# CHRONIC MORPHINE TREATMENT INDUCES HETEROLOGOUS ALTERATIONS IN ACUTE KINASE-DEPENDENT DESENSITIZATION

By:

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**CERTIFICATE OF APPROVAL**

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# List of Abbreviations:

| | |
|---|---|
| AAV | adeno-associated virus |
| ACSF | artificial cerebrospinal fluid |
| ANOVA | analysis of variance |
| BAPTA | 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid |
| βCNA | β-chlornaltrexamine |
| CMP101 | compound 101 |
| DOR | ∂-opioid receptor |
| exWT | expressed wild type |
| GIRK | G protein-coupled inwardly rectifying potassium channel |
| GPCR | G protein-coupled receptor |
| GRK | G protein-coupled receptor kinase |
| GTP | guanosine 5'-triphosphate |
| JNK | c-Jun N-terminal kinase |
| KO | knock out |
| KOR | κ-opioid receptor |
| LC | locus coeruleus |
| ME | [$Met^5$]enkephalin |
| MOR | μ-opioid receptor |
| MTA | morphine treated animal |
| NMDAR | N-methyl-D-aspartic acid receptor |
| PKC | protein kinase C |
| SST | somatostatin |
| SSTR | somatostatin receptor |
| TPD | total phosphorylation-deficient |
| WT | wild type |

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## Abstract:

Opioids such as morphine are still the most effective analgesics for many types of pain. However, clinical utility of opioids is limited because long-term use leads to analgesic tolerance, such that higher concentrations of opioids are needed to achieve the same analgesic effect. This is problematic since tolerance to the life-threatening respiratory depressive effects of opioids develops slower than to the analgesic effects of opioids. Thus, opioid tolerance increases risk for overdose and death. An enormous amount of work has therefore been devoted to understanding opioid tolerance. The mu-opioid receptor (MOR) is the primary receptor involved in the analgesic effects of opioids and there is increasing evidence that agonist-mediated regulation of MOR in single neurons contributes to analgesic tolerance. Work done in the locus coeruleus (LC) has shown that acute desensitization, or the rapid loss of MOR-effector coupling during sustained agonist exposure, may contribute to long-term analgesic opioid tolerance. In addition, phosphorylation of amino acid residues on the C-terminus of MOR is a necessary step in acute desensitization. Although mutation of all 11 phosphorylation sites on the C-terminal tail of MOR to alanine blocks acute desensitization and cellular tolerance, the kinases involved in both of these processes remained uncertain. Therefore, the aim of this dissertation project was to determine the kinases involved in MOR acute desensitization and cellular tolerance with the ultimate goal of identifying cellular mechanisms that may contribute to analgesic tolerance. To do this, a combination of pharmacological inhibition of specific kinase activity and whole-cell electrophysiology to measure potassium conductance in single LC neurons was used. Acute desensitization was measured in acute brain slices from naïve and morphine treated rats. Based on established measures, an increase in desensitization and/or reduced recovery from desensitization in slices from morphine treated animals was considered indicative of cellular

tolerance. Results showed that the GRK2/3 inhibitor, CMP101, significantly blocked acute desensitization of MOR in LC neurons in slices from naïve animals. Interestingly, CMP101 was not sufficient to block acute desensitization in slices from morphine treated animals. In addition, while PKC or JNK inhibitors (Go6976 and SP600125 respectively) did not block acute desensitization in slices from naïve animals, all three inhibitors in combination nearly abolished acute desensitization in slices from animals treated chronically with morphine. These results indicate that chronic morphine treatment induced an adaptation in the kinase regulation of acute desensitization such that additional kinases, including PKC and JNK, contributed to desensitization. This altered regulation of acute desensitization by PKC and JNK may be one mechanism underlying the augmentation of desensitization and reduced recovery from desensitization seen in animals chronically treated with morphine. Similar results were found when looking at desensitization of the somatostatin receptor in LC neurons, indicating that chronic morphine treatment induces heterologous adaptations in the kinase regulation of desensitization that affect more than just opioid receptors. If this same adaptation occurs in other brain areas involved in analgesia then this may be one mechanism involved in analgesic tolerance. In addition, that chronic morphine treatment affected somatostatin receptor signaling may also have important functional consequences for how chronic morphine treatment affects non-opioid receptors in opioid-sensitive neurons. Future work should investigate the mechanism underlying this PKC- and JNK-dependent cellular tolerance and if it is conserved across cell types, brain regions, effector systems, and for other commonly used opioid agonists such as fentanyl.

# Chapter 1: Introduction

## The Opioid Crisis and Tolerance

Opioids, such as morphine, have been used to treat pain since the mid 1800s and are still the most effective analgesics for many types of pain. However, long-term use of opioids is complicated by the development of analgesic tolerance, such that higher doses of opioids are needed to achieve the same analgesic effect. This requirement for escalating doses of opioids is problematic for multiple reasons. Opioids not only exert pain-relieving effects, but also cause debilitating side effects such as constipation, nausea, and respiratory depression, making long-term opioid use difficult. In spite of these side effects, many opioid users also find it difficult to stop opioid use, due to the rewarding effects of opioids, the withdrawal symptoms that occur upon cessation of opioid treatment, or a combination thereof. Of patients prescribed an opioid, many misuse them and nearly 1 in 10 develop an opioid use disorder (Vowles et al., 2015).

Increasing doses of opioids are also problematic as this increases the risk for overdose. In late 2017, the opioid crisis was declared a public health emergency due to the rapid rise in deaths from opioid overdose (Jalal et al., 2018). The mortality rate has nearly doubled since 2010 despite there being greater public awareness along with measures implemented to combat the opioid epidemic. The CDC reported that more than 47,000 Americans died from opioid overdose in 2017, with 128 people dying every day. Every 13 minutes an American dies from opioid overdose (Hagemeier, 2018).

The main reason that patients die from opioid overdose is not due to general neurological depression as is seen with barbiturate overdose, but instead is due to opioids acting on respiratory neurons to cause respiratory depression. In addition, the development of tolerance to the respiratory depressive effects of opioids is slower than to the analgesic effects of opioids (Levitt

and Williams, 2018; Ling et al., 1989; Paronis and Woods, 1997). Thus, higher doses of opioids increase the risk for overdose and death. Because of this, a major goal of opioid research has been to understand the mechanisms underlying tolerance at both the cellular and behavioral level. A better understanding of opioid tolerance will facilitate the development of safer opioid drugs that reduce side effects such as the development of tolerance.

## The μ-Opioid Receptor

Opioids exert both their therapeutic and deleterious effects by binding to opioid receptors, which are expressed abundantly throughout the central nervous system. There are several types of opioid receptors, each encoded by distinct structural genes, including μ-, κ-, δ-opioid receptors (MOR, KOR, and DOR respectively), as well as the nociceptin receptor. MOR, encoded by OPRM1, is the primary opioid receptor involved in the analgesic action of opioids (Williams et al., 2013). Morphine-induced actions, including analgesia and tolerance, are absent in MOR KO mice (Le Merrer et al., 2009; Matthes et al., 1996). Therefore, in order to elucidate mechanisms underlying opioid tolerance, it is critical that we understand how MOR is regulated under drug naïve and chronic treatment conditions.

MOR, like all opioid receptors, is a G protein-coupled receptor (GPCR). GPCRs are 7 transmembrane receptors that have their N-terminus in the extracellular space and the C-terminus in the intracellular compartment. GPCRs are activated following agonist-binding, which causes a conformational change in the receptor allowing heterotrimeric G-proteins to bind and become activated. G-proteins consist of three components, including the α, β, and γ subunits. Prior to activation, all three G-protein subunits are bound together with a GDP bound to the α subunit. Upon GPCR activation, inactive G-proteins bind to the receptor, allowing a GDP to be replaced by a GTP on the α subunit. This then causes the α subunit, bound to a GTP, and the β and γ

subunits, remaining bound to each other, to dissociate from the receptor to go on to activate downstream effectors (**Figure 1.1, 1.2**). Numerous G-proteins can be activated per agonist-binding event, allowing for amplification of signaling (Gurevich and Gurevich, 2019).

There are several types of α subunits including, Gαi, Gαo, Gαs, Gαq, etc., distinguished by the downstream signaling they transduce. Opioid receptors couple to Gαi and Gαo G-proteins, which are "inhibitory" G-proteins. Activation of Gαi/o G-proteins leads to a decrease in cAMP production due to inhibition of adenylyl cyclase (**Figure 1.1**). This is in opposition to Gαs G-proteins which stimulate cAMP production by activating adenylyl cyclase (Gurevich and Gurevich, 2019).

Gαi/o proteins also go on to initiate mitogen-activated protein kinase (MAPK) cascades, including extracellular regulated kinase (ERK), Jun N-terminal kinase (JNK), and p38 pathways, all of which have many targets including nuclear transcription factors. Gαi/o proteins can also lead to the activation of Src kinases, a family of non-receptor tyrosine kinases (**Figure 1.1**). Gβγ subunits, upon dissociation from the α subunits, go on to affect other downstream effectors,

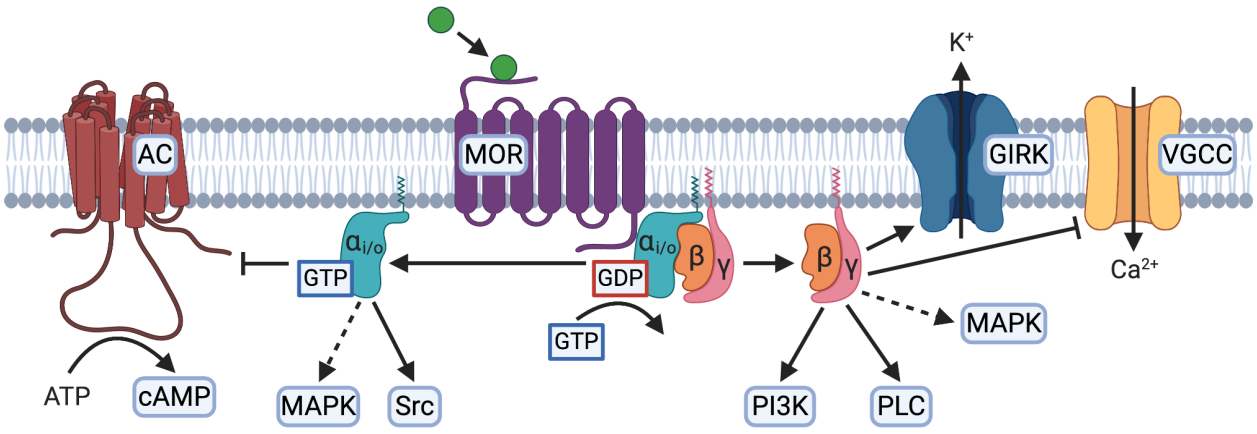


**Figure 1.1: Activation of MOR leads to activation or inhibition of different effectors by activated Gα and/or Gβγ subunits.** Agonist-induced MOR activation leads to activation of G-proteins by replacement of the GDP for a GDTP. Activated Gαi/o G-proteins lead to inhibition of cAMP production and activation of MAPK and Src pathways. Dissociated Gβγ subunits go on to inhibit voltage-gated $Ca^{2+}$ channels (VGCCs), activate G protein-coupled inwardly-rectifying potassium channels (GIRKs), and initiate MAPK, PI3K, and PLC signaling pathways.

including activation of G protein-coupled inwardly-rectifying potassium channels (GIRKs), which hyperpolarizes the cell, and inhibition of voltage-gated calcium channels (VGCCs), which leads to inhibition of transmitter release in presynaptic axon terminals. Gβγ subunits can also initiate signaling pathways that include MAPK, phosphoinositide 3-kinase (PI3K), and phospholipase C (PLC) pathways (**Figure 1.1**; Bailey et al., 2006; Goldsmith et al., 2013; Gurevich and Gurevich, 2019; Narita et al., 2004; Pena et al., 2018; Takeda et al., 1999; Williams et al., 2013; Zhu and Birnbaumer, 1996).

In addition to the agonist-induced activation of G-proteins, agonist-induced MOR activation also leads to phosphorylation of the intracellular region of MOR by G protein-coupled receptor kinases (GRKs, **Figure 1.2**). Phosphorylation by GRKs is thought to require agonist-induced activation of MOR since maximal GRK2/3 recruitment requires free Gβγ and because inactive G-protein binding to receptors may block access to phosphorylation sites (Gurevich and Gurevich, 2019; Stoeber et al., 2020; Williams et al., 2013). Phosphorylation of MOR by GRKs consequently increases the receptor's affinity for arrestins (non-visual β-arrestin1 or β-arrestin2). Arrestins are scaffolding proteins involved in receptor trafficking, by which receptors are internalized via clathrin-coated pits and transported to the endosome, where they are either sent to the lysosome for degradation or recycled back to the plasma membrane (**Figure 1.2**). Although arrestin binding and internalization could presumably turn off certain types of signaling through steric hinderance (e.g. arrestins sterically occluding G-protein activation by MOR) or removal from the membrane (e.g. blocking membrane delimited Gβγ-activation of GIRKs), arrestins can also act as scaffolds to initiate other signaling pathways, including MAPK (**Figure 1.2**), and we now know that GPCRs are capable of signaling from endosomes (Gurevich and Gurevich, 2019; Lobingier and von Zastrow, 2019; Williams et al., 2013).

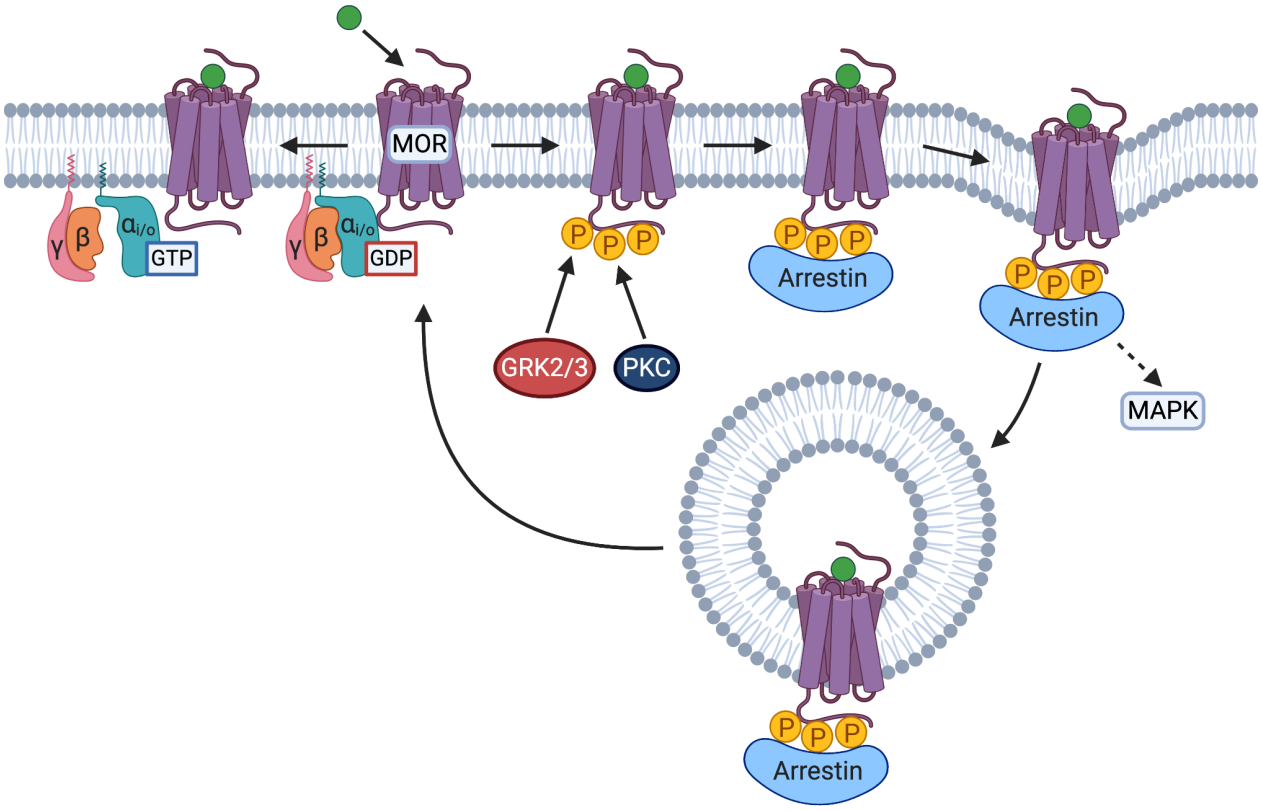


**Figure 1.2: Agonist-induced regulation of MOR.** Agonist-induced activation of MOR leads to phosphorylation of the intracellular region by kinases such as G protein-coupled receptor kinases (GRKs) and protein kinase C (PKC), which increases the receptors affinity for arrestins. Arrestins regulate receptor internalization and act as a scaffold for the initiation of signaling pathways including MAPK cascades. Internalized receptors are either degraded are recycled back to the plasma membrane.

Another early event following activation and phosphorylation of MOR is acute desensitization, or the rapid (seconds to minutes) loss of MOR-effector coupling that occurs during sustained exposure to agonist. Recovery from desensitization occurs in about 40-60 min and is thought to involve dephosphorylation and recycling of functional receptors back to the cell surface (Dang and Williams, 2004; Harris and Williams, 1991; Virk and Williams, 2008; Williams et al., 2013). Acute MOR desensitization may be a critical step in the development of tolerance and has therefore been a major focus of opioid research. However, while considerable study has been devoted to understanding acute MOR desensitization and tolerance, a clear

understanding of the mechanisms underlying these processes is incomplete. In addition, it is likely that opioid tolerance is a complex process that involves various regulatory mechanisms that occur at both the level of individual opioid-responsive neurons as well as at the level of neural circuits (Birdsong and Williams, 2020; Williams et al., 2013).

It is thought that many mechanisms that contribute to tolerance occur by homeostasis, such that opioid-responsive neurons and circuits adapt to the prolonged opioid receptor activation in order to normalize net activity. These homeostatic adaptations may include regulatory processes that directly reduce opioid response/sensitivity, as well as processes that indirectly reduce opioid responses by engaging opposing or compensatory mechanisms and signaling pathways. Upon cessation of opioid use, these adaptations are unmasked and result in many of the withdrawal symptoms experienced by opioid users (e.g. dysphoria, hyperalgesia, and gut hypermotility), underscoring the importance of a complete understanding of these processes (Christie, 2008; Williams et al., 2001; Williams et al., 2013). Examples of potential adaptations identified in the literature that could contribute to opioid tolerance include: increased desensitization, reduced recovery from desensitization, reduced recycling after endocytosis, changes in agonist affinity and potency, and an upregulation of adenylyl cyclase activity after chronic opioids (Birdsong et al., 2015; Birdsong et al., 2013; Dang et al., 2011; Dang and Williams, 2004; Ingram et al., 2008; Ingram et al., 1998; Quillinan et al., 2011; Williams, 2014; Williams et al., 2001; Williams et al., 2013). However, the mechanisms underlying these adaptations are unclear. Other possible adaptations, such as downregulation of MORs or changes in internalization after chronic treatment, have been discounted due to lack of evidence (Quillinan et al., 2011; Trafton and Basbaum, 2004).

## Measuring Tolerance

While an immense amount of study at the behavioral, cellular, and molecular levels has been devoted to understanding opioid tolerance, a complete picture of how receptor-activation leads to tolerance is lacking. Many potential mechanisms that may contribute to tolerance have been identified. However, differences between studies often make comparisons between results problematic, making it difficult to resolve a clear, mechanistic understanding of opioid tolerance. Thus, when measuring opioid tolerance, it is important to consider the level of analysis (e.g. behavioral vs. cellular), brain region, cellular compartment (e.g. soma vs axon terminals), agonist, and the technique that is used (Birdsong and Williams, 2020; Williams et al., 2013).

Several approaches have been applied in order to better understand opioid tolerance, including measuring analgesic tolerance *in vivo* (e.g. rodent models of opioid tolerance) and measuring cellular tolerance in cell lines, primary neuronal cultures, or native neurons in tissue preparations. Each level of analysis offers different advantages and disadvantages and can answer different types of questions.

Analgesic tolerance can be studied in rodent models using two different noxious stimuli, including the hot-plate test (supraspinal nociception) and the warm-water tail-withdrawal test (spinal nociception). For both measures, a decrease in the latency of removal from the respective stimulus after chronic versus acute opioid treatment is indicative of analgesic tolerance (Heinricher and Ingram, 2008). These behavioral studies can provide valuable information on the extent of tolerance that develops as a result of treatment with various opioid agonists as well as how different genetic or pharmacological manipulations can affect analgesic tolerance in whole animals. However, results from these *in* vivo experiments can be hard to interpret since they involve a complex circuit response that could be affected at many sites between activation of

MOR and the motor response. Observed effects could involve mechanisms in opioid-sensitive neurons themselves or indirect effects onto opioid-sensitive neurons, making it impossible to separate circuit-level adaptations from adaptations in single cells. Thus, the underlying cellular and molecular mechanisms occurring in single neurons are difficult to resolve (Birdsong and Williams, 2020; Williams et al., 2013).

While it is clear that analgesic tolerance is a problem at the systems level, studies that showed reduced opioid sensitivity in cultured cells along with studies that identified reduced opioid responsiveness in *ex vivo* preparations from tolerant animals make it clear that opioid tolerance occurs at the cellular level as well (Bailey and Connor, 2005; Christie et al., 1987; Williams et al., 2013). Thus, cellular tolerance refers to cellular-level adaptations that result in decreased MOR-effector coupling after chronic opioid treatment. In contrast to acute desensitization, cellular tolerance occurs over a longer time period of several hours to days or weeks and can last well after drug removal. While it is not clear how cellular tolerance relates to analgesic tolerance, it is evident that cellular level processes contribute to opioid tolerance (Williams et al., 2013). Thus, it is critical to understand mechanisms at the cellular level in order to have a full understanding of opioid tolerance.

To better understand MOR regulatory processes that contribute to tolerance, a lot of work has been done using expression studies in cell lines (i.e. HEK293, AtT20) and primary neuronal cultures. These studies have provided valuable information about how MOR is regulated (i.e. phosphorylation sites, etc.). However, while cultured cells offer some advantages (i.e. agonists readily wash from the preparation), how these results translate to native neurons is still being established since receptor number and concentrations of signaling or regulatory components may differ across cell types. Studies have shown that altering receptor number or amounts of

signaling and regulatory components present in cells can affect opioid signaling, efficacy, and potency (Williams et al., 2013). For example, the phosphorylation and internalization induced by morphine can be enhanced by overexpression of GRKs and arrestins, showing that concentrations of proteins can affect regulatory processes that may contribute to tolerance (Miess et al., 2018; Whistler and von Zastrow, 1998; Zhang et al., 1998).

One main way that MOR signaling, desensitization, and cellular tolerance are measured in native neurons is with whole-cell electrophysiology in intact brain slices from rodents. Three common electrophysiological measures used include activation of potassium current (GIRKs), inhibition of $Ca^{2+}$ current (VGCCs), and inhibition of transmitter release, with activation of potassium current being the most common. Like mentioned above, MORs couple to GIRK channels. When MOR is activated, this activates G-proteins, causing the Gβγ subunit to dissociate and bind to GIRK channels, causing an outward potassium current that can be measured using whole-cell electrophysiology (**Figure 1.1, 2.1A-B**). Therefore, measuring GIRK currents allows for a real-time readout of MOR activation and desensitization in response to agonist. Established measures of cellular tolerance using this technique include: an increase in desensitization, reduced recovery from desensitization, reduced sensitivity to morphine, and a rightward shift in opioid concentration-response curves in acute brain slices from rodents treated chronically with opioids (Birdsong and Williams, 2020; Williams et al., 2013).

In addition, it is also important to consider cell type and brain region when measuring cellular opioid tolerance. Studies have shown that MOR regulatory processes, including internalization and desensitization, can vary across cell types and brain regions. For example, while there is robust desensitization in LC neurons, there is very little desensitization in neurons of the Kölliker-Fuse (KF), a brainstem region that regulates aspects of respiration (Levitt and

Williams, 2018). Therefore, it is possible that different brain regions contribute differentially to opioid tolerance and that mechanisms that contribute to tolerance could differ across cell type and brain region. As mentioned above, one reason mechanisms of MOR regulation may differ depending on cell type/brain region is because of differences in signaling components present in cells (Williams et al., 2013). This highlights the importance of studying processes that could contribute to opioid tolerance in native neurons across various opioid-sensitive brain regions.

In addition to evidence for cell-type and brain-region specific receptor regulation, there is also evidence that regulatory mechanisms can even differ between cellular compartments. MORs that are localized in different parts of the cell (e.g. soma, dendrites, axon terminals) have distinct functional actions. MORs located in the somatodendritic compartment decrease excitability, while those located in the axon terminals inhibit transmitter release (Birdsong and Williams, 2020). In addition, although desensitization can be readily observed in the postsynaptic compartment, acute MOR desensitization to the inhibition of transmitter release is lacking in the presynaptic axon terminals (Blanchet and Luscher, 2002; Fox and Hentges, 2017; Fyfe et al., 2010; Pennock et al., 2012; Pennock and Hentges, 2011; Pennock and Hentges, 2016). However, even though desensitization is not observed in the presynaptic compartment, long-term tolerance to the inhibition of transmitter release does occur (Fyfe et al., 2010; Hack et al., 2003; North and Vitek, 1980; Schulz et al., 1980; Williams et al., 2001). Recent evidence indicates that the lack of apparent presynaptic desensitization results from the fast diffusion of active receptors along the cell surface to release sites, effectively mitigating presynaptic inhibition from loss of functional receptors (Jullie et al., 2020).

Furthermore, in addition to activating signaling at the plasma membrane, GPCRs can also signal from intracellular compartments (e.g. Golgi, nucleus, endosomes). Receptors localized in

the nucleus or Golgi are activated by membrane-permeable agonists or agonists transported inside the cell. Intracellular signaling may also occur at endosomes after agonist-induced internalization. Importantly, activation of intracellular receptors can initiate distinct signaling pathways compared to that of receptors at the cell surface. Various agonists may preferentially activate these different types of signaling depending on their ability to permeate the membrane or induce internalization (Fernandez et al., 2020; Lobingier and von Zastrow, 2019).

Thus, another major consideration when measuring tolerance is the agonist used. Many agonists are used to measure MOR activity, including partial-agonists such as morphine, as well as full agonists such as: the highly potent synthetic opioid fentanyl, the high specificity synthetic opioid peptide [D-Ala$^2$, N-MePhe$^4$,Gly-ol]enkephalin (DAMGO), and the more promiscuous endogenous opioid peptide [Met]$^5$enkephalin (ME). Agonists can differ in their pharmacokinetic/ pharmacodynamic properties as well as their efficacy, or ability to induce a response. Within a system, full agonists cause a maximal response with partial agonists only producing a sub-maximal response (Fernandez et al., 2020, Williams et al., 2013). Evidence suggests that low-efficacy agonists (e.g. morphine) produce more pronounced analgesic tolerance, as well as larger rightward shifts in concentration-response curves, than high-efficacy agonists (e.g. fentanyl, Christie et al., 1987; Duttaroy and Yoburn, 1995; Grecksch et al., 2006; Sosnowski and Yaksh, 1990; Walker and Young, 2001). However, another important consideration is that full agonists only need to activate a small portion of the total receptor population in order to produce a maximal response, while partial agonists must occupy a larger fraction of the total receptor population to exert their maximal response. This phenomenon of receptor reserve (or spare receptors) can vary between cell types and for different signaling pathways and thus can also affect measures of opioid signaling (Fernandez et al., 2020; Williams et al., 2013).

There is also a lot of evidence for biased agonism in the sense that various agonists differentially activate signaling cascades and regulatory mechanisms, possibly through differential coupling to signaling proteins (e.g. G-proteins, arrestins, GRKs). For example, morphine induces less phosphorylation and internalization than full agonists, such as DAMGO or ME (Miess et al., 2018; Williams et al., 2013). There is also evidence that different MOR agonists preferentially activate Gαi versus Gαo G-proteins (McPherson et al., 2018). Numerous studies have also indicated that different agonists result in differential regulation by kinases (Chu et al., 2010; Johnson et al., 2006; Kliewer et al., 2019; Kuhar et al., 2015; Melief et al., 2010; Yousuf et al., 2015). Therefore, mechanisms underlying tolerance may differ between agonists. Although it has been difficult in some cases to determine whether agonists differ in their efficacy for signaling generally or if different agonists induce different types of signaling (Gillis et al., 2020), it is clear that the agonist used can affect measures of MOR regulation and tolerance.

Given the differences highlighted above, the technique used to measure opioid tolerance is also very important since various techniques will allow you to measure at distinct levels (e.g. behavioral vs cellular assays) and in different cellular compartments (e.g. postsynaptic vs presynaptic terminal). In addition, assay conditions, such as model used (e.g. rat vs mouse vs cell line), cell type examined, response measured (e.g. GIRK currents vs inhibition of cAMP production), chosen dosing paradigm, and agonist used (e.g. partial vs full agonist) may also affect observed results. Measures of tolerance may differ depending on the effector examined. In addition, the time course of the response measured can affect interpretation of results. While activation of GIRK currents is fast and allows for reliable measures of desensitization in isolation from some regulatory processes, other measures, such as phosphorylation of MAPK endpoints or inhibition of adenylyl cyclase, are slower and may include multiple processes that could include

desensitization, internalization, recovery from desensitization, and even recycling, confounding results (Williams et al., 2013).

Thus, in order to determine cellular-based mechanisms that contribute to opioid tolerance, experiments in this study were conducted at the cellular level by using whole-cell electrophysiology to measure real-time ME-induced activation of potassium conductance in the soma of single locus coeruleus (LC) neurons in acute brain slices from naïve and morphine treated rats. This technique allows for measurement of acute desensitization separate from other processes. In addition, animals were treated with morphine using a protocol known to induce robust tolerance (80 mg/kg/day for 6-7 days using osmotic pumps; Arttamangkul et al., 2018). Lastly, a bulk of the studies looking at acute MOR desensitization have been conducted in the LC. Although the LC is likely not involved in analgesia, it contains homogenous MOR expression without expression of most other opioid receptors (i.e. DORs, KORs), making it an ideal area to study MOR regulation (Williams et al., 2013).

## Phosphorylation, Acute Desensitization, and Tolerance

### C-terminal Phosphorylation Sites

As mentioned above, there is increasing evidence that acute MOR desensitization is an early step leading to long-term tolerance to opioids. In addition, it is widely accepted that phosphorylation of residues in the intracellular region of MOR by kinases such as GRKs is a necessary step in acute desensitization (Williams et al., 2013). There are about 20 potential phosphorylation sites on the intracellular loops and C-terminal tail of MOR that could contribute to MOR desensitization (**Figure 1.3**; Connor et al., 2004; Koch and Hollt, 2008). Site-directed mutagenesis of residues, phosphosite-specific antibodies, and quantitative mass spectrometric analysis of phosphorylated residues were used to determine the relative importance of these

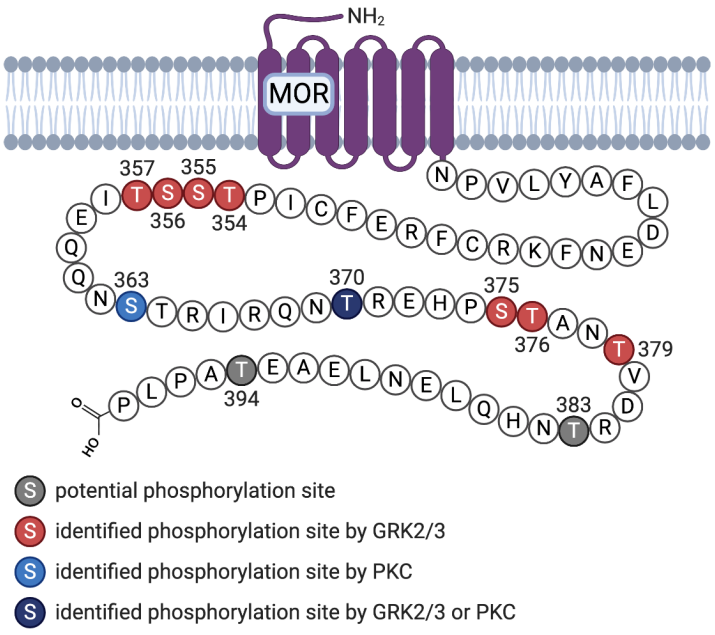


**Figure 1.3: Potential and Identified Phosphorylation Sites on the C-terminus of MOR.** Agonist-induced activation of the μ-opioid receptor (MOR) results in phosphorylation of sites on the C-terminus. Potential phosphorylation sites are shaded gray, phosphorylation sites identified to be phosphorylated by GRK2/3 are shown in red, phosphorylation sites identified to be phosphorylated by PKC are shown in light blue, and phosphorylation sites identified to be phosphorylated by either GRK2/3 or PKC are shown in navy (Williams et al., 2013; Just et al., 2013).

residues in MOR regulation. These studies have shown that high-efficacy agonists, such as DAMGO and fentanyl, very efficiently and rapidly (less than 2 min at 37°C) phosphorylate residues on the C-terminal region of MOR (Thr370 through Thr379), while low-efficacy agonists, such as morphine, phosphorylate the same residues less efficiently (Doll et al., 2011; Lau et al., 2011; Miess et al., 2018; Mouledous et al., 2015; Schulz et al., 2004). However, morphine-induced phosphorylation could be enhanced by overexpression of GRK2/3 (Doll et al., 2011; Miess et al., 2018; Zhang et al., 1998).

These studies, along with studies measuring pan-phosphorylation induced by high-efficacy agonists, have established two main clusters, amino acid residues 354 to 357 (TSST) and 375 to 379 (STANT), that are phosphorylated by GRK2/3 following agonist application (**Figure 1.3**; Chen et al., 2013; Doll et al., 2011; Just et al., 2013; Lau et al., 2011; Miess et al., 2018; Wang, 2000; Wang et al., 2002). In addition, mutation of the STANT cluster, or even Ser375 alone, prevented or reduced the rate and extent of phosphorylation at other C-terminal residues (Doll et al., 2011; Just et al., 2013; Miess et al., 2018). This indicates a hierarchical pattern of phosphorylation where phosphorylation of Ser375 facilitates further phosphorylation

at other residues. Mutation of the TSST cluster also slightly reduced the rate of phosphorylation at other residues, further demonstrating the interconnectivity of the C-terminal phosphorylation sites (Miess et al., 2018).

In addition to the two main clusters mentioned above, there are four additional phosphorylation sites (Ser363, Thr370, Thr383, and Thr394), constituting 11 total phosphorylation sites on the C-terminal tail of MOR (**Figure 1.3**; Williams et al., 2013). Mutational studies have implicated Thr394 in agonist-induced phosphorylation of MOR (Deng et al., 2000; Wolf et al., 1999). In transfected HEK293 cells, Ser363 was constitutively phosphorylated with no significant increase by opioid agonists (Doll et al., 2011). In addition, Ser363 could be phosphorylated by PKC (**Figure 1.3**; Chen et al., 2013; Feng et al., 2011) and constitutive Ser363 phosphorylation required ongoing PKC activity (Illing et al., 2014). Thr370 could also be phosphorylated by stimulation of PKC activity in addition to DAMGO-induced phosphorylation and GRK2/3 activity (**Figure 1.3**; Doll et al., 2011; Doll et al., 2012; Illing et al., 2014; Just et al., 2013). One study also showed that Thr370 could be phosphorylated by CAMKII (Chen et al., 2013).

More recently, studies using phosphorylation-deficient mutant MORs have established that phosphorylation at all 11 sites contributes to acute desensitization and long-term tolerance. Acute desensitization was reduced for phosphorylation-deficient STANT mutants expressed in rat LC neurons, AtT20 cells, and HEK293 cells (Arttamangkul et al., 2019; Birdsong et al., 2015; Miess et al., 2018; Yousuf et al., 2015). Phosphorylation-deficient MORs in which all 11 phosphorylation sites are mutated to alanine nearly abolish acute desensitization in ATt20 cells and LC neurons in tissue from both naïve and morphine treated animals (Arttamangkul et al., 2018; Kliewer et al., 2019; Yousuf et al., 2015). In addition, analgesic tolerance was greatly

reduced for knockin mice expressing phosphorylation-deficient MORs (10S/T-A and 11S/T-A, Kliewer et al., 2019). Thus, measures of acute desensitization, cellular tolerance, and analgesic tolerance are all reduced by mutation of MOR C-terminal phosphorylation sites, indicating that phosphorylation of the C-terminus of MOR is critical for acute MOR desensitization and long-term tolerance. However, how each of the two main phosphorylation site clusters, TSST and STANT, as well as the 4 additional phosphorylation sites on the C-terminus differentially contribute to acute desensitization and tolerance is unclear. Thus, this study examined acute desensitization and two separate measures of cellular tolerance for mutant MORs, with alanine or glutamate mutation of different C-terminal phosphorylation sites, virally expressed in the LC of MOR KO rats.

**Phosphorylation by GRKs**

In addition, the kinase(s) underlying phosphorylation-dependent acute desensitization and tolerance remain poorly understood. Phosphorylation of C-termini by kinases like GRKs has profound effects on MOR function, regulating receptor internalization, desensitization, and agonist affinity (Birdsong et al., 2015; Williams et al., 2013). Indeed, the canonical mechanism for acute MOR desensitization is through GRK activity (Gurevich and Gurevich, 2019). Consistent with this, many studies have shown that acute MOR desensitization is attenuated when GRK function is disrupted. Nonvisual GRKs consist of two subfamilies, GRK2/3 and GRK4/5/6 (Gurevich et al., 2012). Phosphorylation by mostly GRK2 and GRK3 has been implicated in acute MOR desensitization *in vitro* (Johnson et al., 2006; Kovoor et al., 1998; Li and Wang, 2001; Zhang et al., 1998), with some evidence for the involvement of GRK5 (Doll et al., 2012; Just et al., 2013). In addition, viral expression of a dominant negative GRK2 in LC neurons reduced DAMGO-induced acute desensitization (Bailey et al., 2009b; but see Quillinan

et al., 2011). More recently, acute MOR desensitization by multiple opioid agonists was blocked in LC neurons by the GRK2/3 inhibitor, CMP101 (Lowe et al., 2015). In addition, one measure of chronic morphine-induced cellular tolerance to the activation of GIRK conductance in mouse LC neurons (i.e. reduced recovery from desensitization) was reduced by GRK2 inhibition (Dang et al., 2011; Quillinan et al., 2011). Analgesic tolerance was also reduced for some opioid agonists in GRK3 knockout mice (Melief et al., 2010; Terman et al., 2004). Resolving the relative contributions of GRK2 and GRK3 to desensitization and tolerance have been difficult, however, since the GRK2 knockout mouse is embryonic lethal (Peppel et al., 1997).

**Phosphorylation by PKC**

In addition to phosphorylation by GRKs, there is also evidence that MORs, or MOR signaling components, are also phosphorylated by non-GRK kinases, including PKC, JNK, CAMKII, and MAPK (Chen et al., 2013; Koch and Hollt, 2008; Liu and Anand, 2001; Schmidt et al., 2000), which may contribute to both desensitization and tolerance. As mentioned above, studies using phosphosite-specifc antibodies and mass spectrometry showed that MOR can be directly phosphorylated by PKC at Ser363 and Thr370 *in* vitro (Chen et al., 2013; Doll et al., 2011; Feng et al., 2011). In addition, inhibition of PKC in HEK293 cells decreased the basal level of MOR phosphorylation, indicating that MOR is pre-phosphorylated by PKC instead of in response to agonist activation (Johnson et al., 2006). However, while MOR may be phosphorylated directly by PKC, it is unknown if other kinases act directly on MOR or whether they act sequentially. It is possible that kinases phosphorylate other non-kinase proteins involved in MOR desensitization (Williams et al., 2013).

There is a lot of evidence for the involvement of PKC in mechanisms of acute desensitization and tolerance that may be agonist-dependent. Morphine- but not DAMGO- or

ME-induced MOR desensitization was reduced by inhibition of PKC in HEK293 and ATt20 cells (Johnson et al., 2006; Yousuf et al., 2015). In addition, PKC activation was necessary for morphine-induced (but not DAMGO) MOR desensitization in HEK293 cells (Chu et al., 2010). Activation of PKC activity also enhanced acute desensitization induced by ME and morphine as well as short- and long-term cellular tolerance to morphine in LC neurons (Arttamangkul et al., 2015; Arttamangkul et al., 2018; Bailey et al., 2004; Bailey et al., 2009a; Bailey et al., 2006). Another study in the LC showed that following chronic morphine treatment, one component of acute desensitization involved PKC, but another was insensitive to inhibition of PKC or JNK (Levitt and Williams, 2012). *In vivo* studies showed that morphine-induced tolerance can be reduced with administration of PKC inhibitors or elimination of PKC through mutagenesis (Bohn et al., 2002; Granados-Soto et al., 2000; Hua et al., 2002; Hull et al., 2010; Inoue and Ueda, 2000; Newton et al., 2007; Smith et al., 2003; Smith et al., 1999). In addition, it appears that ongoing PKC activity is necessary to maintain morphine tolerance since PKC inhibition reversed morphine-induced analgesic tolerance even after it developed and cellular tolerance was reversed with a PKC inhibitor in LC neurons (Bailey et al., 2009a; Granados-Soto et al., 2000; Smith et al., 1999).

**Phosphorylation by JNK**

Other studies have indicated that JNK activity may be involved in MOR regulation. Morphine-induced acute MOR desensitization in the dorsal root ganglion was mediated by JNK (Mittal et al., 2012). In addition, while short-term analgesic tolerance induced by fentanyl administration was GRK3/arrestin-dependent, short-term analgesic tolerance following morphine administration involved JNK2 (Kuhar et al., 2015; Melief et al., 2010). Intrathecal injections of a

JNK inhibitor throughout chronic morphine treatment also reduced long-term analgesic tolerance to morphine (Chen et al., 2008).

**Phosphorylation by Other Kinases**

There is also some evidence for alternative mechanisms of acute desensitization involving other kinases, including CaMKII and ERK1/2. In *X. laevis* oocytes expressing mutants of a putative CaMKII phosphorylation site in the third intracellular loop of MOR (S261A/S266A), the rate of DAMGO-induced desensitization was slowed (Koch et al., 1997). In heterologous expression systems, MAPK inhibition reduced agonist-induced activation of MAPK as well as agonist-induced phosphorylation and desensitization of MOR (Polakiewicz et al., 1998; Schmidt et al., 2000). In LC neurons, ME-induced desensitization could be mediated by at least two separate mechanisms, one involving GRK2-β-arrestin2 and the other involving ERK1/2 activity (Dang et al., 2009). However, there are conflicting reports on whether ERK1/2 contributes to measures of morphine tolerance. Inhibition of ERK activity *in vivo* did not alter short- or long-term analgesic tolerance to morphine (Mouledous, et al., 2007; Melief et al., 2010; but see Chen et al., 2008). One study even showed enhanced morphine tolerance after disruption of ERK1/2 signaling in the PAG (Macey et al., 2009). Therefore, how ERK activity contributes to acute desensitization and tolerance is still undefined. Little is known about the involvement of other sites on the intracellular loops of MOR or other kinases in acute desensitization and tolerance as well (Williams et al., 2013).

Together, these studies suggest that GRKs, along with non-GRK kinases, may contribute to mechanisms of acute desensitization and tolerance. Indeed, chronic morphine induced increases in the phosphorylation of MAPKs, including p38-, ERK-, and JNK-IR in L4 DRG neurons (Chen et al., 2008). Acute desensitization in LC neurons is augmented and the recovery

from desensitization is prolonged in slices taken from morphine treated animals (Arttamangkul et al., 2018; Dang and Christie, 2012; Dang and Williams, 2004; Dang and Williams, 2005; Levitt and Williams, 2012; Quillinan et al., 2011). The mechanism that underlies this augmentation is not understood but regulation of MOR by additional kinases may be one mechanism underlying this measure of cellular tolerance. The present study sought to determine the kinases involved in acute desensitization and tolerance using a combination of whole-cell electrophysiology and pharmacological inhibition of select kinase activity.

## Acute Desensitization, Internalization, and Tolerance

As mentioned above, phosphorylation of the C-terminal tail of MOR by GRKs increases the receptors affinity for arrestins, leading to receptor internalization. Studies looking at disruption of arrestin translocation and internalization in cultured cells have further indicated that phosphorylation of Ser375 is a crucial residue for arrestin recruitment and internalization (El Kouhen et al., 2001; Williams et al., 2013). Similar results were reported using a S375A mutant (Schulz et al., 2004). Additional studies found that mutations of Thr376 and Ser379 GRK phosphorylation sites also blocked arrestin recruitment and internalization, suggesting that multi-site phosphorylation of the $^{375}$STANT$^{379}$ cluster is required for efficient receptor internalization (Just et al., 2013; Lau et al., 2011). Recent studies validated this result, showing that arrestin recruitment and internalization measured with a BRET assay in HEK293 cells was blocked for a phosphorylation-deficient STANT mutant (Miess et al., 2018). In addition, arrestin recruitment and internalization is reduced or eliminated with inhibition of GRK2/3 activity by either siRNA or CMP101 in HEK293 cells (Doll et al., 2012; Lowe et al., 2015; Miess et al., 2018). Inhibition of MAPK activity has also been shown to reduce internalization (Polakiewicz et al., 1998; Schmidt et al., 2000). However, although it is clear that phosphorylation of the STANT cluster

by GRK2/3 is necessary for efficient arrestin recruitment and internalization, it should be noted that there is evidence that some GPCRs can recruit arrestins in the absence of phosphorylation (Gurevich and Gurevich, 2019).

Although receptor phosphorylation is known to be a key initial step for both acute desensitization and internalization, making it tempting to link the two processes, it has been established that they are separate processes. Robust acute desensitization precedes internalization, with acute desensitization saturating in about 3-5 min and internalization reaching a steady state in about 30 minutes (Arttamangkul et al., 2008; Arttamangkul et al., 2006; Borgland et al., 2003; Johnson et al., 2006; Law et al., 2000; Tanowitz et al., 2008; Tanowitz and von Zastrow, 2003). Acute desensitization also proceeds when internalization is blocked (Arttamangkul et al., 2006; Dang et al., 2011; Johnson et al., 2005). In addition, it was also thought that receptor internalization is necessary for resensitization of MORs. However, we now know that dephosphorylation and recovery from desensitization of MOR do not require receptor internalization (Dang and Christie, 2012). Upon agonist removal, dephosphorylation of Ser375 and Thr370 and recovery from desensitization was rapid at the cell surface when internalization was blocked regardless of the agonist used (Arttamangkul et al., 2006; Dang et al., 2011; Doll et al., 2011; Doll et al., 2012; Quillinan et al., 2011).

Although desensitization and internalization are not mechanistically or functionally equivalent, it is still unclear for MOR whether phosphorylation itself desensitizes receptor signaling or whether it causes desensitization by initiating arrestin binding. Arrestin binding presumably blocks G-protein binding through steric hinderance and has a similar time course to acute desensitization (saturating in about 3-5 minutes; McPherson et al., 2010; Molinari et al., 2010; Oakley et al., 2000). However, it is also possible that phosphorylation induces a receptor

conformation that is inaccessible to G-proteins. A clear understanding has been difficult due to an inability to measure arrestin-dependent processes electrophysiologically and contradictions between studies. Some of the studies looking at phosphorylation and involvement of GRK2/3 mentioned above have also reported that acute desensitization is attenuated under conditions where arrestin binding is disrupted or absent (Williams et al., 2013). For example, in gene expression studies in *X. laevis* oocytes, acute MOR desensitization required coexpression of both GRK3 and β-arrestin2 (Kovoor et al., 1997). Desensitization was also blocked in mouse embryonic fibroblast (MEF) cells from β-arrestin2 or β-arrestin1 and 2 knockout mice (Chu et al., 2008). However, there are other studies opposing these findings that show desensitization persists when arrestin activity is reduced or eliminated. Acute desensitization of MOR coupling to VGCCs in sensory neurons and GIRKs in LC neurons was unaffected in the β-arrestin2 knockout (Arttamangkul et al., 2008; Walwyn et al., 2007).

In addition, it is also unclear how or if arrestin binding and internalization contribute to cellular or analgesic tolerance. One measure of cellular tolerance (shift in concentration response curve) to the inhibition of $Ca^{2+}$ currents in PAG neurons was not observed in tissue from β-arrestin2 KO mice treated chronically with morphine (Connor et al., 2015). Another measure of cellular tolerance (reduced recovery from desensitization) was absent in LC neurons in slices from β-arrestin2 KO mice treated chronically with morphine (Dang et al., 2011, Quillinan et al., 2011). However, interpretation of recovery rates in the β-arrestin2 KO is complicated since β-arrestin2 could contribute to both desensitization and receptor recycling. It is possible that blocking internalization allows receptors to resensitize faster at the membrane. Indeed, dephosphorylation at the plasma membrane is rapid when internalization is blocked (Doll et al., 2011). Analgesic tolerance, as well as tolerance to DAMGO-induced GTPγS binding, was

reduced in β-arrestin2 KO mice (Bohn et al., 2000; Bohn et al., 2002). However, morphine-induced tolerance has also been shown to be attenuated under conditions where internalization is enhanced (Kim et al., 2008). It is unknown how enhancing internalization may lessen tolerance. It is possible that removal from the membrane turns off certain types of signaling, limiting induction of adaptations that lead to tolerance. It is unclear whether agonists that robustly induce arrestin binding and internalization produce less tolerance than agonists that weakly recruit arrestin and induce internalization when matched for intrinsic efficacy. Further complicating interpretation, while arrestin could contribute to MOR-uncoupling through steric hinderance or removal of MORs from the membrane through internalization, it could also contribute to long-term tolerance through induction of signaling via its scaffolding function or through MOR signaling from the endosome (Lobingier and von Zastrow, 2019; Williams et al., 2013).

Thus, more work is required to determine the role of arrestins in acute desensitization and/or tolerance. Many recent studies where acute desensitization or cellular tolerance was blocked used phosphorylation-deficient and therefore G-protein biased receptors incapable of efficiently binding arrestin, making it impossible to separate effects due to phosphorylation versus arrestin binding and internalization (Arttamangkul et al., 2018; Kliewer et al., 2019). Determining the kinases involved in arrestin binding/internalization versus desensitization may offer some mechanistic insights. In order to gain a clearer picture of events leading to the development of opioid tolerance, this study aimed to investigate the kinases involved in both MOR internalization and acute desensitization before and after chronic morphine treatment.

## Heterologous Effects

Acute desensitization and tolerance can be homologous, being restricted to MOR or MOR signaling itself, as has been described above. However, acute desensitization and tolerance

can also be heterologous, affecting other receptors/signaling cascade components. Other GPCRs, such as Gαi-coupled α2-adrenergic or somatostatin receptors, share signaling cascade components with MOR (Williams et al., 2013). Therefore, it is of great interest whether acute or chronic application of opioids can affect the signaling of such GPCRs. It is still unclear whether mechanisms that contribute to opioid tolerance affect not only MOR regulation but also the regulation of other receptors in opioid-sensitive neurons.

Opioid-induced acute desensitization has been observed to be homologous (Bailey et al., 2004; Bailey et al., 2009b; Dang et al., 2011; Dang et al., 2009; Harris and Williams, 1991). Loss of function after chronic opioid treatment was also restricted to MOR with no decrease at other GPCRs that couple to the same effectors (Bailey et al., 2009a; Christie et al., 1987; Connor et al., 1999), indicating that desensitization and tolerance mechanisms occur independently of GIRKs. However, heterologous desensitization of α2-adrenergic receptor-dependent currents after MOR activation has been reported in LC neurons (Blanchet and Luscher, 2002; Blanchet et al., 2003; Dang et al., 2012; Llorente et al., 2012). There is also evidence that acute desensitization of MOR results in heterologous desensitization of the somatostatin receptor (SSTR, Fiorillo and Williams, 1996; Yousuf et al., 2015). Heterologous short-term tolerance has also been reported in some cell types (Tan et al., 2003; Tan et al., 2009; Walwyn et al., 2006). However, mechanisms underlying possible heterologous desensitization and tolerance are unknown. Some evidence implicates PKC in mechanisms of heterologous desensitization (Chu et al., 2010; Yousuf et al. 2015). Mechanisms involving β-arrestin2, ERK1/2, and Src have also been implicated in heterologous desensitization (Dang et al., 2012). Thus, it is still unclear if and how acute MOR desensitization and tolerance heterologously desensitize other GPCR signaling.

In addition to MORs, LC neurons also contain somatostatin receptors (SSTRs, SSTR2) that couple to the same population of GIRK channels (Fiorillo and Williams, 1996). Similar to MOR, agonist-induced activation of SSTRs leads to phosphorylation of the intracellular region of the receptor by kinases including GRKs and PKC (**Figure 1.4**). In addition, truncation experiments indicated that phosphorylation of sites on the C-terminus of SSTR contributes to acute homologous SSTR desensitization (Cole and Schindler, 2000; Gunther et al., 2018). Therefore, this study also sought to determine whether acute or chronic activation of MOR reduced somatostatin-induced activation of SSTRs.

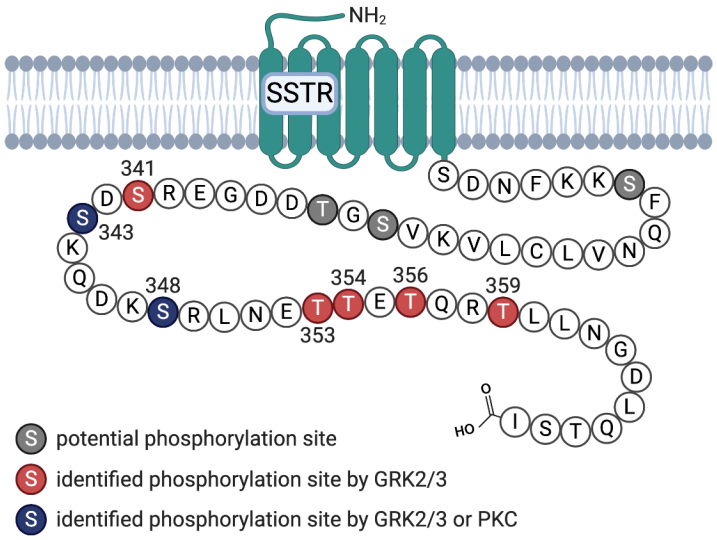


**Figure 1.4: Potential and Identified Phosphorylation Sites on the C-terminus of the Somatostatin Receptor.** Agonist-induced activation of the somatostatin receptor (SSTR) results in phosphorylation of sites on the C-terminus. Potential phosphorylation sites are shaded gray, phosphorylation sites identified to be phosphorylated by GRK2/3 are shown in navy, and phosphorylation sites identified to be phosphorylated by either GRK2/3 or PKC are shown in red (Günther et al., 2018).

## Summary

In order to gain a clearer picture of cellular events leading to the development of opioid tolerance, this dissertation aimed to investigate the kinases involved in MOR internalization and acute desensitization of MORs and SSTRs before and after chronic morphine treatment. Chapter 3 first describes how MOR C-terminal phosphorylation sites might contribute differentially to acute desensitization versus cellular tolerance. Chapter 4 describes evidence for a heterologous adaptation in the kinase regulation of acute desensitization such that, in addition to GRK2/3, PKC and JNK activity contribute to acute desensitization following chronic morphine treatment.

Chapter 5 presents additional experiments conducted in order to begin to tease apart mechanisms underlying phosphorylation-dependent desensitization. Chapter 6 closes with a discussion of the interpretation of these results within the context of opioid tolerance and GPCR signaling more generally. The work presented in this dissertation demonstrates one heterologous adaptation induced by chronic morphine treatment that may contribute to cellular tolerance and provides a new appreciation for how chronic morphine treatment may affect non-opioid receptors.

# Chapter 2: Materials and Methods

## Animals:

Adult male and female rats with ages between 5 – 8 weeks were used for the following experiments. Both wildtype Sprague-Dawley and μ-opioid receptor knockout (MOR KO) rats on a Sprague-Dawley background were used. Wildtype Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). MOR KO Sprague-Dawley rats were used as described in Arttamangkul et al. (2019). All animal experiments were conducted in accordance with the National Institutes of Health guidelines and with approval from the Institutional Animal Care and Use Committee of the Oregon Health & Science University (Portland, OR).

## Microinjections:

MOR KO rats (P24-30) were anesthetized with isoflurane and placed in a stereotaxic frame for microinjection of adeno associated virus type 2 (AAV2) encoding either wildtype MORs (exWT, AAV2-CAG-SS-GFP-MOR-WT-WPRE-SV40pA) or total phosphorylation-deficient (TPD, AAV2-CAG-SS-GFP-MOR-TPD-WPRE-SV40pA), STANT 3A (AAV2-CAG-SS-GFP-MOR-STANTWPRE-SV40pA), STANT 7A (STANT-7A, AAV2-CAG-SSGFP-MOR- STANT-7A -WPRE-SV40pA), TSST 4A (AAV2-CAG-SS-GFP-MOR-TSST-4A-WPRE-SV40pA), or TSST 4E (AAV2-CAG-SS-GFP-MOR-TSST-4EWPRE-SV40pA) mutant MORs in the locus coeruleus (LC). A GTP was fused to the N-terminus of each construct with a human prolactin hormone signal sequence. 200 nL of virus (2.06 x $10^{13}$ vg/ml) was injected at 0.1 μl/min, bilaterally in the LC (AP: -9.72 mm, ML: ±1.25 mm, DV: -6.95 mm, from bregma) using a computer controlled stereotaxic frame (Neurostar, Tubingen, Germany). Viruses were obtained from Virovek (Hayward, CA). Electrophysiology experiments were carried out 2-4 weeks following injection.

Chronic Morphine Treatment:

Both drug naïve and morphine treated animals (MTA) were used. Rats (5-6 weeks) were treated with morphine sulfate (National Institute on Drug Abuse, Bethesda, MD) continuously released from osmotic pumps. Osmotic pumps (2ML1, Alzet, Cupertino, CA) were filled with the required concentration of morphine sulfate in water to deliver 80 mg/kg/day for 6-7 days. This dose was used in order to maintain a circulating concentration of at least 1 μM. Osmotic pumps were implanted subcutaneously in the mid-scapular region of rats maintained on isoflurane anesthesia and remained in the animals until they were used for experiments 6 or 7 days later.

Pharmacology:

| **Drug:** | **Abbr:** | **Acts on:** | **Purchased from:** |
|---|---|---|---|
| [Met]$^5$enkephalin | ME | MOR/DOR agonist | Sigma-Aldrich (St Louis, MO) |
| Somatostatin | SST | SSTR agonist | ProSpec (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel |
| UK14304 tartate | UK | α2-adrenergic receptor agonist | Tocris (Bio-Techne Corp., Minneapolis, MN) |
| Idazoxan | Ida | α2-adrenergic receptor antagonist | Sigma-Aldrich |
| β-chlornaltrexamine | β-CNA | MOR/KOR/DOR irreversible antagonist | Sigma-Aldrich |
| (+)MK-801 | - | NMDAR blocker | Hello Bio (Princeton, NJ) |
| Compound 101 | CMP101 | Inhibits GRK2/3 | Hello Bio |
| Go6976 | - | Inhibits PKCα and PKCβ | Tocris |
| SP600125 | - | Inhibits JNK | Tocris |
| Staurosporine | Stp | Inhibits kinases besides GRKs | LC Laboratories (Woburn, MA) |

Somatostatin and ME (10 mM) were dissolved in water, diluted to the appropriate concentration in ACSF and applied by superfusion. Go6976, SP600125, and staurosporine (all 10 mM) were dissolved in DMSO. CMP101 was first dissolved in a small amount of DMSO (10% of final volume), sonicated, and then brought to its final volume with 20% (2-Hydroxypropyl)-β-cyclo-dextrin (Sigma-Aldrich, St. Louis, MO) and sonicated again to create a 10 mM solution. β-CNA (10 mM) was dissolved in methanol and used in ACSF.

## Electrophysiology:

### Tissue Preparation:

Rats were deeply anesthetized and euthanized by cardiac percussion. Brains were extracted and blocked, removing the posterior half of the brain. The brain was then fixed onto the vibratome stage (Krazy Glue) and placed in the vibratome chamber with warm (34°C) ACSF containing (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 1.2 $NaH_2PO_4$, 21.4 $NAHCO_3$, and 11 D-glucose with +MK-801 (10 μM). Horizontal brain slices (260 μm) containing the locus coeruleus (LC) were then made using a vibratome (VT 1200S; Leica, Nussloch, Germany). The LC is a brainstem area with homogenous MOR expression, making it a good area to study MORs. The LC was identified based on its proximity to the fourth ventricle. Slices were allowed to recover in warm ACSF containing +MK-801 (10 μM) for at least 30 minutes and then hemisected and stored in glass vials with warm (34°C), oxygenated (95% $O_2$/5% $CO_2$) ACSF until used.

### Whole-cell Recording:

Whole-cell recordings from LC neurons were obtained with an Axopatch 200B amplifier (Axon Instruments) in voltage-clamp mode ($V_{hold}$ = -60mV). Recording pipettes (World Precision Instruments, Saratosa, FL) with a resistance of 1.5-2 MΩ were filled with a potassium-based

internal solution containing BAPTA (in mM): 115 potassium methanesulfonate or potassium methyl sulfate (Alfa Aesar, Ward Hill, MA), 20 KCl, 1.5 $MgCl_2$, 5 HEPES(K), 10 BAPTA, 2 Mg-ATP, 0.2 Na-GTP, pH 7.4, and 275–280 mOsM. Immediately after gaining access to the cell, membrane capacitance, series resistance, and input resistance were measured with the application of square test pulses (+2 mV for 50 ms) averaged before computation using Axograph X (Axon Instruments, version 1.5.4). Only recordings where the series resistance remained <15 MΩ were included. Data were collected at 400 Hz with PowerLab (Chart Version 8.1.8; AD Instruments, Colorado Springs, CO).

Drug Perfusions and Incubations:

Agonists, including ME, somatostatin, and UK14304, and antagonists, including idazoxan, were applied via bath perfusion at a rate of about 2 mL/minute. For experiments using kinase inhibitor(s), slices were incubated in a higher concentration of inhibitor(s) diluted in ACSF for at least 1 hour prior to recording. Inhibitors were also included in the bath and drug perfusion solutions at lower concentrations. For experiments with partial removal of receptors with the irreversible antagonist, β-CNA, slices were incubated in ACSF containing β-CNA (30 – 100 nM) for 5 minutes before placement on the rig for recording.

## Measuring Desensitization and Cellular Tolerance:

MOR Desensitization and Tolerance:

Agonist-induced activation of MORs results in activation of G protein-coupled inwardly-rectifying potassium channels (GIRKs) through binding of dissociated Gβγ subunits (**Figure 2.1A**). Activation of GIRKs results in an outward potassium current that can be measured using whole-cell recording (**Figure 2.1B**, Birdsong and Williams, 2020). Therefore, GIRK currents

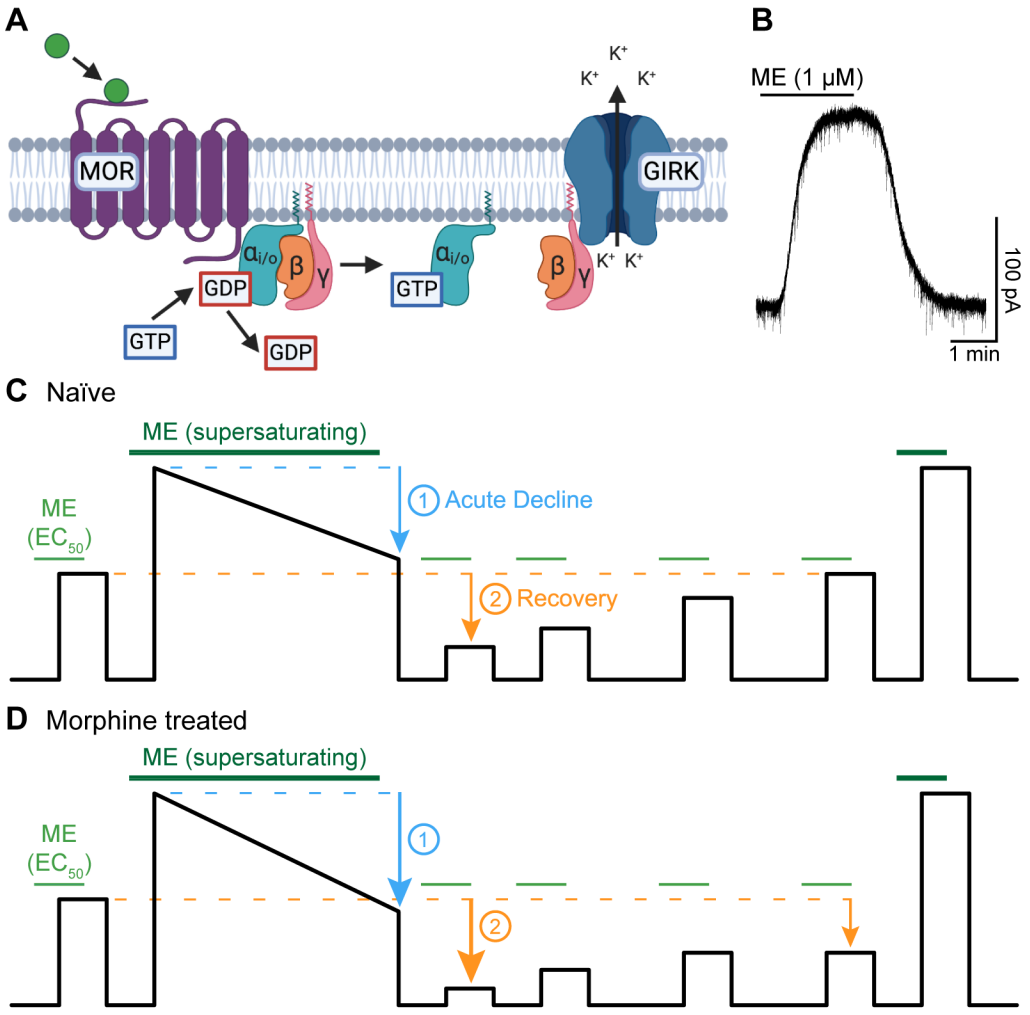


**Figure 2.1: Potassium channel currents as a readout for μ-opioid receptor activation and desensitization.** (A) Schematic for how activation of μ-opioid receptors (MORs) leads to activation of G protein-coupled inwardly rectifying potassium (GIRK) channels. (B) Example trace from a whole-cell recording of a locus coeruleus (LC) neuron showing the GIRK current induced by the endogenous opioid peptide [Met]$^5$enkephalin (ME, 1 μM). (C) Stick diagram showing the protocol used to measure acute desensitization in naïve animals via (1) acute decline and (2) recovery from desensitization using $EC_{50}$ concentrations of agonist. (D) Stick diagram showing the protocol used to measure acute desensitization in morphine treated animals. Measures of cellular tolerance are indicated by the differences between (C) and (D): increase in desensitization for both (1) and (2, left arrow) and reduced recovery from desensitization (2, right arrow).

were used as a readout for MOR activation. Recording of GIRK currents allows for a real-time readout of receptor signaling and therefore was also used to measure desensitization. The endogenous opioid peptide, [Met]$^5$enkephalin, which activates both μ- and ∂-opioid receptors,

was used because of its ability to easily wash from the slice and because the LC does not contain ∂-opioid receptors. Morphine does not wash from the slice and therefore could not be used for these experiments.

Acute desensitization was measured in two commonly used ways that are not necessarily reflective of the same process (Birdsong and Williams, 2020). The first measure is the decline in the peak current during the continuous application of a supersaturating concentration of agonist (30 μM ME, 10 min) and is referred to as acute decline (**Figure 2.1C, (1)**). The second measure is the decrease in the peak current evoked by an $EC_{50}$ concentration of agonist (300 nM ME) 5 min after desensitization **(1)** relative to the current induced by the $EC_{50}$ concentration before desensitization (**Figure 2.1C, (2)**). The $EC_{50}$ concentration was also applied at 10, 20, and 30 minute timepoints in order to measure the recovery from desensitization over time. The saturating concentration of ME was also applied at the end of each experiment in order to confirm that there had not been rundown during the experiment.

Acute desensitization was also measured in morphine treated animals in order to determine measures of tolerance (**Figure 2.1D**). For all experiments using tissue from tolerant animals, brain slices were prepared in morphine-free solutions such that they were in a state of acute withdrawal. It has been established that after chronic morphine treatment, there is an increase in acute desensitization (as measured by acute decline **(1)** and recovery from desensitization **(2), Figure 2.1D**) and reduced recovery from desensitization **(2)**. Both of these are considered measures of cellular tolerance. Another measure of tolerance was also used that takes advantage of the partial agonist, morphine, which is more sensitive to changes in coupling efficacy (Birdsong and Williams, 2020). For this measure, morphine (1 μM) was bath applied until the

current reached a steady state, after which a supersaturating concentration of ME (30 μM) was immediately applied. Then the ratio of the morphine-induced current relative to the current induced by ME was determined in both naïve and morphine treated animals. A decrease in this ratio in morphine treated animals was considered indicative of cellular tolerance.

SSTR Desensitization:

In order to determine the heterologous effects of chronic morphine treatment, the somatostatin receptor (SSTR) was examined. LC neurons express MORs and SSTRs in the same neurons and SSTRs activate the same population of GIRK channels (Fiorillo and Williams, 1996). The endogenous peptide agonist, somatostatin (SST), was used. While SST does not wash well from the slice, limiting the ability to measure the second measure of desensitization for this receptor, there were no known alternative agonists that wash well from the slice. Therefore, acute desensitization of SSTR could only be measured using the decline in the peak current during the application of a saturating concentration of SST (20 μM). To obtain a baseline at the end of each experiment, $BaCl_2$ (100 μM) was used to reverse the current by blocking potassium conductance.

Concentration-response Curves:

For construction of concentration-response curves, ME-induced currents were normalized to the peak current induced by a saturating concentration of the α2-adrenergic receptor agonist, UK14304 (UK, 3 μM) followed by the antagonist idazoxan (ida, 1 μM) since α2-adrenergic receptors couple to the same population of GIRK channels (Williams et al., 2013). A range of concentrations of ME was used (100 nM – 30 μM) in order to create a logarithmic scale of ME-induced MOR activation in LC neurons.

Internalization and Imaging:

To visualize trafficking of virally expressed wildtype MORs, acute brain slices (260 μm) were prepared and then incubated with an anti-GFP nanobody conjugated to Alexa594 (Nb-A594, 10 mg/ml, 30-45 min). Images were captured before and after application of a saturating concentration of ME (30 μM, 10 min) using a 2-photon microscope. A z-series of 10 sections was acquired at 1-μm intervals so the whole neuron could be qualitatively compared.

Data Computation and Analysis:

Analysis was performed using GraphPad Prism 6 (GraphPad Software, version 6.0d, San Diego, CA) based on number of technical replicates (number of slices). Values are presented as average ± S.D. Statistical comparisons were made using one-way or two-way ANOVA, along with multiple comparison adjusted Tukey's post hoc tests, as appropriate. For all experiments $P < 0.05$ is considered significant.

# Chapter 3: Separation of Acute Desensitization and Long-term Tolerance of μ-Opioid Receptors is Determined by the Degree of C-terminal Phosphorylation

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**Abstract**

Phosphorylation of sites on the C-terminus of the mu opioid receptor (MOR) result in the induction of acute desensitization that is thought to be a precursor for the development of long-term tolerance. Alanine mutations of all 11 phosphorylation sites on the C-terminus of MORs almost completely abolished desensitization and one measure of tolerance in locus coeruleus neurons when these phosphorylation deficient MORs were virally expressed in MOR knockout rats. The present work identifies specific residues that underlie acute desensitization, receptor internalization and tolerance. Four MORs variants with different alanine or glutamate mutations in the C-terminus were examined. Alanine mutations in the sequence between amino acids 375 and 379 (STANT-3A) and the sequence between amino acids 363 and 394 having 4 additional alanine substitutions (STANT+7A), reduced desensitization and two measures of long term tolerance. Following chronic morphine treatment, alanine mutations in the sequence between 354 and 357 (TSST-4A) blocked one measure of long-term tolerance (increased acute desensitization and slowed recovery from desensitization) but did not change a second (decreased sensitivity to morphine). With the expression of receptors having glutamate substitutions in the TSST sequence (TSST-4E), an increase in acute desensitization was present after chronic morphine treatment but the sensitivity to morphine was not changed. The results show that all 11 phosphorylation sites contribute, in varying degrees, to acute desensitization and long-term tolerance. That acute desensitization and tolerance are not necessarily linked illustrates the complexity of events that are triggered by chronic treatment with morphine.

## Significance

This work shows that the degree of the phosphorylation sites on the C-terminus of the mu opioid receptor alters acute desensitization, internalization and measures of long term tolerance to morphine. The primary conclusion is that the degree of phosphorylation on the 11 possible sites of the C-terminus has different roles for expression of the multiple adaptive mechanisms that follow acute and long term agonist activation. Although the idea that acute desensitization and tolerance are intimately linked is generally supported, these results indicate that disruption of one phosphorylation cassette of the C-terminus, TSST (354-357) distinguishes the two processes.

## Introduction

The C-terminus of the mu opioid receptor (MOR) has multiple phosphorylation sites that are implicated in the mechanisms of receptor desensitization and trafficking. Two cassettes on the MOR C-terminus were efficiently phosphorylated after treatment with a potent agonist (Wang et al., 2002; Lau et al., 2011; Chen et al., 2013; Just et al., 2013; Miess et al., 2018; Doll et al., 2011). When the residues from 375 to 379 (STANT) were mutated to alanine, arrestin recruitment and internalization were blocked (Lau et al., 2011). Residues in the second cassette ranging from 354 to 357 (TSST) were also efficiently phosphorylated, but internalization was unaffected. The electrophysiological consequences of alanine mutations in each cassette, was examined using viral expression in neurons of the thalamus and locus coeruleus (LC) in MOR knockout mice (Birdsong et al., 2015). The results indicated that mutations of all serine and threonine residues in the individual cassettes had small effects on acute desensitization. With mutations of the residues in both cassettes acute desensitization was reduced (Birdsong et al., 2015; Yousuf et el., 2015). The functional consequences of mutations in other potential

phosphorylation sites (S-363, T-370, -383 and -394) have not been examined. Phosphorylation of S-363 is thought to be constitutive or PKC dependent while phosphorylation of T-370 can be induced by agonists (reviewed in Williams et al, 2013). There is no evidence showing phosphorylated forms at T-383 and T-394 by mass spectrometry (Lau et al., 2011). Acute desensitization measured in locus coeruleus neurons was almost completely eliminated in a knock in mouse where all 11 C-terminus phosphorylation sites or the 10 sites minus T-394 mutated to alanine (Kliewer et al., 2019). Likewise acute desensitization and the development of long term tolerance were blocked following viral expression of phosphorylation deficient MORs in locus coeruleus neurons of MOR knockout rats (Arttamangkul et al., 2018).

The present study examined acute desensitization in slices from untreated and morphine treated animals in each of four mutant MORs—STANT-3A (S375-T379 to AAANA), STANT-7A (S363A, T370A, 375-379 – AAANA, T383A, T394A), TSST-4A (354-375 to AAAA) and TSST-4E (354-357 to EEEE). Each receptor contained an N-terminus GFP tag that, when coupled with a fluorescent nanobody, was used to assess trafficking (Arttamangkul et al., 2018). Internalization of the STANT-3A and STANT-7A mutant receptors was blocked as was previously found in HEK cells (Birdsong et al., 2015). Acute desensitization was maintained in the STANT-3A and TSST mutant receptors and blocked in the STANT-7A receptors. Two measures of tolerance were examined following chronic treatment. First, the current induced by morphine was reduced in experiments with wild type and TSST-4A receptors whereas there was no change in slices expressing the STANT-3A and TSST-4E receptors. The second assay used the time course of recovery from acute desensitization as a measure of the development of tolerance. The recovery from acute desensitization was decreased in slices expressing wild type receptors following chronic morphine treatment (Dang & Williams, 2004; Quillinan et al., 2011;

Arttamangkul et al., 2018) but in slices expressing each of the mutant MORs recovery was not different between untreated and morphine treated animals. The results suggest that phosphorylation of the C-terminus of the MOR contribute to the prolongation of the recovery from desensitization that is a hallmark of tolerance found in experiments from wild type animals.

**Materials and Methods**

*Drugs* - Morphine sulfate and morphine alkaloid were obtained from the National Institute on Drug Abuse (NIDA), Neuroscience Center (Bethesda, MD). Naloxone was purchased from Abcam (Cambridge, MA), MK-801, from Hello Bio (Princeton, NJ), UK14304 tartrate, from Tocris (Bio-Techne Corp. Minneapolis, MN). Potassium methanesulfonate was from Alfa Aesar (Ward Hill, MA). [$Met^5$] enkephalin (ME) was from Sigma (St. Louis, MO).

Morphine alkaloid was converted to salt form with 0.1 M HCl and made up a stock solution in water. The working solution was diluted in artificial cerebrospinal fluid (ACSF) and applied by superfusion. Naloxone (1 mM) was dissolved in water, diluted to 1 μM in ACSF and applied by superfusion. Bestatin (10 μM) and thiorphan (1 μM) was included in solutions containing ME to limit peptidase induced breakdown.

*Animals*– All animal experiments were conducted in accordance with the National Institutes of Health guidelines and with approval from the Institutional Animal Care and Use Committee of the Oregon Health & Science University (Portland, OR). Adult (180 – 300 g or 5-6 weeks) male and female Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). MOR-knockout Sprague-Dawley rats were obtained from Horizon (St. Louis, MO). Homozygous animals were bred in house.

*Microinjection protocol*- Microinjections of virus into the locus coeruleus was carried out as previously described (Arttamangkul et al., 2018). MOR-knockout animals (24-30 days) were anesthetized with isofluorane (Terrell®, Piramal Clinical Care, Inc., Bethlehem, PA) and placed in a stereotaxic frame. Viral particles containing adeno associated virus type 2 for the expression of mutant MORs (STANT-3A, AAV2-CAG-SS-GFP-MOR-STANT-WPRE-SV40pA, $2.06x10^{13}$ vg/ml), (STANT-7A, AAV2-CAG-SS-GFP-MOR- STANT-7A -WPRE-SV40pA, $2.06x10^{13}$ vg/ml), (TSST-4A AAV2-CAG-SS-GFP-MOR-TSST-4A-WPRE-SV40pA, $2.06x10^{13}$ vg/ml) and (TSST-4E, AAV2-CAG-SS-GFP-MOR-TSST-4E-WPRE-SV40pA, $2.06x10^{13}$ vg/ml). The N-terminus of each construct was fused to GFP with a human prolactin hormone signal sequence. All viruses were obtained from Virovek (Hayward, CA). Injections of 200 nl at the rate of 0.1 µl/min were done bilaterally at ±1.25 mm lateral from the midline and -9.72 mm from the bregma at a depth of 6.95 mm from the top of the skull using computer controlled stereotaxic Neurostar (Kähnerweg, Germany). Experiments were carried out 2-4 weeks following the injection.

*Animal treatment protocols*-Rats (5-6 weeks) were treated with morphine sulfate using osmotic pumps (2ML1, Alzet, Cupertino, CA) as described previously (Quillinan et al., 2011). Rats were anesthetized and the pumps were implanted subcutaneously.

*Tissue preparation* – Horizontal slices containing locus coeruleus (LC) neurons were prepared as described previously (Williams and North, 1984). Briefly, rats were killed and the brain was removed, blocked and mounted in a vibratome chamber (VT 1200S, Leica, Nussloch, Germany). Horizontal slices (250-300 µm) were prepared in warm (34°C) artificial cerebrospinal fluid (ACSF, in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 1.2 $NaH_2PO_4$, 11 D-glucose and 21.4 $NaHCO_3$ and 0.01 (+) MK-801 (equilibrated with 95% O2/ 5% CO2, Matheson, Basking Ridge,

NJ). Slices were kept in solution with (+)MK-801 for at least 30 min and then stored in glass vials with oxygenated (95% O2/ 5% CO2) ACSF at 34°C until used.

*Electrophysiology* –Slices were hemisected and transferred to the recording chamber which was superfused with 34°C ACSF at a rate of 1.5 - 2 ml/min. Whole-cell recordings were made from LC neurons with an Axopatch-1D amplifier in voltage-clamp mode ($V_{hold}$ = -60 mV). Recording pipettes (1.7 – 2.1 MΩ, World Precision Instruments, Saratosa, FL) used an internal solution of (in mM): 115 potassium methanesulfonate or potassium methyl sulfate, 20 KCl, 1.5 $MgCl_2$, 5 HEPES(K), 10 BAPTA, 2 Mg-ATP, 0.2 Na-GTP, pH 7.4, 275-280 mOsM. Recordings where the series resistance was <15 MΩ were terminated. Data were collected at 400 Hz with PowerLab (Chart version 5.4.2; AD Instruments, Colorado Springs, CO).

*MOR-GFP Trafficking*-Brain slices (240 μm) from the virally injected rats were prepared as previously described. Slices were visualized with an Olympus Macroview fluorescent microscope for GFP expression in the LC area and to visualize plasma membrane associated receptors slices were incubated in a solution of anti-GFP nanobody Alexa594 (Nb-A594, 10 μg/mL, 30-45 min, Arttamangkul et al., 2018). Images were captured with an upright microscope (Olympus, Center Valley, PA.) equipped with a custom-built two-photon apparatus and a 60x water immersion lens (Olympus LUMFI, NA1.1, Center Valley, PA). The dye was excited at 810 nm. Data were acquired and collected using Scan Image Software (Pologruto et al., 2003). Images were taken at a magnification where a single neuron filled the field of view. A z-series of 10 sections was collected at 1 μm intervals. With this protocol, the whole neuron was qualitatively compared. Drugs were applied by perfusion at the rate of 1.5 ml/min. All experiments were done at 35˚C.

*Data Analysis* - Analysis was performed using KaleidaGraph (Synergy software). Values are presented as mean±SD. Statistical comparisons were made using unpaired t-tests or a two-way ANOVA, as appropriate. Comparisons with $p<0.05$ were considered significant.

**Results**

As in previous work, [Met]$^5$enkephalin (ME, 0.3 μM and 30 μM) was applied in each experiment. In slices from wild type animals, the initial current amplitude induced by ME (0.3 μM) was about 60% of that induced by the saturating concentration of ME (30 μM). The initial amplitude of the currents induced by ME (0.3/30 μM) measured in slices from virally expressed receptors varied but were similar in amplitude to the currents (in pA) measured in wild type animals (TABLE 1, ANOVA, Dunnett post hoc)

**Trafficking**

Previous results showed that virally expressed wild type receptors were efficiently internalized during the application of ME (30 μM, 10 min) whereas internalization of receptor where all phorphorylation sites on the C terminus were mutated to alanine was not observed (Arttamangkul et al., 2018). In the present study internalization of the receptors with specific mutations was examined using 2-photon microscopy. Each mutant receptor had an extracellular GFP-tag such that plasma membrane associated receptors were labeled with an anti-GFP nanobody conjugated with alexa594 (Figure 1). A z-stack of images of single cells was collected before and after treatment of slices with ME (30 μM, 10 min). There was a distinct qualitative change in the receptor distribution in slices with neurons expressing the TSST-4A (S/T354-357A) and TSST-4E (S/T354-357E) receptors. Before treatment with ME the receptors lined the

plasma membrane and following treatment with ME receptors were internalized and the pattern became punctate (Figure 1). The distribution of STANT-3A (S/T375-377A, T379A) receptors was relatively unchanged. To quantify the increase in intracellular fluorescence the z-stack was compressed with a z-projection and a region of interest just inside of the plasma membrane of the soma was drawn (Supplemental Figure 1). The increase in fluorescence measured in arbitrary fluorescence units (AFU) in that region of interest following treatment with ME was taken as the internalized receptors. By this rough measure there was an increase in fluorescence with the TSST-4A (95% confidence level = 255±168 AFU, n-8) and TSST-4E (95% confidence level = 166±117 AFU, n=7) receptors and little change in the STANT-3A receptors (95% confidence level = 31±33 AFU, n=14). Internalization was statistically different between the STANT-3A and both TSST-4A and TSST-4E receptors (ANOVA, Student-Newman-Keuls post hoc). Given that the alanine mutations of the STANT sequence (375-379) largely blocks arrestin recruitment it was not surprising that the application of ME did not induce detectable internalization (Lau et al., 2011).

**Acute Desensitization**

The current induced by ME (0.3 μM) was measured before and following a 10 min application of ME (30 μM). The degree of desensitization was determined by the relative amplitude of the current induced by ME (0.3 μM) following a 5 min wash of the saturating concentration of ME compared to the amplitude of the initial current induced by ME (0.3 μM). Recovery from desensitization was determined by measuring the current amplitude induced by ME (0.3 μM) over time (Figure 2). As reported previously, recovery from desensitization in slices from wild type animals occurred over a period of 20-35 min.

**Coupling efficiency using a partial agonist, morphine, to measure tolerance**

The relative amplitudes of the currents induced by morphine and ME were compared (Figure 2D). Morphine (1 μM) was applied until the current reached a steady state. The solution was then switched immediately to a saturating concentration of ME (30 μM). The ratio of the morphine/ME current was determined in slices from untreated and morphine treated animals (Figure 2E). This protocol takes advantage of the fact that morphine is a partial agonist and therefore is more sensitive to changes in coupling efficiency and thus was used as a second measure of the tolerance induced by treatment of animals with morphine (Christie et al., 1987; Levitt, Williams, 2012).

Animals were treated with morphine (80 mg/kg/day) for 6-7 days. Brain slices were prepared and maintained in morphine free solutions such that the preparations were in a state of acute withdrawal. First, the degree of tolerance was examined using the current induced by ME (0.3 μM) measured before and following the application of ME (30 μM, 10 min, Table 1). The recovery of the current induced by ME (0.3 μM) was examined over 20-35 min. As reported previously the recovery from desensitization was slowed in slices from morphine treated animals (Figure 2, Dang, Williams, 2004; Quillinan et al., 2011; Arttamangkul et al., 2018). Second, the current induced by morphine (1 μM) relative to the peak ME current was reduced in slices from morphine treated animals (Figure 2, $p<0.05$ Mann-Whitney U test). This simple assay is therefore a reasonable measure of tolerance and is particularly valuable for experiments where the viral expression of receptors in the knockout animals can be variable. A summary of the results with this measure is illustrated in the plots of the amplitude of the current induced by morphine (1 μM) against the current induced by ME (30 μM, wild type, Figure 3A). The current

induced by morphine was smaller than that induced by ME in slices from morphine treated animals over a wide range of current amplitudes.

The same protocols were used to measure tolerance, desensitization and the recovery from desensitization in slices taken from animals expressing each of the mutant receptors.

**STANT-3A**

Application of a saturating concentration of ME resulted in desensitization of the STANT-3A receptor. The decline in current during the application of ME (30 μM, 10 min) was not different from that in slices from wild type animals ($p>0.05$, ANOVA, Dunnett post hoc). The decrease in the current induced by ME (0.3 μM) following the saturating concentration was reduced compared to the wild type (Figure 4, $p<0.05$ ANOVA Dunnett post hoc). This result was expected based on previous work in the mouse (Birdsong et al., 2015). The phosphorylation of S375 is thought to be an initial step leading to sequential phosphorylation events on the C-terminus. The observation that desensitization persisted indicates that this sequence is not the sole area that underlies acute desensitization.

In slices from morphine treated animals the decline from the peak current induced by ME (30 μM, 10 min) was not different from that in slices from untreated animals (Table 1, $p>0.05$, unpaired T-test). Likewise the recovery from desensitization was the same in slices from morphine treated and untreated animals (Figure 4C). Thus, in spite of the acute desensitization seen with this receptor, there was no apparent induction of tolerance as measured by an increase in desensitization or a prolongation of the time it takes to recover from desensitization. The ratio of the morphine/ME current was also not different between experiments carried out in untreated and morphine treated animals (Figure 4E). The current amplitudes (morphine/ME) for individual

cells in slices from untreated and morphine treated animals overlapped over a range of current amplitudes. Thus by two measures, mutation of the STANT sequence in the MOR blocked the development of tolerance.

Finally, given that treatment of the slices with ME (30 μM, 10 min) did not induce detectable internalization, one conclusion from these experiments could be that trafficking and/or arrestin recruitment is a necessary step in the development of long-term tolerance.

**STANT-7A**

There was little or no decline in the current induced by ME (30 μM), or the ME (0.3 μM) current following washout of the saturating concentration in slices expressing the STANT-7A receptor (Table 1, Figure 5). Thus by the inclusion of 4 additional alanine mutations, the STANT-7A resulted in a near complete block of desensitization, as was found in STANT-3A receptors.

In slices from morphine treated animals there was a substantial increase in desensitization as determined by the decrease in the current induced by ME (0.3 μM) 5 min following the wash of the saturating concentration (Table 1, Figure 5). The recovery from desensitization in slices from animals treated with morphine was near complete after washing for 10 min (Figure 5). The decline from the peak current induced by ME (30 μM, 10 min) was slightly smaller than in slices from morphine treated animals (Table 1, MTA=0.83±0.09, n=14, Untreated= 0.73±0.10, n=9, p=0.03, unpaired T-test). Chronic morphine treatment therefore resulted in a facilitation of acute desensitization measured by the decrease in current induced by ME (0.3 μM) that was short lasting and not present prior to the morphine treatment. Thus mutation of the 4 additional residues blocked acute desensitization in slices from untreated animals but did not eliminate at least one adaptive mechanism induced by chronic morphine. The results suggest that the 4

remaining phosphorylation sites play a role in the mechanisms induced by chronic morphine treatment.

**TSST-4A**

Acute desensitization and trafficking of this receptor closely resembled that measured in slices from wild type animals and experiments with wild type receptors expressed in the knockout animals (Figure 1 and 6). Following chronic morphine treatment, the decline from the peak current induced by ME (30 μM, 10 min) was not different from slices from untreated animals (untreated 0.53±0.13 of the peak n=7, MTA 0.60±0.05 of the peak n=5). There was also no difference in the decrease in the ME (0.3 μM) current or the time course or extent of recovery from desensitization following washout of the saturating concentration of ME (Figure 6B&C). Thus by one measure (acute desensitization) chronic morphine had no effect. However the current induced by morphine (morphine/ME) was reduced in slices taken from morphine treated animals (Figure 6D&E). Thus the second measure of tolerance was the same as that measured in slices taken from morphine treated wild type animals (compare Figures 2E and 6E) suggesting that the link between acute desensitization and the reduced sensitivity to morphine was broken.

**TSST-4E**

Previous work suggested that by mimicking phosphorylation of the TSST sequence with glutamic acid residues (TSST-4E), a desensitized state of the receptor was induced (Birdsong et al., 2015). The initial current amplitude induced by ME (0.3 μM) was not different from cells expressing TSST-4A or wild type receptors (TSST-4E=286±102 pA, n=7, WT=279±96, n=7, Table 1, $p>0.05$, ANOVA, Dunnett post hoc). Likewise the current induced by a saturating

concentration of ME (30 μM) was not different (TSST-4E=400±123 pA, n=10: WT 451±95 pA, n=7, Table 1, $p>0.05$, ANOVA, Dunnett post hoc). The decline from the peak current induced by ME (30 μM) was greater in slices from morphine treated animals than in untreated controls (Figure 7D, Table 1 $p<0.05$, unpaired T test). The current induced by ME (0.3 μM) following the wash of the saturating concentration of ME (30 μM) was also smaller in slices from morphine treated animals than in untreated controls (Figure 7D, Table 1, $p<0.05$, unpaired T test). Finally, receptor internalization induced by ME (30 μM, 10 min) was similar to measured in wild type and TSST-4A receptors (Figure 1).

The results indicate that acute desensitization of the TSST-4E receptors in slices from morphine treated animals is facilitated but the recovery from desensitization is complete (Figure 7C, Table 1). Finally the plot of morphine versus ME current amplitudes illustrates the overlap in experiments in slices between untreated and morphine treated animals (Figure 3E, $p>0.05$, unpaired T test). Although acute desensitization was increased in slices taken from morphine treated animals, the recovery was complete and there was no change in the current induced by morphine suggesting that one measure of tolerance of these receptors was blocked but acute desensitization was facilitated. As with the results obtained with the TSST-4A receptors acute desensitization and tolerance appear to be dependent on separate processes.

**Discussion**

The present study measured the opioid induced outward current mediated by GIRKs to examine the functional regulation of MORs by phosphorylation sites on the C-terminus. Alanine mutation of all phosphorylation sites (11S/T-A) on the C-terminus results in a dramatic decrease in acute desensitization and long-term tolerance to opioids (Arttamangkul et al., 2018; Kliewer et al.,

2019). The present study used a series of MORs having selective alanine mutations in different areas of the C-terminus in order to determine the role of acute desensitization in the development of long term tolerance. The results show that each of the mutated receptors affected one or another aspect of MOR signaling. With one exception (STANT-7A), some degree of acute desensitization was present on all receptors and following chronic morphine treatment there was an acute, although transient, desensitization of even that receptor. The unexpected observation was that by using three measures of opioid action to determine the level of tolerance there were distinct differences between the mutant opioid receptors. Thus different phosphorylation sites on the C-terminus have functionally distinct actions following chronic morphine treatment.

**Acute desensitization as a measure of tolerance.**

Following chronic treatment of animals with morphine acute desensitization was augmented and recovery from desensitization was prolonged (Dang, Williams, 2004; Quillinan et al., 2011; Arttamangkul et al., 2018). Acute desensitization was facilitated in slices expressing TSST-4E as in wild type animals but not in STANT-3A, TSST-4A MOR variants. There was a transient facilitation in the STANT-7A variants but that is because there was no desensitization in slices from untreated animals, and the increase in desensitization was small, transient and to the same level as in the STANT-3A variants. Unlike the observation made in wild type animals, the delayed time course of recovery following chronic morphine treatment was not present in any of the mutated receptors. It therefore appears that each of the phosphorylation sites on the C-terminus are necessary for the both the augmented acute desensitization and slowed recovery from desensitization that is induced following chronic morphine treatment. Thus the full complement of phosphorylation sites appears necessary for the development of long term tolerance.

**Uncoupling of signaling as a measure of long-term tolerance.**

As a partial agonist morphine offers a sensitive assay for functional coupling (Christie et al., 1987; Dang, Williams, 2004; Levitt, Williams, 2012; Williams et al., 2014). In slices taken from animals treated chronically with morphine, the morphine-induced current was reduced (Christie et al., 1987; Quillinan et al., 2011). This measure of long-term tolerance was present in some but not all of the mutant MORs tested. With the notable exception of TSST-4E, the MOR mutations that blocked desensitization and internalization (STANT-3A, STANT-7A and Total Phosphorylation Deficient (TPD, Arttamangkul et al., 2018)) also blocked the decrease in the morphine induced current.

**Phosphorylation cassettes**

It is well established that alanine mutations in the STANT-3A sequence decreases arrestin recruitment and receptor internalization (Lau et al., 2011; Just et al., 2013). Measures of tolerance following chronic morphine were also blocked in receptors with the STANT-3A and STANT-7A mutations (this study).

Much less in known about how alanine mutations of the TSST sequence affect MOR function particularly following chronic morphine treatment. The TSST-4A receptors desensitize and internalize ‘normally’ with agonist application. A receptor binding assay that used a fluorescently labeled agonist, DERM-A594, in HEK cells was used to measure the change in agonist affinity following incubation with potent agonists, such as ME (Birdsong et al., 2013). There was a significant long term (1-2 hours) increase in agonist affinity that followed incubation for 20-120 min. The increased affinity was not affected after treatment with pertussis toxin, was present in arrestin2/3 knock cells and there was no change in antagonist affinity. The

increase in agonist affinity was blunted in the TSST-4A receptors particularly with longer agonist incubation times. The results suggested that phosphorylation of the TSST sequence largely blocked one consequence of acute agonist desensitization. Glutamate substitutions in the TSST sequence (TSST-4E) increased agonist affinity in the absence of pre-incubation suggesting that the glutamate substitution largely mimicked the effect of phosphorylation of this sequence (Birdsong et al., 2013).

Previous work reported that there were two components of tolerance induced by chronic morphine treatment, one that was transient (60-90 min) and a second that was long term (>3 hours, Levitt, Williams, 2012). The striking difference between the TSST-4A and TSST-4E receptor function following chronic morphine treatment suggests an important role for these phosphorylation sites in the adaptive changes induced by chronic morphine treatment. Although the idea that acute desensitization and tolerance are intimately linked is generally supported, the results indicate that disruption of the normal role of this (TSST) sequence distinguishes the two processes. Following chronic morphine treatment, long term tolerance as measured by the decrease in the current induced by morphine was maintained with the TSST-4A mutants. There was however no decrease in the current induced by morphine in the TSST-4E mutants in slices taken from morphine treated animals. Thus this measure of long term tolerance to morphine was blocked. The adaptive changes in acute desensitization were different in that acute desensitization following chronic morphine treatment was increased in the TSST-4E receptors, as in wild type receptors. On the other hand, acute desensitization of the TSST-4A receptors was unchanged by chronic morphine treatment. The present results could result from two separate mechanisms that underlie acute desensitization as previously indicated (Dang et al., 2009).

**Summary**

Alanine mutations of different phosphorylation sites on the C-terminus were used to identify receptor dependent signaling by measuring acute desensitization, recovery from desensitization and internalization. Mutations of the STANT sequence blocked all three measures as well as measures of tolerance. Mutations in the TSST sequence had little effect on acute actions but had a modulatory effect on the expression of tolerance.

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Participated in Research Design: Williams, Arttamangkul, Birdsong

Conducted Experiments: Williams, Arttamangkul, Leff, Koita

Contributed new reagents: Arttamangkul

Performed data analysis: Williams

Wrote or contributed to writing of the manuscript: Williams, Arttamangkul, Birdsong

| | I- ME (pA) | | Decline (10 min/Peak) | | ME (0.3 μM), Post/Pre | |
|---|---|---|---|---|---|---|
| | 0.3 μM | 30 μM | Untreated | MTA | Untreated | MTA |
| WT | 279±96 (7) | 451±95 (7) | 0.60±0.16 (7) | 0.51±0.07 (9) | 0.23±0.06 (7) | **0.13±0.08** (9) |
| STANT-3A | 259±137 (9) | 439±134 (9) | 0.79±0.07 (7) | 0.78±0.08 (7) | 0.62±0.14 (10) | 0.56±0.15 (9) |
| STANT-7A | 290±138 (8) | 435±143 (8) | 0.73±0.10 (9) | 0.83±0.09 (14) | 0.82±0.15 (9) | **0.61±0.13 (13)** |
| TSST-4A | 231±82 (8) | 425±123 (8) | 0.53±0.13 (7) | 0.60±0.05 (10) | 0.25±0.14 (6) | 0.33±0.11 (5) |
| TSST-4E | 286±102 (7) | 400±123 (10) | 0.65±0.10 (7) | **0.49±0.13** (10) | 0.41±0.12 (7) | **0.18±0.13** (10) |

**Table 1.** Summary of results. **I-ME (pA)** - The mean amplitude of the currents induced by ME (0.3 and 30 μM) in slices from wild type animals (WT) and each of the mutant MORs. There was no statistical difference from wild type (ANOVA, Dunnett post hoc) indicating that the sensitivity to ME in slices from wild type animals was similar to slices taken from animals expressing each of the mutant receptors. **Decline (10 min/peak)** – Paired comparison between experiments from untreated and morphine treated animals (MTA) showing the decrease in current induced by ME (30 μM) during a 10 min application for each of the mutant receptors. In slices from animals expressing the TSST-4E receptors the decline was increased in morphine treated animals (MTA, $p<0.05$, unpaired T-test). **ME (0.3, post/pre)** – Paired comparison between slices from untreated and morphine treated animals. The ratio of the current induced by ME (0.3 μM) following desensitization normalized to the initial current for each of the mutant receptors. This ratio decreased in wild type, STANT-7A and TSST-4E ($p<0.05$, unpaired T-test).

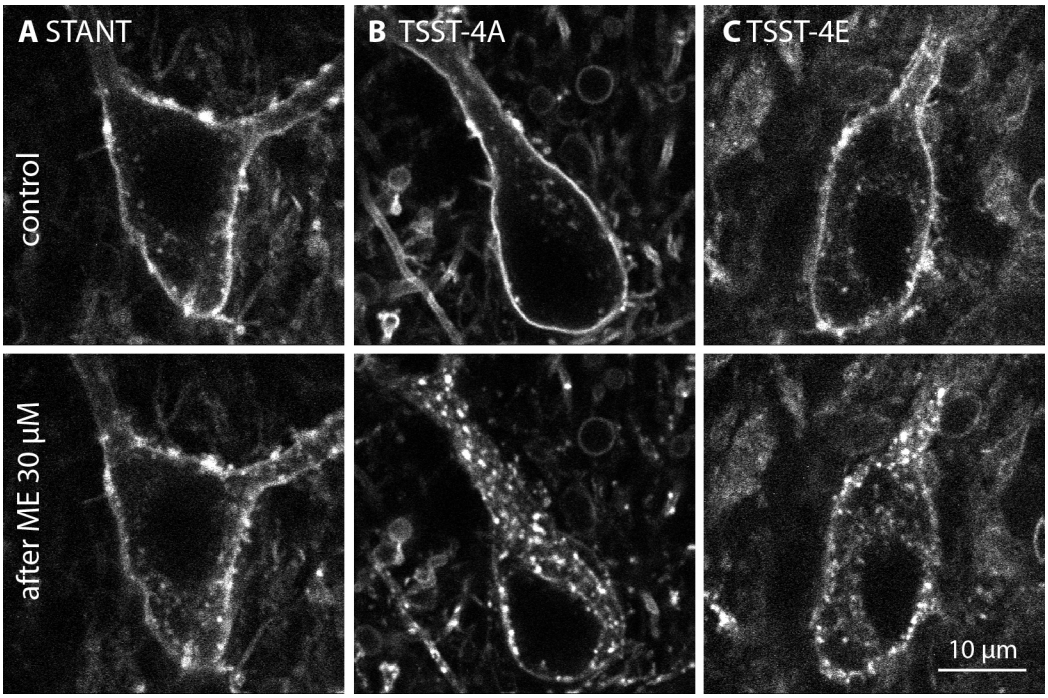



**Figure 1.** Receptor imaging before and after treatment with ME (30 µM, 10 min). An anti-GFP nanobody conjugated with alexa594 was to image the (A) STANT, (B) TSST-4A and (C) TSST-4E receptors expressed in locus coeruleus before (top) and following ME (30 µM, 10 min, bottom).

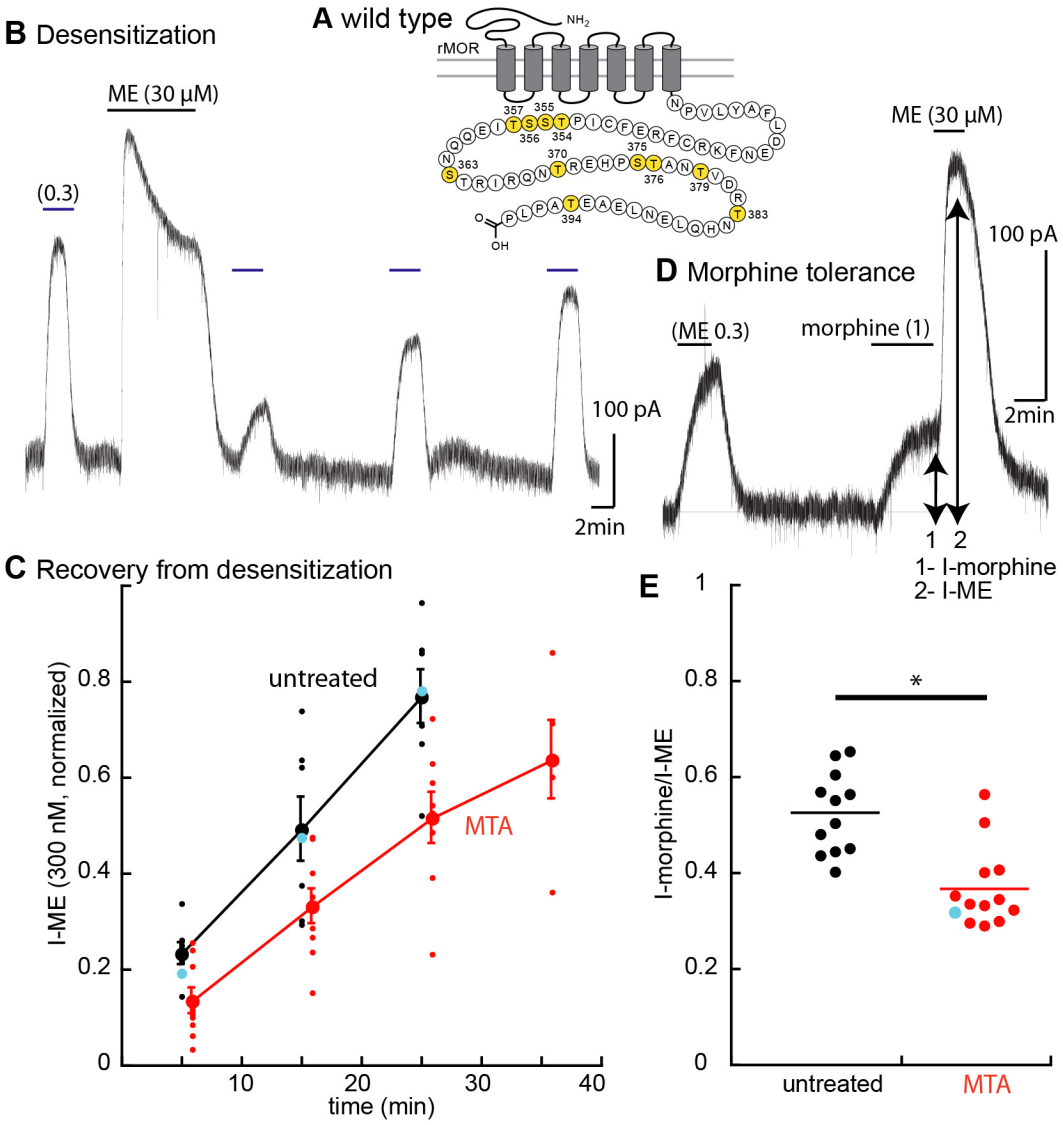



**Figure 2.** Protocols used to measure two forms of tolerance using experiments were carried out in wild type animals. (A) Snake plot illustrates the phosphorylation sites on the C-terminus (yellow,T354, S355, S356, T357, S363, T370, S375,T376, T379, T383, T394). (B) Protocol used to determine acute desensitization and the recovery from desensitization. (C) Summary of results showing the time course of recovery from desensitization slices from untreated (black) and in morphine treated (red) animals (MTA), blue dote indicate the results from the trace in B. (D) Trace shows the current induced by morphine (1 µM, 1) relative to that induced by ME (30 µM, 2). (E) Summary of the results measuring the relative current induced by morphine (1/2) in slices

from untreated animals (95% confidence interval 0.518±0.047) and morphine treated animal (95% confidence level 0.37±0.045) indicating that the morphine induced current is smaller in slices from morphine treated animals. Mann-Whitney U test P<0.001. Blue dot indicates the results from the experiment in D.

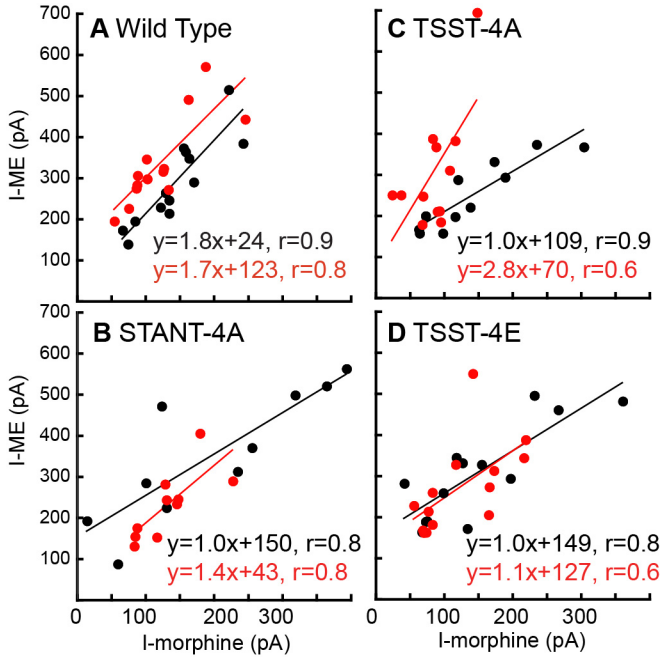



**Figure 3.** Summary of results comparing the current induced by morphine (1 µM) plotted against the current induced by ME (30 µM) in individual cells in slices from untreated (black) and morphine treated (red) animals. A) Cells in slices taken from wild type animals. The current induced by morphine after treatment with morphine is smaller than that in untreated animals. B) Cells taken from animals following expression of the STANT mutant. C) Cells taken from animals following the expression of the TSST-4A mutant. D) Cells in slices taken from animals expressing the TSST-4E mutant.

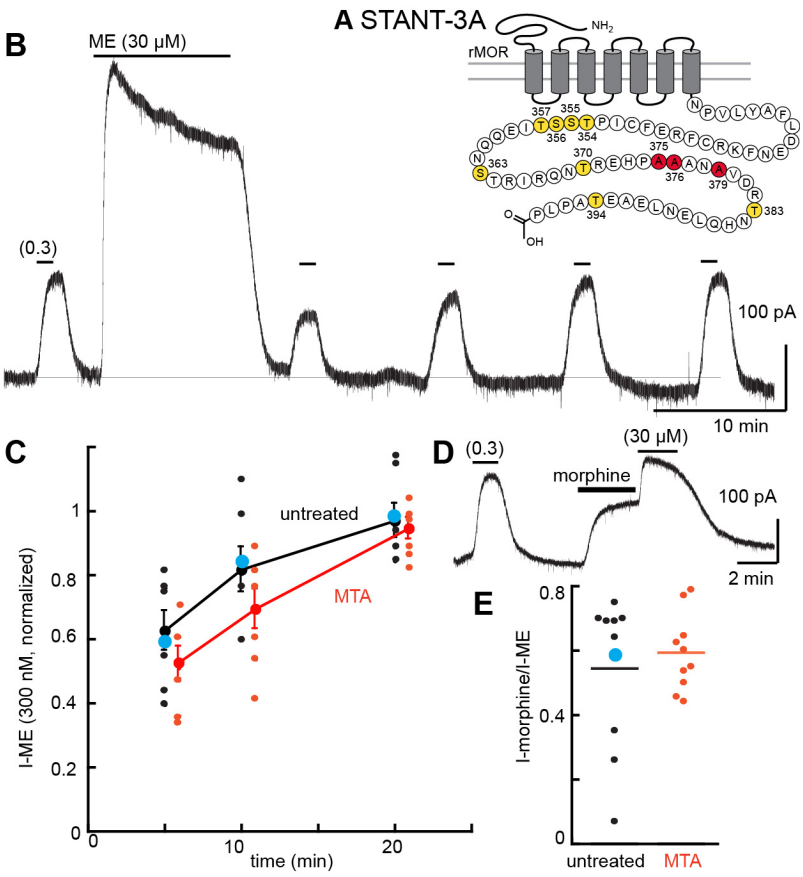



**Figure 4.** Desensitization is decreased and tolerance is blocked in cells expressing the STANT mutant receptor. (A) Snake plot illustrates the sites in the STANT mutant having alanine mutations (red, S375. T376, T379). (B) An experiment taken from a morphine treated animal illustrating the desensitization and recover from desensitization of ME (0.3 µM). (C) Summary of results showing the recovery from desensitization in slices from untreated and morphine treated animals (MTA). Blue dot indicates the results taken from the trace in B. (D) Trace illustrating the morphine (1 µM) current relative to the ME (30 µM) current. Blue dot indicates the result from the experiment illustrated in D. (E) Summary of experiments plotting the current induced by morphine (1 µM) divided by that induced by ME (30 µM, I-morphine/I-ME) indicating that the relative morphine current was unchanged in slices from morphine treated animals. Untreated 0.55±0.23, MTA 0.59±0.12 (95% confidence level).

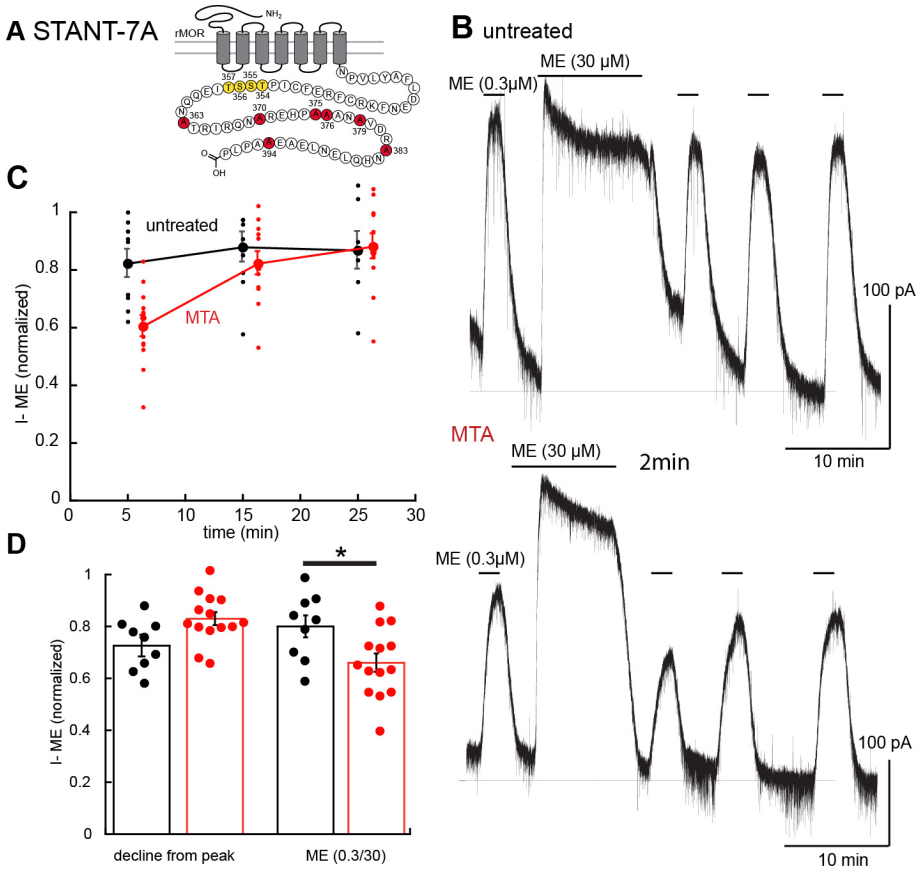



**Figure 5.** Transient desensitization is induced following chronic morphine treatment in the STANT-7A mutant receptors. (A) Snake plot indicates the site (red, S363, T370, S375, T376, T379, T383, T394) with alanine mutations. (B) Illustrates the lack of ME induced desensitization in a slice from an untreated animal (left) and a morphine treated (right) animal. (C) Summary of results showing the transient desensitization that is induced by chronic morphine treatment (MTA, red). (D) Left, shows the decline from the peak current during ME (30 µM, 10 min) in slices from untreated (black) and morphine treated (red) animals. Right shows the ME (0.3 µM) current relative to the peak ME (30 µM) current in slices from untreated and morphine treated animals.

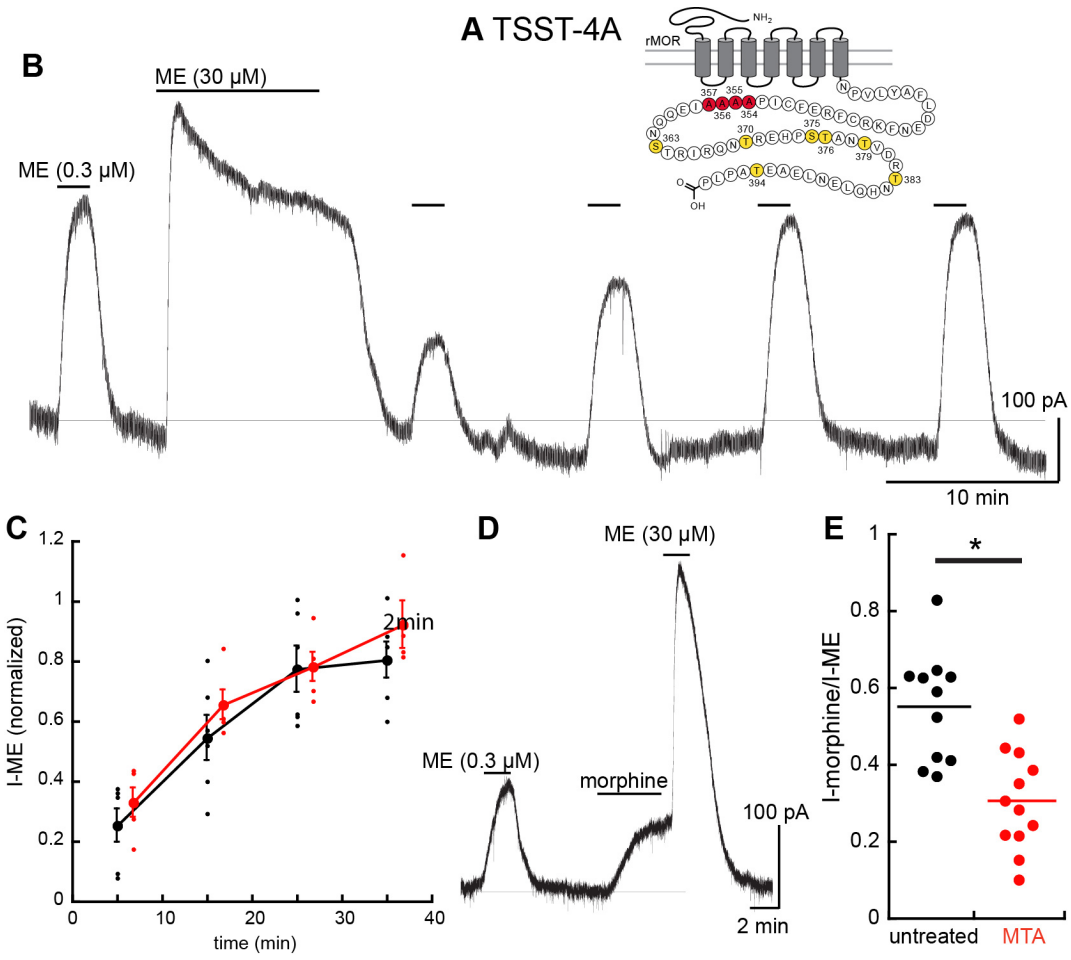



**Figure 6.** The TSST-4A mutant receptors distinguish tolerance measured by two assays, the increase in acute desensitization (blocked) and the decrease in sensitivity to morphine (present). (A) Snake plot illustrating the sites that were mutated to alanine (red, T354, S355, S356. T357). (B) An experiment illustrating the acute desensitization and recovery from desensitization. (C) Summarized results from showing the recovery from desensitization in untreated (black) and morphine treated (red) animals (MTA). Acute desensitization is insensitive to chronic morphine treatment. (D) An experiment in a slice taken from a morphine treated animal illustrating the decreased amplitude of the current induced by morphine. (E) Dot plot shows the relative current induced by morphine (I-morphine/I-ME) in slices from untreated (black) and morphine treated

(red) animals indicating that the relative morphine current was less in slices taken from morphine treated animals. Untreated 0.55±0.14, MTA 0.30±0.13 (95% confidence level).

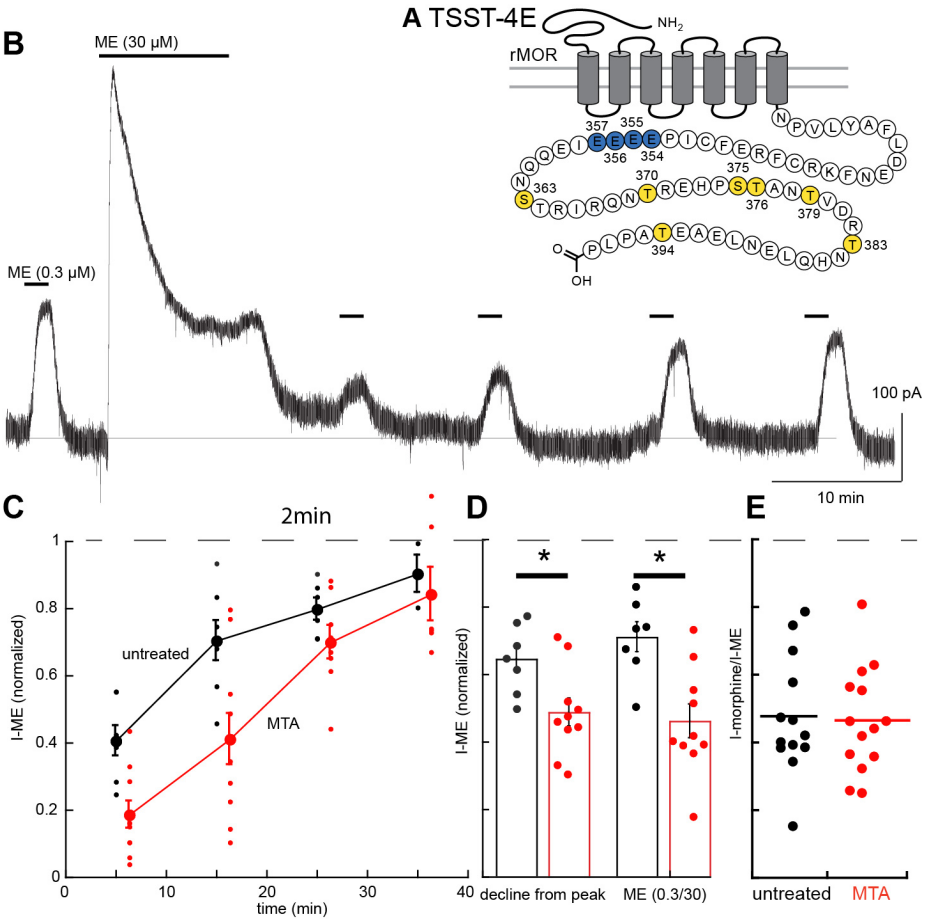
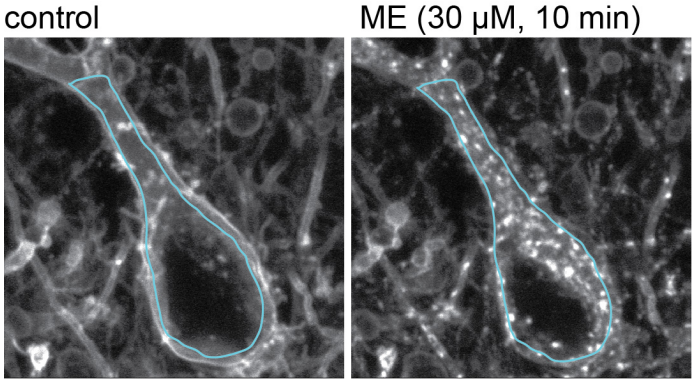



**Figure 7.** Following chronic treatment with morphine, the TSST-4E mutant MOR separates induction of changes in acute desensitization (present) from the decrease in the sensitivity to morphine (blocked). (A) Snake plot shows the sites with glutamate substitutions (blue, T354, S355, S356. T357). (B) An example of acute ME induced desensitization and the recovery from desensitization in a slice from a morphine treated animal. (C) Summary of experiments showing the recovery from desensitization in slices from untreated (black) and morphine treated (red) animals (MTA). (D) Summarized results showing the decline from the peak current induced by

ME (30 µM, 10 min) and the amplitude of the ME (0.3 µM) current relative to the peak current induced by ME (30 µM) in slices from untreated and morphine treated animals. (E) Dot plot of the relative current induced by morphine (1 µM) relative to the peak current induced by ME (30 µM, I-morphine/I-ME) showing that there was no change in the relative morphine current between slices from untreated and morphine treated animals. (Untreated 0.48±0.18, MTA 0.47±0.16, 95% confidence level)




**Supplemental Figure 1.** Illustration of the method used to determine the extent of receptor internalization. A line (blue) was drawn around the inside of the plasma membrane of a single neuron. The fluorescent in raw numbers was measured in fiji. That outline was then pasted on the same cell following treatment with MR (30 µM, 10 min). The fluorescence was once again determined. The increase in fluorescence was taken as a measure of internalization induced by ME.

# Chapter 4: Chronic Treatment with Morphine Disrupts Acute Kinase-Dependent Desensitization of GPCRs

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**Abstract:**

Based on studies using mutations of the μ-opioid receptor (MOR), phosphorylation of multiple sites on the C-terminus has been recognized as a critical step underlying acute desensitization and the development of cellular tolerance. The aim of this study is to explore which kinase(s) mediate desensitization of MOR in brain slices from drug naïve and morphine treated animals. Whole-cell recordings from locus coeruleus neurons were made and the agonist-induced increase in potassium conductance was measured. In slices from naïve animals, pharmacological inhibition of G-protein receptor kinase (GRK2/3) with compound 101 blocked acute desensitization. Following chronic treatment with morphine, compound 101 was less effective at blocking acute desensitization. Compound 101 blocked receptor internalization in tissue from both naïve and morphine treated animals suggesting that GRK2/3 remained active. Kinase inhibitors aimed at blocking PKC and JNK had no effect on desensitization in tissue taken from naïve animals. However, in slices taken from morphine treated animals, the combination of these blockers along with compound 101 was required to block acute desensitization. Acute desensitization of the potassium conductance induced by the somatostatin receptor was also blocked by compound 101 in slices from naïve but not morphine treated animals. As was observed with MOR, it was necessary to use the combination of kinase inhibitors to block desensitization of the somatostatin receptor in slices from morphine treated animals. The results show that chronic treatment with morphine results in a surprising and heterologous adaptation in kinase-dependent desensitization.

**Significance:**

The results show that chronic treatment with morphine induced heterologous adaptations in kinase regulation of GPCR desensitization. Although the canonical mechanism for acute

desensitization through phosphorylation by GRKs is supported in tissue taken from naïve animals, following chronic treatment with morphine, the acute kinase-dependent desensitization of GPCRs is disrupted such that additional kinases, including PKC and JNK, contribute to desensitization.

**Introduction**:

Acute desensitization of many GPCRs is initiated by G protein-coupled receptor kinase (GRK)-dependent phosphorylation (Gurevich and Gurevich, 2019). Most work to date on the μ-opioid receptor (MOR) has focused on phosphospecific antibodies and mutant receptors where phosphorylation sites on the C-terminus have been substituted with alanine (Williams et al., 2013). Two clusters of amino acid residues, 354 to 357 (TSST) and 375 to 379 (STANT), are phosphorylated following application of high efficacy agonists (Chen et al., 2013; Doll et al., 2011; Just et al., 2013; Lau et al., 2011; Miess et al., 2018; Wang et al., 2002). Four additional phosphorylation sites, constituting 11 total phosphorylation sites on the C-terminal tail of MOR, are either phosphorylated constitutively or by activity-dependent kinases, such as PKC (Williams et al., 2013). All 11 sites contribute to acute desensitization and long-term tolerance in varying degrees. Partially phosphorylation-deficient mutant MORs virally expressed in the rat locus coeruleus (LC) blocked internalization and reduced acute desensitization (Arttamangkul et al., 2019; Birdsong et al., 2015; Yousuf et al., 2015). In addition, total phosphorylation-deficient (TPD) MORs, in which all 11 phosphorylation sites are mutated to alanine, nearly abolished acute desensitization in the LC of both naïve and morphine treated animals (Arttamangkul et al., 2018; Kliewer et al., 2019), indicating that C-terminal phosphorylation is critical for MOR desensitization.

Acute desensitization in LC neurons is augmented and the recovery from desensitization is prolonged in slices taken from morphine treated animals (Arttamangkul et al., 2018; Dang and Williams, 2004; Levitt and Williams, 2012; Quillinan et al., 2011). The mechanism that underlies this augmentation is not understood. In slices from naïve animals, inhibition of GRK2/3 with compound 101 blocked one measure of acute desensitization in LC neurons (Lowe et al., 2015). Following chronic morphine treatment, a second component of desensitization that was dependent on PKC was observed (Bailey et al., 2009a; Levitt and Williams, 2012). In addition, spinally-mediated acute analgesic tolerance induced by morphine administration involved JNK2 (Melief et al., 2010). Morphine-induced acute MOR desensitization in the dorsal root ganglion was also mediated by JNK (Mittal et al., 2012). Taken together, these results suggest that chronic treatment with morphine induced the involvement of additional kinase(s) that augment desensitization.

In the present study, the activation of potassium conductance in rat brain slices induced by opioids and somatostatin on LC neurons was used to examine how kinase inhibitors affect acute desensitization before and following chronic treatment with morphine. Although the GRK2/3 inhibitor, compound 101 (CMP101), blocked acute desensitization of MORs and somatostatin receptors in slices from untreated animals, CMP101 did not block desensitization of either receptor in slices from morphine treated animals. While inhibitors of PKC or JNK (Go6976 and SP600125 respectively) did not block acute desensitization in naïve animals, these inhibitors in combination with CMP101 nearly abolished acute desensitization in chronically treated animals. The results indicate that kinase regulation of GPCR desensitization fundamentally changed following chronic morphine treatment.

**Materials and methods**:

Drugs: Morphine sulfate was obtained from the National Institute on Drug Abuse, Neuroscience Center (Bethesda, MD). [Met]$^5$enkephalin (ME), idazoxan, and β-chlornaltrexamine (β-CNA) were from Sigma-Aldrich (St. Louis, MO). Somatostatin was from ProSpec (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel). MK-801 ((5*S*,10*R*)-(+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate) and compound 101 (CMP101, 3-[(4-methyl-5-pyridin-4-yl-1,2,4-triazol-3-yl)methylamino]-N-[[2-(trifluoromethyl)phenyl]methyl]benzamide hydrochloride) were purchased from Hello Bio (Princeton, NJ); Go6976 (3-(13-methyl-5-oxo-6,7-dihydro-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazol-12(13H)-yl)propanenitrile), SP600125 (1,9-Pyrazoloanthrone), and UK14304 tartate (5-Bromo-6-(2-imidazolin-2-ylamino)quinoxaline) from Tocris (Bio-Techne Corp., Minneapolis, MN); and staurosporine from LC Laboratories (Woburn, MA). Potassium methanesulfonate was acquired from Alfa Aesar (Ward Hill, MA).

Somatostatin and ME (10 mM) were dissolved in water, diluted to the appropriate concentration in ACSF and applied by superfusion. Go6976, SP600125, and staurosporine (all 10 mM) were dissolved in DMSO. CMP101 was first dissolved in a small amount of DMSO (10% of final volume), sonicated, and then brought to its final volume with 20% (2-Hydroxypropyl)-β-cyclo-dextrin (Sigma-Aldrich, St. Louis, MO) and sonicated again to create a 10 mM solution. Slices were incubated in inhibitor(s) diluted in ACSF for at least 1 hour prior to recording and inhibitors were included in the bath and drug solutions at lower concentrations. β-CNA (10 mM) was dissolved in methanol and used at 30-100 nM in ACSF.

Animals: Adult rats of both sexes were used with ages between 5 – 8 weeks. Wildtype Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). MOR-knockout

Sprague-Dawley rats were also used as described in Arttamangkul et al. (2019). All animal experiments were conducted in accordance with the National Institutes of Health guidelines and with approval from the Institutional Animal Care and Use Committee of the Oregon Health & Science University (Portland, OR).

Microinjections: Animals (P24-30) were anesthetized with isoflurane and placed in a stereotaxic frame for microinjection of adeno associated virus type 2 (AAV2) encoding either wildtype MORs (exWT, AAV2-CAG-SS-GFP-MOR-WT-WPRE-SV40pA) or total phosphorylation-deficient MORs (TPD, AAV2-CAG-SS-GFP-MOR-TPD-WPRE-SV40pA) in the locus coeruleus (LC) of MOR knockout rats. 200 nL of virus was injected at 0.1 µl/min, bilaterally in the LC (AP: -9.72 mm, ML: ±1.25 mm, DV: -6.95 mm, from bregma) using a computer controlled stereotaxic frame (Neurostar, Tubingen, Germany). Both viruses were obtained from Virovek (Hayward, CA). Electrophysiology experiments were carried out 2-4 weeks following injection.

Chronic Opioid Treatment: Rats (5-6 weeks) were treated with morphine sulfate continuously released from osmotic pumps as described in Quillinan et al. (2011). Osmotic pumps (2ML1, Alzet, Cupertino, CA) were filled with the required concentration of morphine sulfate in water to deliver 80 mg/kg/day. Osmotic pumps were implanted subcutaneously in the mid-scapular region of rats maintained on isoflurane anesthesia and remained in the animals until they were used for experiments 6 or 7 days later.

Tissue Preparation: Horizontal slices (260 µm) containing LC neurons were prepared as previously described (Williams and North, 1984). Rats were deeply anesthetized and euthanized by cardiac percussion. Brains were removed, blocked, and placed in warm (34°C) ACSF

containing (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 1.2 $NaH_2PO_4$, 21.4 $NAHCO_3$, and 11 D-glucose with +MK-801 (10 μM), and cut horizontally (260 μm) using a vibratome (VT 1200S; Leica, Nussloch, Germany). Slices were allowed to recover in warm ACSF containing +MK-801 (10 μM) for at least 30 minutes and then stored in glass vials with warm (34°C), oxygenated (95% $O_2$/5% $CO_2$) ACSF until used.

Electrophysiology: Slices were hemisected and then transferred to the recording chamber, which was continuously superfused with 34ºC carbogenated ACSF at 1.5-2 ml/min. Whole-cell recordings from LC neurons were obtained with an Axopatch 200B amplifier (Axon Instruments) in voltage-clamp mode ($V_{hold}$ = -60mV). Recording pipettes (World Precision Instruments, Saratosa, FL) with a resistance of 1.5-2 MΩ were filled with an internal solution of (in mM): 115 potassium methanesulfonate or potassium methyl sulfate, 20 KCl, 1.5 $MgCl_2$, 5 HEPES(K), 10 BAPTA, 2 Mg-ATP, 0.2 Na-GTP, pH 7.4, and 275–280 mOsM. Only recordings where the series resistance remained <15 MΩ were included. Data were collected at 400 Hz with PowerLab (Chart Version 8.1.8; AD Instruments, Colorado Springs, CO).

Internalization and imaging: Trafficking of virally expressed wildtype MORs was visualized as previously described (Arttamangkul et al., 2019). Briefly, acute brain slices (260 μm) were prepared and then incubated with an anti-GFP nanobody conjugated to Alexa594 (Nb-A594, 10 mg/ml, 30-45 min). Images were captured before and after application of a saturating concentration of ME (30 μM, 10 min) using a 2-photon microscope. A z-series of 10 sections was acquired at 1-μm intervals so the whole neuron could be qualitatively compared.

Data Analysis: For all conditions, animals were used in order to obtain at least 6 technical replicates per group; if more than 6 could be analyzed, all were included. Analysis was

performed using GraphPad Prism 6 (GraphPad Software, version 6.0d, San Diego, CA) based on number of technical replicates (number of slices). Values are presented as average ± S.D. Statistical comparisons were made using one-way or two-way ANOVA, along with multiple comparison adjusted Tukey's post hoc tests, as appropriate. For all experiments $P < 0.05$ was used to describe significance.

**Results:**

**Naïve Animals**

**GRK2/3 is necessary for acute desensitization of MOR in naïve animals**

The selective, membrane-permeable, small-molecule GRK2/3 inhibitor, compound 101 (CMP101, Ikeda et al., 2007; Thal et al., 2011) was used to test the canonical mechanism of MOR desensitization by GRKs. Slices were incubated in CMP101 (30 μM, 1 hour), perfused throughout the recording at a lower concentration (CMP101, 1 μM) and CMP101 (10 μM) was included in drug containing solutions. Recordings were made in brain slices containing the locus coeruleus (LC) to measure the outward current induced by activation of G protein-coupled inwardly-rectifying potassium channels (GIRKs) by [Met]$^5$enkephalin (ME).

Two measures of desensitization that have been examined in the past were also used in the present study. First, the current induced by an $EC_{50}$ concentration of ME (0.3 μM) was measured before and after application of a saturating concentration of ME (30 μM, 10 min). The degree of desensitization was taken as the relative current amplitude induced by ME (0.3 μM) 5 min after washing the saturating concentration of ME (30 μM) compared to the initial current amplitude induced by ME (0.3 μM). Recovery from desensitization was measured with repeated applications of ME (0.3 μM) for 20-40 min following the washout of the saturating concentration of ME (30 μM, 10 min). The decline in the peak current during the application of the saturating

concentration of ME (30 μM, 10 min) was used as a second measure of desensitization, henceforth referred to as acute decline.

ME induced desensitization (30 μM, 10 min) as measured by both acute decline (58.8 ± 6.8% of peak, n = 15, Fig. 1A, E) and the decrease in the current induced by the $EC_{50}$ concentration of ME (0.3 μM) 5 min after washing ME (30 μM, 31.4 ± 9.1%, n = 12, Fig. 1A, D). As reported previously, recovery from desensitization occurred over a period of 20-35 min. However, in the presence of CMP101 (10 μM), both measures of acute desensitization were substantially reduced (Fig. 1C-E). The inhibition of desensitization induced by CMP101 in WT rats, measured by both acute decline and recovery from desensitization, was indistinguishable from that found using mutant receptors having alanine mutations in all 11 C-terminus phosphorylation sites (total phosphorylation-deficient MORs, TPD MORs) expressed in MOR knockout rats (decline: WT CMP101: 83.5 ± 2.4% of peak, n = 6; TPD: 80.2 ± 6.0% of peak, n = 7; P = 0.591; relative current at 5 min: WT CMP101: 78.8 ± 12.1% of initial, n = 6; TPD: 76.9 ± 9.5% of initial, n = 7; P = 0.931; Fig. 1B-E; Arttamangkul et al., 2018). This block was not due to an increased sensitivity to agonist induced by CMP101 as the concentration-response curve for ME was not changed in the presence of CMP101 (Supplemental Fig. 1A, B). These results indicate that GRK2/3 is necessary for acute desensitization in naïve rats. To test whether GRK primarily acted on the phosphorylation sites on the C-terminal tail of MOR, we measured the effect of CMP101 on MOR desensitization in rats expressing TPD MORs. Notably, there was no additional effect of CMP101 on desensitization in slices from rats expressing TPD MORs (Supplemental Fig. 2A-C). Thus, the 11 phosphorylation sites on the C-terminus are the main sites involved in GRK2/3-mediated acute MOR desensitization.

**GRK2/3 is necessary for MOR internalization**

The internalization of MORs induced by efficacious agonists is also known to be dependent on phosphorylation of the C-terminus. Wildtype MORs (exWT MORs), but not TPD MORs, that were virally expressed in the LC of MOR knockout rats were efficiently internalized during application of a saturating concentration of ME (30 μM, 10 min, Arttamangkul et al., 2018). The internalization of virally expressed wildtype MORs was examined in slices from naïve animals in the absence and presence of CMP101. Expressed receptors had an N-terminus GFP-tag such that plasma membrane-associated receptors were immuno-labeled with an anti-GFP nanobody conjugated to Alexa594 and imaged using 2-photon microscopy (Fig. 2). Labeled receptors were visualized before and after application of a saturating concentration of ME (30 μM, 10 min). As in previous studies, MORs were internalized following the application of ME (Fig. 2A, Arttamangkul et al., 2018). When slices were incubated in CMP101 (30 μM, 1 hour) along with the nanobody, internalization of exWT receptors was blocked (Fig. 2B). Thus, phosphorylation of MOR by GRK2/3 is a critical step in the process of internalization as well as desensitization.

**Other kinase inhibitors**

In addition to GRK2/3, other kinases have been shown to play a role in acute MOR desensitization that include PKC and JNK. The acute decline, the decrease in the $EC_{50}$-induced current, and the recovery from ME-induced desensitization were measured in the presence of selective kinase inhibitors for PKC and JNK (Go6976 and SP600125, respectively). Brain slices from wildtype rats were incubated in Go6976 (1 μM) or SP600125 (20 μM) for at least one hour prior to the experiment. Acute desensitization, as measured by acute decline and recovery from desensitization, in the presence of Go6976 did not differ from that in control slices (Fig. 3B-D). Likewise, the JNK inhibitor, SP600125, had no effect on acute desensitization; there was,

however, an unexpected decrease in the recovery from desensitization at 10 min (Fig. 3A, C, D). Experiments were also performed in the presence of the non-selective kinase inhibitor, staurosporine, that does not act on GRKs. Slices were incubated in staurosporine (1 μM) for at least an hour and staurosporine (100 nM) was included in the superfusion solution. As in experiments containing PKC or JNK inhibitors, staurosporine had no effect on acute MOR desensitization or acute decline in naïve animals (relative current at 5 min: 31.6 ± 5.4% of initial, n = 6, P > 0.999; decline: 59.3 ± 8.3% of peak, n = 6, P = 0.999). Thus, GRK2/3 is the major kinase(s) involved in acute MOR desensitization in the LC in slices from naïve animals and PKC or JNK alone are not sufficient to mediate acute desensitization of MOR under naïve conditions.

**Morphine Treated Animals**

In experiments with virally expressed TPD MORs, acute desensitization and signs of tolerance were blocked in morphine treated animals, indicating that phosphorylation of the receptor is necessary for the development of cellular tolerance (Arttamangkul et al., 2018). Previous work indicated that chronic morphine treatment induced a component of desensitization that was PKC-dependent in LC neurons, suggesting chronic morphine treatment altered the kinase regulation of MOR (Levitt and Williams, 2012). In the present study, the change in kinase-dependent modulation of MOR signaling following chronic morphine treatment was examined using kinase inhibitors. Wildtype rats were treated with morphine (80 mg/kg per day) for 6-7 days with osmotic mini pumps. Brain slices were maintained in morphine-free solutions such that they were in a state of acute withdrawal. Acute desensitization was examined in slices from morphine treated animals in the absence and presence of the GRK2/3 inhibitor, CMP101. In slices not treated with CMP101, the recovery from desensitization was slowed as previously reported (Fig. 4A, C; Arttamangkul et al., 2018; Arttamangkul et al., 2019; Dang and Williams, 2004;

Quillinan et al., 2011). In slices that were incubated with CMP101, the acute decline in the peak ME current was blocked, but CMP101 had no effect on the decrease in the current induced by the $EC_{50}$ concentration (ME, 0.3 μM) and the incomplete recovery from desensitization (Fig. 4B-D). Finally, the relative current induced by an $EC_{50}$ concentration of ME (0.3 μM) compared to that induced by a saturating concentration of ME (30 μM) was the same in the absence and presence of CMP101, suggesting that CMP101 did not change the sensitivity to ME (Fig. 4E). Thus, although CMP101 blocked two measures of desensitization in slices from naïve animals, following chronic treatment with morphine it had differing effects on the two forms of desensitization.

This observation could result from a difference in sensitivity of the two measures. A rightward shift in the concentration response curve could decrease the current induced by an $EC_{50}$ but not affect the peak outward current in slices from morphine treated animals. This possibility was examined by measuring the acute decline after partial irreversible block of receptors with the irreversible opioid receptor antagonist, β-chlornaltrexamine (β-CNA, 30-100 nM, 5 min) to reduce receptor reserve. Slices were incubated in β-CNA (100 nM, 5 min) after treatment with CMP101 (30 μM, 1 hr). The block of MORs was normalized to the current induced by the α2-adrenergic receptor agonist, UK14304 (3 μM). The peak current induced by ME (30 μM) decreased from 124.1 ± 10.9% in control (n = 4) to 48.6 ± 16.2% after treatment with β-CNA (n = 12). There was no correlation between the ratio of the peak current induced by ME (30 μM) relative to the peak current induced by UK14304 (3 μM) and the extent of acute decline in the presence of β-CNA (Fig. 5F). In slices from naïve animals treated with β-CNA, CMP101 still blocked acute decline (Fig. 5B, E). However, in slices from morphine treated animals treated with β-CNA, CMP101 no longer blocked the acute decline (Fig. 5D, E). Although CMP101

blocked acute decline in slices from morphine treated animals, it was no longer effective following partial irreversible removal of receptors with β-CNA. Thus, inhibition of GRK2/3 no longer blocked this measure of desensitization.

The internalization of MOR induced by ME was also examined in slices from morphine treated animals incubated with and without CMP101. Slices from MOR knockout animals that expressed wildtype N-terminus linked GFP-MORs (exWT MORs) were incubated in a solution containing anti-GFP nanobodies conjugated with alexa594 (Arttamangkul et al., 2018). Receptor trafficking was visualized with 2-photon microscopy in slices taken from morphine treated animals. Treatment with ME (30 μM, 10 min) induced internalization of the exWT MORs in slices from MTAs but internalization was blocked in slices that were incubated with CMP101 (Fig. 2C, D). Thus, GRK2/3 is necessary for internalization of MOR in both naïve and MTAs. The observation that internalization was blocked by CMP101 but desensitization measured by the recovery from desensitization was not affected further suggests that desensitization and internalization are separate processes (Arttamangkul et al., 2006). The results also indicate that GRK2/3 activity was not eliminated following chronic morphine treatment, suggesting that something other than, or in addition to, GRK2/3 must mediate desensitization in MTAs.

**PKC and JNK contribute to acute desensitization after chronic morphine treatment**

Given that the decrease in the recovery from desensitization following chronic morphine treatment was insensitive to CMP101, the action of PKC and JNK inhibitors on this measure was examined in slices from morphine treated animals. There was no change in the extent or rate of recovery of the current induced by ME (0.3 μM) in slices that were incubated with the JNK inhibitor alone (Fig. 6A, E). In contrast, this measure of desensitization was significantly

attenuated when slices were incubated with both the JNK inhibitor and CMP101 (Fig. 6B, E). When the combination of CMP101 and the PKC inhibitor, Go6976, were examined, there was a small reduction in acute desensitization following chronic morphine treatment (Fig. 6C, E). Finally, when all three kinase inhibitors were applied, a near complete inhibition of desensitization was observed (Fig. 6D, E). In these experiments, the desensitization was the same as that observed in experiments using expression of the GFP-TPD MORs in MOR knockout animals (Arttamangkul et al., 2018). It is interesting to note that neither the PKC nor JNK inhibitor had an additional effect on the acute decline of the current induced by a saturating concentration of ME (30 μM, Fig. 6F).

The non-selective kinase blocker, staurosporine, was used to determine if additional kinase activity contributed to the induction of desensitization in slices from morphine treated animals. Staurosporine alone had no effect on acute desensitization measured by the acute decline (decline: 58.7 ± 5.7% of peak, n = 7, P = 0.264, Fig. 7A, D) or the recovery from desensitization at 5 min (relative current at 5 min: 31.1 ± 5.6% of initial, n = 7, P = 0.173, Fig. 7A, C). Experiments with staurosporine in combination with CMP101 were not significantly different from those using all three specific inhibitors (CMP101, Go6976, and SP600125; relative current at 5 min: 75.2 ± 9.0% of initial, n = 6; P = 0.502; Fig. 6D, E; Fig. 7B-D). This further supports the idea that while kinases other than GRK2/3 are upregulated following chronic treatment, GRK2/3 activity is not downregulated as the block of desensitization still required treatment with CMP101. In addition, although CMP101 no longer blocked the acute decline in slices from morphine treated animals after treatment with β-CNA, the acute decline was blocked in slices from morphine treated animals treated with β-CNA in the presence of CMP101 and staurosporine (30 μM, 1 μM respectively; MTA βCNA CMP101: 50.9 ± 8.7% of peak, n = 8;

MTA βCNA CMP101 staurosporine: 76.4 ± 6.2% of peak, n = 8, P < 0.0001). Taken together, the results indicate that chronic morphine treatment induced an adaptive response in the kinase regulation of MORs that involves GRK2/3, JNK, and PKC.

**Chronic morphine treatment and heterologous desensitization of somatostatin receptors**

Somatostatin activates the same potassium conductance as opioids as determined by occlusion experiments (Fiorillo and Williams, 1996) and is known to be phosphorylated by GRK2/3 (Gunther et al., 2018). Desensitization of the somatostatin receptor was induced by somatostatin (SST, 20 μM, 10 min) and the decline in the peak current during the application was measured. The recovery from desensitization could not be tested because of the extended time it took to wash from the slice preparation. In order to obtain a baseline at the end of the application of SST, $BaCl_2$ (100 μM) was used to reverse current by blocking the potassium conductance.

The inhibition of desensitization induced by the kinase inhibitors was tested in slices from naïve and morphine treated animals. Treatment with SST (20 μM, 10 min) resulted in robust desensitization that was significantly reduced in slices incubated with CMP101 (Fig. 8A, B, F). The desensitization induced by SST was larger in slices taken from morphine treated animals (Fig. 8C, F). The acute decline in the current induced by somatostatin measured in slices from morphine treated animals was insensitive to CMP101 (Fig. 8D, F). The insensitivity to CMP101 was similar to that found with MORs with measures of recovery from desensitization and the acute decline (following treatment with β-CNA). When the desensitization of the somatostatin receptor was examined in the presence of the kinase inhibitors, CMP101, Go6976, and SP600125, desensitization was significantly reduced (Fig. 8E, F). Thus, chronic morphine

treatment induced a heterologous adaptive response on the kinase regulation of both MOR and the somatostatin receptor.

**Discussion**:

The present study examined kinase regulation of MORs and somatostatin (SST) receptors in LC neurons before and following chronic treatment with morphine. Inhibition of GRK2/3 with the selective inhibitor, CMP101, blocked acute MOR and SST desensitization in slices from naïve animals. Following chronic treatment with morphine, the kinase regulation of both MORs and SST receptors changed. Internalization of MORs induced by ME remained sensitive to CMP101, however the slowed recovery from desensitization was insensitive to CMP101. In addition, following the partial irreversible block of MORs in slices from morphine treated animals, CMP101 no longer blocked the acute decline. Thus, measures of acute desensitization and cellular tolerance to ME were insensitive to inhibition of GRK2/3. Although inhibitors of PKC and JNK had no effect on acute MOR desensitization in slices from naïve animals, in combination with CMP101, these inhibitors resulted in a near complete block of desensitization in slices from morphine treated animals. Taken together, the results show that chronic morphine treatment induced a heterologous adaptation in the kinase regulation of two GPCRs.

**Naïve Animals**

Canonically, phosphorylation by GRKs initiates acute homologous desensitization of GPCRs (Gurevich & Gurevich, 2019). As previously reported, inhibition of GRK2/3 with CMP101 blocked both measures of desensitization in slices taken from naïve animals (Lowe et al., 2015). Multiple sites on the C-terminus of MOR are directly phosphorylated by GRK2/3, suggesting that GRK2/3-dependent desensitization is likely through direct phosphorylation of MOR (Chen

et al., 2013; Doll et al., 2011; Just et al., 2013; Lau et al., 2011). The effect of CMP101 on MOR desensitization in WT rats, as measured by both acute decline and recovery from desensitization, was indistinguishable from that of desensitization of expressed TPD MORs, where all 11 phosphorylation sites on the C-terminus of MOR were mutated to alanine. In addition, CMP101 did not alter the small amount of desensitization in slices from rats expressing TPD MORs. The results suggest that the 11 phosphorylation sites on the C terminus underlie GRK2/3-dependent desensitization with little role of additional phosphorylation sites on the intracellular loops.

**Morphine Treated Animals**

While CMP101 blocked two measures of acute MOR desensitization and internalization in slices from naïve animals, it had mixed actions in slices taken from morphine treated animals. The acute decline and internalization of MORs remained sensitive to CMP101 in slices from morphine treated animals. However, the slowed recovery from desensitization measured using the $EC_{50}$ concentration was no longer sensitive to CMP101. It is possible that the two different measures of desensitization are dependent on separate processes (Arttamangkul et al., 2015; Birdsong et al., 2015), although following partial irreversible block of receptors with β-CNA, the acute decline was also insensitive to CMP101. Therefore, while inhibiting the activity of GRK2/3 blocked desensitization in naïve animals, it was not sufficient to block desensitization following chronic morphine treatment. Given CMP101 blocked internalization and the acute decline of the current induced by ME (without preincubation with β-CNA) in morphine treated animals, this suggests GRK2/3 remained active following chronic morphine treatment. There was also no indication that the sensitivity to ME was changed by CMP101 given that there was no change in the ratio of the current induced by ME (0.3/30 μM) in slices taken from both naïve and morphine treated animals.

The results support the idea that following chronic morphine treatment, additional kinases contribute to desensitization. One component of acute desensitization was dependent on PKC following chronic morphine treatment (Bailey et al., 2009a; Levitt and Williams, 2012). In addition, morphine-induced acute MOR desensitization in the dorsal root ganglion (Mittal et al., 2012), spinally-mediated acute analgesic tolerance induced by morphine administration (Melief et al., 2010) and centrally-mediated tolerance to morphine all involved JNK (Kuhar et al., 2015). In the present study, when the combination of staurosporine and CMP101 was used, the results were indistinguishable from those using the combination of selective inhibitors of GRK2/3, PKC, and JNK. Thus, GRK2/3, PKC, and JNK all contribute to desensitization following chronic treatment with morphine.

While inhibitors of GRK2/3, PKC, and JNK blocked the majority of acute desensitization (~80% of initial), a small amount of desensitization remained. This incomplete block could be either incomplete inhibition of kinases due to incomplete penetration or an as yet unknown phosphorylation-independent mechanism of acute desensitization. It is also possible that phosphorylation by kinases not affected by the inhibitors at sites other than the C-terminal tail could be responsible.

It is unclear how PKC and JNK are contributing to desensitization in slices from morphine treated animals. Although GRK2/3 has been shown to directly phosphorylate MOR, there is no evidence for direct phosphorylation of MOR by JNK. Arrestin can act as a scaffold for JNK (Kook et al., 2013; Zhan et al., 2013); however, morphine-induced JNK activation was arrestin-independent and PKC- and Src-dependent (Kuhar et al., 2015). Phosphorylation of MOR by PKC is known but the mechanism that underlies the morphine-induced activation of PKC has not

been characterized (Chen et al., 2013; Doll et al., 2011; Feng et al., 2011; Illing et al., 2014; Wang et al., 2002). The activation of PKC induced by muscarine enhanced desensitization of wildtype and also TPD MORs, indicating that PKC could increase desensitization through a mechanism that is independent of phosphorylation of the C-terminal tail (Arttamangkul et al., 2018). Thus, PKC and JNK could contribute to desensitization indirectly through phosphorylation of a component of the receptor signaling complex, preventing G-protein activation through an alternative process to GRK2/3-mediated desensitization. Heterologous desensitization in HEK293 cells through PKC-dependent phosphorylation of $G\alpha_{i2}$ has been reported (Chu et al., 2010). An alternative possibility is that PKC and/or JNK may act to mediate desensitization through phosphorylation of RGS proteins to turn off signaling, as has been reported in some cell types (Garzon et al., 2005; Ogier-Denis et al., 2000). Lastly, JNK-dependent desensitization resulted from JNK-dependent activation of peroxiredoxin 6 (PRDX6) to generate reactive oxygen species via NADPH oxidase that reduced the palmitoylation of receptor Gαi subunits that impacted MOR signaling (Schattauer et al., 2017).

**Heterologous kinase-dependent signaling following morphine treatment**

Chronic morphine treatment resulted in a heterologous modulation of GRK2/3-induced desensitization of the somatostatin receptor. Desensitization of somatostatin receptors was augmented in tissue taken from morphine treated animals and the inhibition of GRK2/3 by CMP101 was less effective. The co-application of PKC and JNK inhibitors along with CMP101 was required to substantially block somatostatin-induced desensitization in slices taken from morphine treated animals, indicating a heterologous adaptation of kinase regulation following chronic morphine treatment. The somatostatin receptor contains phosphorylation sites on the C-terminus that are phosphorylated by GRK2/3 and PKC, but not JNK (Gunther et al., 2018).

There is also evidence that acute desensitization of MOR in slices from naïve animals results in heterologous desensitization of the somatostatin receptor (Fiorillo and Williams, 1996). The present results suggest that this heterologous action is the result of the recruitment of GRK2/3 to the plasma membrane to affect not only MORs but also SST receptors.

That the desensitization induced by SST was not sensitive to the inhibition induced by CMP101 following chronic morphine treatment is unlike what was observed for the same measure of MOR desensitization. The acute decline in the current induced by ME (30 μM) remained sensitive to inhibition by CMP101. Thus, that measure of desensitization was dependent on GRK2/3. However, following the partial irreversible block of MORs with β-CNA, the acute decline in the current was no longer sensitive to CMP101. Given that a decrease in receptor reserve in LC neurons is induced following chronic morphine (Christie et al., 1987), it seems unlikely a decrease in receptor reserve alone is responsible for the change in sensitivity to CMP101 following treatment with β-CNA. It is clear however, that as was observed with the slowed recovery from desensitization, the acute decline in the current induced by ME (30 μM, following treatment with β-CNA) was not solely dependent on GRK2/3 in slices taken from morphine treated animals.

**Conclusion**

This study demonstrated that chronic morphine treatment induced heterologous adaptations in the kinase regulation of acute desensitization for two GPCRs. This may be one adaptation responsible for the augmentation of desensitization seen in animals chronically treated with morphine and therefore may contribute to behavioral tolerance. The surprising adaptive change

in kinase regulation of GPCRs may have significant functional consequences that are not directly related to opioid receptors.

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**Author Contributions:**

Participated in Research Design: Leff, Williams

Conducted Experiments: Leff, Arttamangkul

Performed data analysis: Leff

Wrote or contributed to writing of the manuscript: Leff, Williams

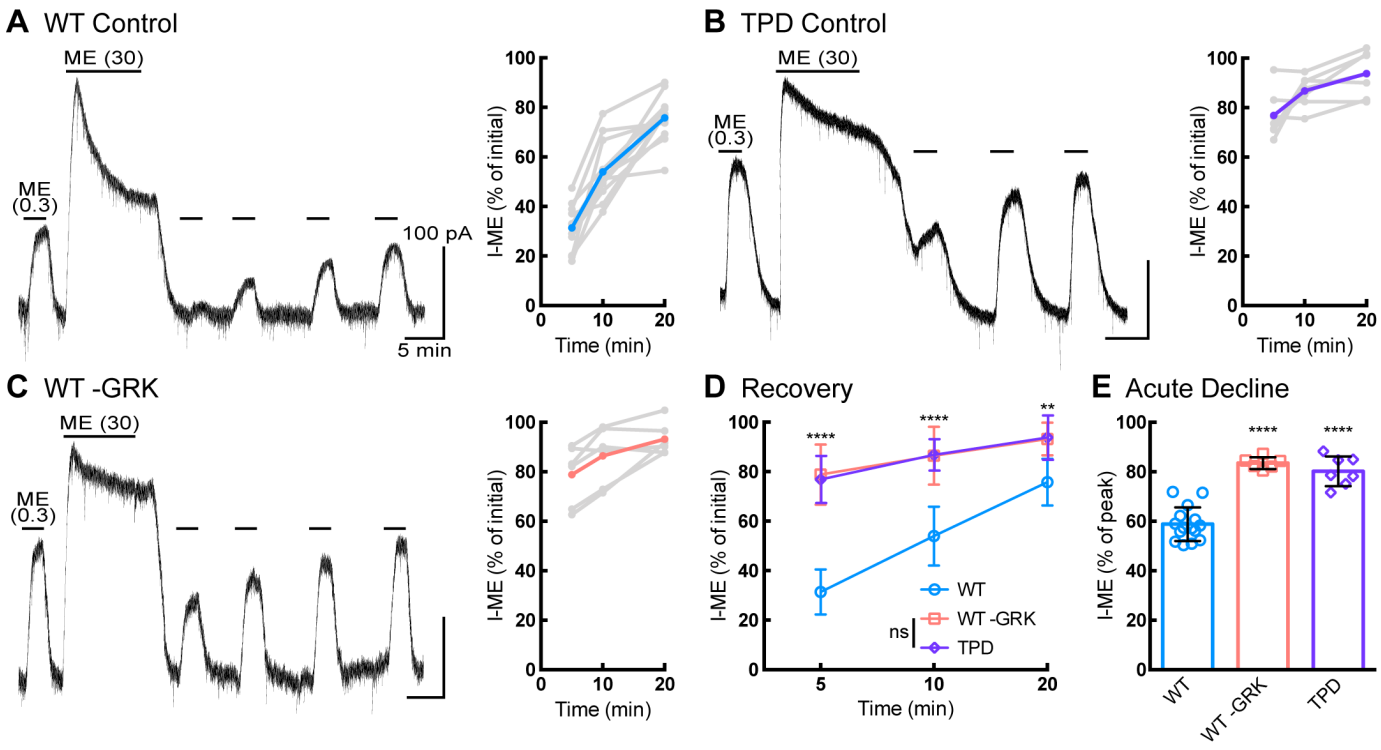



**Figure 1: GRK2/3 is necessary for acute desensitization of MOR in naïve animals.** Example trace showing the G protein-coupled inwardly-rectifying potassium channel (GIRK) currents induced by [Met]$^5$enkephalin (ME, 0.3 µM) before and following application of ME (30 µM, 10 min) and summary of recovery from µ-opioid receptor (MOR) desensitization at 5, 10, and 20 minutes post desensitization in locus coeruleus (LC) slices from (A) wildtype (WT) controls (n = 12 slices, 8 animals), (B) MOR KO animals virally expressing total phosphorylation deficient (TPD) mutant MORs in the LC (n = 7 slices, 7 animals), and (C) WT incubated in the GRK2/3 inhibitor, compound 101 (CMP101, 30 µM, 1 hr, n = 6 slices, 4 animals). CMP101 was also included in the bath (1 µM) and drug solutions (10 µM). Grey lines indicate individual n's and colored lines indicate averages. Current amplitudes following desensitization are normalized as a percentage of the prepulse. Summary graphs showing (D) recovery from desensitization (2way ANOVA, Tukey's post hoc) and (E) acute decline (one-way ANOVA, Tukey's post hoc) for WT

controls (light blue open circles), TPD controls (purple open diamonds), and WT incubated in CMP101 (salmon open squares). Data presented as average ± S.D., ****P < 0.0001.

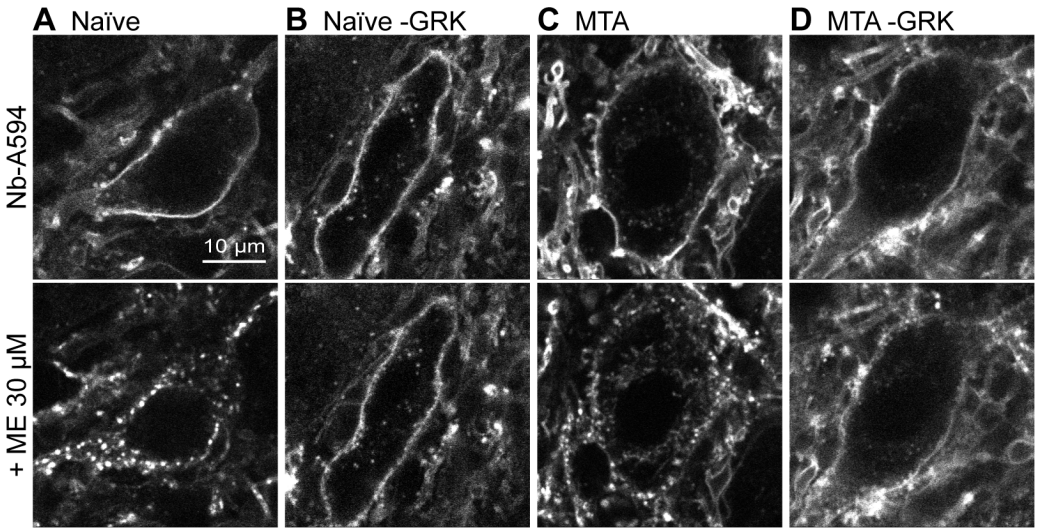



**Figure 2: GRK2/3 inhibitor blocks internalization in both naïve and morphine treated animals.** An anti-GFP nanobody conjugated to alexa594 was used to image expressed WT (exWT) receptors in the LC before (top) and following application of ME (30 µM, 10 min, bottom). Receptor distribution was imaged in both (A, B) naïve and (C, D) MTAs both in the (A, C) absence and (B, D) presence of CMP101 (1 µM). Experiments containing CMP101 were incubated in CMP101 (30 µM, 1 hour) prior to imaging. The exWT receptors internalized and became punctate in slices from (A, bottom) naïve (n = 4 slices, 4 animals) and (C, bottom) MTAs (n = 3 slices, 4 animals) in the absence of CMP101. Receptor trafficking was inhibited in slices both (B, bottom) naïve (n = 6 slices, 4 animals) and (D, bottom) MTAs (n = 6 slices, 4 animals) in the presence of CMP101. Scale bar = 10 µm.

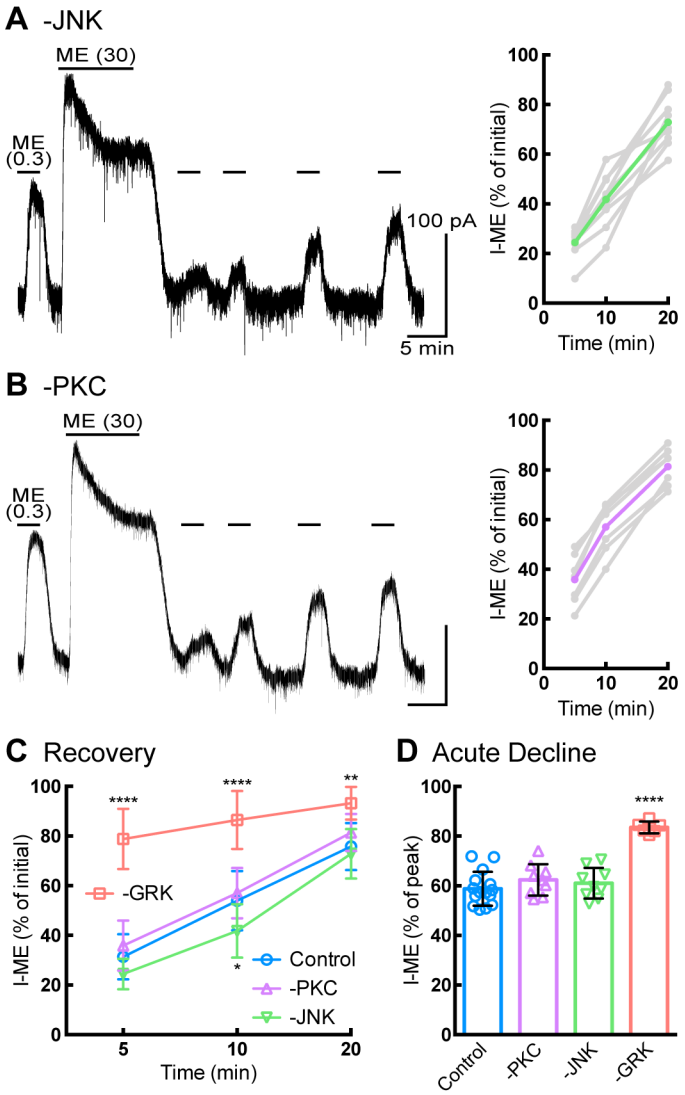



**Figure 3: PKC and JNK kinases not sufficient for acute MOR desensitization in naïve animals.** Example trace showing the currents induced by ME (0.3 µM) before and following application of ME (30 µM, 10 min) and summary of recovery from MOR desensitization at 5, 10, and 20 minutes post desensitization in slices from (A) WT animals incubated in a JNK inhibitor (SP600125, 20 µM, 1 hour, n = 9 slices, 5 animals) or (B) a PKC inhibitor (Go6976, 1 µM, 1 hour, n = 7 slices, 5 animals). Inhibitors were also included in the bath (1 µM, 100 nM respectively) and drug solutions (10 µM, 1 µM respectively). Grey lines indicate individual n's and colored lines indicate averages. Current amplitudes following desensitization are normalized

as a percentage of the prepulse. Summary graphs showing (C) recovery from desensitization (2way ANOVA, Tukey's post hoc) and (D) acute decline (one-way ANOVA, Tukey's post hoc) for WT controls (light blue open circles), WT incubated in SP600125 (light green open upside down triangles), WT incubated in Go6976 (light purple open triangles), and WT incubated in CMP101 (salmon open squares). Data presented as average ± S.D., *P < 0.05, **P < 0.01, ****P < 0.0001.

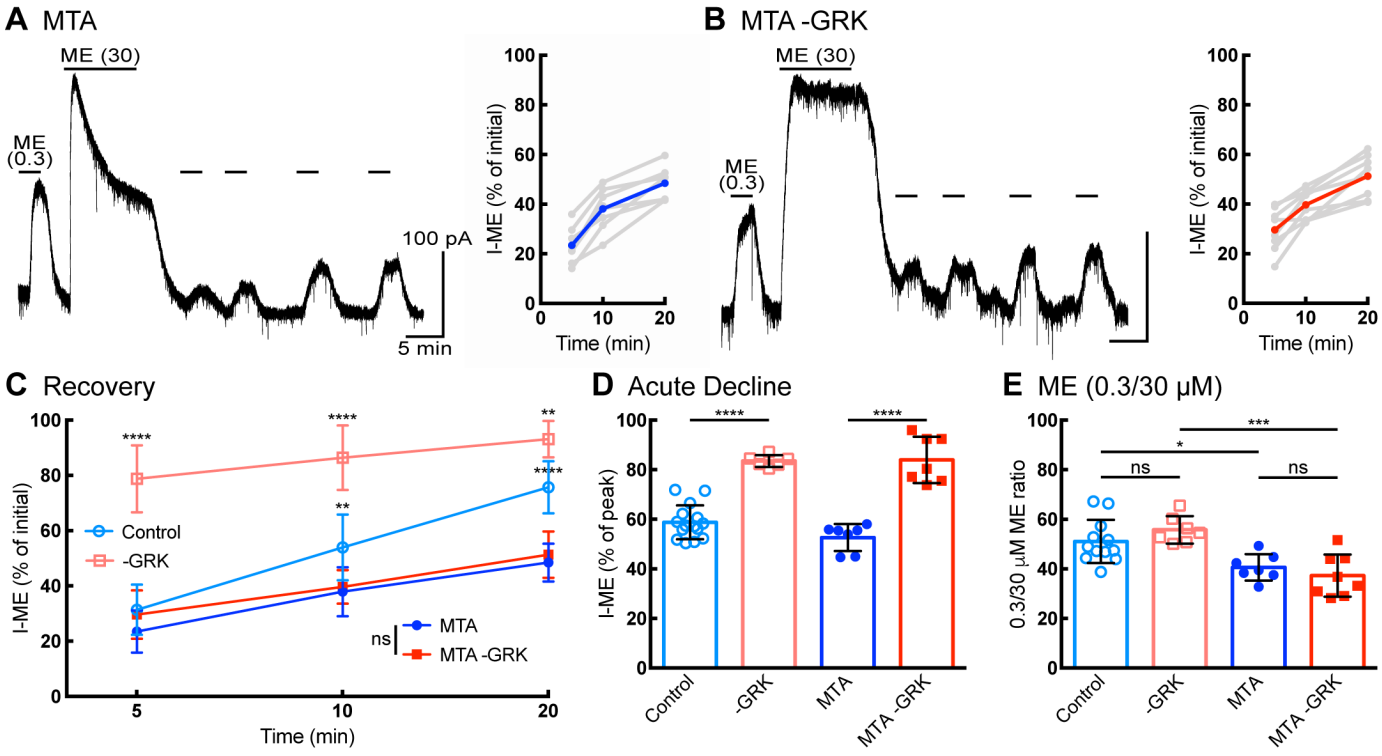



**Figure 4: GRK2/3 inhibitor blocks acute desensitization in naïve but not morphine treated animals.** Experiments were in slices from morphine treated animals (MTAs). Example trace showing the currents induced by ME (0.3 µM) before and following application of ME (30 µM, 10 min) and summary of recovery from MOR desensitization at 5, 10, and 20 minutes post desensitization in slices from (A) WT MTA (n = 7 slices, 6 animals) or (B) WT MTA incubated in CMP101 (30 µM, 1 hour, n = 8 slices, 5 animals). CMP101 was also included in the bath (1

μM) and drug solutions (10 μM). Grey lines indicate individual n's and colored lines indicate averages. Current amplitudes following desensitization are normalized as a percentage of the prepulse. Summary graphs showing (C) recovery from desensitization (2way ANOVA, Tukey's post hoc), (D) acute decline (one-way ANOVA, Tukey's post hoc), and (E) ME 0.3 μM /30 μM ratio (one-way ANOVA, Tukey's post hoc) for WT controls (light blue open circles), WT MTA (dark blue closed circles), WT incubated in CMP101 (salmon open squares), and WT MTA incubated in CMP101 (red closed squares). Data presented as average ± S.D., *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

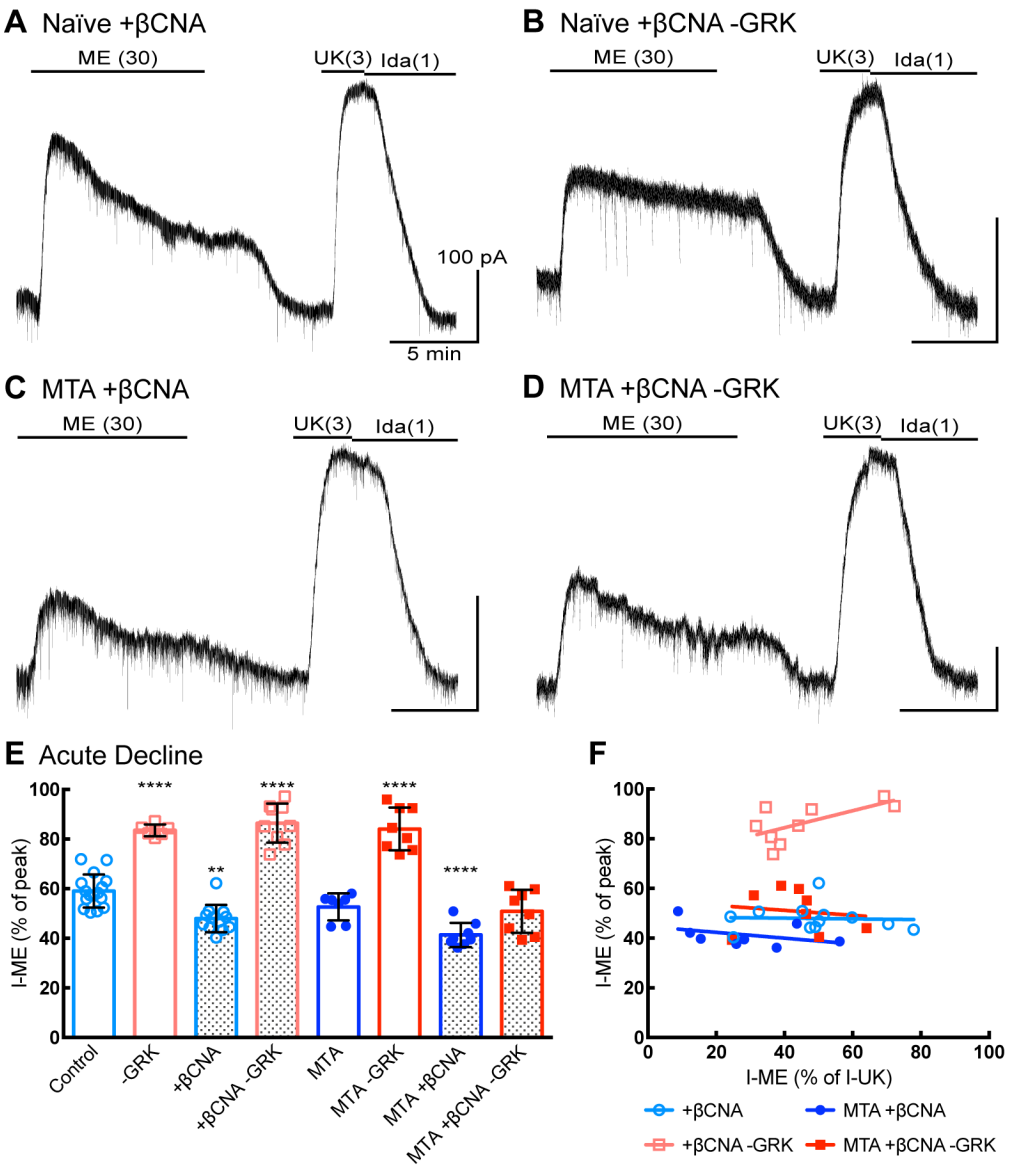



**Figure 5: GRK2/3 inhibitor does not block acute decline in morphine treated animals after partial irreversible block of MORs.** Experiments were performed in slices from both naïve and MTAs. Example trace showing the decline in the peak current during application of ME (30 µM, 10 min) followed by the current induced by UK14304 (UK, 3 µM) and reversed with idazoxan (1 µM) in slices from (A) WT naïve incubated in the irreversible MOR antagonist, β-CNA (30-100 nM, 5 min, n = 12 slices, 4 animals), (B) WT naïve incubated in CMP101 (30 µM, 1 hour) and then β-CNA (30-100 nM, 5 min, n = 9 slices, 6 animals), (C) WT MTA incubated in β-CNA (30-

100 nM, 5 min, n = 8 slices, 4 animals), and (D) WT MTA incubated in CMP101 (30 μM, 1 hour) and then β-CNA (30-100 nM, 5 min, n = 8 slices, 4 animals). CMP101 was also included in the bath (1 μM) and drug solutions (10 μM). Summary graphs showing (E) acute decline (one-way ANOVA, Tukey's post hoc) and (F) the correlation between the ratio of the peak current induced by ME (30 μM) relative to the peak current induced by UK (3 μM) and the extent of acute decline in the presence of β-CNA (linear regression) for WT naïve incubated in β-CNA (light blue open circles, shaded box), WT naïve incubated in CMP101 and then β-CNA (salmon open squares, shaded box), WT MTA incubated in β-CNA (dark blue closed circles, shaded box), and WT MTA incubated in CMP101 and then β-CNA (red closed squares, shaded box). Data presented as average ± S.D., **P < 0.01, ****P < 0.0001.

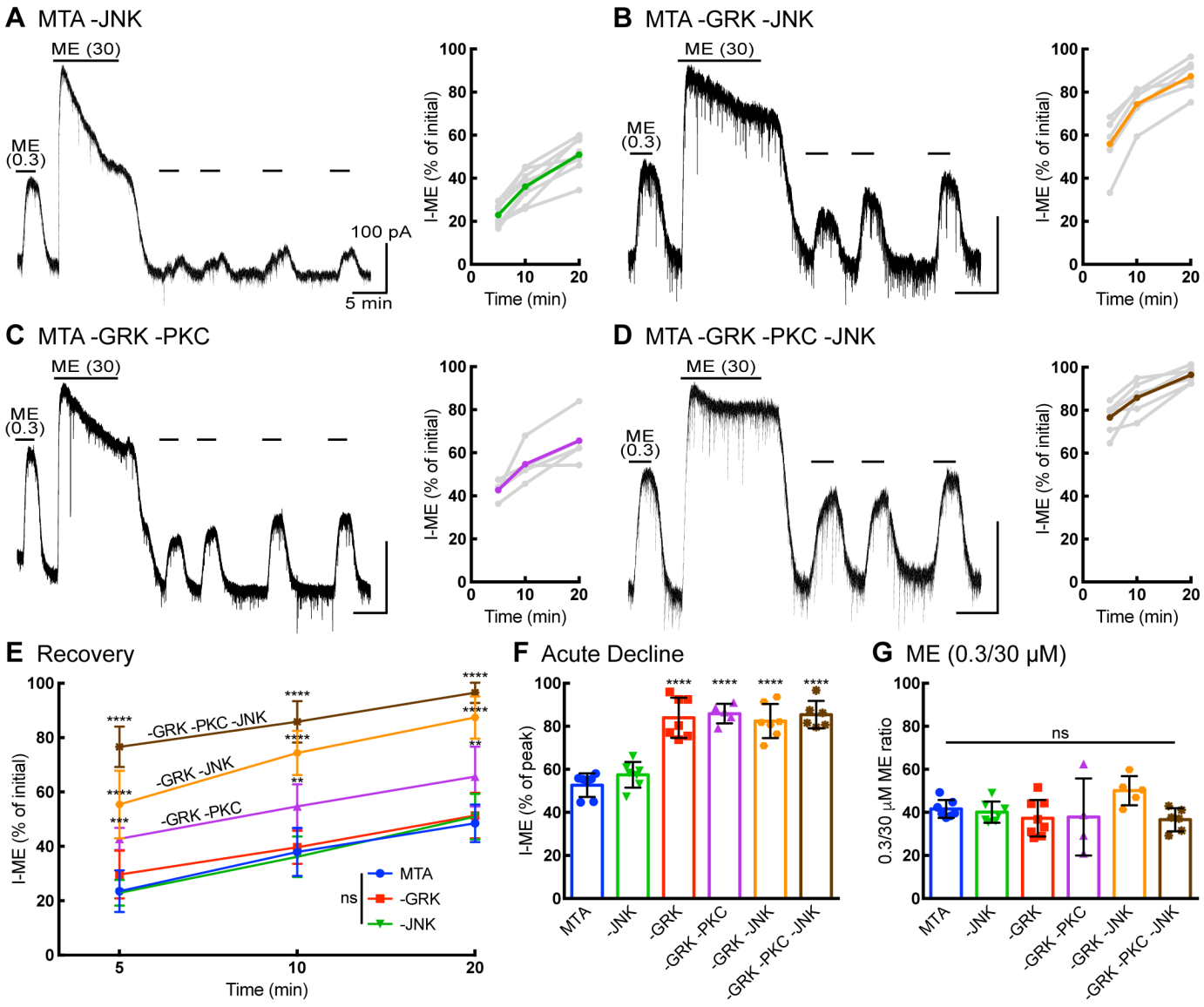



**Figure 6: PKC and JNK are sufficient to cause acute desensitization in morphine treated animals.** Experiments were conducted in slices from MTAs. Example trace showing the currents induced by ME (0.3 µM) before and following application of ME (30 µM, 10 min) and summary of recovery from MOR desensitization at 5, 10, and 20 minutes post desensitization in slices from (A) WT MTA incubated in CMP101 and Go6976 (30 µM, 1 µM, 1 hour, n = 5 slices, 3 animals), (B) WT MTA incubated in CMP101 and SP600125 (30 µM, 20 µM, 1 hour, n = 6 slices, 4 animals), (C) WT MTA incubated in SP600125 (20 µM, 1 hour, n = 8 slices, 6 animals),

and (D) WT MTA incubated in all three kinase inhibitors, CMP101, Go6976, and SP600125 (30 μM, 1 μM, 20 μM, 1 hour, n = 6 slices, 4 animals). Inhibitors were also included in the bath and drug solutions at lower concentrations. Grey lines indicate individual n's and colored lines indicate averages. Current amplitudes following desensitization are normalized as a percentage of the prepulse. Summary graphs showing (E) recovery from desensitization (2way ANOVA, Tukey's post hoc), (F) acute decline (one-way ANOVA, Tukey's post hoc), and (G) ME 0.3 μM /30 μM ratio (one-way ANOVA, Tukey's post hoc) for WT MTA controls (dark blue circles), WT MTA incubated in CMP101 (red squares), WT MTA incubated in SP600125 (green upside down triangles), WT MTA incubated in CMP101 and Go6976 (purple triangles), WT MTA incubated in CMP101 and SP600125 (orange hexagons), and WT MTA incubated in all three inhibitors (brown asterisks). Data presented as average ± S.D., **P < 0.01, ***P < 0.001, ****P < 0.0001.

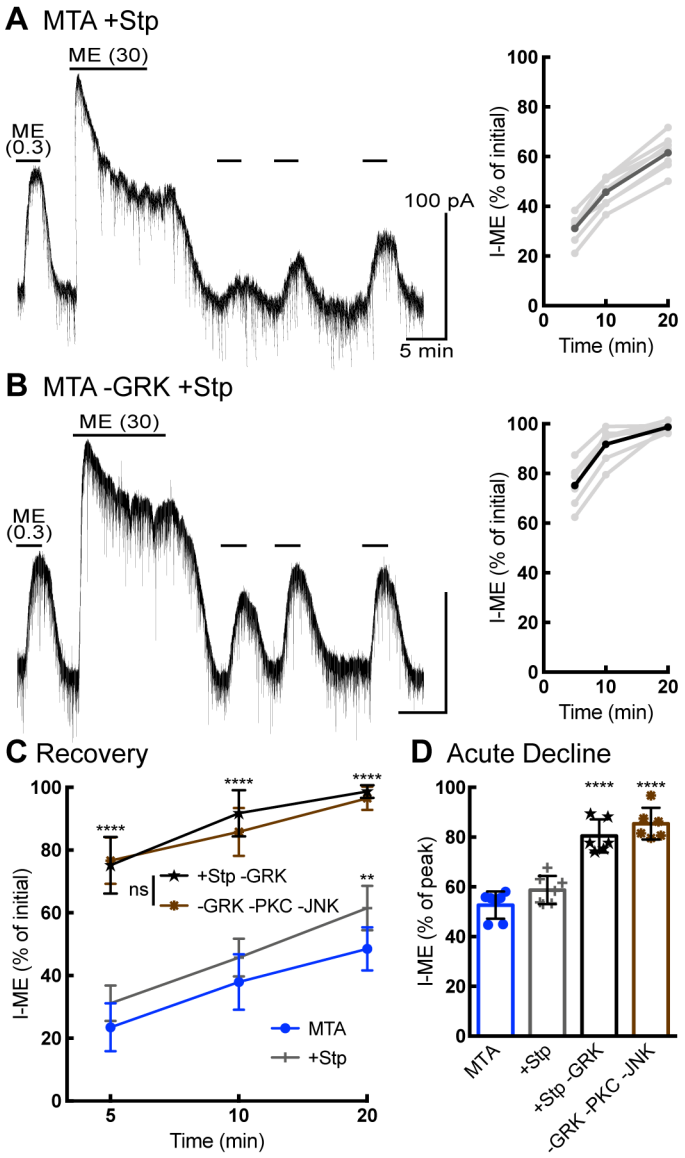



**Figure 7: GRK2/3, PKC, and JNK are the three main kinases involved in acute desensitization in morphine treated animals.** Experiments were conducted in slices taken from MTAs. Example trace showing the currents induced by ME (0.3 μM) before and following application of ME (30 μM, 10 min) and summary of recovery from MOR desensitization at 5, 10, and 20 minutes post desensitization in slices from (A) WT MTA incubated in the nonspecific kinase inhibitor, staurosporine (Stp, 1 μM, 1 hour, n = 7 slices, 4 animals) or (B) WT MTA incubated in staurosporine and CMP101 (1 μM, 30 μM, 1 hour, n = 6 slices, 4 animals). Grey

lines indicate individual n's and colored lines indicate averages. Current amplitudes following desensitization are normalized as a percentage of the prepulse. Summary graphs showing (C) recovery from desensitization (2way ANOVA, Tukey's post hoc) and (D) acute decline (one-way ANOVA, Tukey's post hoc) for WT MTA controls (dark blue circles), WT incubated in staurosporine (dark grey plusses), WT incubated in staurosporine and CMP101 (black stars), and WT incubated in the 3 inhibitors: CMP101, Go6976, and SP600125 (brown asterisks). Data presented as average ± S.D., **P < 0.01, ****P < 0.0001.

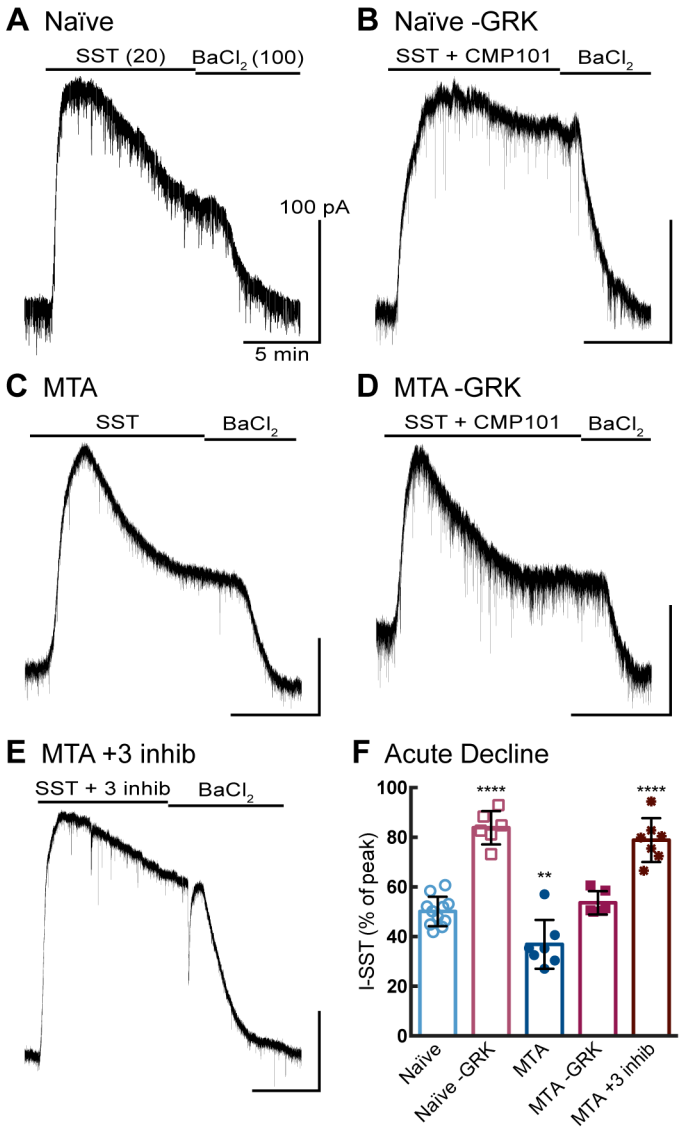
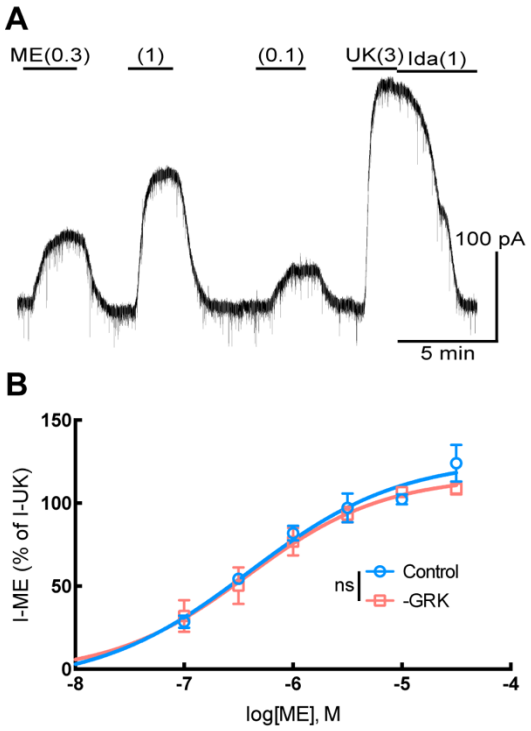



**Figure 8: Chronic treatment with morphine induces a heterologous adaptation in the kinase regulation of acute desensitization.** Example trace showing the decline in current induced by Somatostatin (SST, 20 μM, 10 min) in slices from (A) WT naïve animals (n = 11 slices, 6 animals), (B) WT naïve incubated in CMP101 (30 μM, 1 hour, n = 6 slices, 5 animals), (C) WT MTA (n = 7 slices, 6 animals), (D) WT MTA incubated in CMP101 (30 μM, 1 hour, n = 6 slices, 5 animals), and (E) WT MTA incubated in all 3 inhibitors, CMP101, Go6976, and SP600125 (30 μM, 1 μM, 20 μM, 1 hour, n = 7 slices, 5 animals). CMP101 was also included in

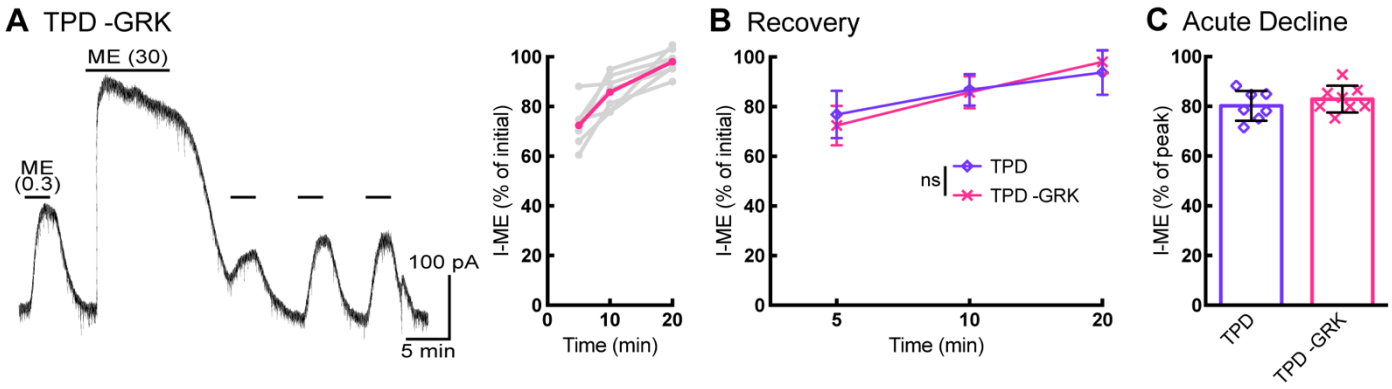
the bath (1 μM) and drug solutions (10 μM) as were CMP101, Go6976, and SP600125 when all 3 were used. (F) Summary graph showing acute decline (one-way ANOVA, Tukey's post hoc) for WT naïve (light blue open circles), WT naïve incubated in CMP101 (light pink open squares), WT MTA (dark blue circles), WT MTA incubated in CMP101 (dark pink squares), and WT MTA incubated in all 3 inhibitors (brown asterisks). Data presented as average ± S.D., **P < 0.01, ****P < 0.0001.

**Supplemental Figures:**




**Supplemental Figure 1: GRK2/3 inhibitor does not alter MOR sensitivity to ME.** (A) Example trace showing currents induced by different concentrations of the MOR agonist ME (0.3, 1, and 0.1 μM) and a saturating concentration of the α2-adrenergic receptor agonist, UK14304 (UK, 3 μM), which was reversed by the antagonist Idazoxan (Ida, 1 μM). (B) Concentration-response curves for WT controls (light blue open circles, n = 3-4/concentration)

and WT incubated in CMP101 (30 µM, 1 hour, salmon open squares, n = 3-4/concentration). CMP101 was also included in the bath (1 µM) and drug solutions (10 µM). ME-induced current responses were normalized as a percentage of the current induced by UK. Curves were fit using nonlinear regression (log[agonist] vs. response – variable slope (four parameters)). There was no significant difference between the curves for any of the parameters (extra sum-of-squares F test, P = 0.5207). Data presented as average ± S.D.




**Supplemental Figure 2: GRK2/3 inhibitor does not further block desensitization in slices from animals expressing TPD MORs.** Example trace showing the currents induced by ME (0.3 µM) before and following application of ME (30 µM, 10 min) and summary of recovery from MOR desensitization at 5, 10, and 20 minutes post desensitization in slices from (A) MOR KO animals virally expressing TPD MORs in the LC and incubated in CMP101 (30 µM, 1 hour, n = 7 slices, 5 animals). CMP101 was also included in the bath (1 µM) and drug solutions (10 µM). Grey lines indicate individual n's and colored lines indicate averages. Current amplitudes following desensitization are normalized as a percentage of the prepulse. Summary graphs showing (B) recovery from desensitization (2way ANOVA, Tukey's post hoc) and (C) acute decline (one-way ANOVA, Tukey's post hoc) for TPD controls (purple open diamonds) and TPD incubated in CMP101 (pink x's). Data presented as average ± S.D.

# Chapter 5: Additional Experiments

## Naïve Animals

So far, this study has shown that the 11 phosphorylation sites on the C-terminus of MOR play varying roles in measures of acute desensitization and cellular tolerance (Chapter 3) and that chronic morphine treatment induces an adaptation in the kinase-regulation of acute desensitization such that JNK and PKC contribute to MOR desensitization (Chapter 4). Thus, mutant receptors (TSST 4A and STANT 3A) used in chapter 3 were again used to further elucidate how the different phosphorylation site clusters contribute to the adaptation observed in chapter 4. Similar to previous experiments, the acute decline, decrease in the $EC_{50}$-induced current, and recovery from ME-induced desensitization were measured in slices from MOR KO animals virally expressing either expressed WT (exWT), TSST 4A, or STANT 3A mutant MORs in the LC. Again, GRK2/3 activity was blocked with the GRK2/3-specific inhibitor, CMP101.

Like for endogenous wildtype MORs, these experiments showed that CMP101 blocked both measures of acute desensitization (acute decline and recovery) for each receptor (exWT, TSST 4A, and STANT 3A) in slices from naïve animals (**Figure 5.1A-E**). Although there were small significant differences in the recovery from desensitization at 5 and 10 min between the TSST 4A and STANT 3A mutants, desensitization of each respective mutant did not significantly differ from that of exWT MORs. Thus, CMP101 blocked desensitization for each receptor to similar degrees (**Figure 5.1A-E**). However, when these results are compared to the extent of desensitization in the absence of CMP101 for each respective receptor as reported in Arttamangkul et al. 2018 and Arttamangkul et al., 2019 (Chapter 3, indicated by dark grey circles in the recovery graphs of **Figure 5.1**), important differences arise.

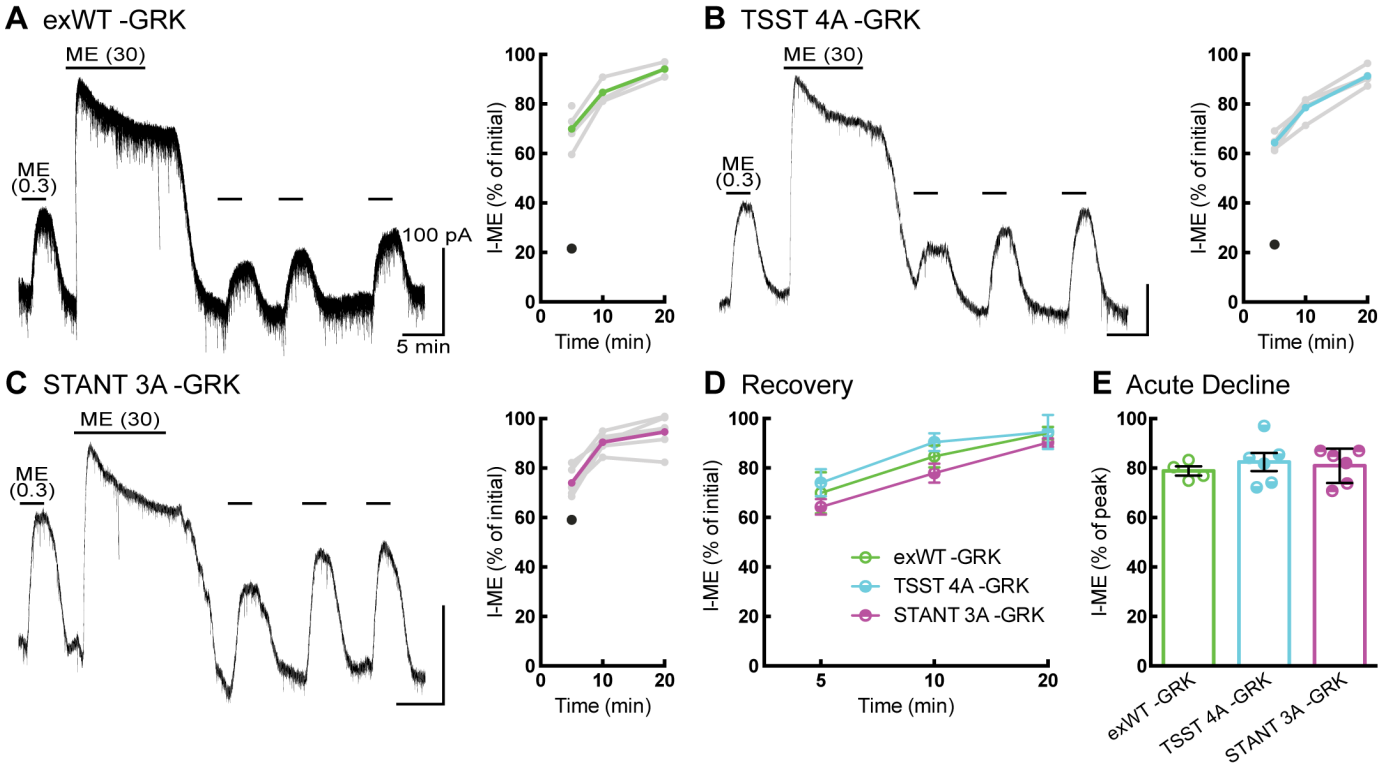



**Figure 5.1: The STANT cluster is more involved in GRK2/3-mediated desensitization than the TSST cluster.** Example trace showing the GIRK currents induced by [Met]$^5$enkephalin (ME, 0.3 µM) before and following application of ME (30 µM, 10 min) and summary of recovery from µ-opioid receptor (MOR) desensitization at 5, 10, and 20 minutes post desensitization in locus coeruleus (LC) slices from MOR KO rats virally expressing (A) wildtype (exWT) MORs in the LC incubated in the GRK2/3 inhibitor, compound 101 (CMP101, 30 µM, 1 hr, n = 4 slices, 2 animals), (B) mutant MORs with alanine mutation of the TSST cluster (TSST 4A) in the LC incubated in CMP101 (n = 6 slices, 3 animals), and (C) mutant MORs with alanine mutation of the STANT cluster (STANT 3A) in the LC incubated in CMP101 (n = 6 slices, 4 animals). CMP101 was also included in the bath (1 µM) and drug solutions (10 µM). Grey lines indicate individual n's and colored lines indicate averages. Current amplitudes following desensitization are normalized as a percentage of the prepulse. The extent of desensitization at 5 min for each respective receptor (exWT, TSST 4A, and STANT 3A) as reported in Arttamangkul et al. (2018, 2019) is shown with a dark gray circle in each recovery graph. Summary graphs showing (D) recovery from desensitization (2way ANOVA, Tukey's post hoc) and (E) acute decline (one-way ANOVA, Tukey's post hoc) for exWT incubated in CMP101 (green open circles), TSST 4A incubated in CMP101 (blue half closed cirlces), and STANT 3A incubated in CMP101 (magenta half closed circles). Data presented as average ± S.D

In the absence of CMP101, desensitization of TSST 4A mutant MORs is similar to that for exWT MORs. Contrastingly, acute desensitization for the STANT 3A mutant was significantly blocked, even in the absence of CMP101, with an additional effect of CMP101 (**Figure 5.1C**). Thus, CMP101 had a larger effect in blocking desensitization of the TSST 4A

mutant when compared to the STANT 3A mutant. Together, these results indicate that the STANT cluster plays a larger role in GRK2/3-mediated desensitization than the TSST cluster.

Morphine Treated Animals

Although acute desensitization was not blocked for the TSST 4A mutant in slices from naïve animals, one measure of cellular tolerance was affected, indicating that TSST is involved in mechanism(s) underlying cellular tolerance. To determine whether the TSST cluster is necessary for the adaptation in kinase regulation observed in Chapter 4, both measures of acute desensitization were again measured in slices from MOR KO animals virally expressing TSST 4A MORs in the LC and chronically treated with morphine for 6-7 days (80 mg/kg/day) using osmotic mini pumps.

Similar to experiments in slices from wildtype animals from Chapter 4, although CMP101 blocked acute desensitization of TSST 4A MORs in slices from naïve animals (**Figure 5.1B, D-E**), it was not sufficient to block acute desensitization of TSST 4A MORs in slices from MTAs as measured by recovery from desensitization, but did block the acute decline (**Figure 5.2A-C**). Thus, there was an increase in desensitization for TSST 4A MORs after chronic morphine treatment that was not dependent on GRK2/3. The recovery from desensitization of TSST 4A MORs in slices from MTAs looks very similar in the absence (Chapter 3, **Figure 6**) and presence of CMP101 (**Figure 5.2B**), with only a small decrease in the extent of desensitization at 5 min by CMP101. Therefore, although the recovery from desensitization is greater for TSST 4A MORs in slices from MTAs when compared to that for wildtype MORs, this is likely due to mutation of TSST rather than CMP101. The lessened effect of CMP101 in blocking desensitization of TSST 4A MORs after chronic morphine treatment could be dependent on PKC and JNK.

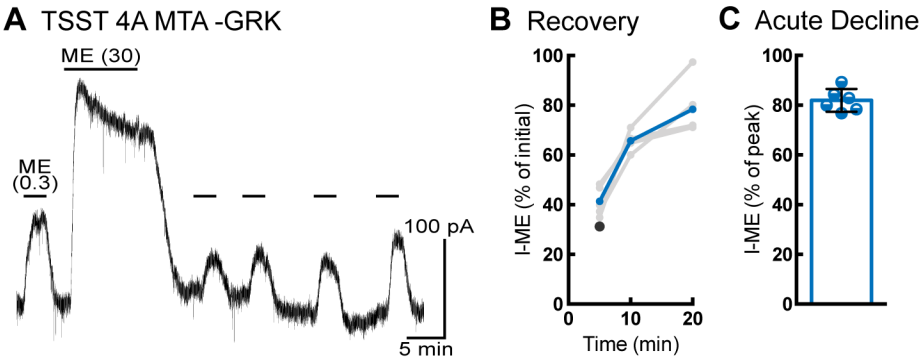



**Figure 5.2: Desensitization in MTAs occurs independently of the TSST cluster.** Experiments were performed in slices from MOR KO rats that were chronically treated with morphine and virally expressing TSST 4A mutant MORs in the LC. Slices were incubated in CMP101 prior to recording (30 µM, 1 hr) and CMP101 was also included in the bath (1 µM) and drug solutions (10 µM). (A) Example trace showing the currents induced by ME (0.3 µM) before and following application of ME (30 µM, 10 min). (B) Summary of recovery from MOR desensitization at 5, 10, and 20 minutes post desensitization (n = 6 slices, 4 animals). Grey lines indicate individual n's and colored lines indicate averages. Current amplitudes following desensitization are normalized as a percentage of the prepulse. The extent of desensitization at 5 min as reported in Arttamangkul et al. (2019) is shown with a dark gray circle in the recovery graph. (C) Summary graph showing acute decline. Data presented as average ± S.D.

The results indicate that JNK and PKC can still induce desensitization in morphine treated MOR KO animals virally expressing TSST 4A mutant MORs. Although it should be confirmed that all three inhibitors block desensitization under these conditions.

Therefore, while it is unclear how PKC and JNK are activated to induce desensitization and how PKC and JNK cause MOR desensitization, these results indicate that phosphorylation of TSST is not necessary for either. This suggests that PKC and JNK do not phosphorylate MOR at TSST to cause desensitization, consistent with literature showing that PKC can phosphorylate MOR at Ser363 or Thr370, and not at the TSST cluster, and that JNK may not phosphorylate MOR directly (Chen et al., 2013; Doll et al., 2011; Feng et al., 2011; Williams et al., 2013). This is also consistent with the results from above showing that STANT contributes more to desensitization than the TSST cluster. Finally, these results also indicate that PKC and JNK are not activated during chronic morphine treatment as a result of phosphorylation of TSST. Thus, although

mutation of TSST reduces one measure of cellular tolerance (Chapter 3), it does not seem to block the adaptation by PKC and JNK observed in Chapter 4.

These experiments should be extended with use of the STANT 3A and STANT 7A mutants. Results could elucidate whether STANT is necessary for the activation of PKC and JNK to cause desensitization and/or whether PKC and JNK act on STANT to cause desensitization. However, interpreting these results may prove difficult since blocking phosphorylation at Ser375/STANT reduces phosphorylation at other sites (Williams et al., 2013).

# Chapter 6: Discussion

The work presented here aimed to understand how phosphorylation by kinases and of specific residues on the C-terminus of MOR contributes to acute desensitization and cellular tolerance of MOR in order to gain a better understanding of MOR regulatory events leading to long-term tolerance to morphine. In Chapter 4, a combination of whole-cell electrophysiology and inhibition of select kinase activity was used to determine the kinases involved in acute desensitization and cellular tolerance. Results showed that chronic morphine treatment shifted the kinase regulation of acute desensitization from mainly GRK2/3-mediated to GRK2/3-, PKC-, and JNK-mediated. Further study is necessary to understand the mechanism underlying this PKC/JNK-dependent cellular tolerance and whether it contributes to clinically-relevant behavioral tolerance. In Chapter 3 and Chapter 5, whole-cell electrophysiology and viral expression of phosphorylation-deficient mutant MORs with alanine or glutamate mutation of C-terminal phosphorylation sites were used to examine the relative role of phosphorylation sites in acute desensitization and cellular tolerance. Results indicated that the different phosphorylation sites contribute to acute desensitization and cellular tolerance in varying degrees. Overall, this study showed that while the phosphorylation sites and kinases involved in acute desensitization and cellular tolerance distinguished the two processes, a chronic morphine-induced increase in desensitization by PKC and JNK contributed to cellular tolerance. This may be one adaptation underlying the increase in desensitization seen after chronic morphine treatment.

## Phosphorylation, Acute Desensitization, and Tolerance

### Kinase Regulation of Acute Desensitization

The canonical mechanism for acute GPCR desensitization is through phosphorylation of C-terminal Ser/Thr residues by GRKs (Gurevich and Gurevich, 2019). Indeed, there is good

evidence that inhibition of GRK2/3 activity reduces or eliminates acute MOR desensitization (Lowe et al., 2015; Williams et al., 2013). This study verified these results, showing that inhibition of GRK2/3 with the high specificity inhibitor, CMP101, blocked two separate measures of acute desensitization in LC neurons in slices from naïve animals. Thus, the canonical mechanism of GPCR desensitization is supported for MOR.

It is clear that GRK2/3 can directly phosphorylate multiple Ser/Thr residues on the C-terminus of MOR (Miess et al., 2018; Williams et al., 2013). Indeed, results from this study indicate that GRK2/3-dependent desensitization is likely through direct phosphorylation of MOR. The effect of CMP101 on MOR desensitization in WT rats, as measured by both acute decline and recovery from desensitization, was indistinguishable from that of desensitization of expressed total-phosphorylation deficient mutant MORs, in which all 11 phosphorylation sites on the C-terminus of MOR are mutated to alanine. Additionally, the effect of CMP101 on desensitization was occluded in slices from rats expressing TPD MORs. These results suggest that the 11 mutated residues are the main sites involved in GRK2/3-dependent desensitization with little role of additional phosphorylation sites on the intracellular loops.

The relative role of GRK2 versus GRK3 cannot be determined from this study since CMP101 inhibits both isoforms. Previous studies have indicated roles for both GRK2 and GRK3 in acute desensitization (Williams et al., 2013). Recently, Lowe et al. (2015) indicated that GRK3 is not solely responsible, suggesting that GRK2 and GRK3 both play a role. Thus it is likely that both GRK2 and GRK3 contribute to desensitization.

Although the majority of acute desensitization was blocked for TPD MORs as well as for WT and TPD MORs in the presence of CMP101, a similarly small amount of desensitization remained unaffected for each. This is in agreement with another study showing that CMP101

was not sufficient to completely block acute desensitization (Lowe et al., 2015). This suggests GRK2/3-independent mechanisms of acute desensitization involving sites other than the 11 on the C-terminus in addition to the canonical mechanism of phosphorylation of the C-terminus of MOR by GRK2/3.

Unlike GRK2/3 inhibition, inhibition of PKC or JNK had no effect on acute desensitization in slices from naïve animals. That GRK2/3 inhibition was sufficient to block the majority of acute desensitization is in contrast to studies that have shown involvement of other kinases in acute MOR desensitization, including PKC, JNK, and ERK1/2 (Birdsong and Williams, 2020; Dang et al., 2009; Levitt and Williams, 2012; Mittal et al., 2012). The reason for the disparity between these studies is unclear. Although this study did not try inhibitors for ERK1/2, Lowe et al. (2015) showed that ERK1/2 inhibition had no effect on desensitization. It cannot be ruled out that other kinase inhibitors in combination with CMP101, or for TPD MORs, could block even more acute desensitization since the present study only used one kinase inhibitor at a time in slices from naïve animals. Given that stimulated PKC activity increased acute MOR desensitization for TPD MORs (Arttamangkul et al., 2018; Bailey et al., 2009a), and that acute MOR desensitization by morphine was only blocked by the combination of mutation of the 11 phosphorylation sites and PKC inhibition (Yousuf et al., 2015), it should be confirmed whether inhibition of PKC and/or JNK (as well as other kinases) further reduces acute desensitization of TPD MORs or wildtype MORs in the presence of CMP101 in slices from naïve animals.

Some differences between previous studies may also be attributed to differences between experimental conditions and design. Many of these studies were conducted in heterologous expression systems where concentrations of signaling or regulatory components may differ from

native neurons. As mentioned, overexpression of regulatory proteins such as GRKs or arrestins can greatly affect measures of MOR activity (Miess et al., 2018; Whistler and von Zastrow, 1998; Zhang et al., 1998). In addition, differences may exist between different effector systems measured and brain area or cell type examined. Further experiments should be completed to see if this canonical mechanism holds true in other cell types and brain regions. It is also possible that GRK2/3 contributes to rapid acute desensitization with other kinases being involved with more prolonged MOR activation such as with short- and long-term cellular and analgesic tolerance. Indeed, previous studies have found involvement of other kinases in short- and long-term tolerance (Birdsong and Williams, 2020).

**Kinase Regulation of Cellular Tolerance**

An increase in acute desensitization and a reduced recovery from desensitization are established measures of cellular tolerance (Birdsong and Williams, 2020). This study therefore also examined how kinase inhibitors affected measures of desensitization in rats treated chronically with morphine. While CMP101 blocked both measures of acute MOR desensitization in slices from naïve animals, it had mixed results in slices from animals treated chronically with morphine. CMP101 blocked the acute decline but had no effect on the more sensitive measure of desensitization, recovery from desensitization measured using $EC_{50}$ concentrations. It is unclear why CMP101 had opposing effects on the two different measures of desensitization. There is some evidence that these two different measures of desensitization actually measure two separate processes (Arttamangkul et al., 2015; Birdsong et al., 2015). However, it is also possible that the reason for this difference is because of the difference in sensitivities between the measures. LC neurons contain significant MOR reserve, which needs to be removed before any decrease in the current induced by a saturating concentration of ME can be detected (Connor et al., 2004).

The present study showed that the acute decline was no longer blocked by CMP101 in slices from morphine treated animals after partial irreversible block of receptors with the irreversible antagonist, β-CNA. These results suggest that the observed dichotomy between the two measures of desensitization can be accounted for by the differences in sensitivity between the measures. However, results are difficult to interpret given that receptor reserve is decreased in LC neurons following chronic morphine treatment (Christie et al., 1987) and that acute decline is clearly evident in slices from naïve animals even without pretreatment with β-CNA. Therefore it seems unlikely that reduced receptor reserve is solely responsible for the change in sensitivity to CMP101 in slices from morphine treated animals after pretreatment with β-CNA. Regardless, the results suggest that while inhibiting GRK2/3 activity blocked desensitization in naïve animals, it was not sufficient to block either the slowed recovery from desensitization or the acute decline in the current induced by ME (30 μM, following treatment with β-CNA) following chronic morphine treatment.

The inability of GRK2/3 inhibitors to block measures of cellular tolerance might suggest that GRK2/3 activity is reduced or eliminated in tissue from animals treated chronically with morphine. However, given that CMP101 blocked internalization (discussed further in the next section) and the acute decline (without preincubation with β-CNA) in slices from morphine treated animals, this suggests GRK2/3 remained active following chronic morphine treatment. However, even though GRK2/3 activity is clearly present in cells after chronic treatment, it is possible that GRK2/3 activity is reduced after chronic morphine treatment. There is opposing evidence in the literature for whether GRK2/3 expression is increased or reduced following chronic morphine (Williams et al., 2013).

Previous studies have supported the idea that following chronic morphine treatment, additional kinases contribute to desensitization and tolerance (Bailey et al, 2009a; Kuhar et al., 2015; Levitt and Williams, 2012; Melief et al., 2010; Mittal et al., 2012). Results from this study showed directly using select kinase inhibitors that GRK2/3, PKC, and JNK all contribute to desensitization following chronic treatment with morphine. Inhibitors for all 3 of these kinases were necessary in order to achieve a near complete block of desensitization in slices from morphine treated animals. Staurosporine or the JNK inhibitor alone had no effect on acute desensitization in slices from MTAs, further supporting the idea that GRK2/3 activity is present following chronic morphine treatment. In addition, desensitization experiments using staurosporine and CMP101 were indistinguishable from those using the three specific inhibitors, indicating that GRK2/3, PKC, and JNK are the main kinases involved in phosphorylation-dependent acute desensitization following chronic morphine treatment.

Results from this study are in contrast to previous studies showing that inhibition of GRK, PKC, and JNK did not block measures of acute desensitization or cellular tolerance (Arttamangkul et al., 2012; Dang et al., 2009; Levitt and Williams, 2012; Williams et al., 2013). However, unlike previous studies, this study pretreated slices with inhibitors for at least an hour prior to recording to ensure sufficient block by inhibitors. In addition, this study found that blocking multiple kinases at the same time was necessary in order to observe the effect. Thus, previous studies could have missed a role for PKC or JNK if GRK2/3 was still active.

The results from this study suggest that chronic morphine treatment induces adaptations in the kinase regulation of acute desensitization that may contribute to long-term tolerance. However, it is unclear how PKC and JNK induce desensitization in animals treated chronically with morphine. Although GRK2/3 can directly phosphorylate residues on the C-terminus of

MOR, there is no evidence for direct phosphorylation of MOR by JNK (Williams et al., 2013). It is known that PKC can directly phosphorylate a couple Ser/Thr residues on the C-terminus of MOR (Ser363 and Thr370; Doll et al., 2011; Feng et al., 2011; Chen et al., 2013). In addition, PKC inhibition was shown to decrease basal levels of MOR phosphorylation in HEK293 cells, suggesting that PKC pre-phosphorylates MOR (Johnson et al., 2006). However, it is still unclear whether agonist-induced activation of MOR induces PKC phosphorylation of MOR.

There is mixed evidence for whether PKC desensitizes MOR through direct phosphorylation of C-terminal residues. Some studies support this idea (Johnson et al., 2006; Feng et al., 2011; Doll et al., 2011), while others indicate that PKC can induce MOR desensitization independent of C-terminal phosphorylation. For example, morphine-induced desensitization was only blocked by the combination of PKC inhibition and mutation of C-terminal phosphorylation sites in AtT20 cells (Yousuf et al., 2015). In addition, Arttamangkul et al. (2018) showed that induced PKC activity could enhance desensitization even for phosphorylation-deficient TPD MORs, indicating that PKC could desensitize MOR without direct phosphorylation of the C-terminus. Therefore, it is also likely that PKC and JNK contribute to MOR desensitization indirectly through phosphorylation of components of MOR signaling downstream of the receptor, preventing G-protein activation through an alternative process to GRK2/3-mediated desensitization. A study by Chu et al. (2010) showed that PKC caused heterologous desensitization in HEK293 cells through phosphorylation of $G\alpha_{i2}$. An alternative possibility is that PKC and/or JNK may act to mediate desensitization through phosphorylation of RGS proteins to turn off signaling, as has been reported in some cell types (Garzon et al., 2005; Ogier-Denis et al., 2000). Lastly, JNK-dependent desensitization resulted from JNK-dependent activation of peroxiredoxin 6 (PRDX6) to generate reactive oxygen species

via NADPH oxidase that reduced the palmitoylation of receptor Gαi subunits in order to reduce MOR signaling (Schattauer et al., 2017).

It is also unknown how PKC and JNK are activated to induce desensitization. It has been shown that arrestin can act as a scaffold for JNK (**Figure 1.2**; Kook et al., 2013; Zahn et al., 2013). However, arrestin-independent mechanisms of opioid-induced JNK activation have also been proposed. Both Gαi/o and Gβγ G-protein subunits have been shown to lead to activation of MAPK signaling including JNK (**Figure 6.1**; Gurevich and Gurevich, 2019). In addition, morphine-induced JNK activation was arrestin-independent and PKC- and Src-dependent, suggesting that JNK activation is downstream of PKC (**Figure 6.1**; Kuhar et al., 2015). Yet, in this study, inhibitors for both PKC and JNK were necessary for a more complete block of desensitization, suggesting that they are activated independently. It has also been shown that DAMGO-induced JNK activation is through Src- or PI3K-dependent mechanisms (**Figure 6.1**; Kam et al., 2004; Zhang et al., 2009).

There is evidence for MOR-dependent agonist-induced activation of PKC, but the mechanism that underlies this activation remains unclear (Johnson et al., 2006; Pena et al., 2018). There is some evidence that MOR-induced activation of PI3K leads to PKC activation (**Figure 6.1**; Goldsmith et al., 2013; Johnson et al., 2006). MOR-induced activation of PLC may also lead to PKC activation (**Figure 6.1**; Gresset et al., 2012; Pena et al., 2018; Zhu and Birnbaumer, 1996). It could also be the case that PKC is activated heterologously, such as through ongoing neuronal activity and consequent increases in intracellular free $Ca^{2+}$ or activity of Gq-coupled receptors (Pena et al., 2018). Indeed, PKC activation through activation of Gq-coupled muscarinic receptors facilitated desensitization of MORs in LC neurons (Arttamangkul et al., 2018; Bailey et al., 2004).

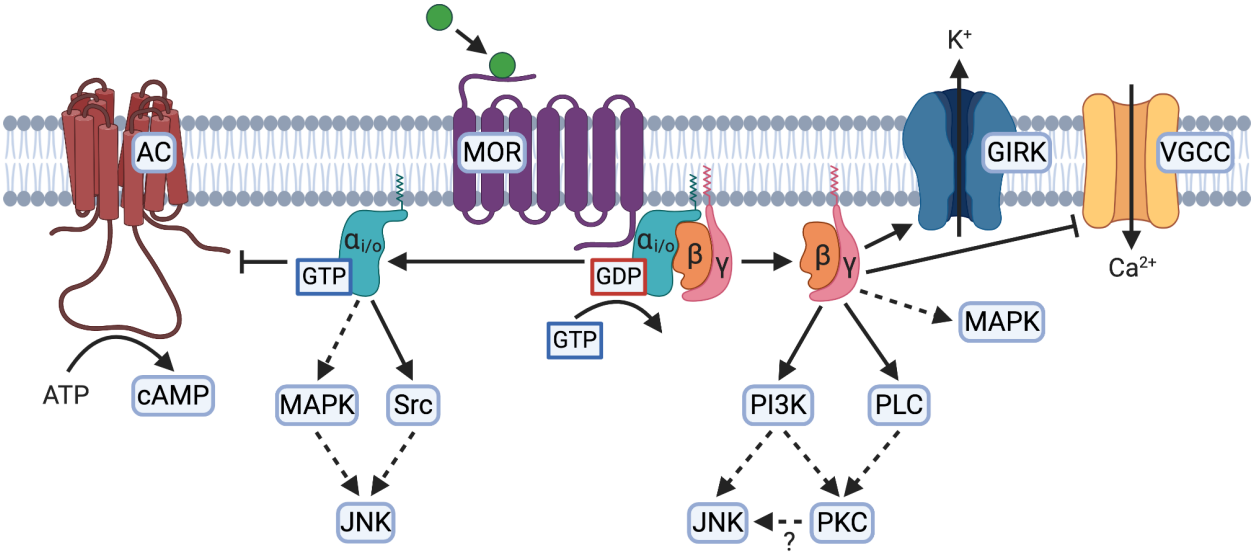



**Figure 6.1: Potential MOR-induced activation of JNK and PKC.** Expanding on Figure 1.1, this figure shows the possible ways that JNK and PKC could be activated by agonist-induced MOR-activation. Solid arrows indicate direct actions with dashed arrows representing indirect actions.

The PKC and JNK isoform(s) responsible for the effects observed in this study are unclear. However, the PKC inhibitor used only blocks the PKCα and PKCβ1 isoforms, indicating that one or both of these are responsible. While the specific JNK isoform(s) involved is less clear, a previous study indicated that JNK2 (and not other isoforms) is involved in analgesic tolerance (Melief et al., 2010).

While inhibitors for GRK2/3, PKC, and JNK, or the nonselective kinase inhibitor, staurosporine, in addition to CMP101, blocked the majority of acute desensitization (~80% of initial), a small amount of desensitization remained unaffected. This incomplete block of desensitization could be either incomplete inhibition of kinases due to incomplete penetration or a yet unknown phosphorylation-independent mechanism of acute desensitization. However, it is also possible that phosphorylation by other kinases not blocked by inhibitors at sites other than the 11 main ones could be responsible (i.e. sites on the intracellular loops). There is previous evidence for the involvement of kinases besides GRK, PKC, and JNK, including ERK1/2 (Dang

et al., 2009; Williams et al., 2013). Although this study found GRK2/3, PKC, and JNK to be the main kinases involved in this adaptation, additional kinases, such as ERK1/2, could also be involved that are either up or downstream of GRK, PKC, and JNK. For example, since kinases phosphorylate numerous targets, including other kinases, it has been hard to decipher whether kinases directly phosphorylate MOR or whether they act sequentially (Williams et al., 2013).

As mentioned, the leftover desensitization after inhibition of GRK2/3, PKC, and JNK, could be a phosphorylation-independent mechanism of acute desensitization. One mechanism proposed in the literature involves GRK sequestration of Gβγ G-proteins in order to turn off signaling (Raveh et al., 2010). While this mechanism could be involved in desensitization, it is likely not responsible for the leftover desensitization observed. CMP101 inhibits GRK2/3 activity by binding to its ATP binding pocket, which overlaps with the Gβγ binding site, and thus CMP101 should also block GRK-sequestration of G-proteins (Thal et al., 2011; Whorton and MacKinnon, 2013). Indeed, CMP101 blocked recruitment of GRK2 to the plasma membrane (Miess et al., 2018). Therefore, other phosphorylation-independent mechanisms of acute desensitization should be investigated. There was also no indication that CMP101 changed the sensitivity to ME given that there was no change in the ratio of the current induced by ME (0.3/30 μM) in slices taken from both naïve and morphine treated animals.

**Involvement of Phosphorylation Sites in Acute Desensitization and Tolerance**

Mutation of MOR C-terminal phosphorylation sites affects measures of acute desensitization, cellular tolerance, and analgesic tolerance (Arttamangkul et al., 2018, Kliewer et al., 2019). In addition to investigating the kinases involved in acute desensitization and cellular tolerance, this study used expression of phosphorylation-deficient mutant MORs in the LC of MOR KO rats to examine the relative roles of MOR C-terminal phosphorylation sites in acute

desensitization and two measures of cellular tolerance. Four mutant MORs with either alanine or glutamate mutations were examined: TSST 4A (T354-T357 to AAAA); TSST 4E (T354-T357 to EEEE); STANT 3A (S375-T379 to AAANA); and STANT 7A (S363A, T370A, S375-T379 to AAANA, T383A, T394A). For the TSST 4A mutant, acute desensitization and one measure of cellular tolerance (decreased sensitivity to morphine) was unaffected, while another measure of cellular tolerance was blocked (increased acute desensitization and reduced recovery from desensitization). For the TSST 4E mutant, acute desensitization persisted with an increase after chronic morphine treatment (one measure of cellular tolerance), while the sensitivity to morphine was not changed (another measure of cellular tolerance). Acute desensitization was reduced for the STANT 3A mutant and nearly abolished for the STANT 7A mutant. The remaining amount of acute desensitization seen for the STANT 7A mutant was similar to that of the TPD mutant in Chapter 4, indicating that TSST may play little role in acute desensitization, consistent with the finding that acute desensitization was unaffected for the TSST 4A mutant. This is also consistent with results from Chapter 5 indicating that phosphorylation of the STANT cluster plays a larger role in GRK2/3-mediated desensitization than that of the TSST cluster. While CMP101 blocked acute desensitization for both the TSST 4A and STANT 3A mutants to similar degrees, it had a larger relative effect in blocking desensitization of the TSST 4A when compared to experiments in the absence of CMP101.

Both measures of cellular tolerance were blocked for the STANT 3A mutant. However, there was a transient increase in acute desensitization for the STANT 7A mutant in slices from morphine treated animals that matched the extent of desensitization for the STANT 3A mutant under chronic conditions. This, together with the finding that mutation of TSST reduced cellular tolerance indicates that phosphorylation of both TSST and STANT contribute to adaptations

resulting in cellular tolerance. Thus, either overall amount of phosphorylation or phosphorylation of specific residues may affect the degree of both acute desensitization and cellular tolerance.

How phosphorylation of specific residues contributes to acute desensitization and tolerance is still unclear. It is possible that signaling proteins such as kinases or arrestins block G-protein coupling by steric hinderance. However, it is also possible that phosphorylation itself of residues on the C-terminus prevents signaling by inducing a conformation of the receptor that is inaccessible to G-proteins. For cellular tolerance the picture is more complicated because phosphorylation of these sites may also induce signaling (i.e. arrestin signaling) that results in long-term adaptations in order to reduce coupling (Birdsong and Williams, 2020; Williams et al., 2013). That the same measure of cellular tolerance used in Chapter 4 (reduced recovery from desensitization) was reduced or blocked for TSST 4A, STANT 3A, and TPD MORs (Chapter 3; Arttamangkul et al., 2018), indicates that phosphorylation of some or all of these residues either directly or indirectly contributes to the increase in desensitization by PKC and JNK seen in slices from morphine treated animals. Results from Chapter 5 showing that acute desensitization persisted for TSST 4A MORs in the presence of CMP101 in slices from morphine treated animals suggests that while phosphorylation of TSST may contribute to some forms of cellular tolerance, it is likely not required for the adaptation by PKC/JNK observed in this study.

While this study has indicated that chronic morphine induces increased acute desensitization by additional kinases that contributes to measures of cellular tolerance, this study has also distinguished acute desensitization and cellular tolerance. Results mentioned above from Chapter 3 indicated that there might be some specificity for which phosphorylation sites regulate acute desensitization versus cellular tolerance. In addition, results from Chapter 4 indicated that different kinases are involved in cellular tolerance compared to acute desensitization. While

phosphorylation-dependent acute desensitization was regulated by GRK2/3, chronic morphine treatment induced an adaptation whereby additional kinases, PKC and JNK, contributed to desensitization. Therefore, acute desensitization and cellular tolerance are not equivalent, but it is likely that increased desensitization after chronic morphine by PKC and JNK contributes to cellular tolerance. This is one of many possible mechanisms by which neurons may adapt in order to reverse long-term activation of MOR by morphine.

**Conclusions and Future Directions**

Phosphorylation of residues on the C-terminus of MOR is critical for acute desensitization and cellular tolerance (Birdsong and Williams, 2020; Arttamangkul et al., 2018). The different phosphorylation sites/clusters (i.e. STANT, TSST, and the 4 additional sites) contributed to acute desensitization and cellular tolerance in varying degrees. In addition, acute desensitization was regulated by GRK2/3, while the involvement of additional kinases, PKC and JNK, contributed to cellular tolerance. Thus, while acute desensitization and cellular tolerance can be distinguished from each other, an increase in MOR desensitization by JNK and PKC after chronic morphine may be one adaptation that contributes to long-term tolerance. While the link between cellular tolerance and analgesic tolerance is unclear, studies showing that analgesic tolerance can be reduced by PKC or JNK inhibitors indicate that this mechanism may contribute to analgesic tolerance (Granados-Soto et al., 2000; Hua et al., 2002; Inoue and Ueda, 2000; Melief et al., 2010; Newton et al., 2007; Smith et al., 2003; Smith et al., 1999). Results from this study are also corroborated by studies showing that tolerance is maintained by kinase activity, such as PKC (Bailey et al., 2009a; Granados-Soto et al., 2000; Smith et al., 1999). Further study should investigate the mechanism underlying the development of PKC/JNK-dependent tolerance as well as how long this adaptation persists following chronic morphine treatment.

The work presented here shows one adaptation induced by chronic morphine measured via GIRK conductance in postsynaptic LC neurons. It is unknown if this same adaptation occurs for other effector systems or in other brain regions/cell types. GIRK conductance, and thus likely G-protein signaling, was affected by the adaptation observed in this study, indicating that this adaptation may affect other types of G-protein signaling as well (e.g. Gα-mediated inhibition of adenylyl cyclase). However, regulatory processes, such as desensitization, that may contribute to tolerance may differ between effector systems (i.e. no observed desensitization to presynaptic inhibition of transmitter release; Fox and Hentges, 2017; Fyfe et al., 2010; Pennock et al., 2012; Pennock and Hentges, 2011). These regulatory processes are also known to vary across brain regions and cell types (i.e. lack of desensitization in KF neurons; Birdsong and Williams, 2020; Levitt and Williams, 2018). Therefore, further study should investigate whether the adaptation observed in this study affects other types of MOR signaling and if it holds true in brain regions involved in analgesia. For example, in the periaqueductal gray (PAG), a brain region known to contribute to analgesia and tolerance and where an increase in desensitization is observed in slices from animals treated chronically with morphine (Ingram et al., 2008). Cellular tolerance occurs in multiple cell types and brain regions (e.g. LC, PAG, trigeminal ganglion neurons, AtT20, HEK293 cells) and for a range of effectors (e.g. activation of GIRKs and GTPγS, inhibition of VGCCs, adenylyl cyclase, and transmitter release), but the mechanisms underlying the observed tolerance is unclear (Birdsong and Williams, 2020; Williams et al., 2013). While it is likely that many mechanisms/adaptations contribute to tolerance, the adaptation observed in this study may contribute to cellular tolerance more broadly than just the LC. If this adaptation holds true in cell culture, this would provide an easy system to further probe this mechanism and suggest that this adaptation is more broadly conserved. Electrophysiology experiments to test for

this adaptation could be completed in AtT20 cells expressing exogenous MORs or cultured and differentiated SH-SY5Y cells with endogenous MORs. Proteomics could also be done if this approach indeed works.

There is also a lot of evidence that mechanisms underlying MOR regulatory processes differ depending on agonist used (Birdsong and Williams, 2020; Williams et al., 2013). For example, full agonists such as DAMGO induce more phosphorylation than partial agonists such as morphine. The reason for this difference is unknown but overexpression of GRKs increases morphine-induced phosphorylation of MOR (Doll et al., 2011; Miess et al., 2018). It may be that agonist efficacy determines efficiency of phosphorylation. It is also possible that different agonists induce different patterns of phosphorylation. It would be interesting to determine whether chronic morphine treatment induces more phosphorylation of the intracellular region of MOR or signaling proteins, such as phospho-JNK. In a previous study, chronic morphine treatment induced increases in phosphorylation of MAPKs, including JNK, in L4 DRG neurons (Chen et al., 2008). This should be repeated to determine if similar increases occur in other brain regions including the LC. Results from this study suggest increased phosphorylation, either of MOR directly or of MOR signaling components. It is possible that while morphine does not induce robust phosphorylation acutely, morphine-induced phosphorylation, possibly by PKC and/or JNK, increases with chronic receptor stimulation, increasing desensitization and contributing to long-term tolerance.

In addition, mechanisms of acute desensitization and tolerance may differ between agonists (Birdsong and Williams, 2020; Williams et al., 2013). For example, while analgesic tolerance to morphine persisted for knockin mice with the S375A mutation, analgesic tolerance to high efficacy agonists, including fentanyl, was diminished (Grecksch et al., 2011; Kliewer et

al., 2019), indicating that distinct phosphorylation sites may play a role in tolerance to different MOR agonists. Multiple studies have also shown that PKC-dependent processes are more associated with morphine-induced MOR desensitization, rather than desensitization induced by full agonists such as DAMGO (Bailey et al., 2009a; Chu et al., 2010; Hull et al., 2010; Johnson et al., 2006; Yousuf et al., 2015). In addition, morphine-induced analgesic tolerance involved JNK while fentanyl-induced analgesic tolerance was mediated by a GRK/arrestin-dependent mechanism (Melief et al., 2010). Therefore, further study should investigate whether the adaptation observed in this study differs or holds true for other agonists with varying efficacy, such as buprenorphine and fentanyl.

## Acute Desensitization, Internalization, and Tolerance

It is well established that phosphorylation of the STANT cluster on the C-terminus of MOR is required for efficient arrestin binding and internalization (Birdsong and Williams, 2020). These results were validated in this study, with internalization persisting for the TSST 4A and TSST 4E mutants but being blocked for the STANT 3A mutant. Previous studies have also found that GRK2/3 inhibition reduces arrestin binding and internalization in HEK293 cells, consistent with evidence that MOR activation induces phosphorylation of STANT by GRKs (Birdsong and Williams, 2020; Lowe et al., 2015; Miess et al., 2018). However, since additional kinases were found to contribute to acute desensitization in slices from morphine treated animals, and since internalization is another process that is dependent on phosphorylation of MOR, this study also sought to determine the kinases involved in internalization under both naïve and chronic morphine treated conditions. While the GRK2/3 inhibitor, CMP101, was only sufficient to block acute desensitization in slices from naïve animals, CMP101 blocked internalization of MOR in slices from both naïve and morphine treated animals. These results suggest that internalization is

mediated by GRK2/3 under naïve and chronic morphine conditions and that although kinases, such as PKC and JNK, contribute to acute desensitization under chronic conditions, they do not largely contribute to internalization. This further establishes internalization and desensitization as separate processes, as has been shown by previous studies (Arttamangkul et al., 2006; Dang et al., 2011; Johnson et al., 2005). This is also consistent with studies showing that the extent of internalization is not increased by chronic morphine treatment (Williams et al,, 2013).

While these studies distinguish acute desensitization and internalization, the role of arrestin in acute desensitization and tolerance of MOR is still unclear. CMP101 blocked desensitization under naïve conditions, but would also block arrestin binding, since efficient arrestin binding relies on phosphorylation of STANT by GRKs (Birdsong and Williams, 2020). Indeed, CMP101 blocked arrestin recruitment in HEK293 cells (Lowe et al., 2015; Miess et al., 2018). Therefore arrestin may or may not be involved in GRK2/3-mediated mechanisms of acute desensitization. However, results from this study suggest that MOR was desensitized by PKC and JNK in the presence of CMP101, indicating that MOR can be desensitized by phosphorylation-dependent arrestin-independent mechanisms. It is unknown if GRK2/3 and PKC/JNK induce desensitization by similar or distinct mechanisms.

Unlike previous studies with expressed phosphorylation-deficient mutant MORs, this study also examined endogenous receptors that are not inherently G-protein biased. Arrestin binding was therefore not blocked during the chronic morphine treatment, and could contribute to cellular tolerance. While PKC and JNK likely do not rely on *acute* agonist-induced arrestin binding to desensitize MOR, arrestin signaling and internalization could contribute to the observed adaptation by inducing increased activation of PKC and JNK during chronic morphine treatment. Indeed, arrestin binding initiates other signaling events, such as MAPK signaling

including JNK (Gurevich and Gurevich, 2019). In addition, there is evidence that mechanisms of tolerance are maintained by kinase activity, such as PKC activity (Bailey et al., 2009a; Granados-Soto et al., 2000; Smith et al., 1999).

**Conclusions and Future Directions**

While phosphorylation of the C-terminus of MOR contributes to both acute desensitization and internalization (Birdsong and Williams, 2020; Williams et al., 2013), the kinases involved distinguish the processes. Although GRK2/3 inhibition blocked both processes under naïve conditions, PKC and JNK activity contributed to acute desensitization, but likely not internalization, in slices from animals treated chronically with morphine. This is in agreement with previous studies showing that acute desensitization can occur in the absence of internalization (Arttamangkul et al., 2006; Dang et al., 2011; Johnson et al., 2005).

However, while arrestin binding is known to be critical for internalization, it is still unclear how arrestin contributes to mechanisms of acute desensitization and cellular tolerance of MOR (Birdsong and Williams, 2020; Williams et al., 2013). Further experiments in the arrestin KO might tell us if arrestin signaling contributes to the adaptation observed in this study. However, the β-arrestin1/β-arrestin2 double knockout is lethal (Williams et al., 2013) and compensation between the two arrestins in single knockouts may confound results. Viral expression of mutant MORs with mutation of phosphorylation sites in the STANT cluster could also be used to determine the role of arrestin in this adaptation since arrestin recruitment and internalization is blocked for the STANT 3A mutant (Chapter 3; Miess et al., 2018). However, results from this study also showed that desensitization is reduced and cellular tolerance is blocked for the STANT 3A mutant, possibly complicating measurements. While this may indicate that arrestin is involved in mechanisms of acute desensitization and tolerance, including

the adaptation found in this study, this relationship should be investigated under conditions where arrestin is blocked without affecting phosphorylation, possibly with a conditional knockout. Transient cellular tolerance was observed for the STANT 7A mutant, indicating arrestin-independent mechanisms of cellular tolerance as well.

## Heterologous Effects

Desensitization of the somatostatin receptor (SSTR) was also examined to determine the selectivity of the adaptation observed for MOR. Desensitization of SSTRs was augmented in slices from morphine treated animals compared to naïve animals. In addition, although CMP101 blocked acute homologous SSTR desensitization in slices from naïve animals, it was much less effective after chronic morphine treatment. Preincubation with the three specific kinase inhibitors for GRK2/3, PKC, and JNK were required to block somatostatin-induced desensitization in slices taken from morphine treated animals. These results indicate that chronic morphine induced a heterologous modulation of GRK2/3-mediated desensitization of SSTR such that after chronic morphine treatment, PKC and JNK contributed to SSTR desensitization. This is in agreement with previous studies showing that acute desensitization of MOR leads to heterologous desensitization of SSTR (Fiorillo and Williams, 1996; Yousuf et al., 2015).

Although it is clear that morphine treatment induced heterologous effects on the somatostatin receptor, results conflicted for measures of acute decline for MOR versus SSTR. While CMP101 blocked the acute decline in the current induced by ME (30 μM) in slices from both naïve and morphine treated animals, the same measure of SSTR desensitization (acute decline) was no longer sensitive to CMP101 following chronic morphine treatment. However, following partial irreversible block of MORs with β-CNA, the acute decline in the current induced by ME was also no longer sensitive to CMP101. Therefore, this difference may be due

to differences in receptor reserve for MOR and SSTR in LC neurons. LC neurons contain significant MOR reserve, which needs to be removed before any decrease in the current induced by a saturating concentration of ME can be detected (Connor et al., 2004). If SSTRs have less receptor reserve in LC neurons then acute desensitization would be detected more easily with a saturating concentration of SST. However, given the confusing nature of the results with β-CNA, another possibility may be that acute decline for MOR is a separate mechanism from that of SSTR and slowed recovery from desensitization.

The present results showed that the adaptation in altered kinase regulation of desensitization following chronic morphine treatment was heterologous, affecting desensitization of both MOR and SSTR. The mechanism for this chronic morphine-induced heterologous desensitization of SSTR is unclear. There is evidence that PKC contributes to heterologous desensitization, but that mechanism is unclear (Chu et al., 2010; Yousuf et al., 2015). The somatostatin receptor, like MOR, contains phosphorylation sites that can be phosphorylated by GRK2/3 and PKC, but not JNK. It is thought that phosphorylation of these sites contributes to homologous desensitization of SSTR (Gunther et al., 2018). Therefore, it is possible that GRK2/3, PKC, and JNK phosphorylate SSTR directly to induce desensitization. However, it is also possible that GRK2/3, PKC, and JNK act indirectly on SSTR, inhibiting signaling components that affect both MOR and SSTR signaling (i.e. Gα subunits, RGS proteins, etc.).

**Conclusions and Future Directions**

The present study indicated that chronic morphine treatment induced a heterologous adaptation of the kinase regulation of desensitization, such that additional kinases, including PKC and JNK, contribute to the desensitization of two GPCRs. Further experiments should investigate the mechanism underlying this heterologous adaptation in kinase regulation.

Chronically treating MOR KO rats virally expressing TPD MORs with morphine and then measuring somatostatin-induced desensitization in the presence and absence of kinase inhibitors like in Chapter 4 would shed some light. This would tell us whether phosphorylation/ internalization/tolerance of MOR is required in order to get the same heterologous effect that is seen in the morphine treated wildtype animals (increased somatostatin desensitization and altered kinase regulation of somatostatin desensitization).

It is also unknown if the adaptation in kinase regulation observed in this study affects other GPCRs in the same neurons. There is mixed evidence for MOR-induced heterologous desensitization of other GPCRs such as α2-adrenergic receptors (Doll et al., 2012; Llorente et al., 2012; Williams et al., 2013). It is also unknown if this heterologous modulation of SSTR desensitization occurs in other cell types and brain regions and for other types of signaling. Further study should investigate whether the adaptation in kinase regulation after chronic morphine is more broadly conserved across cell types, brain regions, and effector systems. That chronic morphine treatment altered the signaling of another GPCR in the same cell has huge implications for how morphine treatment affects the brain. Chronic morphine treatment may have profound functional consequences for non-opioid receptors, including for subsequent signaling pathways and the circuits they occupy.

# Conclusion

The present study demonstrated that chronic morphine treatment induced heterologous adaptations in the kinase regulation of acute desensitization of both MOR and SSTR (**Figure 6.2**). MOR desensitization appears to be mediated acutely by phosphorylation of the C-terminus of MOR by GRK2/3, and following chronic morphine treatment by additional kinases, including PKC and JNK. This may be one adaptation responsible for the augmentation of desensitization

seen in animals chronically treated with morphine (Williams et al., 2013) and therefore may also contribute to behavioral tolerance. Additional studies are required to fully understand this mechanism. Understanding these specific adaptations may facilitate the development of drugs that can reduce tolerance. In addition, that this adaptation was heterologous, affecting the signaling of SSTR, may have profound functional consequences for how chronic morphine treatment affects the brain.

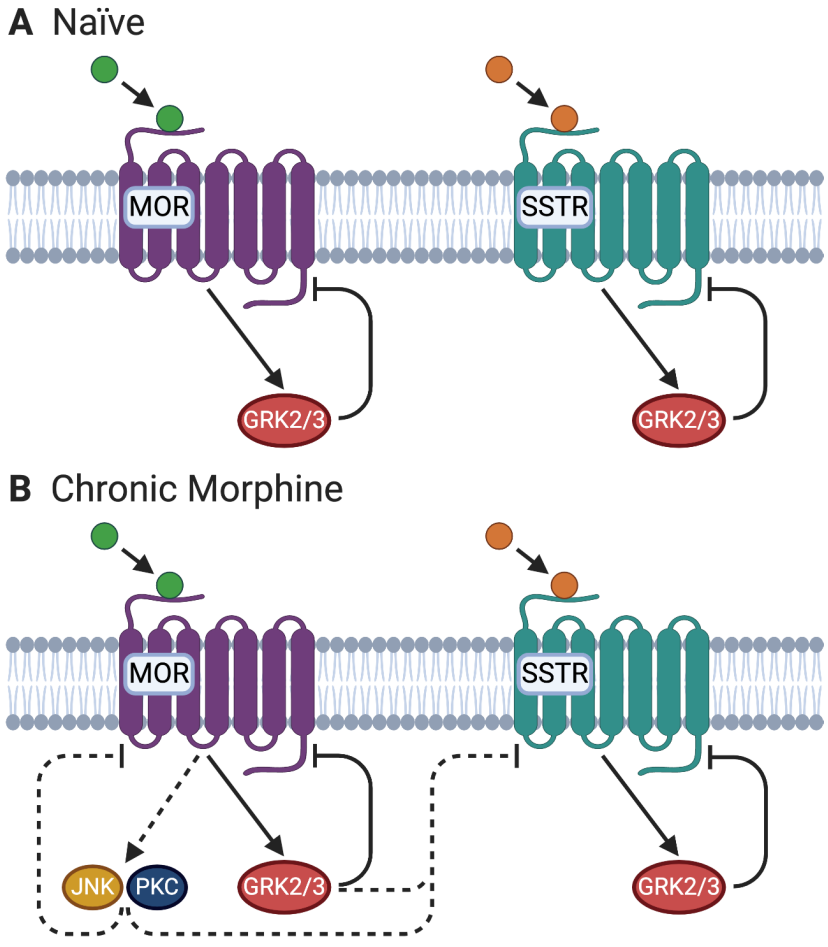



**Figure 6.2: Chronic morphine treatment induces a heterologous adaptation in the kinase regulation of acute desensitization.** (A) In acute brain slices from naïve animals, acute homologous desensitization of MOR and SSTR was mediated by GRK2/3. (B) Following chronic treatment with morphine, the kinase regulation of acute desensitization was altered such that additional kinases, including PKC and JNK, contributed to acute desensitization of MOR and SSTR. Solid arrows indicate direct actions and dashed arrows indicate indirect or unknown mechanisms.

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