhibitors in vivo and ex vivo to determine that PKC inhibition reverses meperidine, morphine, and fentanyl acute tolerance [55]. Therefore, investigations of PKC modulation of opioid signaling and antinociception suggest that this molecular pathway could be important in MOR desensitization and developing opioid tolerance. Inhibition of G-protein activation of PLCβ3 pathways could prevent the activation of PKC that contributes to opioid desensitization.

1.3.3 G-protein receptor kinase (GRK) in opioid signaling.

GRKs are kinases that are recruited by Gβγ to the plasma membrane and phosphorylate MOR in vitro [56, 57]. Opioid receptor activation by a highly selective MOR agonist such as

DAMGO drives receptor-phosphorylation at serine and threonine residues in the c-terminus [18,19]. The same study that defined MOR desensitization by morphine is mediated by PKC concluded that the desensitization of MOR by DAMGO is primarily mediated by GRK [53]. DAMGO-mediated phosphorylation was preferentially catalyzed by GRK2, GRK3, and GRK5 was primarily responsible for morphine-mediated MOR-phosphorylation [58]. Also, inhibition of GRK expression using phosphosite-specific antibodies and siRNA enhanced MOR signaling and reduced receptor internalization [50, 58]. But when GRK inhibitors were tested in an electrophysiological model of acute tolerance, it only blunted the tolerance provoked by DAMGO activation [55]. In vivo opioid acute-tolerance and inhibition of GRK determined that pharmacological treatment with a GRK inhibitor blocks tolerance developed by DAMGO, but not morphine or fentanyl [55].

Phosphorylation of MOR's c-tail by kinases such as GRK rapidly desensitizes the receptor and creates a "barcode" that assists in the recruitment β-arrestin proteins (β-arr) to the receptor to induce internalization [59, 60]. β-arr signaling is heavily implicated in negative regulation of receptor response [60]. β-arrestin2 knock-out mice have been used to show that deletion of β-arr2 increases the antinociceptive response of opioids [61, 62] and blunted opioid tolerance using a subcutaneous morphine pellet implanted for 3 days [63]. β-arr signaling pathways depend on G-protein activation of GRK, but some studies controversially suggest that β-arr signaling can also be activated independently of G-proteins.

Interestingly, recent studies have introduced the notion of crosstalk between Gβγ and $G\alpha_q$ signaling pathways through GRK. Structural information reveals that $G\alpha_q$ can interact with GRK2/3, but the functional purpose of this interaction remains unknown [64, 65]. This study used cellular sensors and GPCR activation in HEK-293 cells to dissect this functional model of

G-protein crosstalk with different GRK isoforms [66]. They determined that recruitment of GRK2 by Gβγ desensitizes $G\alpha_q$ -dependent intracellular Ca^{2+} release by competitively binding to $G\alpha_q$ and preventing PLCβ activation [66]. This study proposed a functional mechanism for G-protein crosstalk between Gβγ and $G\alpha_q$ through GRK to limit the activation of PLCβ signaling pathway. These observations suggest that crosstalk of G-proteins after receptor activation might have a role in the differences observed between phosphorylation between PKC and GRK in opioid signaling.

1.4 Proposed pharmacological strategies to improve opioid pain treatment.

Opioid research aims to improve pain treatment in the clinic by increasing the analgesic effects of current and new opioid ligands and reducing unwanted opioid effects such as dependence, tolerance, constipation, and addiction. Some examples of novel pharmacological strategies for opioid research are positive allosteric modulation, mixed-efficacy agonists, and biased agonists. Still, all these innovative scientific ideas aim to improve therapeutics for better pain management without detrimental consequences by separating the therapeutic effects and the undesired side effects of opioid agonist.

Positive allosteric modulation (PAM) is based on the idea that targeting an allosteric site, as opposed to the orthosteric–canonical binding site for GPCR ligands, will increase the affinity and potency of an agonist. Identifying positive allosteric modulators of MOR could improve pain alleviation and dampen unwanted side effects [67]. Class A GPCRs have a highly conserved sodium binding site that favors the inactive state of the receptor that has been the target for PAM development. Positive allosteric modulators of MOR disrupt this sodium binding in the receptor priming an active state to improve agonist responses. One possible therapeutic target is to use positive allosteric modulators to increase the potency of endogenous opioid neuropeptides for

analgesia [24]. A study showed that positive allosteric modulator BMS-986122 increases in vitro MOR affinity and in vivo has antinociceptive effects on its own, but when co-administered with an opioid ligand, it increased antinociception [68].

Mixed-efficacy opioid ligands are multifunctional ligands that can work as agonists and antagonists for specific opioid receptors [69, 70]. Opioid receptors have similar and different pharmacological effects—for example, MOR, DOR, and KOR activation lead to antinociception with different efficacy requirements leading to DOR and KOR agonist not eliciting enough pain relief for moderate-to-severe pain like MOR agonist do. However, the DOR agonist has lower abuse liability than MOR agonist, and KOR activation has dysphoric effects that could counteract MOR-induced euphoria. Mixed-efficacy ligands could also work as antagonists for certain opioid receptors creating the opportunity to create favorable pharmacological combinations to improve antinociception. A study of compound VRP26, a MOR-agonist, and DOR-antagonist characterized using in vitro binding affinity assays, produces in vivo antinociception but no significant tolerance or physical dependence after chronic administration compared with fentanyl treatment [69]. Another study characterized AAH8 with a similar pharmacological profile as VRP26 with similar acute antinociceptive effects, but less tolerance development and dependence and less rewarding than morphine [70].

The approach of bias agonism consists of creating ligands that can activate a GPCR in a manner that favors G-protein signaling pathways and avoids β-arr signaling that negatively impacts receptor signaling [60]. Targeting MOR activation with a biased agonist would theoretically activate G-protein transduction considered pro-antinociceptive, and would prevent β-arr signaling, thought to block antinociceptive opioid effects. β-arr mouse knock-out models and pharmacological inhibition support this approach [61-63, 71]. One limitation of this

approach is that β-arr signaling is partly regulated by G-protein activation. Altogether, these approaches focus on targeting signaling outcomes at the level of receptor activation. However, this thesis focuses on a different strategy that targets G-protein signaling directly, bypassing the receptor.

1.5 Biasing Gβγ-signaling using small molecules as an option for improving opioid treatment for pain.

1.5.1 Small molecule gallein influences Gβγ-signaling.

Our laboratory has developed a novel approach using small molecules to target selected Gβγ protein-protein interactions (PPI) to avoid unwanted signaling pathways. Two well-used prototypical small molecules bind to Gβγ; M119 and gallein [72, 73]. These small molecules were discovered in a competition screen assay with peptide SIGK for binding to the Gβγ subunit [74, 75]. Gallein and M119 are structurally similar, and binding to Gβγ is slowly reversible [73]. These molecules bind to a specific area of the heterodimer Gβγ called the "hot spot" [73-76]. The established mechanism of action of gallein is that it interferes with selective PPI while preventing the interaction of others [75, 77]. Using specific PPI, small molecules do not completely inhibit the functional activity of the subunit itself but disrupt the interaction of Gβγ with specific effectors without interfering with the general signaling cascade.

This principle was tested in opioid-mediated antinociception, proposing that small molecules like gallein promote the activated form of Gβγ to interact and activate signaling pathways that lead to antinociception while preventing the interactions that oppose it. Gallein and M119 treatment alone does not have significant effects on acute antinociception, but pretreatment with M119 –via intracerebroventricular injection (i.c.v.)—with morphine resulted in a leftward shift in morphine dose-response curve using the WWTW assay to measure

antinociception [72, 73]. However, M119 did not potentiate morphine antinociception in PLCβ3 KO mice [73]. M119 also decreased the development of acute morphine tolerance and reduced withdrawal jumps upon naloxone injection [72]. Another study showed the same opioid potentiating effect as M119 when gallein administered i.c.v. or systemically via intraperitoneal injection (i.p.) increased opioid response in antinociception, but it did not change other opioid-related behavior such as locomotion, conditioning, constipation, and respiration [78]. These findings suggest that targeting this pathway could improve the antinociceptive effects of MOR opioid ligands such as morphine. It has been shown that gallein can mechanistically achieve this because, when bound to Gβγ, it allows for the interaction of necessary effectors such as VGCC and GIRK but prevents the interaction of effectors that negatively modulate opioid response such as PLCβ3 and GRK [76, 77].

1.5.2 Application of Gβγ-signaling bias to improve MOR-dependent antinociception.

G-protein signaling bias consists of guiding signaling pathways by selectively inhibiting a subset of effectors [60, 76]. In the case of GPCRs, the Gβγ subunit is a dynamic protein that modulates a range of cellular functions serving as a molecular master key. Small molecules binding to a specific surface area of Gβγ could be used to allow selective activation of pro-antinociceptive signaling pathways and avoid those that oppose it. Animal studies show that small molecule gallein can potentiate opioid antinociception without altering other opioid effects. Since there is a connection between Gβγ-dependent kinase activation and opioid desensitization, this approach could be used to prevent antinociceptive tolerance developed by prolonged administration of opioid ligands. Small molecules that target Gβγ-signaling have been used to improve the outcome of other preclinical disease models, such as a decrease in heart failure, inflammation by immune cells, and fibrosis [76].

In the next data chapters, we will explore a molecular mechanism for MOR-dependent activation of Gβγ-PLCβ signaling pathways and the therapeutic benefit of gallein treatment in developing opioid tolerance. The first data chapter investigates the undefined mechanism of PLCβ activation by MOR. It is hypothesized that PLCβ activation by MOR depends on Gβγ signaling, but this mechanism has not been demonstrated in a cellular system. As previously discussed, PLCβ is regulated synergistically by Gβγ and $G\alpha_q$ but brings into question if Gβγ-signaling alone is sufficient for a cellular response. Therefore, we proposed a mechanism of PLCβ activation by MOR that requires coincident $G\alpha_q$ signaling activation to produce PLC activation and subsequent feedback inhibition of MOR signaling. We tested this mechanism of PLCβ activation in different translational systems of MOR activation. We started with exploring synergistic activation of PLCβ by $G\alpha_q$ -coupled receptors with MOR in HEK-293 cells using a fluorescent biosensor that measures DAG production. Then this coactivation of PLCβ by G-proteins was tested in opioid-mediated inhibition of GABA release in the PAG by inhibiting either Gβγ pathways using gallein or a $G\alpha_q$ -signaling inhibitor. Lastly, we completed our studies using in vivo testing of opioid-mediated antinociception using gallein or $G\alpha_q$ -signaling inhibitor as pretreatment to morphine.

In data chapter 2, we investigate the impact of gallein treatment on opioid tolerance. We proposed that biasing Gβγ-signaling using gallein treatment will improve opioid-antinociception in tolerance developed by repeated administration of morphine. We used two different paradigms of gallein treatment to test its impact on the development of opioid tolerance and opioid-antinociception in a tolerant state. To test our hypothesis, we used repeated injections of morphine for 6 days to develop opioid tolerance in vivo and tested gallein as co-treatment during the repeated morphine period and as a pretreatment to morphine after repeated exposure. We also

tested the role of PLCβ3 in developing opioid tolerance and in gallein's effects in morphine antinociception in an opioid-tolerant state.

Overall, the approaches proposed in this thesis will deepen the understanding of the molecular mechanism of gallein in the potentiation of opioid-antinociception and the therapeutic value of biasing Gβγ-signaling as a strategy to improve the treatment of pain and understanding of the underlying molecular pathways involved.

A. No gallein treatment

B. Gallein treatment

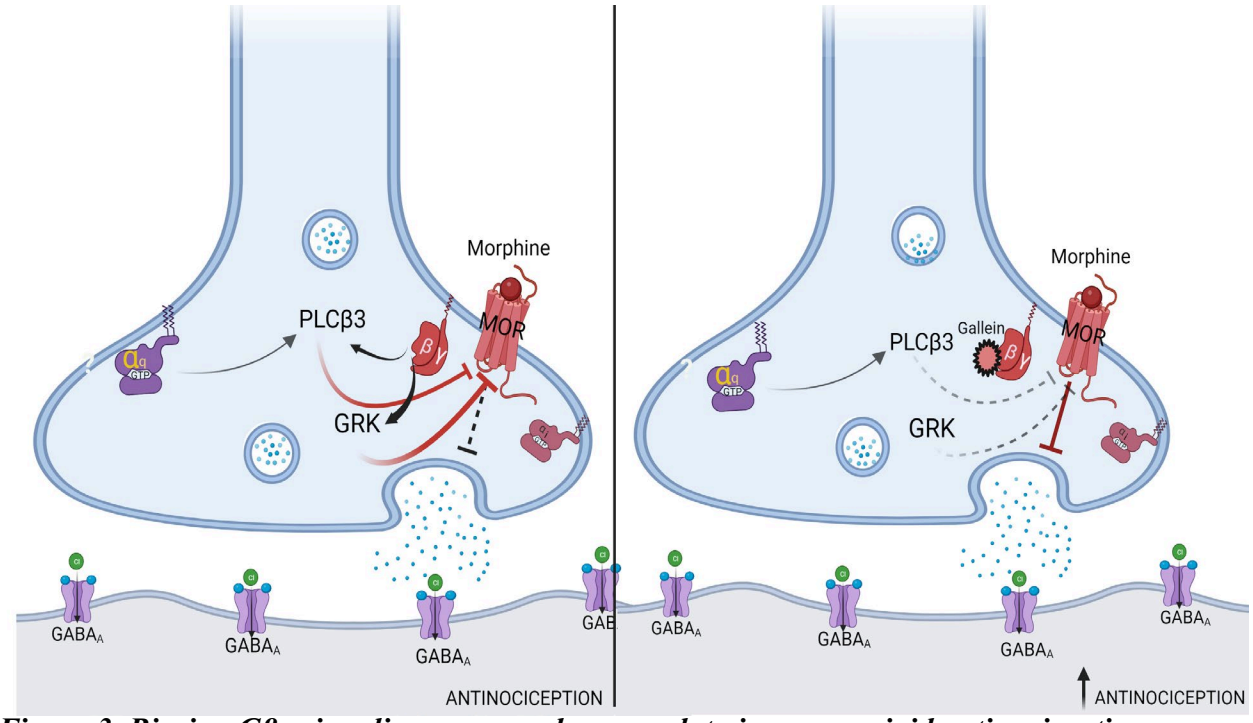

Figure 3. Biasing Gβγ-signaling as a novel approach to improve opioid antinociception.

Panel **A** shows that after MOR activation by morphine, the Gβγ-dependent activation of PLCβ and GRK signaling pathways inhibits opioid signaling that produces opioid antinociception. We propose that treatment of gallein binds to Gβγ and prevents PPI with PLCβ and GRK that results in a stronger opioid-mediated antinociceptive response (**B**). The inhibition of these two pathway may also contribute to the reduction of opioid tolerance after or during chronic opioid treatment.

Chapter 2 Coincident Regulation of PLCβ Signaling by Gq-coupled and μOpioid Receptors Opposes Opioid-mediated Antinociception

2.1 Introduction

Pain management is an important problem worldwide. The current frontline approach for clinical pain-management is the use of opioid analgesics. While these compounds are highly effective, they come with substantial drawbacks. Prolonged use of MOR agonist results in the development of tolerance and physical dependence, which severely limits their use in the treatment of chronic pain. The reinforcing effects of MOR agonists cause opioid abuse liability. Severe respiratory depression as a result of opioid overdose is the major cause of opioid related deaths.

The primary analgesic target of opioids is MOR. MORs are G protein-coupled receptors (GPCRs) that are expressed in both pre- and postsynaptic locations throughout the nervous system and can activate many different signaling pathways. As GPCRs, MORs activate G proteins and are desensitized and/or internalized through recruitment of β-arrestins [79-81]. One approach to improving opioid analgesics has been to find strategies that improve potency and efficacy of opioid agonists while limiting MOR desensitization and internalization [82, 83]. Work by our laboratory has identified phospholipase C signaling as a process that limits the antinociceptive effects of MOR agonists [84] and that pharmacological attenuation or blockade of activation of this pathway enhances the potency of opioid analgesics in mice [85-90].

Phospholipase-C is the upstream enzyme responsible for PKC activation, and this pathway can be activated by GPCRs. PKC has been implicated in adaptations involved in

morphine tolerance through alterations in MOR signaling [91-95], but the mechanisms for upstream regulation of PKC activation in the opioid system have not been examined. Our laboratory has been interested in understanding the mechanisms for activation of phospholipase-Cβ by opioid receptors. Since PLCβ3 is activated by Gβγ subunits released from Gi-coupled receptors [96-98] we hypothesized that PLCβ3 would be activated downstream of MOR via a Gβγ-dependent signal transduction pathway. Indeed, inhibition of Gβγ signaling with M119 or gallein enhanced the antinociceptive effects of morphine in wild type (wt) mice but not in PLCβ3$^{-/-}$ mice [85].

Here we further explored potential mechanisms for MOR-dependent PLC activation and the relevance of these mechanisms to presynaptic opioid-dependent inhibition of neurotransmitter (GABA) release, and to antinociception in mice. In vitro, MOR activation alone did not stimulate PLC signaling, but rather, required coincident activation of a Gq coupled receptor, consistent with the previously described property of PLCβ3 as a coincidence detector for $G\alpha_q$ and Gi signaling [99]. In PAG brain slices, inhibition of either Gβγ or $G\alpha_q$ signaling through PLCβ3 enhanced opioid-dependent inhibition of neurotransmitter (GABA) release. Finally, blockade of either $G\alpha_q$ or Gβγ in mice enhanced morphine-dependent antinociception in mice. These data show that MOR signaling is inhibited in presynaptic terminals through a PLCβ3-dependent mechanism that utilizes coincident inputs from Gq-coupled receptors and MOR to modulate antinociception.

2.2 Materials and Methods

Reagents.

Gallein (Tocris, Minneapolis MN), myrGq-CT inhibitor and scrambled peptide (GenScript USA Inc., Piscataway NJ), YM-254890 (MedChemExpress MCE, Monmouth Junction NJ), [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-Enkephalin acetate salt (DAMGO) (Sigma-Aldrich, St. Louis MO), morphine (Henry Schein, Melville NY), carbachol (Montana Molecular, Bozeman MT), Adenosine-5' triphosphate (ATP) (Sigma-Aldrich, St. Louis MO) Ser-Phe-Leu-Leu-Arg-Asn-amide trifluoroacetate salt (PAR1-AP) (Sigma-Aldrich, St. Louis MO), Pertussis toxin (PTX) (Sigma-Aldrich, St. Louis MO).

Animals.

All animal procedures were conducted at the University of Michigan according to National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with approval of Institutional Animal Care and Use Committee (IACUC) at the University of Michigan. Wild type C57BL/6 mice purchased from Envigo (Indianapolis, IN), and from an in-house breeding colony were used for these studies. PLCβ3-/- mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were bred on a C57BL/6 background. Mice were housed with a maximum of five animals per cage in clear polypropylene cages with corn cob bedding and Nestlets as enrichment. Animals were housed in specific pathogen–free rooms maintained between 68°F and 72°F and between 30% and 70% humidity and a 12-hour light/dark cycle (lights on at 7 A.M. and lights off at 7 P.M.) light/dark cycle with free access to food (Lab Diets, St. Louis, MO; 5L0D) and water. Experiments were conducted in the housing room during the light cycle. All mice were used between 8 and 15 weeks of age and weighed 19–26 g. A

combination of male and female mice was used in gallein experiments, but male mice were used for myrGq-inhibitor experiments. Mice were tested only once with a single dose of drug, and all analyses were between-subject.

Electrophysiology studies were done at Oregon Health & Science University (OHSU). These studies used male and female wildtype C57BL/6 mice and PLCβ3$^{-/-}$ mice and wildtype littermates. Mice were group housed with unlimited access to food and water. Lights were maintained on a 12 h light/dark cycle (lights on at 7:00 A.M.). Mice were sacrificed and cellular recordings were conducted during the light phase of this cycle. The Institutional Animal Care and Use Committee at Oregon Health & Science University approved all experimental procedures. Experiments were conducted in accordance with the United States National Research Council *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Maintenance of HEK293 cell culture and Stable MOR-FLAG HEK293 cell culture.

Human Embryonic Kidney (HEK293) cells were grown in DMEM medium (Corning, Corning NY) with 4.5 g/L glucose, L-glutamine and sodium pyruvate with 100U penicillin/streptomycin and research grade 10% Fetal Bovine Serum (FBS) (ThermoFisher scientific, Pittsburgh, PA). Cells were maintained in a 5% CO_2 humid atmosphere at 37°C. HEK293 cells stably expressing MOR-FLAG tagged receptor were obtained from the Puthenveedu laboratory at the University of Michigan and were maintained with addition of 50mg/mL Geneticin (G418 Sulfate) (ThermoFisher Scientific, Rochester NY).

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 (8-24hrs) 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 (50µL of 500,000 cells/mL per well) with BacMam transduction reaction (100µL per well).

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 120µL of warmed Gibco Dulbecco's Phosphate Buffered Saline (DPBS) with Ca^{2+} and Mg^{2+} (Life Technologies Co, Grand Island, NY). Cells at 80% confluency were kept in the dark inside the plate reader and incubated in DPBS for 10m before agonist was added. Baseline fluorescence at 540 nM was measured each second for 30s followed by 15 µL of agonist added simultaneously to each well of the plate. Fluorescence intensity measurements were aquired every second for 230s. 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.

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 24h before the assay.

Immunocytochemistry (ICC).

Transfected cells and stable MOR-FLAG cells were plated in a 20 mm glass bottom cell culture dish (Wuxi NEST Biotechnology, China). Cells were allowed to adhere and then fixed with 4% PFA for 15 min and then incubated with 10% normal goat serum in PBS containing 0.1% Triton X100 (PBS-T) for 1 hr at room temperature. Anti-FLAG primary antibody DYKDDDK tag polyclonal antibody (Invitrogen, Rockford IL) was incubated at a dilution of 1:1000 in 2% goat serum in PBS-T overnight at 4C°. After three washes with PBS-T, cells were incubated with secondary antibody goat anti-rabbit Alexa Fluor 568 (Life Technologies, Carlsbad CA) at a dilution of 1:1000 in PBS-T for 1.5 hr at room temperature. After three washes with PBS-T cells were imaged using confocal microscopy at 63 x.

Electrophysiological recordings.

Mice (postnatal day >25) were anesthetized with isoflurane, brains were removed, and brain slices containing the vlPAG were cut with a vibratome (180–220 μm thick) in sucrose cutting buffer containing the following: 75 mM NaCl, 2.5 mM KCl, 0.1 mM $CaCl_2$, 6 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM dextrose, 50 mM sucrose and placed in a holding chamber with artificial cerebral spinal fluid (ACSF) containing the following: 126 mM NaCl, 21.4 mM $NaHCO_3$, 11.1 mM dextrose, 2.5 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 1.2 mM NaH_2PO_4, pH 7.35, and equilibrated with 95% O_2/5% CO_2 until moved into a recording

chamber. In experiments using gallein and myrGαq-CT inhibitors, slices were incubated for at least 30 min in ACSF plus inhibitor before recording. Recordings were made with electrodes pulled to 2–4 MOhm resistance with an internal solution consisting of the following: 140 mM CsCl, 10 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.3 mM $CaCl_2$, 4 mM MgATP, and 3 mM NaGTP, pH 7.4. Junction potentials of 5 mV were corrected at the beginning of the experiments. Access resistance was monitored throughout the experiments. Neurons were voltage-clamped at -70 mV. Miniature inhibitory post-synaptic currents (mIPSCs) were collected in the presence of tetrotodotoxin (500 nM). Evoked inhibitory postsynaptic currents (eIPSCs) were stimulated with bipolar stimulating electrodes placed ~50-100 μm away from recording site. A paired-pulse stimulation paradigm was used (two pulses (2 ms) at 50-100 ms intervals) and paired-pulse ratios (PPRs= Pulse 2/Pulse 1) were determined. Data were collected with Axopatch 200B microelectrode amplifier (Molecular Devices) at 5 kHz and low-pass filtered at 2 kHz. Currents were digitized with InstruTECH ITC-18 (HEKA), collected via AxoGraph data acquisition software and analyzed using AxoGraph (Axograph Scientific).

Intracerebroventricular (i.c.v.) pre-treatment injection.

I.c.v. injection was done after baseline withdrawal latencies were taken and before morphine injection. Hamilton syringes of 10μL with a 26 G (catalog #7804-03; Point #4, 12° bevel) needle were used, with a custom-made stopper that allowed 4mm of the needle to enter the skull. Mice were anesthetized using isoflurane until they were no longer responsive to noxious stimuli and breathing slowed down to one inhale per second. Injection is free handed utilizing the ears and eyes for orientation to target the lateral ventricles of the brain. The needle was inserted through the skull using published methods [100]. Immediately after the

experimental procedure, mice are euthanized to confirm injection site. When the needle enters the skull, 3μL of solution is injected into the ventricles, then after 30 seconds the needle is carefully removed. Mice were placed back in their home cage to recover from isoflurane anesthesia. Following recovery from anesthesia, mice were able to move and groom in a normal manner. Gallein was administered 30 min prior to morphine; MyrGα_q-CT peptide was administered 60 min prior to morphine.

Warm Water Tail Withdrawal.

Withdrawal latencies were determined by briefly placing a mouse into a cylindrical plastic restrainer and immersing 2–3 cm of the tail tip into a water bath maintained at 55°C. The latency to tail withdrawal or rapidly flicking the tail back and forth was recorded with a maximum cut-off time of 15 seconds to prevent tissue damage; baseline latencies, 2-3s for 55ºC, were consistent for each assay. Mice were briefly habituated to handling and restrainer, injected with saline (i.p.), and 30 min later withdrawal latencies were recorded (BL, baseline withdrawal latency). Thirty min after i.c.v. injections, withdrawal latencies were recorded, and then mice were injected with 3.2 mg/kg morphine (i.p.). Withdrawal latencies were recorded 30-, 60-, 90-, and 120-min post-morphine injection.

Data analysis

For HEK cell and animal experiments, mean and standard error of the mean (SEM) were calculated for each data set. Where indicated, unpaired student's two-tailed t-tests, one-way ANOVAs with Tukey's or Dunnett's multiple comparisons tests, or two-way ANOVAs with Sidak's multiple comparisons tests were conducted for all analyses involving the comparison of group means as indicated in the figure legends. Concentration dependent curves were fitted using

non-linear regression. AUC calculations are after baseline subtraction (vehicle). % Max Response was calculated as a percentage of the maximal response to PDBu of separately analyzed wells in the same plate after vehicle subtraction. All analyses were performed using Prism 9 (Graphpad, San Diego, CA). Statistical significance was accepted at $P < 0.05$.

Each electrophysiological recording from a single neuron is treated as an individual observation because the vlPAG contains heterogenous cell populations; however, all datasets contain recordings from at least three separate animals. Drug effects were reversed by specific antagonists, and peak drug effects were measured as an increase in current from the average of baseline and washout or the presence of antagonists. Differences between groups were assessed using Student's t-test or ANOVA when appropriate (significance is denoted as $*p < 0.05$). All data are expressed as mean and standard error of the mean (SEM) except figure 6 where data are mean and standard deviation (SD). Data were analyzed with Prism 9 (GraphPad Software).

2.3 Results

2.1.1 MOR does not activate PLCβ in HEK293 cells.

To measure activation of PLC in cells, HEK 293 cells were transduced with a protein kinase C-based fluorescent reporter that detects PLC-dependent diacylglycerol (DAG) production as an increase in overall fluorescence intensity (Green UP DAG assay, Montana Molecular, [101]. The sensor was expressed efficiently in the cytoplasm of the cells (Fig. 4A). Addition of the phorbol ester PDBu as a positive control that binds directly to C1 domain of the sensor produced a strong sustained increase in fluorescence (Fig. 4A). To measure GPCR-dependent PLC activation we transduced cells with the Gq-coupled M1 muscarinic acetylcholine receptor and stimulated cells with the muscarinic agonist carbachol. After establishing baseline

fluorescence, addition of carbachol produced a strong time-dependent increase in fluorescence (Fig 4B). To measure MOR-dependent activation of PLC activity, cells transduced with the reporter were transfected with N-terminally flag tagged MOR. MOR was detected at the plasma membrane (PM) by immunocytochemistry in the majority of the cells (Fig 4C). Surprisingly, addition of saturating concentrations of DAMGO or morphine produced no detectable PLC activation (Fig. 4D).

We considered the possibility that the DAG sensor needs to be targeted to the PM to detect local PLC activation and DAG production [102]. To target the DAG sensor to the PM, a PM targeting CAAX sequence was fused to the C-terminus of a DAG sensor that responds with decreasing fluorescence intensity upon DAG binding (CAAX-Green Down DAG assay, Montana Molecular). Localization to the PM was confirmed by monitoring GFP fluorescence of the sensor transduced into HEK293 cells (Fig 4E). Activation of transfected MOR with either DAMGO or morphine produced no detectable change in reporter fluorescence, while transduced M1 muscarinic receptors produced robust DAG accumulation (Fig. 4F).

2.1.2 Synergistic stimulation of PLC activity by muscarinic Gq-coupled receptors and MOR in HEK293 cells.

Synergistic activation of PLCβ3 has been implicated in cross-talk between Gi and Gq coupled receptor-dependent activation of PI hydrolysis in cells [36, 103]. To test if low level stimulation of $G\alpha_q$ signaling could reveal MOR-dependent stimulation of DAG production downstream of PLC activity, we co-expressed the M1-muscarinic receptor with MOR in HEK293 cells and stimulated with either a subsaturating concentration (100 nM) of carbachol alone, or carbachol with saturating concentrations of either DAMGO (100 nM) or morphine (1 μM). As before, treatment of cells with either DAMGO or morphine did not activate the DAG

reporter while 100 nM carbachol led to a small increase in DAG production. When cells were co-stimulated with DAMGO and carbachol, or morphine and carbachol together, DAG production was strongly increased relative to the signal with carbachol alone (Fig 5A). Traces were quantified and data plotted in Figure 5B. Since DAMGO and morphine gave no response on their own, anything greater than the carbachol alone response is greater than additive and thus synergistic. To confirm that DAMGO and Morphine components of the synergistic responses were Gi-dependent, cells were pretreated with pertussis toxin (PTX), followed by addition of agonists. Treatment with PTX eliminated the DAMGO or morphine-dependent components of the response without affecting the response to carbachol (Fig 5 C, D and E).

2.1.3 Cooperation of MOR with Gq-coupled receptors in PLC activation is generalizable.

To explore synergy with endogenous $G\alpha_q$-coupled receptors we used HEK293 cells with stable expression of MOR but without transfected M1-muscarinic receptor. HEK293 cells have been reported to endogenously express the M3 muscarinic receptor [104]. Stimulation with saturating concentrations of carbachol (50 μM) gave a barely detectable signal likely due to low level endogenous expression of the M3 receptor (Fig. 6A and B). However, co-stimulation with carbachol and DAMGO resulted in strong PLC activation (Fig 6A and B). We performed concentration response analysis for both DAMGO and morphine in the presence of a fixed 50 μM concentration of carbachol (a representative experiment for DAMGO is shown in figure 6C). EC50s for DAMGO and morphine were calculated from multiple experiments (Fig 6D). HEK293 cells have also been reported to endogenously express other Gq coupled receptors including P2Y11 and P2Y12 purinergic receptors and the protease activated receptor F2R (PAR-1) [104]. Stimulation of HEK293 cells with either the purinergic agonist ATP (100 μM) or the PAR-1 agonist PAR-1 activating peptide (PAR1AP) (3 μM) resulted in very low levels of

detectable DAG production that was strongly enhanced in the presence of DAMGO (Fig 6, E-H). Both PAR and purinergic receptors can also couple to other G proteins, thus it remains possible that other G proteins, including $G_{12/13}$, could be involved in this process. Nevertheless, the most straightforward interpretation of the data, consistent with prior literature, is a model where low level Gq activation, regardless of the nature of the activating receptor, synergizes with MOR to reveal MOR-dependent PLC activation in HEK293 cells.

2.1.4 DAMGO-mediated inhibition of GABA release in the PAG is greater in PLCβ3 KO mice or with blockade of $G\alpha_q$ or $G\beta\gamma$ signaling.

To examine the role of synergistic PLCβ activation via Gq and MOR-dependent Gi/Gβγ signaling in a physiological setting, we blocked each of these components individually in PAG brain slices. Inhibition of GABA release by presynaptic MORs in the PAG produces antinociception in the descending pain pathway [7, 105, 106]. Since the only PLCβ isoform that is synergistically regulated by $G\alpha_q$ and Gi/Gβγ is PLCβ3, and since MOR-mediated antinociception is enhanced in PLCβ3$^{-/-}$ mice, we first tested whether MOR-dependent inhibition of GABA release was potentiated in vlPAG slices from these mice. Evoked GABAergic inhibitory postsynaptic currents (eIPSCs) were isolated in the presence of NBQX, an inhibitor of AMPA glutamate receptor-mediated synaptic currents (Fig. 7A). The concentration-response curve for DAMGO-mediated inhibition of the GABAergic eIPSCs was shifted to the left in recordings from PLCβ3$^{-/-}$ slices compared to recordings from PLCβ3$^{+/+}$ slices (Fig. 7B). Thus, lower concentrations of DAMGO were sufficient to inhibit GABAergic eIPSCs when PLCβ3 was deleted.

To test whether inhibition of Gβγ signaling could potentiate MOR-dependent inhibition of GABA release, we incubated slices from WT mice with gallein (Fig. 7C). Gallein is an inhibitor of Gβγ that selectively blocks activation of a subset of effectors including PLCβ3 (Bonacci et al. 2006). We have previously demonstrated that gallein enhances the antinociceptive potency of morphine in mice [85, 86, 88, 89, 107] supporting the idea that gallein inhibits PLCβ3 activation by Gβγ without inhibiting interaction of Gβγ with other targets relevant to MOR actions including Ca^{2+} and K^{+} channels. Gallein (10 μM) potentiated inhibition at various concentrations of DAMGO leading to a left shift in the DAMGO concentration-response curve with a minor effect on efficacy (Fig 7C). Gallein also potentiated the ability of DAMGO (50 nM) to inhibit the frequency of miniature inhibitory postsynaptic currents (mIPSCs) (SEM 43 ± 4%, n = 6) compared to control (SEM 6 ± 4%, n = 5), without changing mIPSC amplitude (SEM 2 ± 5%) indicating a presynaptic effect of gallein on MOR signaling. Gallein had no effect in slices from PLCβ3$^{-/-}$ mice (Fig 7D) indicating that gallein enhances MOR-dependent inhibition of GABA release through blockade of Gβγ-dependent regulation of PLCβ3. This provides evidence for a synaptic mechanism underlying gallein's ability to enhance the nociceptive potency of morphine in mice.

To examine the role of $G\alpha_q$ signaling in the PAG we used a myristoylated peptide from the C terminus of $G\alpha_q$ (myr$G\alpha_q$-CT) that competes for Gα subunit interactions with endogenous GPCRs to prevent G protein activation [108]. Slices pretreated with myr$G\alpha_q$-CT revealed DAMGO-dependent inhibition of eIPSCs at 50 nM DAMGO to an extent similar to treatment with gallein (Fig 8A). A similar potentiation was produced after incubating slices in a small molecule inhibitor of $G\alpha_q$ YM-254890 (500 nM) [109]. The paired pulse ratio (PPRs) for eIPSCs in the presence of DAMGO compared to baseline were changed in both inhibitors (myr$G\alpha_q$-CT:

t(5) = 5.4, p = 0.003; YM-254890: t(5) = 3.1, p = 0.03) indicating that the DAMGO-mediated inhibition is via presynaptic MORs. Neither gallein nor myrGα_q-CT had any effect on DAMGO dependent inhibition of eIPSCs in slices from PLC$\beta3^{-/-}$ mice (Fig 8B) or in the absence of DAMGO stimulation (Fig 8C). These results indicate that blocking either G$\beta\gamma$ or Gα_q is sufficient to enhance MOR inhibition of GABA release at low concentrations of a MOR agonist, and that these G proteins dampen MOR signaling in vlPAG terminals via PLCβ3.

Since either Gα_q or G$\beta\gamma$ inhibition alone is sufficient to enhance MOR potency, these data together indicate that signaling via both subunits simultaneously is required to maintain inhibition of MOR-dependent regulation of neurotransmitter release via PLCβ3.

2.1.5 Gq signaling and antinociception in mice.

Since simultaneous activation of PLCβ3 by Gα_q and G$\beta\gamma$ is required for inhibition of MOR-dependent regulation of GABA release in the PAG we examined whether either inhibition of Gα_q or G$\beta\gamma$ is sufficient to enhance MOR-dependent antinociception. As discussed above, we have previously demonstrated that G$\beta\gamma$ inhibition with gallein enhances morphine-dependent antinociception [85, 86]. To test whether Gq inhibition *in vivo* in the PAG would enhance MOR-dependent antinociception, we injected mice i.c.v. with either myrGα_q-CT or control myrGα_q-scrambled peptide, or with gallein as a reference, and measured morphine effects (3.2 mg/kg) in the WWTW assay. At this dose, morphine alone had very little, if any, effect on tail withdrawal latencies as compared with baseline (BL) and Post-ICV withdrawal latencies. As previously described [86], gallein (100 nmoles) had no effect on antinoception in the absence of morphine (post-ICV on graph), but strongly increased the effects of morphine, in terms of magnitude and duration of antinociception (Fig. 6A). Similarly, mice injected with myrGα_q-CT did not have

altered withdrawal latencies compared with BL latencies and compared with myrGα_q-scrambled control peptide alone but showed enhanced morphine-induced antinociception compared to DMSO (vehicle) or myrGα_q-scrambled peptide injected mice (Fig. 9B).

These data, together with prior data demonstrating that MOR-dependent antinociception is enhanced in PLC$\beta3^{-/-}$ mice, support the idea that Gα_q signaling in cooperation with G$\beta\gamma$ signaling via PLCβ3 in the CNS, opposes MOR-dependent antinociception. This model explains how blockade of any of these components enhances morphine-dependent antinociception in vivo.

2.4 Discussion

Previous work identified negative regulatory effects of PLCβ3 on opioid antinociception [84]. Our prior studies showed that inhibitors of G$\beta\gamma$ (M119 and gallein) enhance opioid-mediated antinociception [85, 86, 90] and that the effects of M119 were occluded in mice with PLCβ3 deletion supporting the idea that gallein and M119 block G$\beta\gamma$-PLCβ3 interactions [85]. Based on this information we proposed that G$\beta\gamma$ released from Gi-coupled MORs activates PLCβ3 which opposes MOR-stimulated analgesia. Importantly, our results presented here show that opioids do not appreciably activate PLC, and subsequent DAG production, on their own unless there is coincident signaling from Gq-coupled receptors. Cross-talk between Gq coupled receptors and MOR has previously been described for regulation of Ca^{2+} signaling in MOR expressing cell lines, but the mechanism for this cross-talk, and its role in MOR biology has not been clearly defined [110-114]. Here we provide evidence for synergistic regulation of PLC activation by Gq and Gi/G$\beta\gamma$ signaling MOR in HEK293 cells and show that this coincident detection of PLC activation operates in PAG synapses, a critical brain region involved in MOR-dependent antinociception. Finally, we show that G$\beta\gamma$ and Gq signaling both oppose MOR-

dependent antinociception in mice. These data, together with previous data from PLCβ3$^{-/-}$ mice implicate PLCβ3 as a source of Gq-MOR crosstalk in the CNS for MOR-dependent antinociception.

PLCβ3, but not PLCβ1 or PLCβ4, is activated by Gβγ, and the vast majority of effector regulation by Gβγ subunits occur downstream of Gi-coupled receptors [89, 115]. PLCβ3 is unique in that it is strongly synergistically regulated by $G\alpha_q$ and Gβγ and it was proposed that this could serve as a coincidence detector for cells to respond to simultaneous signals from Gi and Gq coupled receptors [99, 103]. This was initially demonstrated in detailed in vitro biochemical reconstitution experiments and later confirmed downstream of Gq and Gi coupled receptors in bone marrow derived macrophages and in NIH3T3 cells. A recent study confirmed and extended these observations with a broader range of receptors [36].

In the HEK cell-based studies we tested several examples of Gq-coupled receptors and observed synergistic activation of PLC indicating that the negative regulation exerted on MORs originates from the biochemical properties of PLCβ3. Thus, we propose that any Gq-coupled receptor would synergize with MOR in this system. The myr$G\alpha_q$-CT used as an inhibitor of Gq signaling in these studies does not inhibit $G\alpha_q$ directly, but rather competes for interactions between Gq-coupled GPCRs and $G\alpha_q$ preventing activation of $G\alpha_q$ by GPCRs. The effectiveness of this inhibitor in our experiments indicates that tonic Gq coupled receptor activation in the PAG is limiting MOR-mediated analgesia via this mechanism. Future experiments will determine the nature of this receptor or possibly multiple receptors. A recent C. elegans screen identified GPR139 as a Gq-coupled GPCR that opposes opioid analgesia [32], and is one possible candidate.

Contrasting with our results, Halls et al. reported that morphine, but not DAMGO, activated a plasma membrane targeted PKC sensor, pmCKAR, without a requirement for coincident Gq activation [116]. We see robust responses to both DAMGO and morphine, in the presence of a Gq stimulus, regardless of the localization of the sensor. It is possible that DAG sensor used in our study is less sensitive than pmCKAR. CKAR is a PKCα based FRET reporter which contains both Ca^{2+} and DAG binding sites that interact cooperatively which may sensitize CKAR to local generation of DAG in the presence of elevated Ca^{2+}. It is also possible that in the HEK cell line used in that study there is a tonic Gq signal that does not translate across different HEK cell lines. Thus, while MOR may stimulate low level PLC activation in the absence of Gq signaling, coincident Gq activation results in robust MOR-dependent PLC activation that we demonstrate to have physiological relevance.

One strategy to avoid development of opioid tolerance for treatment of chronic pain and to reduce the potential for addiction would be to lower the doses of morphine needed to produce analgesia. We and others have previously shown that inhibition of Gβγ subunits increases the antinociceptive potency of morphine without enhancing side effects such as constipation and respiratory depression [90] suggesting that inhibition of a subset of effectors downstream of Gβγ is a possible strategy to reduce morphine doses required for pain management [89]. Targeting $G\alpha_q$ signaling is a possible alternative strategy. Since $G\alpha_q$ signaling appears to be tonically activated by a yet to be unidentified Gq-coupled GPCR, either an antagonist or inverse agonist targeting this receptor would likely enhance the antinociceptive effects of morphine.

Highly potent MOR agonists such as fentanyl already exist and are very dangerous drugs. Since these drugs target MOR itself, their potency with respect to causing side effects severely limits their usefulness. MOR-dependent G protein activation is relatively cell context

independent, while signaling downstream of G protein activation is highly cell context dependent. Gβγ signaling depends on the cell type specific expression of Gβγ-regulated effectors and cell-type specific responses to regulation of those receptors [89, 117, 118]. Thus, targeting PLCβ3 or its regulators may enhance the potency of MOR antinociceptive effects relative to side effects because the neurons responsible for antinociception may have different downstream signaling responses that are more sensitive to PLCβ3 than the neurons responsible for respiratory depression or constipation. The signaling mechanisms downstream of PLCβ3 that oppose opioid analgesia have not yet been identified. One possibility is through PKC-dependent phosphorylation of key targets. Both of these issues will be the subject of further investigation.

Dr. Susan Ingram performed ex vivo experiments and data analysis.

2.2 Figures

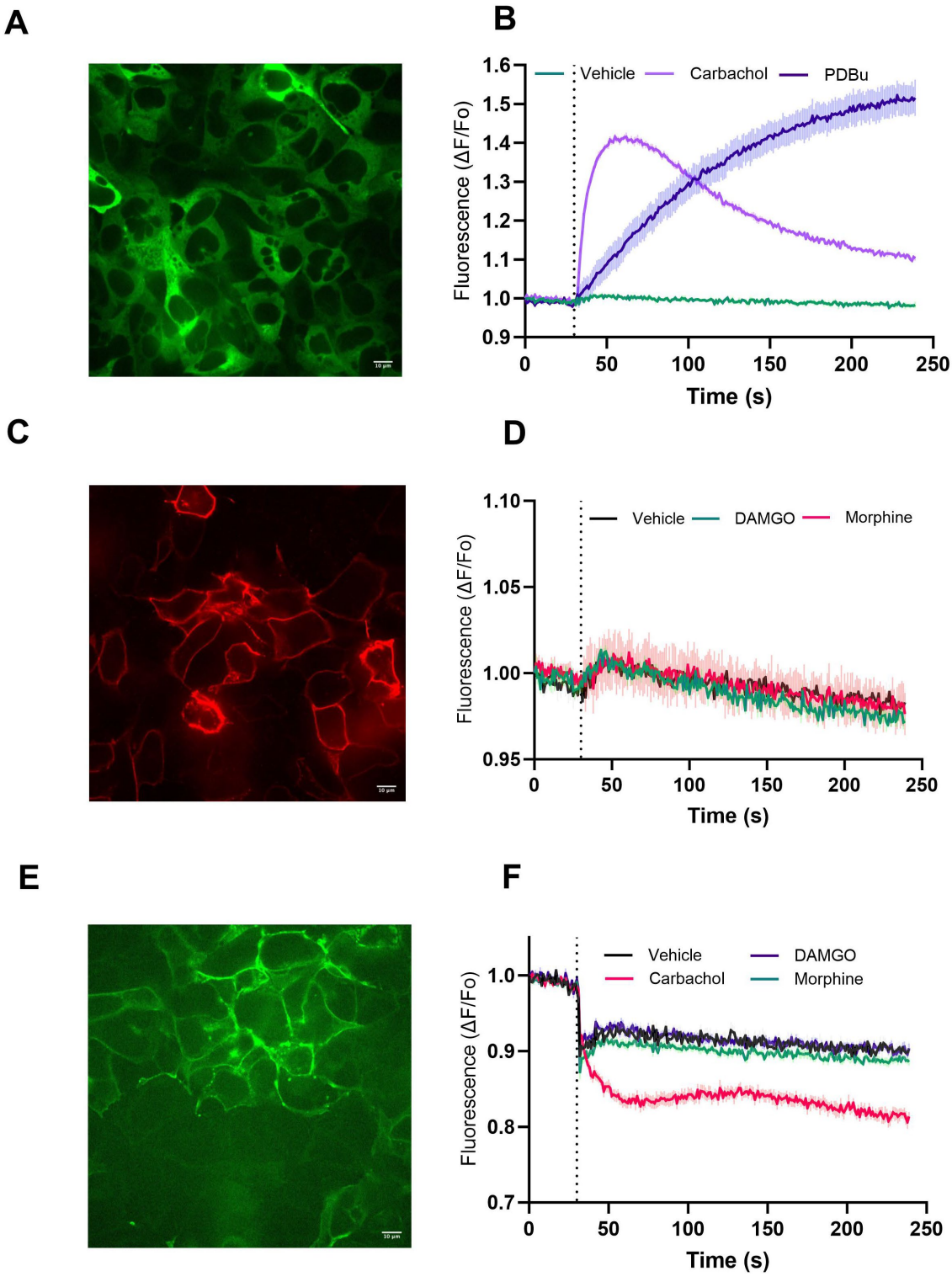

Figure 4. MOR activation alone does not stimulate detectable DAG production.

A. Field of HEK293 cells showing transduction of the DAG reporter. HEK293 cells were transduced with a fluorescent DAG reporter (DAG-up, Montana Molecular) and M1 muscarinic acetylcholine receptor (M1R). **B**. At the dotted line, DPBS vehicle (Veh), PDBu or 50 μM carbachol (Carb) were added and the change in fluorescence intensity across the entire well of a 96 well plate relative to baseline F_o was monitored. **C.** Cells were transduced with the DAG reporter, transfected with flag-MOR. Cells were fixed and stained with an anti-flag antibody. **D.** Cells transduced with the DAG reporter and transfected with flag-MOR were treated with DPBS (veh), 1 μM DAMGO or 10 μM Morphine (Morph) as in B. **E.** Cells were transduced with the CAAX-DAG reporter (DAG-down, Montana Molecular) and M1R, and transfected with MOR. Shown is a field of live cells showing plasma membrane localization of the reporter. **F.** Cells expressing CAAX-DAG reporter, M1R and MOR were treated with the indicated agonists and fluorescence intensity was measured. For B, D and F, each trace is the mean +/-SEM of 3-4 separately transduced wells in a 96 well plate, representative of at least 3 separate experiments. All baseline traces were normalized to 1. The initial downward deflections at the dotted line in traces in F are artifacts associated with compound/vehicle addition.

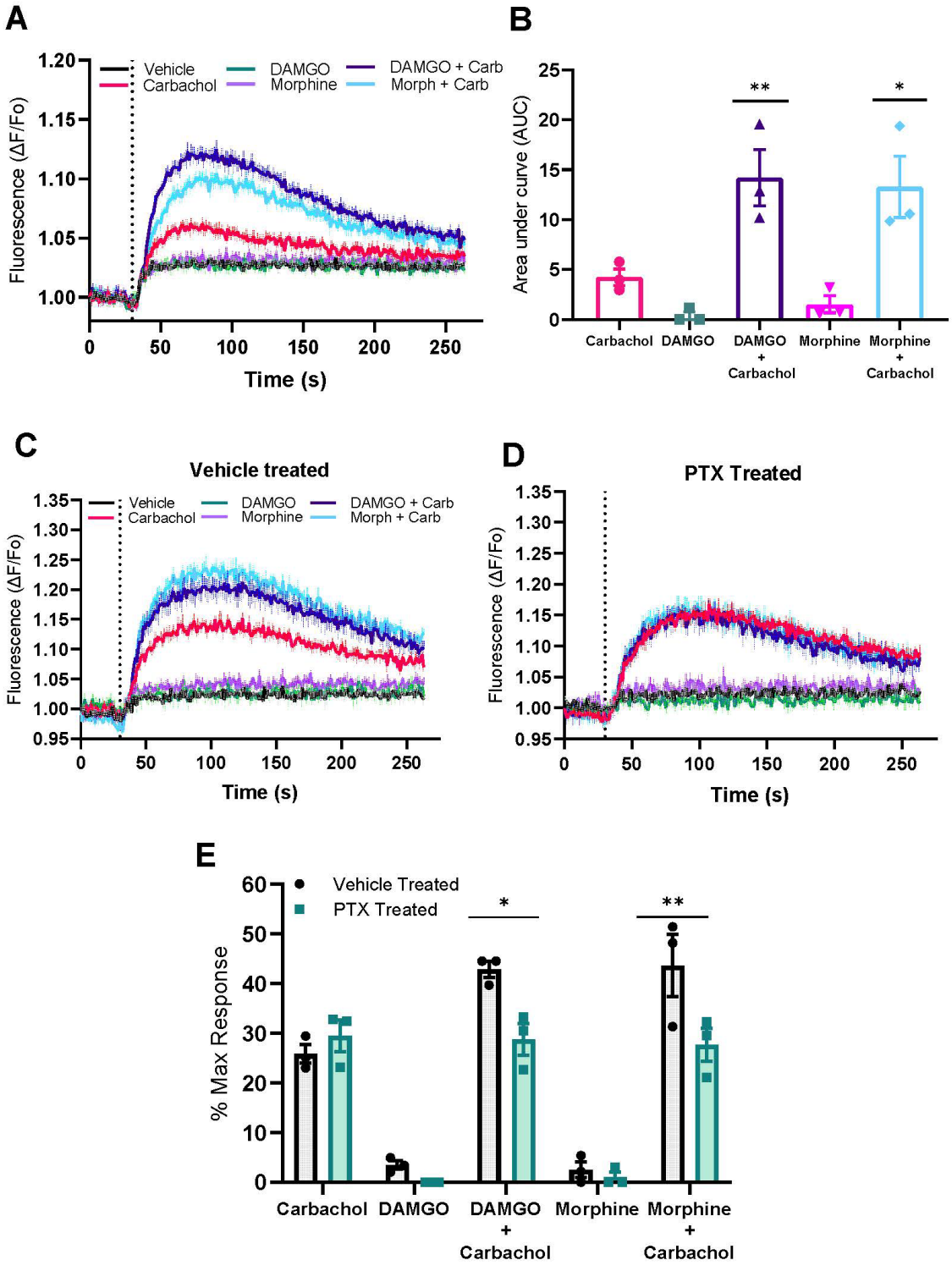

Figure 5. Coactivation of MOR and Gq-coupled muscarinic receptors reveals synergistic PLC activation.

A. HEK293 cells stably expressing flag-MOR were transduced with the DAG reporter and M1 receptor as in Fig 1A and B. Cells were treated (compounds added at the dashed vertical line) with DPBS, 100 nM Carbachol, 500 nM DAMGO, 1 μM Morphine, Carbachol+DAMGO, or Carbachol+morphine at the same concentrations. **B.** To quantify these responses the area under the curve (AUC) with vehicle subtracted for each curve was calculated; combined treatment is compared with individual treatments, one way ANOVA $F(4,10)=11.5$, $P=0.0009$. **C. and D.** Stable MOR, HEK293 cells transduced, transfected, and treated as in A were treated for 16h without and with 100 ng/mL PTX for 16h. **E.** Peak DAG production at each concentration of agonist relative to maximum PDBu-dependent DAG production was calculated from 3 independent experiments, each performed with four replicates. All data are +/- SEM. P values were calculated with an ordinary one-way ANOVA with Tukey's post hoc test and two-way ANOVA with Sidak's post hoc test. *$P<0.05$ and **$P<0.005$.

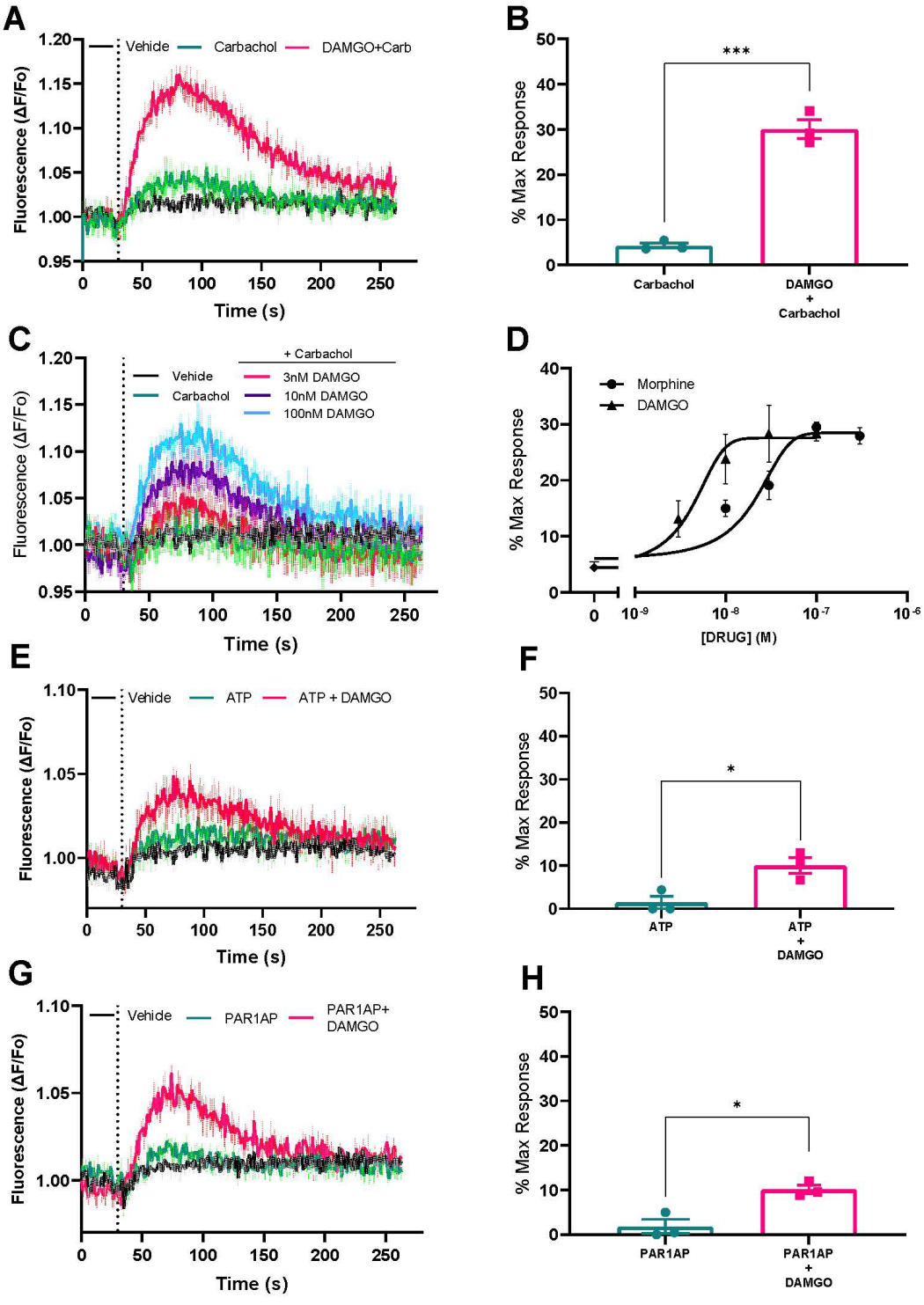

Figure 6. Gq synergy with MOR for PLC activation is independent of the nature of the stimulating Gq-coupled GPCR.

A. HEK cells stably expressing MOR, and transduced with the DAG reporter, without transduction of the M1 muscarinic receptor, were treated with vehicle, 50 μM Carbachol, or 50 μM Carbachol+100 nM DAMGO. Data are mean +/-SEM from one representative experiment. **B.** Peak DAG production relative to maximum PDBu-dependent DAG production was calculated from 3 independent experiments each performed in four independent wells as in A; unpaired t-test t (4)=12.03, P=0.0003. **C.** HEK293 cells stably expressing MOR were as in A treated with vehicle, 50 μM carbachol, or carbachol + varying concentrations of DAMGO. Representative traces from 1 experiment with 4 replicates each condition. **D.** Peak DAG production at each concentration of agonist relative to maximum PDBu-dependent DAG production was calculated from 3 independent experiments each performed in four independent wells for each concentration. Non-linear regression curve fitting; morphine EC_{50}= 17nM [95%CI 7-27nM]; DAMGO EC_{50}= 3.8nM [95%CI 1.6-7.7nM] **E.** Experiments were performed as in A except 100 μM ATP instead of carbachol was used as the agonist for activation of Gq. Data are mean +/-SEM from one representative experiment. **F.** Peak DAG production relative to maximum PDBu-dependent DAG production was calculated from 3 independent experiments each performed in four independent wells as in E; unpaired t-test t(4)=3.7, P=0.02. **G.** Experiments were performed as in A except 3 μM PAR1AP (SSFLRN) was used as the agonist for activation of Gq. Data are mean +/-SEM from one representative experiment. **H.** Peak DAG production relative to maximum PDBu-dependent DAG production was calculated from 3 independent experiments each performed in four independent wells as in G; unpaired t-test t(4) =4.45, P=0.01. *P<0.05, ***P<0.0005.

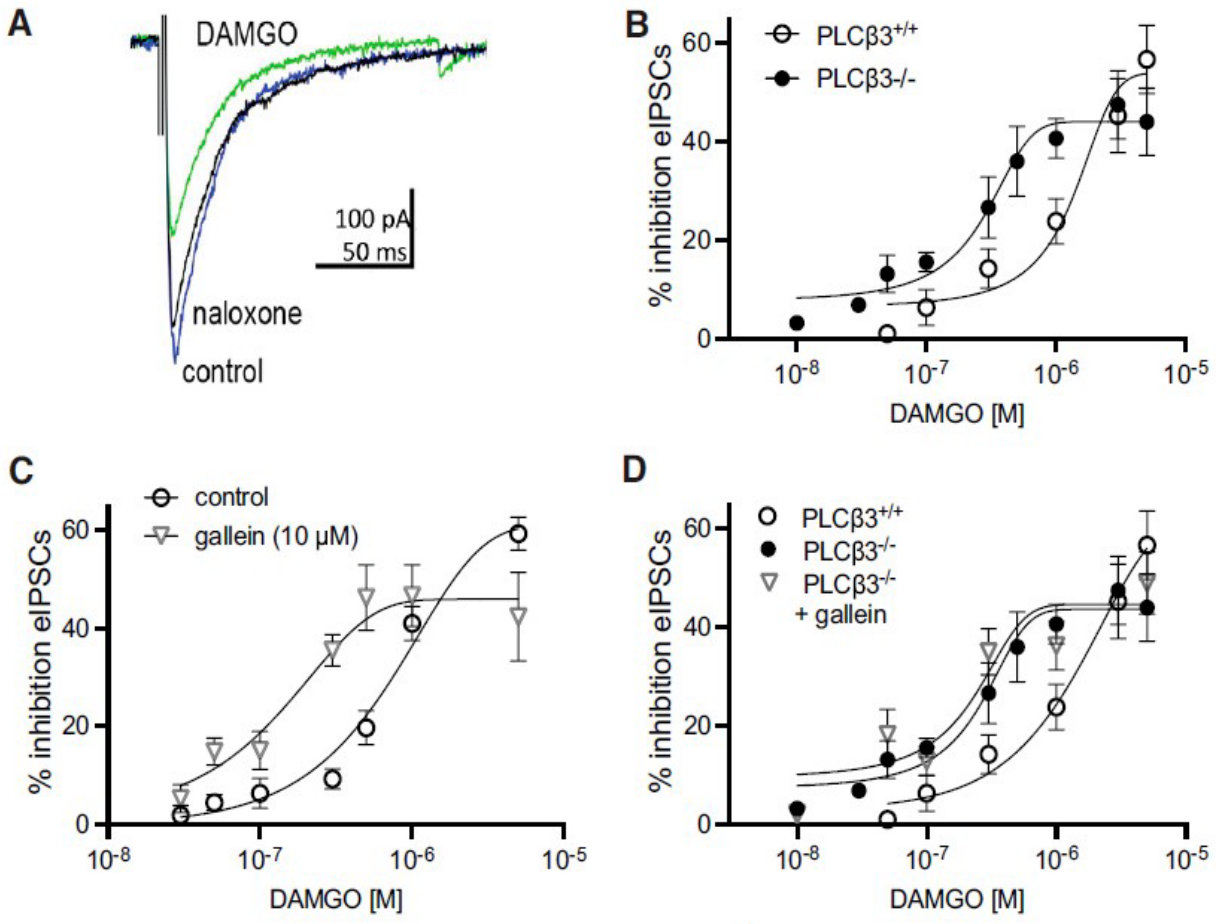

Figure 7. DAMGO inhibition of GABAergic eIPSCs is potentiated in slices from PLCβ3$^{-/-}$ mice and with Gβγ inhibition.

A. Representative eIPSCs from a recording from a slice from a WT mouse showing the effect of DAMGO (1 μM) and reversal with naloxone. **B.** Concentration-response curves for DAMGO-mediated inhibition in slices from WT compared to PLCβ3$^{-/-}$ mice. The EC50 for DAMGO is shifted to the left in slices from PLCβ3$^{-/-}$ mice (238 nM; 95% CI 140-376 nM) compared to WT mice (1.3 μM; 95% CI 732nM- 2.8 μM; $F_{(1,79)} = 22.8$, $p < 0.0001$. Recordings were from 4-6 cells from at least 3 mice per data point. **C.** Gallein shifted the DAMGO concentration response curve in slices from PLCβ3$^{+/+}$ mice (control: 0.8 μM [95%CI 0.52-1.54 μM], gallein: 0.12 μM [95%CI 0.08-0.18 μM]). **D.** Gallein does not shift the DAMGO concentration-response curve in slices from PLCβ3$^{-/-}$ mice. EC50 = 162 nM (95% CI 81 – 367 nM). PLCβ3$^{+/+}$ and PLCβ3$^{-/-}$ curves are the same as in B. Recordings were from 4-6 cells from at least 3 mice per data point. Data are mean +/-SEM.

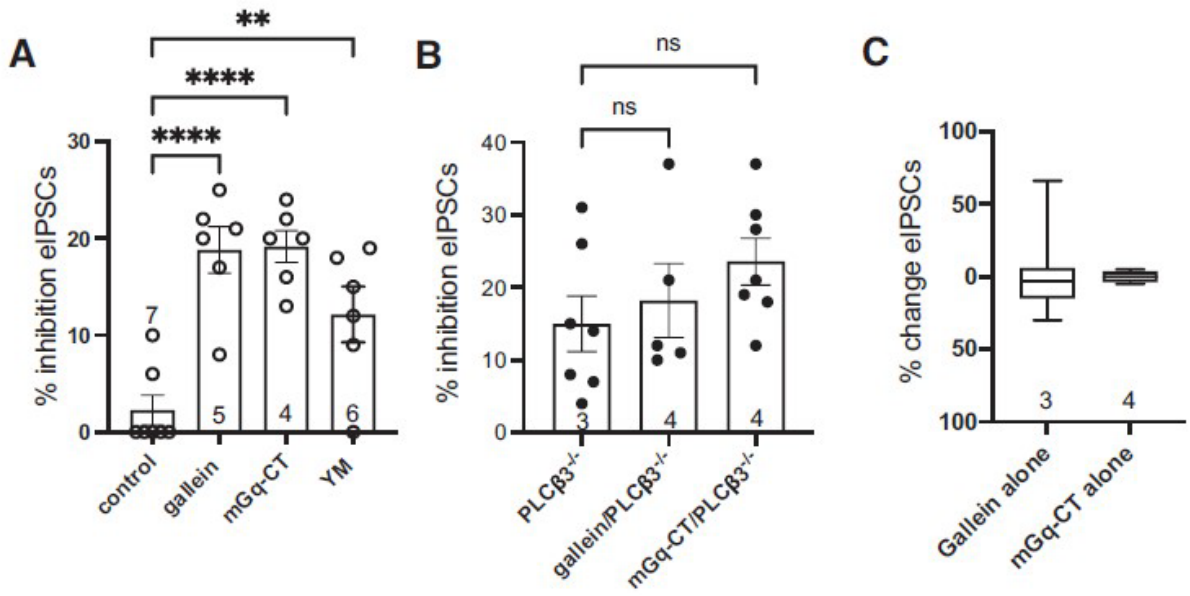

Figure 8. Gq inhibition potentiates DAMGO inhibition of GABAergic eIPSCs in PAG slices.

A. Bar graph comparing effects of Gβγ and Gq inhibitors on inhibition of eIPSCs produced by a single dose of DAMGO (50 nM). All samples were preincubated with either vehicle control (DMSO) or the indicated inhibitors at 10 μM for 30 min, followed by addition of DAMGO. One way ANOVA, $F(3,21) = 14.4$, $p<0.0001$; Dunnett's multiple comparisons, **$p<0.01$, ****$p<0.0001$. **B.** Gallein (10 μM) and mGq-CT (10 μM) had no effect on DAMGO-dependent inhibition of eIPSCs in PAG slices isolated from PLCβ3$^{-/-}$ mice. Experiments were peformed as in A except PAG slices from PLCβ3-/- mice were used. Symbols denote number of recordings and numbers in bars denote number of animals. **C.** Gallein (10 μM) and mGq-CT (10 μM) had no effect on eIPSCs in the absence of MOR activation. eIPSCs were measured before and after Data are mean +/-SEM of all. recordings, Numbers in bar graphs indicate the number of animals tested.

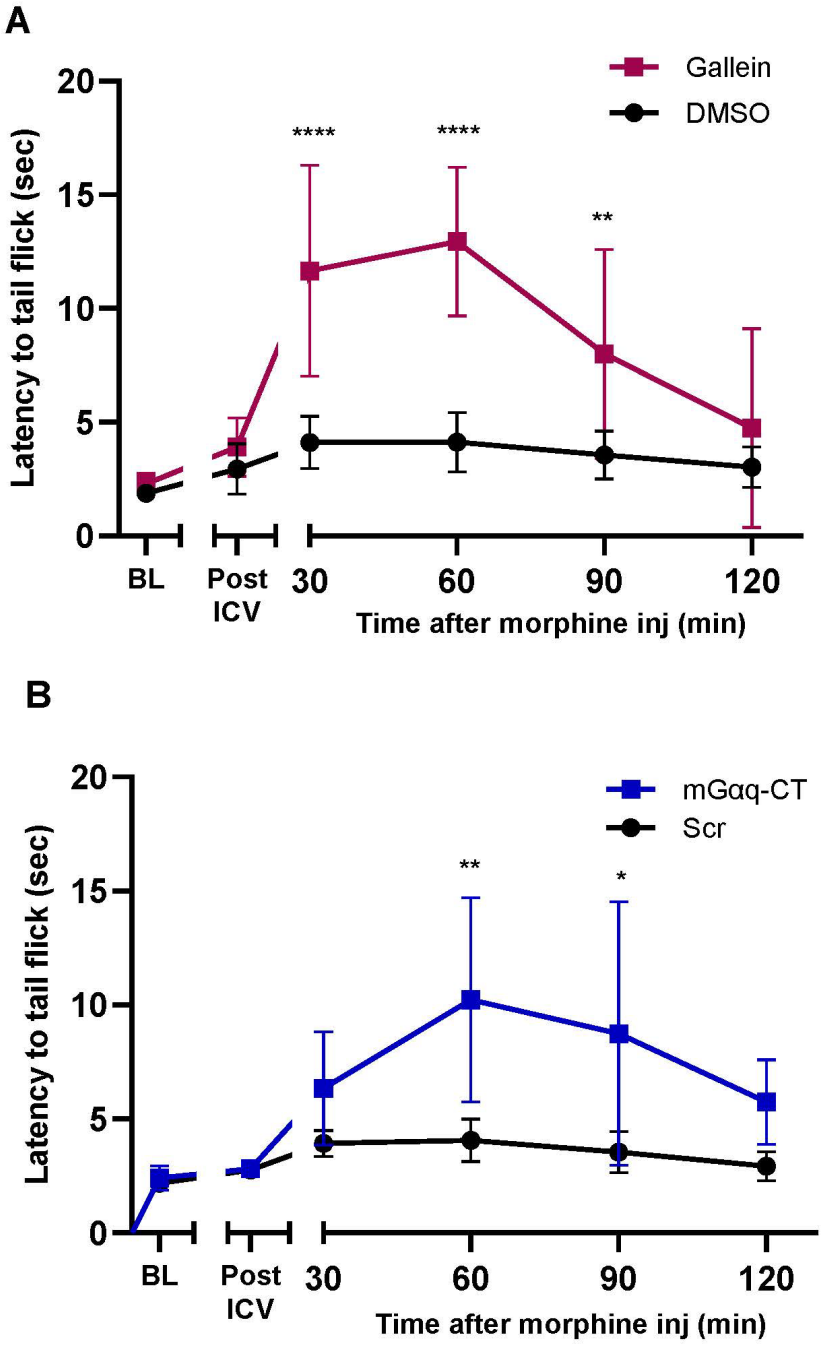

Figure 9. Gβγ and Gq inhibition enhance morphine-induced antinociception in mice.

A. Mice were injected i.c.v. with gallein (100 nmoles) or DMSO (8 mice per condition) and allowed to recover for 30 min and post-ICV tail flick latency was measured. 3.2 mg/kg morphine was then injected at time 0 and tail flick latency was measured at the indicated times; mixed effects two way ANOVA with Sidak's multiple comparisons, significant interaction of time X pretreatment effect F(5,69)=9.2 P=<0.0001 **B.** Same as A except mGα_q-CT (5 male mice)

or myrGα_q-scrambled (4 male mice) were injected i.c.v. at 30 nmoles each; mixed effects two way ANOVA with Sidak's multiple comparisons, significant interaction of time X pretreatment effect F(5,35)=2.78, P=0.03. Data was analyzed with a mixed effects ANOVA followed by Sidak's multiple comparisons test. *P<0.05, **P<0.005, ****P<0.0001 at each time point comparing treatment to control (gallein vs. DMSO) or (m$G\alpha_q$-CT vs. Scr peptide). Data are mean +/-SD.

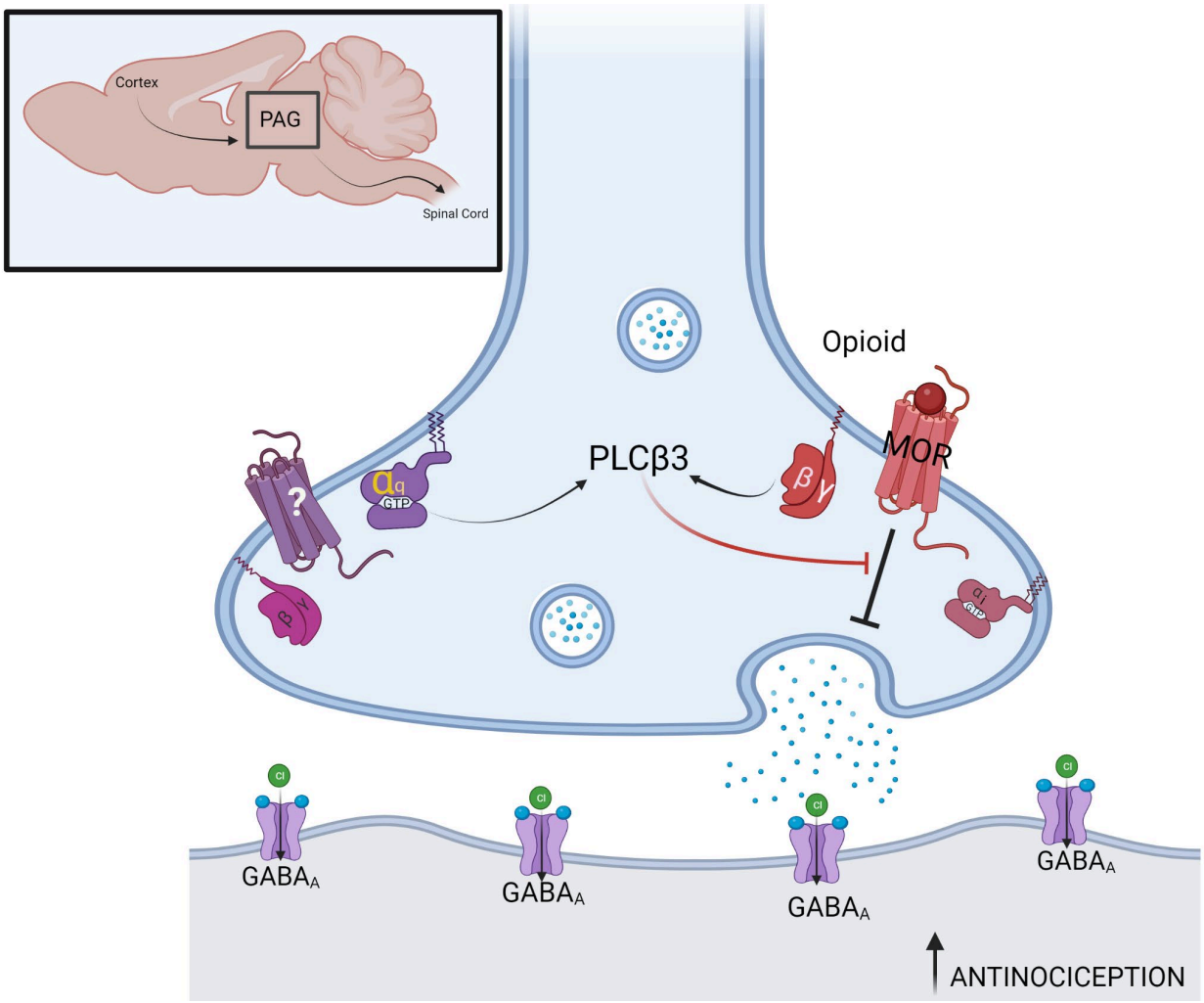

Figure 10. Model for mechanism of MOR -Gq coincidence detection in feedback inhibition of MOR-dependent antinociception in presynaptic PAG input neurons in the descending pain pathway

Pictured is a GABAergic synapse between a PAG input and output neuron. The boxed inset shows the anatomic location of the PAG in the rodent brain with inputs from the cortex, and outputs to the spinal cord. MOR activation in the presynaptic neuron inhibits GABA release resulting in activation of output neurons that ultimately suppress afferent pain transmission in the spinal cord. PLCβ3 activation suppresses MOR actions in presynaptic neuron, and activation of PLCβ3 requires inputs from both Gi/βγ from MOR and Gq from an unknown Gq-coupled

receptor. Since PLCβ3 activation requires simultaneous $G\alpha_q$ and Gβγ binding, blockade of either $G\alpha_q$ or Gβγ is sufficient to relieve the PLC-dependent inhibition of MOR signaling leading to enhanced MOR potency and increased antinociception. Figure created with Biorender.com.

Chapter 3 Treatment of Gallein Alters Development of Opioid Tolerance and Potentiates Opioid-antinociception in a Tolerant State by Gβγ-signaling Bias.

3.1 Introduction

μ-opioid receptor (MOR) agonists are powerful analgesics used in the clinic to treat pain; however, the use of opioid analgesics induces acute adverse effects and other conditions resulting from chronic use. Prolonged use of MOR agonist results in the development of opioid tolerance [59, 119], opioid-induced bowel dysfunction (OIBD)[120], and opioid use disorder (OUD) [59], which impose a substantial limitation on the use of opioids for chronic pain treatment. Opioid tolerance manifests as a decreased response to the analgesic effects of MOR agonists and the need to increase dosing to achieve a therapeutic effect [59, 121]. Therefore, novel treatments to decrease the development of tolerance to the analgesic effects of MOR agonists are needed to improve pain treatment.

The MOR is a G-protein coupled receptor (GPCR) coupled to $\alpha_{i/o}$ G protein subtypes[1]. MOR activation leads to GTP-dependent dissociation of $G\alpha_{i/o}$ and Gβγ subunits that results in direct binding and regulation of their respective effectors[122]. Signaling by Gβγ subunits is necessary for opioid-mediated antinociception through regulation of G-protein inwardly rectifying potassium (GIRK) ion channels [123], voltage-gated calcium channels (VGCC) [9, 124], and SNARE proteins[5, 10, 11]. However, Gβγ subunits also activate signaling pathways that act as negative regulators limiting the antinociceptive effects of MOR activation, such as phospholipase-Cβ3 (PLCβ3) [22, 23, 78] and G-protein receptor kinases (GRK2/3) [5, 125-127].

Therefore, one possible approach to improving the analgesic effects of and decreasing tolerance development to opioid analgesics is to alter the pathways that negatively regulate opioid receptor signaling.

Currently, most scientific approaches to improve opioid treatment for pain rely on the direct targeting of MOR; however, our alternate proposition is to guide or bias the downstream Gβγ signaling pathways after receptor activation by a MOR agonist [76, 128]. Gallein is a small molecule that binds to Gβγ and inhibits interactions with select effectors by occupying a portion of the effector interaction surface [5, 75-77]. For example, gallein-bound Gβγ can activate GIRK channels but not other effectors, such as PLCβ or GRK2, since GIRKs and PLCβ/GRK2 bind to different interaction surfaces on Gβγ [5, 75–77]. We previously demonstrated that gallein administration enhances MOR agonist-mediated antinociception through selective inhibition of PLCβ3 signaling, sparing other Gβγ targets necessary for MOR-antinociceptive effects [22, 23, 73]. Consistent with a mechanism involving gallein-dependent inhibition of Gβγ-PLCβ3 interactions, PLCβ3 KO mice were more sensitive to the antinociceptive effects of morphine than wild-type mice in the warm water tail withdrawal (WWTW) assay, and gallein was ineffective in PLCβ3$^{-/-}$ mice [23, 73]. PLCβ3 is an enzyme that hydrolyzes PIP2 to produce IP3 and diacylglycerol (DAG) and is synergistically activated by $G\alpha_q$ and Gβγ subunits downstream of GPCRs [21]. We recently demonstrated that inhibiting Gβγ signaling with gallein, or inhibiting $G\alpha_q$ signaling, enhances opioid potency at presynaptic MOR receptors *ex vivo* in mouse periaqueductal grey brain slices and increases the potency of morphine *in vivo* in the WWTW assay [129]. However, systemic administration of gallein did not enhance any other opioid receptor-mediated physiological effects such as locomotion, constipation, respiratory depression, reward, acute tolerance, and withdrawal [72, 78]. Together, these data led to the

hypothesis that activation of the PLCβ3 signaling pathway is a negative regulator of MOR antinociceptive signaling, limiting the ability of MOR agonists to produce antinociception.

Tolerance to MOR agonists results from prolonged MOR desensitization, resulting in a loss of receptor function following phosphorylation and internalization of the receptor[41, 59, 119]. It has been demonstrated that Gβγ blockade with gallein prevents the development of acute tolerance to the antinociceptive effects of morphine [72]. This current study evaluates the effects of pharmacological inhibition of specific Gβγ subunit signaling on the development of tolerance following chronic morphine injections and on the antinociceptive effects of morphine in opioid-tolerant mice.

3.2 Materials and Methods

Reagents.

Gallein (Tocris, Minneapolis, MN) was dissolved in a vehicle solution containing 5% dimethyl-sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO), 10% laboratory-grade ethoxylated castor oil (Thermo Fisher, Waltham, MA), and 85% sterile water. To prepare gallein solution for systemic administration, powered gallein is first dissolved and sonicated in DMSO for 5 min, and then castor oil is mixed with the DMSO-gallein solution and homogenized by vortexing. Lastly, water is added to the previously described solution to be mixed and sonicated for 10 min. The final solution yields a concentration of 5 mg/ml gallein and is used within 6 hours of being made at room temperature. Gallein is administered by intraperitoneal (i.p.) injection in a volume of 10-20 ml/kg. Morphine (Henry Schein, Melville, NY) was diluted in saline and administered i.p. or subcutaneously (s.c.) in a volume of 10ml/kg.

Animals

All animal procedures were conducted at the University of Michigan according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and with the approval of Institutional Animal Care and Use Committee at the University of Michigan. Wild-type C57BL/6N mice purchased from Envigo (Indianapolis, IN) and produced from an in-house breeding colony were used for these studies. PLCβ3 knock-out mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were bred in-house on a C57BL/6N background. Mice were housed with a maximum of 5 animals per cage in clear polypropylene cages with corn cob bedding and nestlets as enrichment. Animals were housed in specific pathogen-free rooms maintained between 68°F and 72°F and between 30% and 70% humidity and a 12-hour light/dark cycle (lights on at 7 AM and lights off at 7 PM) with free access to food (Laboratory Diets, St. Louis, MO; 5L0D) and water. Experiments were conducted in the housing room during the light cycle. All mice were used between 8 and 15 weeks of age and weighed 19 to 28 g. Male mice were used for these studies. Mice were used in only one treatment condition.

Warm water tail withdrawal, morphine dose-response curves, and morphine time course

To measure withdrawal latencies, mice were placed briefly into a cylindrical plastic restrainer, and 2 to 3 cm of the tail tip was immersed into a water bath maintained at 55°C. Latency to tail withdrawal or rapidly flicking the tail back and forth was recorded with a maximum cut-off time of 15 seconds to prevent tissue damage. Baseline latencies, 1 to 3 seconds for 55°C, were consistent for each assay. At the start of the experiment, mice were briefly habituated to handling and restraining, then given saline by intraperitoneal injection, and after 30 minutes, withdrawal latencies were recorded (baseline withdrawal latency). Dose-response curves were generated by administering cumulative doses of morphine with one dose administered every 30 minutes by i.p.

injection with doses of 1, 2.2, 6.8, 22, and 24 mg/kg morphine to generate final cumulative doses of 1, 3.2, 10, 32, and 56 mg/kg morphine, and latencies were measured at 30 minutes after each dose of morphine. For time course experiments, baseline tail-flick latencies are taken as previously described. After baseline, each mouse is injected with a bolus dose of 3.2 mg/kg morphine, then every hour, latencies for tail-flick are collected for a total of 3 hours.

Development of opioid tolerance

To induce opioid tolerance, mice were injected subcutaneously three times per day for five consecutive days with either saline, 3.2 mg/kg morphine, or 10 mg/kg morphine at 8 AM, 1 PM, and 6 PM. A morphine dose-effect curve (DRC) was evaluated prior to the start of chronic treatment of morphine or saline. A final morphine dose-response curve is collected on the morning of day six. Treatment of gallein or vehicle was given either: 1) after the initial morphine dose effect curve determination on day 1 and with the noon morphine injection on day 3 or 2) on day five instead of the evening morphine injection.

Data analysis

Mean and standard error of the mean (S.E.M) were calculated for each treatment group. Where indicated, one-way ANOVA with multiple comparison, 2-way ANOVA with Sidak's multiple comparisons tests, or 3-way ANOVA with Tukey multiple comparisons were conducted for all analyses involving the comparison of group means as indicated in the figure legends. Dose-dependent curves were fitted using nonlinear regression using [agonist] vs. normalized response model with variable slopes with restriction in ED50 > 0 and Hillslope shared and <3 (GraphPad Prism 9n, San Diego, CA). ED_{50} values were reported based on these analyses. The percentage

of maximal possible effect (%MPE) was calculated using the difference of each latency value and individual baseline latency divided by the difference of maximal latency cut-off (15 seconds) and baseline of each mouse. Analyses comparing treatment are between-subject, and studies comparing days one and six of repeated dosing are within-subject. Statistical significance was accepted at $P < 0.05$.

3.3 Results

3.3.1 Duration of action of gallein

A previous study showed that systemic injection of 100 mg/kg gallein (i.p.) potentiated acute morphine-mediated antinociception using the WWTW assay [78]. To better understand the duration of action of gallein, we administered one injection of 100 mg/kg gallein i.p. 0.5, 24, 48, and 72 hr prior to 3.2 mg/kg morphine s.c. and measured tail withdrawal latencies from a 55ºC water bath. Gallein administered 30 min before 3.2 mg/kg morphine increased tail withdrawal latencies compared with vehicle pretreatment consistent with previous findings (figure 11A). A separate cohort of mice was used to evaluate the antinociceptive effects of 3.2 mg/kg morphine s.c. 24, 48, and 72 hr following administration of gallein. A single systemic dose of 100 mg/kg of gallein given 24 hours prior robustly potentiated the antinociceptive effects of 3.2 mg/kg morphine (figure 11B). At 48-hour gallein postadministration, there was a slight but nonsignificant increase in the effects of morphine (figure 1C), which entirely dissipated by 72 hr (figure 11D).

3.3.2 Gallein-mediated potentiation of morphine-induced antinociception is dose-dependent at 30 min and 24 hr postadministration.

To further understand the gallein dosing regimen needed for our studies, we evaluated the potentiation of morphine by gallein at two different doses—100 mg/kg i.p. and 50 mg/kg i.p.—and the effect of these doses at 30 min and 24 hr after administration. At 30 min and 24 hr pretreatment, 50 mg/kg gallein did not significantly potentiate the effects of morphine (figure

12A, 12C). However, 30 min and 24 hr pretreatment with 100 mg/kg gallein produced a 1.8- and 2.7-fold leftward shift in the morphine dose-effect curve (figure 12B, D), respectively.

3.3.3 Gallein treatment decreases the development of opioid tolerance.

To evaluate the effects of gallein on the development of morphine tolerance, we gave gallein or vehicle to mice treated with repeated saline, 3.2 mg/kg, or 10 mg/kg morphine 3X per day for five days. Gallein (50 mg/kg) or vehicle was given 2hr after determination of the initial morphine dose-effect curve and 48 hr later. This dose of gallein was selected because it did not alter the acute effects of morphine (figure 12A,12C). Morphine dose-effect curves were re-determined on day 6. The development of opioid tolerance results in a rightward shift in the morphine dose-response curve between day 1 and day 6.

Vehicle treatment in repeated morphine groups induced a significant rightward shift in the morphine dose-response curve compared with the morphine dose-response curve determined on day 1 (figure 13A). Vehicle treatment in the repeated saline group produced a slight rightward shift (1.4-fold) in the morphine dose curve, possibly due to repeated handling and/or behavioral adaptions (figures A and C). Repeated administration of 3.2 or 10 mg/kg morphine with vehicle treatment produced a 3.3-fold and 4.4-fold rightward shift, respectively, in the morphine dose-effect curve, demonstrating development of tolerance to the antinociceptive effects of morphine (figures A and C). However, treatment of gallein strongly and significantly decreased the rightward shift following chronic morphine treatment resulting in an effect similar to that observed with repeated saline administration (figure 13B). The rightward shifts in the morphine dose-response curves observed on day 6 of gallein-treated groups with repeated dosing with 3.2 mg/kg of morphine and 10 mg/kg of morphine were similar to that seen with saline repeated treatment (1.9, 1.7, 1.9-fold, respectively). Statistically, vehicle treatment showed a dose-

dependent increase of ED_{50} values between day 1 and day 6 in morphine-treated groups, indicating development of tolerance to morphine (figure 13C). In contrast, the ED_{50} values did not significantly differ between day 1 and day 6 with gallein treatment regardless of the chronic morphine dose (figure 13C). Thus, utilizing just two doses of 50 mg/kg i.p. gallein was enough to decrease opioid tolerance developed due to chronic morphine treatment.

3.3.4 Gallein potentiates morphine-induced antinociception in an opioid-tolerant state.

To evaluate the opioid-sparing effects of gallein in an opioid-tolerant state, mice were treated with chronic 10 mg/kg of morphine following determination of initial morphine dose-response curve on day 1. On day 5, fifteen hours before evaluating the morphine dose-effect curve, mice were treated with vehicle, 50, or 100 mg/kg gallein (i.p.). Figure 14A compares the day 6 test dose-response curves between 15 hr pretreatment of vehicle, 50 mg/kg, and 100 mg/kg gallein. Pretreatment of 100 mg/kg gallein shifted the curve 1.9-fold leftwards compared with vehicle treatment, but no significant shift (0.7-fold) was observed in mice treated with 50 mg/kg gallein (figure 4A). The ED_{50} value on day 6 following pretreatment with 100 mg/kg gallein injection of (17 mg/kg) is significantly lower than the ED_{50} in the vehicle group (32 mg/kg) and the 50 mg/kg gallein pretreatment group (46 mg/kg) (figure 14B). These data show that gallein increases the potency and efficacy of morphine in mice that are opioid tolerant.

3.3.5 The involvement of PLCβ3 in the development of opioid tolerance.

Our previous data suggest that PLCβ3 is involved in the potentiation of acute morphine by gallein, so we tested PLCβ3 involvement in the development of opioid tolerance. Morphine dose-effect curves were evaluated in PLCβ3 KO animals and WT litter mates before and after repeated treatment with 10 mg/kg morphine. After comparing the dose-dependent response of

PLCβ3 KO and WT on day 1, no significant difference in the antinociceptive response of morphine was detected (figure 15A). Treatment of repeated morphine created a similar rightward shift in the morphine dose-effect curves in both genotypes by day 6; repeated morphine in PLCβ3 WT litter mates produced a 5.2-fold rightward shift, and in PLCβ3 KO mice (figure 15A), a 4.9-fold rightward shift was observed. However, 56 mg/kg morphine on day 6 was more effective in PLCβ3 KO than WT littermates, resulting in a lower ED_{50} value in PLCβ3 KO (32 mg/kg) than WT (41 mg/kg) (figure 15B). We followed these observations by testing 15 hr pretreatment of 100 mg/kg gallein in PLCβ3 KO mice to assess if the ability of gallein to potentiate the antinociceptive effects of morphine in an opioid-tolerant state is PLCβ3-mediated. Pretreatment of 100 mg/kg gallein 15 hr before morphine dose-response curve on day 6 in PLCβ3 KO mice did not alter tolerance developed by day 6 compared to vehicle treatment. In figure 15C, the comparison of pretreatment effect between 100 mg/kg gallein and vehicle in PLCβ3 WT mice is 2.3-fold, whereas pretreatment of gallein compared with vehicle in PLCβ3 KO, is reduced to 1.3-fold (figure 15D). Thus, by comparing the ED_{50} values generated by vehicle pretreatment of day 6 showed a significant difference between genotypes – 36 mg/kg morphine for PLCβ3 WT and 21.9 mg/kg morphine for PLCβ3 KO (figure 15D). On the other hand, gallein pretreatment did not make a difference on day 6 ED_{50} value between genotypes – 15.8 mg/kg morphine for PLCβ3 WT and 16.5 mg/kg morphine for PLCβ3 KO demonstrating that 15 hr gallein pretreatment in PLCβ3 KO mice does not further potentiate antinociception in an opioid-tolerant state (figure 15C).

3.4 Discussion

One goal of opioid research is to increase the therapeutic benefit of MOR agonists by decreasing the severity of unwanted effects, such as opioid tolerance. Previous studies

demonstrated that administration of gallein enhanced the acute antinociception of morphine in the tail-flick assay [22, 72, 73, 76, 78, 129]. This potentiating effect by gallein has been attributed to the blockade of PLCβ3 signaling downstream of MOR [22, 73, 129]. Notably, while gallein increases MOR agonist-induced antinociceptive effects, it does not change other MOR-stimulated behaviors such as locomotion, reward, constipation, respiration, withdrawal, and acute tolerance[72, 78]. These findings suggest that preventing activation of the Gβγ-PLCβ3 pathway could increase opioid-induced pain relief without altering unwanted side effects, and this could be accomplished by gallein's ability as a small molecule to bias Gβγ signaling downstream of MOR [76, 128]. To continue the investigation of Gβγ signaling bias in opioid treatment of pain, this study investigates the effects of gallein in chronic morphine treatment and tolerance development.

We found that gallein has a prolonged duration of action (~24 hr), potentiating morphine-induced antinociceptive effects for 24 hr after administration (figure 1). This is consistent with previous work showing that gallein has a long duration of action in the regulation of neutrophil function [130]. We also observed that these potentiating effects are dose-dependent since 50 mg/kg does not significantly potentiate morphine antinociception, but 100 mg/kg gallein does (figure 2). Gallein has a plasma half-life of 1-2 hours [76]; however, the protein binding-off rate is slow (k_{off}=0.0003s^{-1})[76, 77]. Since gallein molecules remain bound to Gβγ long enough, this could play a role in gallein's ability to have opioid-potentiating effects 24 hr after administration. Chronic administration of morphine and cotreatment of a sub-effective dose of gallein (i.e., 50 mg/kg gallein did not potentiate the antinociceptive effects of morphine) prevented the development of opioid tolerance (figure 3). Gallein treatment blunted the development of opioid tolerance. This finding indicates that inhibition of Gβγ-dependent pathways may block the

cellular adaptations that occur during chronic activation of MOR. Another relevant finding is that gallein enhances the antinociceptive actions of morphine in an opioid-tolerant state. Fifteen hours pretreatment with a large dose of gallein (100 mg/kg) was sufficient to potentiate the antinociceptive effects of morphine even when the system has developed opioid tolerance. Therefore, Gβγ signaling bias harnesses the potential to prevent opioid tolerance and potentiate antinociception in a tolerant physiological state.

Other studies have explored molecular mechanisms that contribute to the development of tolerance to the antinociceptive effects of MOR agonists. One of the mechanisms contributing to the downregulation of MOR at the plasma membrane is phosphorylation and internalization. Phosphorylation of MOR is mediated by serine/threonine kinases such as G-protein receptor kinases (GRK) and protein kinase C (PKC) [53, 55, 131]. The Schulz group created a mouse line that expresses MOR with ten amino acid serine/threonine mutations to alanine out of the 11 phosphorylatable sites of the c-tail (MOR 10S/T-A) [51]; these mutations prevent the phosphorylation of the MOR c-tail by kinases. These animals show blunted opioid tolerance development to pain in the hot plate assay after exposure to chronic opioids using a subcutaneous osmotic pump [51]. This demonstrates the importance of receptor phosphorylation for developing opioid tolerance after chronic treatment.

Furthermore, downstream of PLCβ signaling, PKC activation directly phosphorylates the c-tail of MOR [52]. There is evidence that PKC is involved in the desensitization of MOR by morphine activation [53, 95]. One study determined that morphine induces rapid desensitization of MOR by PKC in rat locus coeruleus [53]. Another study supporting the idea that PKC has a role in the induction of opioid tolerance used kinase inhibitors *in vivo* and *ex vivo* to determine that PKC inhibition reverses meperidine, morphine, and fentanyl-induced acute tolerance [55].

On the other hand, GRKs phosphorylate MOR and induce G-protein-mediated recruitment of β-arrestin proteins and receptor internalization and desensitization [59]. Inhibition of GRK only blunted the tolerance stimulated by DAMGO but not by morphine [55]. β-arrestin2 knock-out mice show a blunted tolerance to opioid-induced antinociception in the hot plate assay using a subcutaneous morphine pellet implanted for 3 days [63]. Based on these observations and our studies, we hypothesize that gallein could act via either blockade of Gβγ regulation of the PLC/PKC pathway and GRK by gallein.

Since opioid tolerance is a major limitation in the treatment of chronic pain using MOR agonists, an important goal to achieve is to either prevent long-term opioid tolerance or retain agonist effectiveness in the treatment of pain. Thus, in this study, we explored possible underlying molecular mechanisms that drive gallein's effects in the development of opioid tolerance and the ability to potentiate morphine-induced antinociception in an opioid-tolerant state. First, to determine if PLCβ3 signaling influences development of opioid tolerance, a PLCβ3 global knock-out mouse line was used. We observed that the morphine dose-dependent response between genotypes on day 1 is not different between KO and WT genotypes (figure 5A, B). These results diverge from previously published data showing that acute morphine treatment is more potent in PLCβ3 KO mice [23], which could be due to differences in the route of gallein and morphine administration or physiological compensatory mechanisms. Then, we further demonstrated that PLCβ3 KO animals develop morphine tolerance similarly to WT animals arguing that PLCβ3 is not involved in chronic tolerance development (figure 5). Interestingly, in PLCβ3 KO animals, treatment with gallein did not further potentiate opioid-induced antinociception in a tolerant state compared with the vehicle-treated group (figure 5C). These results suggest that PLCβ3 signaling is crucial for gallein's potentiation of opioid antinociception

in a tolerant state but not for gallein-dependent prevention of the development of opioid tolerance. Additionally, gallein binding to Gβγ disrupts the interactions between Gβγ-GRK [73, 132]. In previous studies, the physiological importance of Gβγ-GRK inhibition was determined using gallein in a heart failure study [133] and in osteoarthritic animals [132]. Treatment of gallein in heart failure models improved cardiac function and decreased cardiac hypertrophy [133]. The proposed model for how gallein decreased heart failure hypertrophy is through the blockade of Gβγ-GRK signaling, preventing desensitization of β-adrenergic and α2-adrenergic receptors triggered by elevated sympathetic feedback during heart failure. Since evidence suggests that gallein influences signaling of both Gβγ-PLCβ3 and Gβγ-GRK2/3 downstream of MOR activation, this might indicate that gallein is acting through a dual-inhibition effect on these two pathways. This could explain all gallein's effects on opioid tolerance observed in this study; this hypothesis will be further examined in the future by dissecting these effects in opioid tolerance following kinase inhibition (GRK2/3 and PKC).

Another future direction for this study is to determine these interactions in female mice, the development of tolerance using other MOR agonists with different potency-efficacy profiles, different pain states, and the potential influence of gallein in kinase signaling (signaling biasing). Other molecular mechanisms that could influence opioid tolerance are the cellular adaptations by $G\alpha_i$ signaling, prolonged suppression of cAMP, and disruption of adenylate cyclase (AC)-cAMP-protein kinase A signaling cascade[59, 121].

In summary, our study utilizes gallein as a pharmacological adjunct to morphine chronic administration to determine if targeting Gβγ signaling is a potential novel therapeutic approach for preventing or alleviating opioid tolerance. Our results allude to two different strategies for improving opioid treatment for pain using inhibition of selective Gβγ-signaling pathways. First,

gallein blocked the development of morphine antinociceptive tolerance in a model of chronic opioid administration. Secondly, gallein increases the potency of morphine in alleviating pain in a system that has developed tolerance retaining the same efficiency at higher doses creating opioid-sparing effects. From a molecular perspective, this study highlights the differential roles of PLCβ3 activation and potentially GRK in tolerance and antinociception. The most common way to refer to tolerance is the need to increase the dosage to meet the same effect that was experienced at the start of treatment, and this study shows that cotreatment and pretreatment of gallein to morphine prevents the necessity to increase treatment dosage. This study highlights the benefits of small molecule therapy in opioid pain treatment by contributing to the safe use of opioids as analgesics.

3.5 Figures

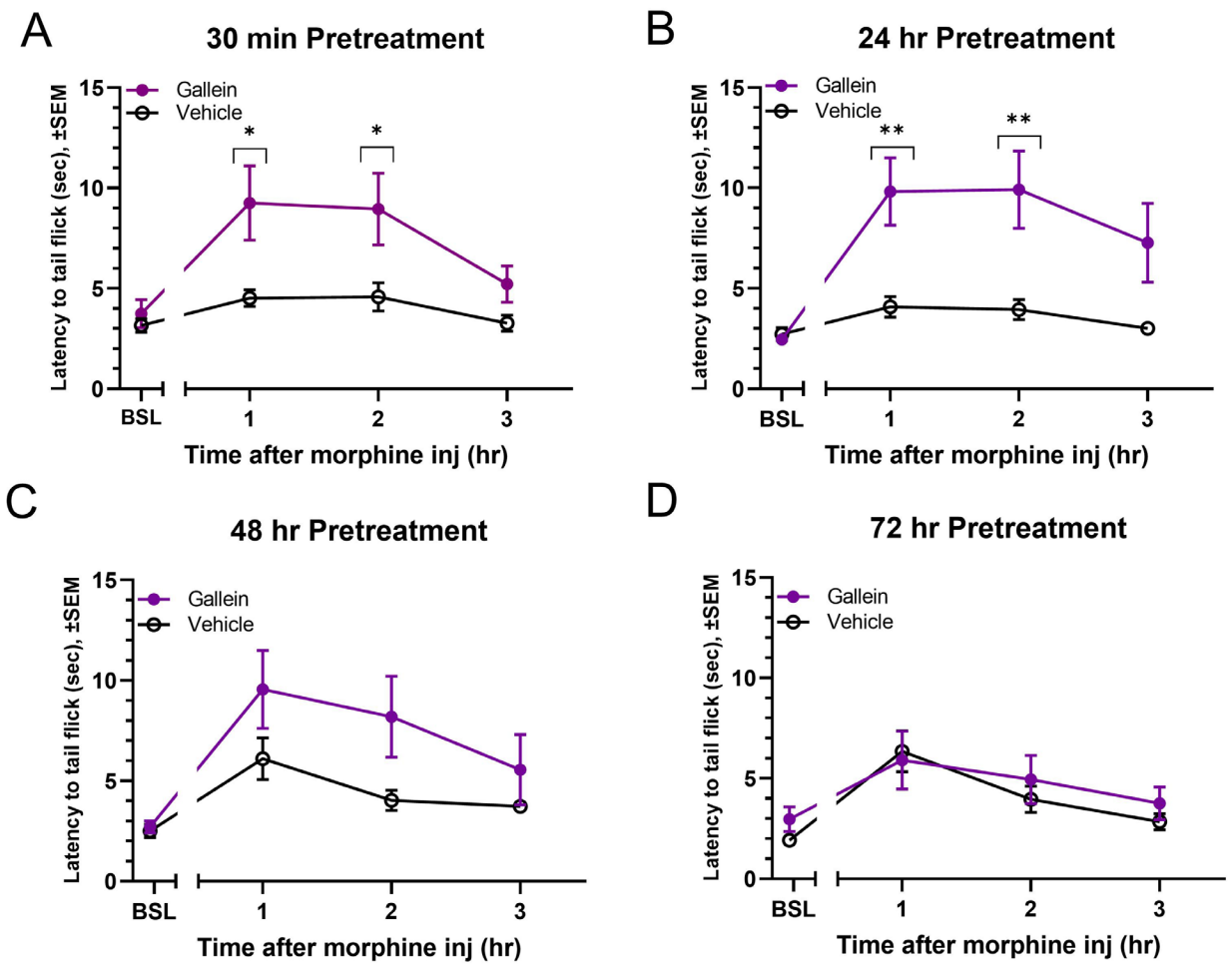

Figure 11. Gallein produces prolonged potentiating effects in morphine-induced antinociception.

A single dose of gallein 100 mg/kg i.p. is tested in a morphine (3.2mg/kg, i.p.) time course for 3 hr. The antinociceptive effects of morphine were enhanced following gallein treatment given at 30 min and 24 hr, but not at 48 or 72 hr, prior to morphine. **A.** With the 30 min gallein pretreatment, there is a significant interaction of time X gallein pretreatment ($F(3,33)=3.34$, $P=0.031$) and significant main effects of time ($F(3,33)=10.42$, $P<0.0001$) and gallein pretreatment ($F(1,11)=5.10$, $P=0.045$). **B.** At 24 hr pretreatment, there is a significant interaction ($F(3,39)=5.16$, $P=0.0042$), and significant main effects of time ($F(3,3)=10.39$ $P<0.0001$), and gallein pretreatment ($F(1,13)=8.03$, $P=0.0141$). **C.** At 48 hr pretreatment, there is no significant interaction ($F(3,39)=2.36$, $P=0.09$) and no significance to gallein treatment ($F(1,13)=2.299$, $P=0.02$), but the main effect of time is significant ($F(3,39)=14.88$, $P<0.0001$). **D.** Similarly, at 72 hr pretreatment, there is no significant interaction ($F(3,42)=0.696$, $P=0.56$) or main effect of gallein treatment ($F(1,14)=0.39$, $P=0.54$), but there is a significant main effect of time ($F(3,42)$

=14.05, P<0.0001). Significant Sidak posthoc multiple comparison are indicated by P*<0.05, P**<0.005.

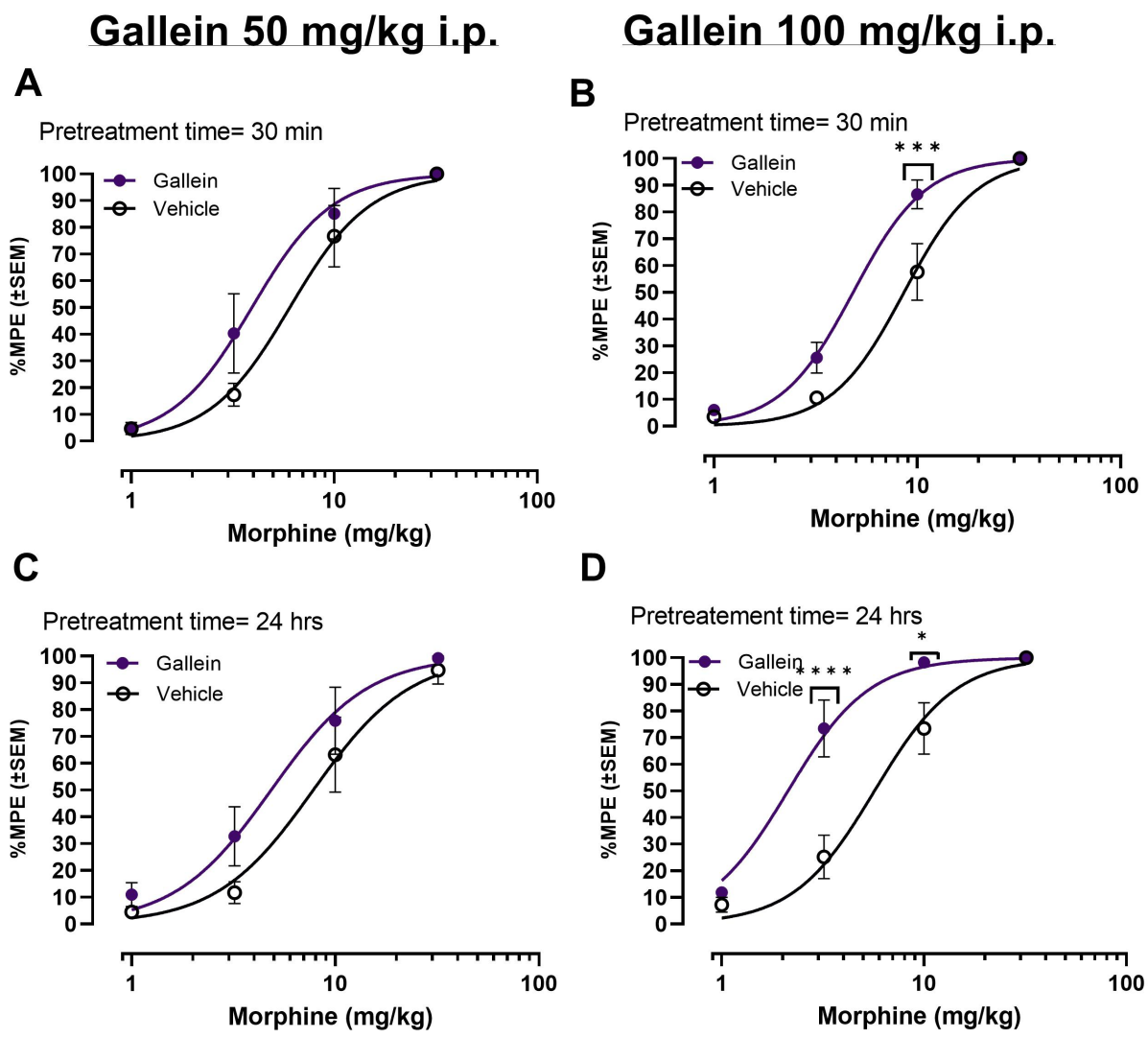

Figure 12. Dose-dependent gallein-induced potentiation with 30 min and 24 hr pretreatment.

A. C. Pretreatment with 50mg/kg gallein i.p. did not potentiate the effects of morphine at either time point tested (30 min and 24hr). No significant interaction between morphine dose X gallein pretreatment at 30 min F (3,30) =1.13, P=0.35 or 24 hr F(3,30)=0.665, P=0.58, but the effect of morphine dose is significant at 30 min F(3,30)=75.93, P<0.001 and at 24 hr F(3,30)=80.04, P<0.001. **B. D**. However, pretreatment of 100 mg/kg gallein significantly potentiated the effects of morphine when it was given as a 30 min and 24 hr pretreatment. Pretreatment of 100 mg/kg gallein 30 min prior to a morphine dose effect curve showed a significant interaction morphine dose X gallein treatment (F (3,33) =4.52, P=0.009) and significant main effects of gallein pretreatment (F (1,11) =9.53, P=0.010) and morphine dose (F (3,33) =202.1, P<0.0001). Pretreatment of 100 mg/kg gallein 24 hr prior to a morphine dose effect curve showed significant interaction morphine dose X gallein pretreatment (F (3,42) =8.61, P=0.0001) and significant main effects of gallein pretreatment (F (1,14) =12.78, P=0.003)and morphine dose (F (3,42)

=116.3, P<0.0001). Significant Sidak posthoc multiple comparison are indicated by P*<0.05 , P***<0.0005, P****<0.00005.

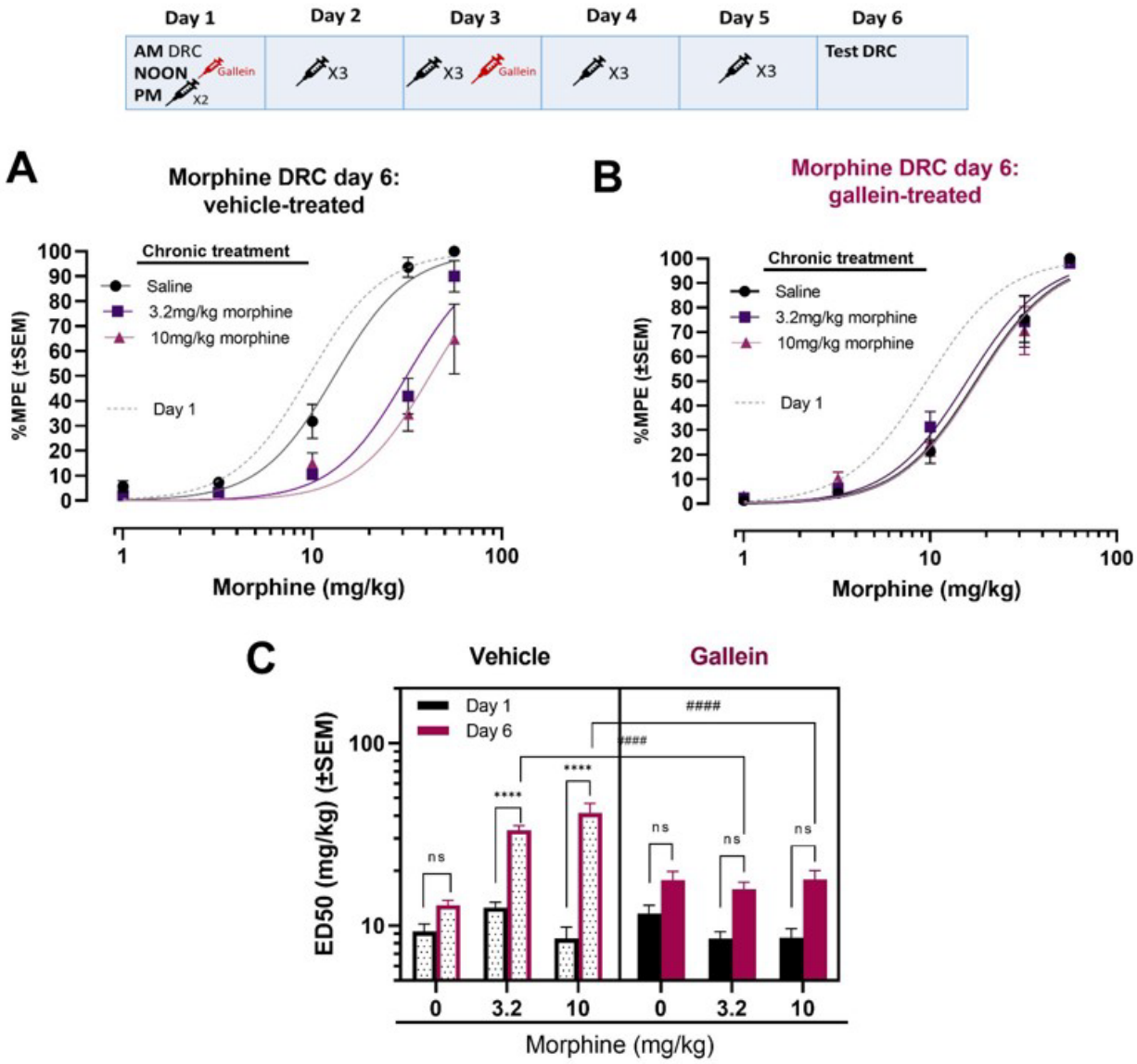

Figure 13. Treatment of gallein decreases the development of opioid tolerance.

A. Repeated administration of 3.2 mg/kg and 10 mg/kg morphine produces rightward shifts in the morphine dose effect curve (DRC) evaluated on day 6. **B**. Gallein 50mg/kg, i.p. on day 1 and day 3 of tolerance development decreases the rightward shift after repeated morphine treatment in the morphine dose response observed on day 6. **C**. The decrease in opioid tolerance by gallein treatment is reflected by comparing ED_{50} values generated on day 1 and day 6 on each repeated morphine treatment. There is a significant 3-way interaction between, +/- galleinXchronic treatmentday ($F(2,60) = 9.87$, $P=0.0002$), a significant interaction between +/- galleinXchronic treatment ($F(2,60)=16.91$, $P<0.0001$), a significant interaction of chronic treatmentXday ($F(2,60)=15.03$, $P<0.0001$), and a significant interaction between +/- galleinXday ($F(1,60)=22.93$, $P<0.0001$). Significant Tukey's posthoc multiple comparison are indicated by P*<0.05, P***<0.0005, P****, P####<0.00005.

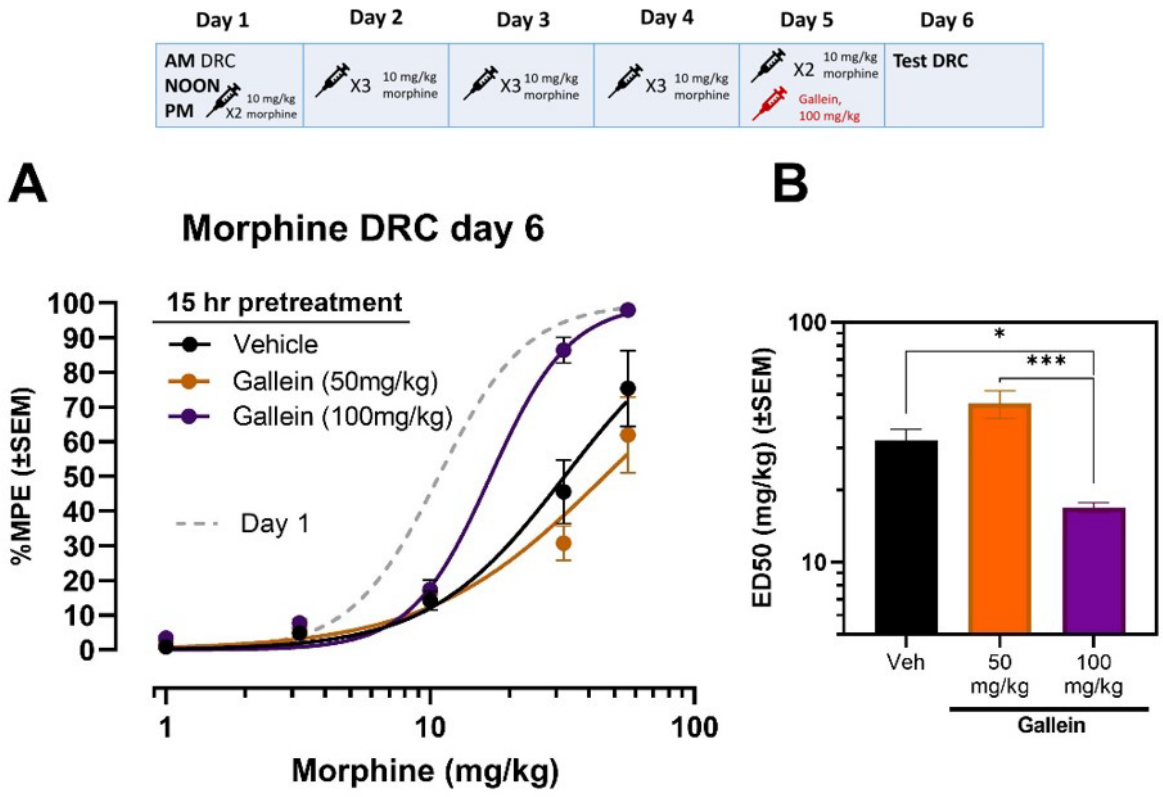

Figure 14. Gallein potentiates morphine-induced antinociception in opioid-tolerant mice.

A dose of gallein 100 mg/kg i.p. replaces the last dose of morphine on day 5 in the evening. **A.** On test day, gallein treatment shifts the dose-response curve of morphine to the right compared to vehicle treatment, and 50 mg/kg i.p. gallein dose on day 5 is insufficient to potentiate morphine antinociception. When day 6 morphine DRC is compared between pretreatments, there is a significant interaction between morphine dose and pretreatment $F(8,64) = 10.14$, $P<0.0001$ with significant main effect of morphine dose $F(4,64) = 193.2$, $P<0.0001$ and significant main effect of pretreatment $F(2,16) = 9.76$, $P=0.0017$. **B.** When ED50 values generated by day 6 morphine DRC are compared between pretreatments, 100 mg/kg gallein significantly reduced the day 6 ED50 value compared with vehicle or 50 mg/kg gallein with significant main effect of treatment $F(2, 16) = 14.22$, $P=0.0003$. Significant Sidak posthoc multiple comparison are indicated by P*<0.05, ***P<0.0005. P****<0.00004. 2-way ANOVA for dose-response curves, one-way ANOVA for ED50 values.

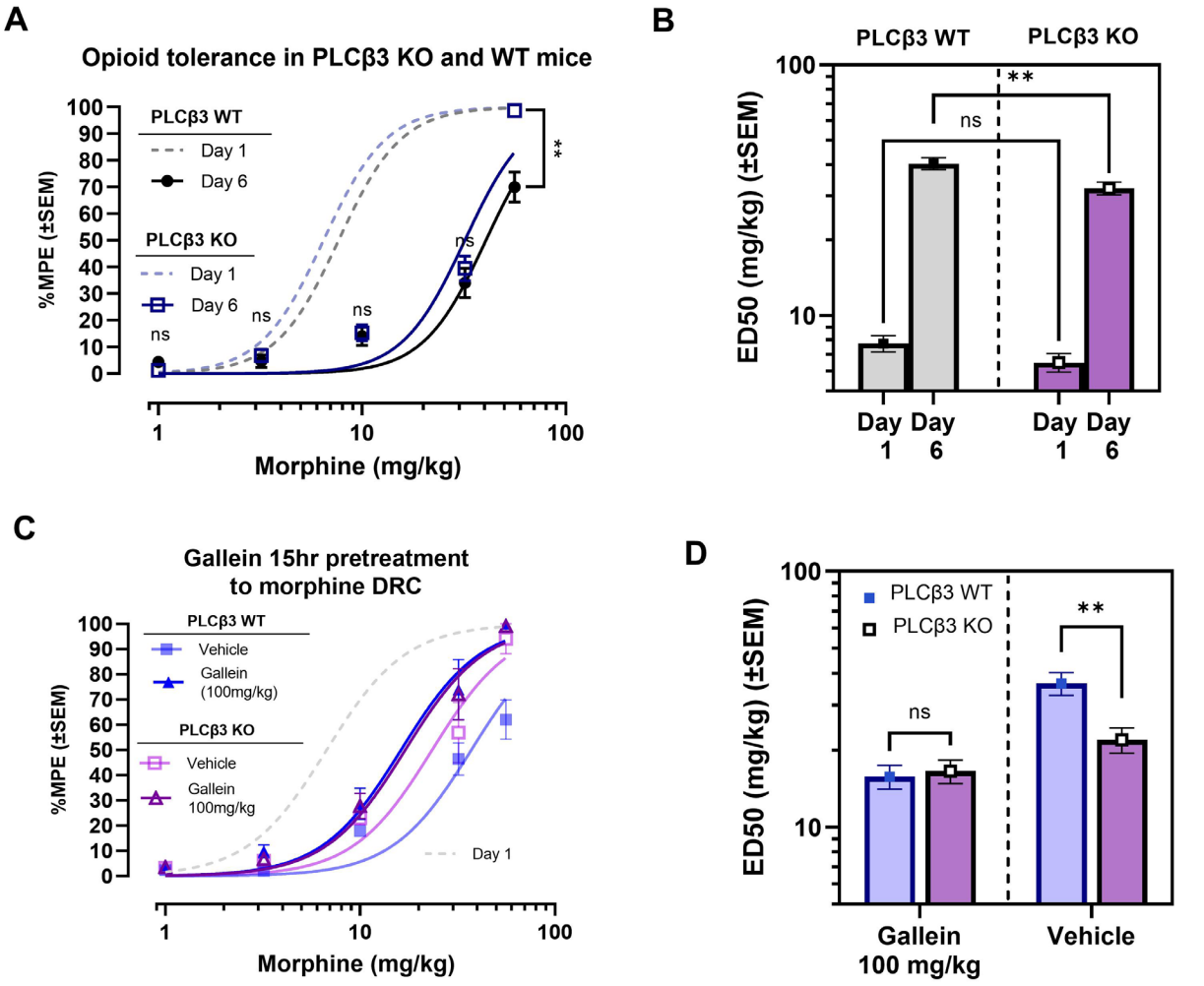

Figure 15. PLCβ3 is needed for gallein-potentiation in opioid tolerant state.

A. Repeated administration of 10 mg/kg morphine was tested in PLCβ3 KO mice and WT littermates to test development of opioid tolerance. Day 1 and day 6 morphine DRC show a similar development of opioid tolerance by day 6 throughout morphine doses tested except for the highest dose (56 mg/kg). There is a significant 3-way interaction between morphine dose X genotype X day tested $F(4, 40) = 3.076$, $P=0.0267$, but no significant interaction between morphine dose X genotype $F(4, 40) = 2.039$, $P=0.1072$ nor genotype X Day tested $F(1, 10) =1.049$, $P=0.33$. **B**. By comparing the ED50 values generated by morphine DRCs show a significant difference on day 6 in between genotypes with a significant interaction genotype X day tested $F(1, 20) =5.37$, $P= 0.0313$. **C.** By comparing 15 hr pretreatment of 100 mg/kg gallein between PLCb3 KO and WT littermates, gallein pretreatment does not have an effect compared to vehicle. However, gallein treatment shifted day 6 morphine DRC to the right compared with vehicle in WT littermates with significant 3 way interaction dose of morphine X genotype X

pretreatment F (4, 72) =3.26, P=0.016 and genotype X pretreatment F (1, 18) =13.02, P=0.002, but no significant interaction between dose of morphine X genotype F (4, 72) =1.46, P=0.224, nor dose of morphine X pretreatment F (4, 72) =1.34, P= 0.264. **D.** ED50 values collected from day 6 morphine DRC show that there is a significant difference between pretreatment in PLCb3 WT, but not in PLCb3 KO with significant interaction between pretreatment X genotype F (1, 19) = 9.97, P=0.005 with main effect of genotype F (1, 19)= 8.024, P=0.0106. Significant Sidak posthoc multiple comparison are indicated by P*<0.05, **P<0.005.

Chapter 4 Discussion

4.1 Significance

By improving pharmacological treatment to reduce pain and minimize the adverse effects of current therapies will enhance human health and well-being. This thesis proposes an approach to enhance opioid analgesia by targeting the G-protein Gβγ subunit after MOR activation, bypassing the receptor, and guiding downstream signaling using small molecules. The overall hypothesis is that biasing Gβγ signaling using small molecules preserves interactions between Gβγ and effectors that promote MOR-depedent antinociception but blocks molecular pathways that negatively regulate opioid signaling. The small molecule gallein has been used as a tool to manipulate Gβγ signaling in different cellular and in vivo models across different systems to investigate Gβγ molecular pathways and its use to improve pathophysiological systems like cardiovascular hypertrophy, inflammation, cancer, and opioid antinociception. In this body of work, we 1) investigate the activation of PLCβ by MOR in different translational models of MOR signaling and 2) determine the impact of gallein treatment on the development of MOR agonist tolerance in vivo.

First, we aimed to determine a molecular mechanism in which activation of MOR leads to PLCβ activation in cellular, ex vivo, and in vivo models. Gallein's effect in MOR agonist potentiation was presumed to be partially dependent on the blockade of Gβγ-PLCβ based on in vivo testing of PLCβ3 knock-out models. However, the molecular mechanism of MOR-dependent PLCβ activation was not defined. Our findings showed that MOR signaling alone could not activate PLCβ through Gβγ, but MOR signaling needs coincident activation of Gαq

signaling to activate PLCβ. These results were followed by ex vivo and in vivo testing of selective inhibition of PLCβ activation by Gβγ or $G\alpha_q$ signaling, which improved both MOR-dependent GABA release inhibition and in vivo antinociception.

Secondly, we explored how treatment with gallein modified tolerance development to the antinociceptive effects of morphine and uncovered a molecular mechanism through Gβγ-PLCβ inhibition. Our data demonstrate that gallein treatment during repeated opioid administration decreases opioid tolerance, and gallein pretreatment improves antinociception in an opioid-tolerant state. But, Gβγ-PLβ3 inhibition by gallein only accounted for the potentiation of opioid antinociception in a tolerant state. Overall, our studies augment the significance of further exploring the manipulation of Gβγ-signaling as an approach to improve opioid pain relief and help overcome unwanted opioid effects.

4.2 Coactivation of MOR and $G\alpha_q$-coupled receptor results in synergistic PLCβ activation and modulates opioid signaling in antinociception.

In chapter 2, we investigate the mechanism for activation of PLCβ downstream of MOR activation. Since MOR is a $G\alpha_i$-coupled receptor, it is proposed that MOR-induced PLCβ activation is dependent on the Gβγ subunit, but there was no concrete evidence that Gβγ-dependent activation of PLCβ occurs downstream of MOR in a cellular context. We showed that MOR-dependent activation of PLCβ requires synergistic activation of Gβγ and $G\alpha_q$ signaling across cellular, ex vivo, and in vivo models of MOR activation. For cellular MOR-dependent activation of PLCβ, a fluorescent biosensor that measures DAG production downstream of PLCβ activity was used in HEK293 cells. The results showed that MOR activation alone does not activate PLCβ, but activation of MOR paired with weak $G\alpha_q$ activation via M1 muscarinic receptor stimulation– and other $G\alpha_q$-coupled receptors in HEK293 cells— leads to robust

synergistic activation of PLCβ. We then tested whether this synergistic activation of PLCβ applies to more intact physiological systems including in ex vivo slice preparations and in vivo models of opioid antinociception. Selective inhibitors were used to block either Gβγ signaling or Gαq signaling in PAG-GABAergic synapses and in an in vivo model of morphine antinociception. In the PAG slice preparation, using gallein or a $G\alpha_q$-inhibitor increased inhibition of MOR-dependent GABA release using subeffective concentration of DAMGO. Also, using PAG tissue lacking PLCβ3, we showed that gallein could not further potentiate inhibition of MOR-dependent GABA release supporting that gallein works through the inhibition of Gβγ-PLCβ signaling in this model. To test our molecular model of PLCβ synergistic activation by Gβγ and Gαq in vivo, we used different pretreatments with gallein or $G\alpha_q$-inhibitor with a low dose of morphine. Our results showed that inhibition of either Gβγ or $G\alpha_q$ signaling increases morphine antinociception over time. Previous in vivo studies showed that PLCβ signaling opposes opioid antinociception, and this study uncovers an underlying mechanism in which PLCβ needs coincident activation of MOR and $G\alpha_q$ to oppose opioid signaling.

This study contributes to the main hypothesis by determining a molecular mechanism in which blockade of Gβγ interactions and PLCβ with gallein improves the antinociceptive response of opioid agonists like morphine. From a translational point of view, this study showed that manipulating Gβγ-signaling through different molecular and physiological elements of opioid signaling could be beneficial in treating pain. This work –in collaboration with Dr. Susan Ingram, who performed the ex vivo experiments – resulted in a publication in the journal of Molecular Pharmacology in 2022 [129].

4.2.1 Future directions and alternative strategies.

The findings of this chapter bring insight into the value of understanding how targeting Gβγ signaling using small molecules could be applied to opioid antinociception. This study demonstrates how MOR activation leads to PLCβ signaling, but we still do not fully understand how PLCβ feedback inhibits MOR signaling. We hypothesize that downstream activation of PKC might be the principal contributor to PLCβ-dependent opioid opposition. We aim to explore the PLCβ-dependent activation of PKC in a similar approach to Sanchez et al. 2022, in which we explore PKC activation of MOR-dependent PLCβ signaling and how it impacts opioid signaling in cellular, ex vivo, and vivo models. But there is the possibility that other molecular mechanisms downstream of PLCβ contribute to the opposition of opioid signaling. Besides PKC-dependent MOR desensitization, PLCβ activation leads to IP_3-dependent intracellular Ca^{2+} increase that could alter Ca^{2+}-dependent molecular mechanisms [134, 135], and PIP_2 depletion of the plasma membrane disturbs GIRK function [33]. Teasing apart how the PLCβ3 enzymatic reaction affects MOR signaling could expand the understanding of how PLCβ modulation could improve opioid antinociception. In Sanchez et al. 2022, it was determined that the blockade of $G\alpha_q$ signaling increased in vivo opioid antinociception, so this could create another pharmacological target. Antagonizing an unidentified $G\alpha_q$-coupled receptor that tonically stimulates PLCβ in the PAG or other modulatory pain pathways could augment MOR agonist antinociceptive response.

4.2.2 Other in vivo models of opioid antinociception.

The in vivo assay used to test opioid antinociception throughout these studies is WWTW. The tail-flick response is dependent on a reflex-arc-response at the level of the spinal column, but at the same time, supraspinal descending modulatory inputs to the spinal cord impact periphery nociception. There is a possibility that Gβγ-signaling regulation by gallein not only

targets PAG descending modulatory inputs by MOR activation but could also be acting at the level of the spinal afferent neurons. Gallein might be increasing opioid-antinociception by improving signaling by opioid receptors located on DRG. The WWTW has limitations to understanding the circuitry involved in gallein's morphine-potentiating effects. Pharmacological use of opioid agonists that are peripherally restricted could allow us to separate supraspinal descending inputs from spinal responses for gallein's mechanism of action.

Other in vivo assays use withdrawal behavior to respond to nociceptive stimuli to measure opioid-dependent antinociception such as hot-plate, von Frey or nitroglycerin-induced hyperalgesia. Testing gallein pretreatment in other antinociceptive models could identify limitations of gallein as a therapeutic molecule in the opioid treatment of pain and identify gallein's effect on other states of sensory hypersensitivity. Lastly, further pharmacological exploration of gallein pretreatment in combination with other opioid agonists with different efficacies and potencies will provide more insight into how biasing Gβγ-signaling using small molecules is most effective in potentiating opioid antinociception.

4.2.3 Gαq-coupled receptor screening in PAG.

Studies from Sanchez et al. 2020 suggest that targeting $G\alpha_q$ signaling could improve opioid antinociceptive response, yet receptors mediating tonic $G\alpha_q$ activation in the PAG need to be identified. Antagonizing a $G\alpha_q$-coupled receptor that tonically opposes opioid response in the PAG would be a possible strategy to enhance opioid potency. To address this possibility, we screened for GPCRs expressed in the PAG to find $G\alpha_q$-coupled receptors that might synergize with MOR to activate PLCβ3. RNA samples were extracted from mouse PAG tested in a qPCR microarray to screen for more than 350 human GPCRs. Table 2 shows a list of class B and C GPCRs expressed in the PAG based on our screen, the α-subunit it couples to, and the threshold

cycle (CT values above 20 were taken into consideration) as a quantitative measure. The $G\alpha_q$-receptors identified on this initial screen are bolded in the table. The next steps to follow based on these data is to test presynaptic opioid response in combination with antagonist for each of the Gαq-coupled receptors of interest, then follow it up with in vivo assessment of opioid antinociceptive response using antagonists or inverse agonists of that receptor. Using this approach would allow for indirectly targeting the endogenous opioid system through $G\alpha_q$-PLCβ regulation instead of using small molecules that target Gβγ-signaling. Another interesting experimental route is to use these same techniques to test if there is an upregulation of $G\alpha_q$-receptors in the PAG after animals experience chronic pain and test if inhibition of those receptors improves acute or chronic antinociception.

	Gene name	Receptor name	G-protein Coupling	CT value
1	GRM7-Hs00356067_m1	glutamate receptor, metabotropic 7	$G\alpha_i$	29.366
2	**HCRTR1-Hs00173513_m1**	**hypocretin (orexin) receptor 1**	**$G\alpha_q$ or** $G\alpha_i$	**30.440**
3	SSTR2-Hs00265624_s1	somatostatin receptor 2	$G\alpha_i$	32.554
4	ADORA1-Hs00181231_m1	adenosine A1 receptor	$G\alpha_i$	33.131
5	GPR3-Hs00270991_s1	G protein-coupled receptor 3	$G\alpha_s$	33.132
6	**NMBR-Hs00159627_m1**	**neuromedin B receptor**	**$G\alpha_q$**	**33.310**
7	**HTR2A-Hs00167241_m1**	**5-hydroxytryptamine (serotonin) receptor 2A**	**$G\alpha_q$**	**33.686**
8	ADRA2A-Hs00265081_s1	adrenergic, alpha-2A-, receptor	$G\alpha_i$	34.205
9	GPR27-Hs00251809_s1	G protein-coupled receptor 27	$G\alpha_s$	34.286
10	OPRM1-Hs00168570_m1	opioid receptor, mu 1	$G\alpha_i$	34.933
11	DRD5-Hs00361234_s1	dopamine receptor D5	$G\alpha_s$	34.983
12	**NTSR2-Hs00173858_m1**	**neurotensin receptor 2**	**$G\alpha_q$**	**35.231**
13	HTR5A-Hs00225153_m1	5-hydroxytryptamine (serotonin) receptor 5A	$G\alpha_i$	35.691
14	GALR1-Hs00175668_m1	galanin receptor 1	$G\alpha_i$	35.697
15	**EDG2-Hs00173500_m1**	**endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2 LPAR2**	**$G\alpha_q$ or** $G\alpha_i$	**37.056**
16	ADRB1-Hs00265096_s1	adrenergic, beta-1-, receptor	$G\alpha_s$	38.197
17	DRD2-Hs00241436_m1	dopamine receptor D2	$G\alpha_i$	39.744

Table 1. GPCRs expressed in mouse PAG.

GPCRs expressed in mouse PAG based on qPCR-microarray GPCR screening. Each row contains GPCR's gene and receptor name, the α-subunit it couples to, and the CT value based on qPCR analysis. The $G\alpha_q$-receptors identified on this initial screen are bolded in the table.

4.3 Biasing Gβγ-signaling by small molecule gallein decreases development of opioid tolerance and improves antinociception in opioid-tolerant state.

Data chapter 3 investigates the impact of gallein treatment in the development of opioid tolerance after chronic morphine administration. In previous studies, gallein pretreatment has potentiated morphine's antinociceptive response in acute behavioral models based on tail-flick response. We hypothesized that gallein treatment during chronic administration with morphine would improve the antinociceptive response by attenuating opioid tolerance. We tested gallein treatment during a paradigm of 5-day repeated administration of morphine to measure opioid tolerance development. In a separate experiment, we tested gallein pretreatment to morphine in an opioid-tolerant state produced by repeated administration of morphine. Our studies showed that gallein treatment not only decreases MOR agonist tolerance developed at the end of chronic morphine treatment but also potentiates morphine antinociception in an opioid-tolerant state. To tease apart the molecular mechanism for gallein's effect on opioid tolerance, we determined that PLCβ3 is necessary for gallein to potentiate morphine antinociception in a tolerant state and has a smaller implication in the development of opioid tolerance, meaning that there are other signaling mechanisms driving the development of opioid tolerance. Overall, these studies show that small molecules like gallein could have a beneficial outcome in decreasing opioid tolerance after chronic treatment and enhancing opioid antinociception even in an opioid-tolerant state.

4.3.1 Future directions and alternative strategies.

Although these studies determined the role of PLCβ in gallein-potentiating effects in an opioid-tolerant state, we have not determined the remaining molecular mechanism for gallein's mechanism of action that decreases opioid tolerance development. We hypothesize that disruption of GRK recruitment by gallein has a role in this effect based on previous evidence of

gallein disrupting Gβγ-GRK interactions and evidence of GRK phosphorylation of MOR c-tail initiates receptor desensitization and internalization. To determine the role of GRK inhibition by gallein in opioid tolerance, we will explore the comparison of treatment with gallein combined with GRK inhibitors or treatment in a knock-out model of GRK in repeated administration of morphine. A biochemical approach to differentiate the phosphorylation of MOR by GRK or PKC could be performed using specific antibodies of phosphorylated MOR. An additional in vivo approach to investigate gallein's influence on kinase function is to utilize phosphorylation-deficient MOR to test gallein in chronic opioid treatment. The 10S/T-A MOR mutation hinders phosphorylation of the receptor's c-tail by kinases. In another section, we will discuss the use of 10S/T-A MOR mice to pinpoint an important piece of gallein's mechanism of action for opioid potentiation.

Gallein's ability to decrease opioid tolerance and increase antinociception in a tolerant state provides an approach that could be used in the clinic to lessen this undesired opioid effect. However, we do not address how a constant state of pain could influence the development of opioid tolerance and if gallein will remain effective in this model. Whether or not gallein improves MOR agonist-induced antinociception in opioid tolerance combined with a state of prolonged pain could solidify the benefit of biasing Gβγ-signaling by small molecules to enhance opioid pain treatment. Another limitation of our in vivo tolerance studies is that the data collected are from experiments testing only male mice. Female mice have different sensitivities to opioid antinociception and opioid tolerance development. In the next section we discuss in vivo sex differences that were observed in some of our studies.

4.3.2 In vivo sex differences were identified throughout studies.

In vivo published data or data presented in this thesis are primarily from male mice. Although data collected from female mice is not presented, we identified sex differences with different pretreatments. In this section, examples of these sex differences will be discussed. First, we found sex differences in the effect of $G\alpha_q$-inhibitor pretreatment on morphine. In data chapter 2, we used a myristoylated peptide that competitively binds to the site of the receptor where $G\alpha_q$ is recruited ($mG\alpha_q$-CT), and it was tested in PAG slices and in vivo antinociception. Pretreatment with 30nmol Myr$G\alpha_q$CT i.c.v. to morphine (3.2 mg/kg) enhanced the antinociceptive effects in male mice but did not have an effect in female mice compared with control pretreatment (A). Secondly, in data chapter 3, we used systemic injection to test gallein's effect on opioid tolerance. Dose-dependent systemic gallein pretreatment had different opioid-potentiating effects in the morphine dose-response curve between males and females. A lower dose of 50 mg/kg gallein 30 min before morphine treatment does not shift the morphine dose-response curve in female mice, but pretreatment in males produces a leftward shift (B). A higher dose of 100 mg/kg gallein used as pretreatment produces a leftward shift in the morphine dose-response curve in both sexes but is more pronounced in male animals (C).

A more extensive study is needed to understand these sex differences better and aim to determine if these differences are dependent on the treatment route of administration, hormonal differences, molecular regulation, or brain circuitry related, and how it could influence the use of small molecules for enhanced opioid antinociception.

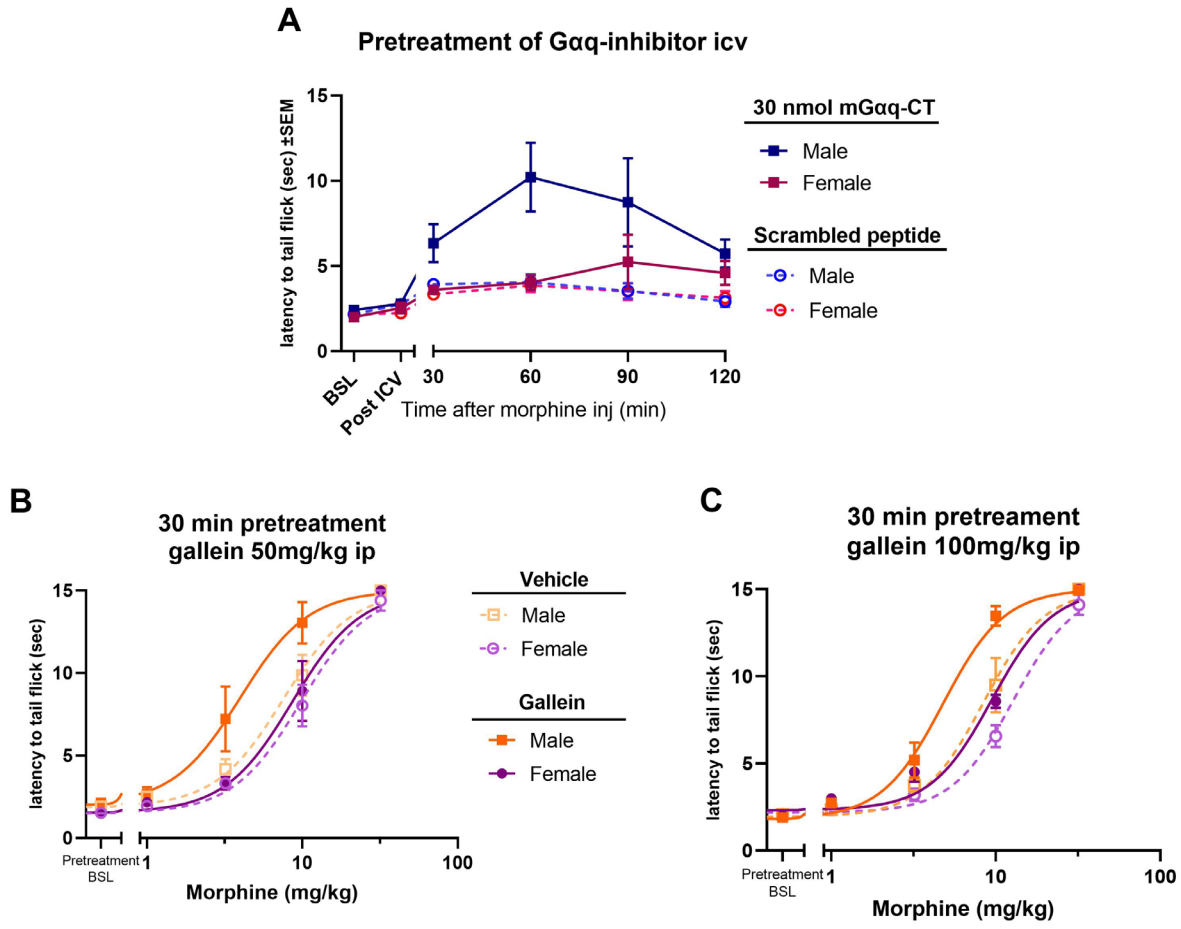

Figure 16. Effects of systemic administration of gallein and centrally administered Gq-inhibitor are different across sexes.

A. i.c.v. pretreatment of Gq-inhibitor was not as effective in females compared with males. Pretreatment of mGaq-CT has a significant effect F (1,14) = 10.25, p=0.0064, and the differences between sexes is also significant F (1,14) = 5.69, p=0.0317. But no significant interaction between pretreatmentXsex F (1,14) = 4.12, p=0.0618, and no 3-way interaction between timeXpretreatmentXsex F (5,70) = 1.942, p=0.0983. **B**. Systemic pretreatment of 50 mg/kg gallein 30 min before morphine DRC is not significantly different from vehicle F (1,16) = 2.8, p=0.113, but differences between sexes are significantly different F (1,16) = 7.714, p=0.0135. But no significant interaction between pretreatmentXsex F (1,16) = 0.9496, p=0.344, and no 3-way interaction between morphine-doseXpretreatmentXsex F (6,96) = 0.945, p=0.4669. **C.** Systemic pretreatment of 100 mg/kg gallein 30 min before morphine DRC is significantly different from vehicle F (1,15) = 7.788, p=0.0137, and differences between sexes are significantly different F (1,15) = 5.615, p=0.0316. But no significant interaction between pretreatmentXsex F (1,15) = 0.175, p=0.6814, and no 3-way interaction between morphine-doseXpretreatmentXsex F (6,90) = 0.973, p=0.4480.

4.4 Next steps to understand gallein's molecular mechanism of action in opioid-potentiation.

Most of the studies with gallein in opioid antinociception have been in relation to Gβγ-PLCβ interactions. Gallein tested in PLCβ3 KO mice and in PAG slices does not enhance opioid response, suggesting that PLCβ3 signaling is necessary for gallein PPI disruption to potentiate opioid antinociception. We predict that this effect is based on preventing PKC activation by gallein. However, gallein has been shown to alter the activation of GRK in a cardiac model of heart failure [133], and the impact that gallein has on GRK activity in a model of opioid response has not been explored. Kinase activity is important for MOR signaling regulation because phosphorylation of the c-tail leads to receptor desensitization. Results from our tolerance experiments suggest that PLCβ signaling is not the only pathway that gallein might be influencing. We hypothesize that gallein might have a dual effect in both regulation of PKC and GRK, and this could be further explored by comparing treatment with PKC and GRK inhibitors to gallein in molecular and electrophysiological models. In vivo testing is challenging because it will require a specific dosing regimen from 2 different pretreatments – gallein and kinase inhibitor—to morphine. To overcome challenges in in vivo dosing and target effects, we began exploring gallein pretreatment in animals with 10S/T-A MOR mutation.

Overall, the necessity to better understand the molecular mechanism of action in which gallein increases opioid antinociception and decreases opioid tolerance is integral to supporting Gβγ-bias signaling as a strategy to advance current therapies for pain treatment. Understanding the pathways that gallein regulate is a significant aim to accomplish because, even if targeting Gβγ is not feasible for human use, targeting pathways inhibited by gallein can lead to other strategies to improve opioid treatment for pain.

4.4.1 Gallein does not potentiate opioid antinociception in 10S/T-A MOR mutation.

Our proposed hypothesis for gallein's opioid-potentiating mechanism of action is due to the interruption of kinase-dependent desensitization of MOR. To test this hypothesis, we used i.c.v. pretreatment with gallein to cumulative doses of morphine in animals with 10S/T-A MOR mutation. We expect that if gallein's opioid-potentiating effects are dependent on kinase activity, then gallein might not be able to further potentiate opioid-antinociception in animals with phosphorylation-deficient MOR. Our results show that 10S/T-A MOR mutation prevented gallein's ability to increase morphine antinociception potency in WWTW. Gallein pretreatment to morphine dose-response curve in WT littermates shifts the curve to the left (A); however, in heterozygous and homozygous 10S/T-A MOR, gallein is ineffective (B,C).

We found sex differences in 10S/T-A MOR males and females responses to morphine and gallein pretreatment. Kliewer et al. reported enhanced opioid antinociception in mice with 10S/T-A MOR mutation but did not report data from female mice with the 10S/T-A MOR mutation for acute opioid-sensitivity. So, we compared opioid treatment between males and females, showing that males have an enhanced response to the treatment of morphine alone compared with females. Although, males and females have different sensitivities to morphine, gallein pretreatment does not enhance morphine-mediated antinociception in both sexes (D). This data suggests that gallein's mechanism of action is dependent on endogenous kinase activity, and through inhibition of PKC and GRK phosphorylation of MOR, it enhances acute and chronic opioid antinociceptive response.

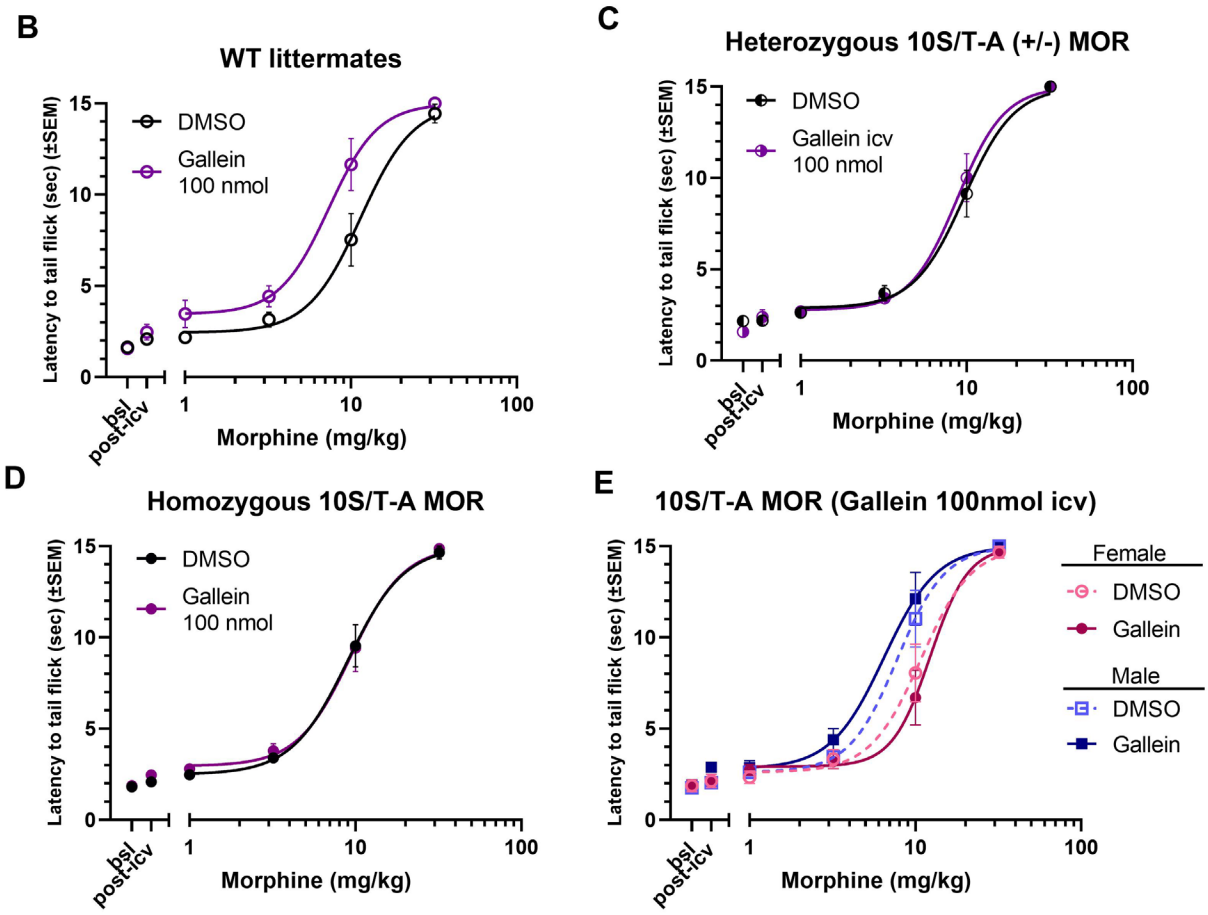

Figure 17. 10S/T-A MOR mutation prevents gallein's opioid-potentiating effects.

Pretreatment of 100nmol gallein i.c.v. or DMSO was tested in 10S/T-A MOR WT littermate, heterozygous and homozygous mutation. Pretreatment +/- gallein was significant in WT littermates $F(1,14) = 6.77$, $p = 0.021$ with significant interaction of pretreatmentXmorphine-dose $F(5,70) = 0.0345$, $p = 0.0345$. However, gallein pretreatment was not significantly different in heterozygous and homozygous 10S/T-A MOR; $F(1,13) = 0.01668$, $p = 0.8992$ and $F(1,22) = 0.248$, $p = 0.6233$, respectively. When 10S/T-A MOR group is separated by sex, pretreatment of gallein remains unsignificant $F(1,20) = 0.229$, $p = 0.637$, but the sex effect is significantly different $F(1,20) = 6.817$, $p = 0.0167$, with significant interaction between sexXmorphine-dose $F(5,100) = 6.255$, $p < 0.0001$, and no 3-way significant interaction pretreamentXsexXmorphine-dose $F(5,100) = 0.5776$.

4.5 Conclusions and closing remarks.

The opioid epidemic in the U.S. has created a medical necessity to improve how pain is treated, focusing on decreasing opioid misuse, tolerance and dependency. The use of opioids is a common way to treat pain in the clinic, but to meet the current necessity for better pain treatment, efforts to decrease unwanted opioid effects such as addiction and tolerance are one major goal of opioid research. Thus, understanding the mechanism of action of gallein that results in enhanced opioid antinociception is an important objective for considering Gβγ-bias signaling as a viable approach for better pain treatment. In addition to understanding gallein's mechanism of action, it is important to produce better small molecules with strong gallein-like effects in opioid antinociception with better potency, fewer unwanted effects, and more bioavailable. In parallel to studies presented in this thesis, the Smrcka, Jutkiewicz, and Ingram laboratories have worked in a collaborative drug discovery project to find the next generation of small molecules that selectively target Gβγ signaling that improve opioid response. This project has consisted of high throughput biochemical screening and medium throughput screening using functional cellular assays followed by a screening in PAG slices and in vivo opioid antinociception to identify refined small molecules that could surpass the use of gallein in the investigation of Gβγ signaling.

In these studies, we determined that targeting G-protein's interaction with designated effectors can have beneficial outcomes; targeting Gβγ downstream of MOR activation allows signaling necessary for opioid antinociception and blocks inhibitory pathways like receptor-desensitization by kinases. We propose that targeting Gβγ signaling in a therapeutic model would improve opioid analgesia acutely and chronically, preventing the development of tolerance.

Biasing Gβγ-signaling could be used in the clinic to treat pain for extended periods of time without losing analgesic efficiency after chronic opioid treatment, and it could help those patients that are already tolerant to opioids avoid the use of large doses of opioids, decreasing chances of respiratory depression. Overall, biasing Gβγ-signaling has the potential to enhance the pain-relieving effects of common opioids used in the clinic and reduce unwanted side effects like tolerance as a strategy to improve opioid treatment for pain.

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