Effects of Chronic Morphine Treatment on Pre- and Postsynaptic Thalamo-Cortico-Striatal Mu-Opioid Receptor Signaling

by

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Dedication

To my mom, who showed me I can do anything I set my mind to, and who would be so proud of all that I have accomplished.

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Abstract

Opioids are regarded as the most effective therapy for pain. However, their clinical use is limited by their dangerous side effects, high abuse liability, and the rapid development of tolerance to their analgesic properties, whereby with prolonged use, increasing doses are required to achieve the same degree of pain relief. Despite widespread opioid use, the cellular and circuit adaptations that drive tolerance and addiction are not fully understood. One challenge in establishing the adaptations relevant to these processes is the widespread expression of the mu-opioid receptor (MOR), the receptor through which effects of clinical opioids are primarily mediated, throughout the nervous system.

The goal of this dissertation is to characterize how chronic exposure to morphine, a prototypical opioid, alters MOR signaling within the somatic and presynaptic compartments of a physiologically relevant thalamo-cortico-striatal circuit, and determine the mechanisms underlying the observed effects. Glutamatergic neurons originating in the medial thalamus (MThal) send projections to the dorsomedial striatum (DMS) and anterior cingulate cortex (ACC). These interconnected brain regions are involved in mediating pain perception and reward behaviors, but how chronic opioid exposure alters synaptic transmission within this circuitry is not well-studied. In chapter 2, I compare chronic morphine effects on subsequent MOR signaling at MThal cell bodies and MThal-DMS presynaptic terminals in male and female mice. I demonstrate that chronic morphine treatment induces cellular tolerance at MThal cell bodies, where subsequent morphine responses are diminished, but facilitation at MThal-DMS terminals,

where morphine responses are enhanced. Moreover, presynaptic facilitation is sex-specific, occurring only in male mice, while tolerance at cell bodies occurs in both sexes. Using MOR phosphorylation deficient mice, I demonstrate that MThal-DMS facilitation appears to be driven by MOR phosphorylation, a critical regulatory process. In chapter 3, I examine chronic morphine effects at MThal-ACC terminals innervating both excitatory and inhibitory pathways. In contrast to our findings in chapter 2, chronic morphine treatment at these terminals induces presynaptic tolerance, rather than facilitation. Again, these effects are sex-specific, where tolerance within the inhibitory pathway is only seen in male mice but tolerance within the excitatory pathway is seen in both sexes, and mediated by receptor phosphorylation.

At MThal cell bodies, a fraction of cells did not respond to morphine. This observation motivated me in chapter 4 to explore whether functional MThal projections to the DMS arise from both MOR-expressing and MOR-lacking subpopulations. After confirming this to be the case, I examined how chronic morphine treatment differentially alters MOR signaling within these subpopulations separately to determine whether the adaptations driving the facilitation observed in chapter 2 were cell autonomous or circuit-level. I report that at MOR-expressing terminals, chronic morphine induces tolerance, rather than facilitation, with no apparent changes in signaling at MOR-lacking terminals. Taken together with my previous findings, this suggests the presence of multiple opposing adaptations in MOR signaling at MThal-DMS synapses; chronic morphine induces tolerance at the level of the cellular- or receptor-level while simultaneously inducing adaptations elsewhere in the circuit that mediate facilitation.

Overall, the work presented here provides insight into chronic morphine-induced adaptations at different subcellular compartments within a physiologically relevant thalamo-cortico-striatal circuit. Moreover, it highlights the complexity with which chronic opioid

exposure alters physiology to produce behavioral outcomes. These effects are not ubiquitous, but rather depend on multiple factors such as sex, brain region, and the microenvironment of the circuit.

Chapter 1
Introduction

1.1 Opioids for the treatment of pain and opioid misuse

An estimated 1 in 5 adults in the United States experience chronic pain annually (Yong et al., 2022). Chronic pain is a debilitating condition which can damage physical and psychological well-being and disrupt day-to-day functioning. In addition to its severe detrimental impact on overall health, , chronic pain poses a substantial economic burden, costing upwards of $560 billion per year in medical costs and lost work time in the U.S. (Cohen et al., 2021). Because chronic pain often has no known cause or persists even after recovery from an injury, treating the underlying causes is difficult and managing symptoms is often the goal. Opioids are the most effective treatment for chronic and severe pain. Unfortunately, long-term use of opioids for pain management can cause serious complications due to adverse acute side effects (such as respiratory depression and constipation) and the high potential for addiction and misuse (Harned & Sloan, 2016). These risks are compounded by the fact that prolonged use readily produces tolerance to opioid analgesia, or pain relief, whereby increasing doses are required to achieve the desired therapeutic effect. In fact, the increased prescribing of opioids such as morphine and oxycodone for pain beginning in the 1990s has given rise to an unprecedented opioid epidemic in the United States, marked by dramatic increases in the prevalence of opioid use disorder (OUD) and overdose deaths. During the initial years of the epidemic, overdose deaths were primarily driven by prescription opioids. As a result, the Centers for Disease Control and Prevention established guidelines to limit the number of opioids being prescribed. Despite this reduction in

the legal dispensation of opioids, the crisis has been worsening since its inception, due in large part to the increasing and uncontrolled presence of highly potent synthetic opioids such as fentanyl on the streets (Volkow & Blanco, 2021). More recently, the trend of increasing opioid overdose deaths was accelerated following the onset of the COVID-19 pandemic, with opioid-related deaths jumping an estimated 31% nationally from 2019 to 2020 (Hedegaard et al., 2021). These alarming statistics highlight the urgent need for a better understanding of how chronic opioid use alters pain and reward systems, and the development of safer therapeutics to treat chronic and severe pain. As such, much scientific research has been done to characterize the pharmacological action of opioids on pain signaling and the physiological adaptations that arise from repeated opioid use.

1.2 The endogenous opioid system

Opioids exert their therapeutic effects (i.e., analgesia) and their undesired effects (i.e., constipation, respiratory depression, and euphoria) through activity at opioid receptors located throughout the nervous system. Three subtypes of opioid receptors have been identified- the mu-, delta-, and kappa-opioid receptors (MOR, DOR, and KOR, respectively) (Connor & Christie, 1999). Opioid receptors are the targets of three classes of endogenous opioid peptides released from neurons- β-endorphin, enkephalins, and dynorphins. These ligands have different binding affinities for each receptor subtype, where β-endorphin binds preferentially to MOR, met- and leu-enkephalin bind preferentially to DOR, and dynorphin binds preferentially to KOR (Akil et al., 1998). Signaling induced by endogenous opioids is tightly regulated and is part of the body's natural response to external stimuli. It plays an important role in numerous physiological functions, including stress regulation (Drolet et al., 2001), autonomic control (Faden, 1984), and,

most notably, pain perception (Corder et al., 2018). Endogenous opioid peptides may be released in specific brain regions in response to a given stimulus and rapidly degraded by peptidases present in the tissue, resulting in high spatial and temporal control of signaling. Exogenous opioids act on this system in a much less regulated manner, causing receptor activation broadly throughout the nervous system and for a prolonged duration until metabolized or cleared from the tissue (Burford et al., 2015). The uncontrolled manner in which exogenous opioids act at opioid receptors may induce dysregulation and dysfunction of the system when taken repeatedly or at high doses.

The development of knockout mice lacking genes that encode opioid receptor subtypes has greatly contributed to our understanding of the different contributions of MOR, DOR, and KOR in mediating opioid effects (Kieffer & Gavériaux-Ruff, 2002). Mice lacking MOR do not display analgesia, reward, or physical dependence in response to morphine, indicating that MOR, specifically, is the primary mediator of opioid analgesia as well as the undesirable properties of opioids (Matthes et al., 1996; Sora et al., 2001; Sora et al., 1999)**.** Thus, understanding MOR signaling and regulation has been the focus of much opioid research and will be the primary focus of this dissertation.

1.3 Opioid receptor signaling, regulation, and tolerance

Opioid receptors belong to the G protein-coupled receptor (GPCR) class of receptors, a superfamily of proteins composed of 7 transmembrane domains that respond to extracellular stimuli to elicit an intracellular response through coupling to heterotrimeric G proteins. GPCRs are classified based on which family of Gα proteins they couple to. Opioid receptors couple to $G\alpha_{i/o}$, which generally have an inhibitory effect on cells in which they are expressed. In an

inactive state, receptors are associated with heterotrimeric G proteins consisting of the α and βγ subunits. Activation by an opioid agonist induces a conformational change in the receptor which promotes the exchange of GDP bound to the Gα subunit for GTP, causing the Gα and Gβγ subunits to dissociate from one another. These proteins act at various intracellular effectors, including inhibition of voltage-gated calcium channels and adenylyl cyclase (AC), and activation of inward-rectifying potassium channels, to ultimately decrease cellular excitability (Syrovatkina et al., 2016). Following agonist activation, opioid receptors undergo phosphorylation at serine (S) and threonine (T) residues on the receptor C-terminal tail, primarily by G protein-receptor kinase (GRK) 2/3. Receptor phosphorylation recruits β-arrestins, which terminate receptor signaling by preventing further association of receptors with G proteins and promoting receptor endocytosis. Once internalized, receptors are either recycled back to the plasma membrane or trafficked into lysosomes and degraded (Williams et al., 2001).

Opioid analgesic tolerance was traditionally thought to arise from prolonged phosphorylation and internalization of MORs, resulting in both a functional uncoupling of surface MORs from their G proteins and a reduction in the number of MORs at the plasma membrane available for signaling. In this hypothesized model, sustained agonist activation of MOR first results in acute receptor desensitization, occurring on the order of seconds to minutes, where surface receptors are unable to signal via G proteins due to phosphorylation by GRKs and subsequent β-arrestin recruitment to the C-terminus. Eventually this process gives rise to receptor tolerance, occurring over hours to days, where the number of surface MORs is decreased due to continuous receptor internalization and therefore fewer receptors are available for signaling (Dang & Christie, 2012).

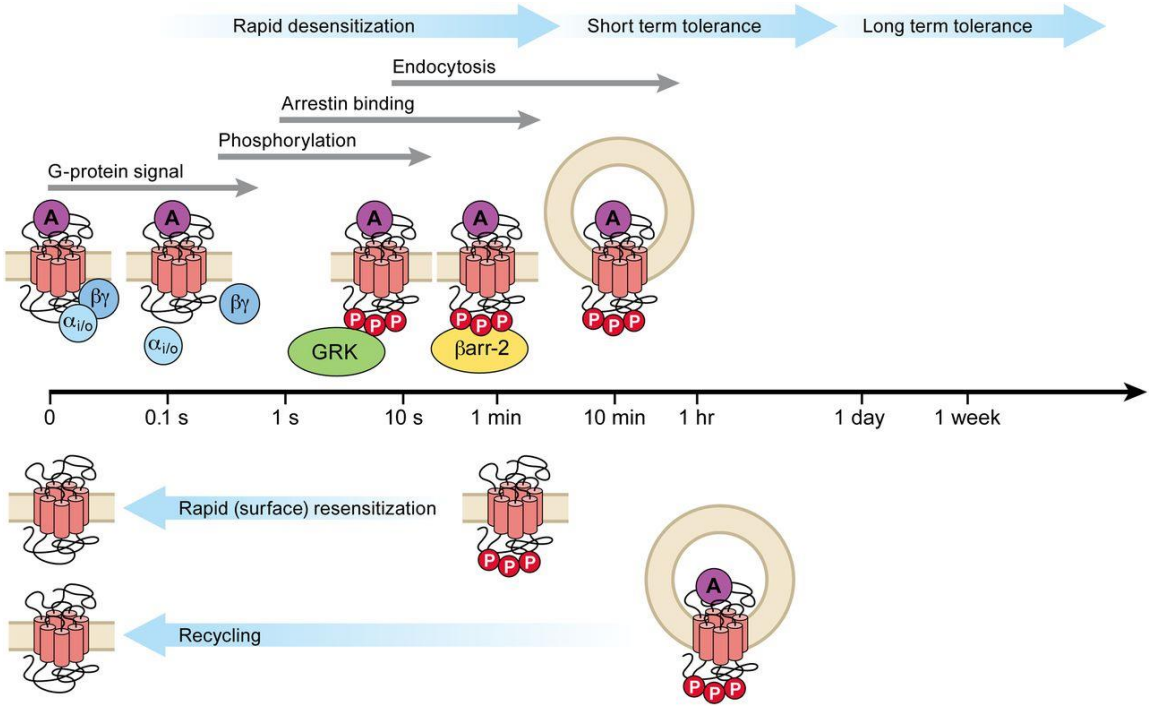

Figure 1.1. Acute receptor desensitization and long-term receptor tolerance induced by prolonged opioid exposure. Acute desensitization occurs on the order of seconds to minutes and results from receptor phosphorylation, β-arrestin binding, and internalization. Long-term tolerance occurs on the order of hours to days and results from functional uncoupling and downregulation of receptors due to multiple regulatory processes. This figure is adapted from (Williams et al., 2013).

Acute receptor desensitization and long-term receptor tolerance have been demonstrated in multiple expression systems, and phosphorylation has been shown to play a critical role in both processes. In cultured cells and *ex vivo* brain slices, acute responses are diminished upon continuous exposure to MOR agonists at saturating concentrations over the course of several minutes (Bailey et al., 2003; Harris & Williams, 1991; Osborne & Williams, 1995; Zhang et al., 1996). This is defined as desensitization and is eliminated when phosphorylation is blocked by either inhibition of GRK2/3 (Leff et al., 2020; Lowe et al., 2015) or mutation of phosphorylation sites (Arttamangkul et al., 2018; Birdsong et al., 2015; Just et al., 2013; Yousuf et al., 2015). In

cultured cells, pretreatment with opioid agonists reduces agonist-mediated G-protein activation (Breivogel et al., 1997; Elliott et al., 1997) and adenylyl cyclase inhibition (Puttfarcken et al., 1988). In brain slices of well-characterized regions involved in mediating opioid effects, such as the locus coeruleus (LC) and periaqueductal gray (PAG), efficacy of MOR agonists to activate potassium conductance at the cell body is decreased in animals chronically treated with morphine (Bagley et al., 2005; Christie et al., 1987; Levitt & Williams, 2018). This observed cellular tolerance is absent in brain slices from chronically morphine treated β-arrestin2 knockout mice (Connor et al., 2015; Quillinan et al., 2011) and rats expressing phosphorylation-deficient MORs (Arttamangkul et al., 2018). The role of MOR phosphorylation in driving chronic opioid effects within relatively understudied circuits related to pain and reward processing will be a focus of the research discussed in this dissertation.

Receptor phosphorylation and uncoupling, however, are not the only mechanisms driving opioid analgesic tolerance. For one, the degree to which MORs are phosphorylated and internalized following activation is highly agonist dependent. For example, morphine is a prototypical opioid clinically but has unique signaling properties at the receptor. While high efficacy agonists such as DAMGO and etorphine produce robust phosphorylation and internalization, morphine is a partial agonist and produces very little (Arden et al., 1995; Zhang et al., 1998). These observations are counter to the previously described model considering that morphine induces greater levels of opioid analgesic tolerance when compared to equianalgesic doses of higher efficacy agonists (Duttaroy & Yoburn, 1995; Madia et al., 2009).

It has therefore been postulated that rather than promoting tolerance, receptor internalization protects receptors from becoming tolerant through dephosphorylation and resensitization. Supporting this model, following internalization MORs are efficiently recycled

back to the plasma membrane rather than degraded by lysosomes (Tanowitz & von Zastrow, 2003). The splice variant MOR1B has a slow rate of desensitization but a rapid rate of endocytosis and recycling compared with other MOR variants, indicating that receptor internalization may in fact counteract desensitization (Wolf et al., 1999). This idea is in agreement with the Relative Activity Versus Endocytosis (RAVE) hypothesis, which proposes that the magnitude of signal transduced by ligand binding to MOR is a function of the relative activity (RA) of the receptor-ligand pair versus the degree to which the ligand promotes endocytosis (VE). Agonists that do not efficiently induce endocytosis display prolonged signaling and produce greater tolerance as a result. According to the RAVE hypothesis, morphine effectively produces tolerance because although a partial agonist, it activates MOR to a high degree relative to the amount of endocytosis it promotes. In contrast, agonists such as DAMGO are highly efficacious but are also efficient at inducing endocytosis and thus produce less tolerance (Martini & Whistler, 2007).

While the canonical mechanism of agonist-induced MOR phosphorylation involves GRK 2/3, phosphorylation by other kinases has also been reported. The phosphorylation pattern and kinase-dependence appear to be agonist-specific, providing another possible explanation for why some agonists produce little internalization but strong tolerance. There are 11 identified phosphorylation sites on the MOR C-terminal tail. High-efficacy agonists like DAMGO and [Met^5]-enkephalin (ME) induce a sequential pattern of phosphorylation, whereby phosphorylation of S^{375} occurs first, followed by T^{370} and T^{379}, and phosphorylation of T^{376} and the $^{354}TSST^{357}$ motif occur at a slower rate. Phosphorylation at each of these residues is primarily mediated by GRK2/3 (Williams et al., 2013). In contrast, morphine induces phosphorylation of S^{375} primarily by GRK5, but little or no phosphorylation of the other residues f(Doll et al., 2012;

Schulz et al., 2004). Evidence also suggests that protein kinase C (PKC), calcium-calmodulin kinase II (CaMKII), and c-J N-terminal kinase (JNK) can directly phosphorylate MOR and that activity of PKC is involved in morphine-mediated acute desensitization (Chen et al., 2013; Johnson et al., 2006). However, it is not yet clear whether MOR activation by morphine directly induces receptor phosphorylation by PKC, or if PKC is simply involved in constitutive phosphorylation and plays an indirect role in mediating acute desensitization by morphine. Chronic morphine exposure also appears to alter kinase regulation of MOR signaling. In naïve rats, desensitization induced by ME is mediated by GRK2/3, but in rats chronically treated with morphine, GRK2/3, PKC, and JNK all appear to be involved in acute receptor desensitization (Leff et al., 2020). Thus, multiple kinases appear to have some involvement in regulating MOR signaling and may contribute to receptor tolerance upon prolonged opioid exposure.

1.4 Downstream adaptations induced by chronic opioid exposure

Beyond the level of the receptor, prolonged exposure to MOR agonists is also known to induce cellular-, synaptic, and circuit-level adaptations. Because these adaptations typically act counter to opioid effects, they enhance neuronal excitability in the absence of drug and are therefore thought to contribute to withdrawal as well as tolerance. However, establishing direct connections between downstream adaptations and their behavioral consequences has been challenging and it is not clear which of these adaptations are drivers of opioid tolerance and/or withdrawal.

At the cellular level, perhaps the best characterized downstream adaptation is AC superactivation. AC catalyzes the production of cyclic AMP (cAMP) from adenosine triphosphate (ATP). As with other $G_{i/o}$ coupled GPCRs, acute activation of opioid receptors

inhibits AC via activity of associated Gα proteins, reducing cAMP concentrations, protein kinase A (PKA) activity, and ultimately neuronal excitability (Chan & Lutfy, 2016). During sustained opioid exposure, AC activity and cAMP levels are initially decreased but eventually return to normal, and basal and stimulated cAMP levels are elevated upon opioid cessation (Nevo et al., 1998; Sharma et al., 1975; Watts & Neve, 2005). As a result, downstream effectors of cAMP become tolerant and are unable to be effectively regulated by morphine (Christie, 2008). Understanding precisely how these downstream adaptations contribute to opioid analgesic tolerance is challenging due to the complexity with which cAMP regulates cell signaling. Changes in cAMP levels and subsequent enzyme activity affect a range of cellular processes, including gene transcription, ion channel gating, and exocytosis (Kopperud et al., 2003; Seino & Shibasaki, 2005). Furthermore, there are 10 different AC isoforms, three of which (ACI, ACII, and ACV) are primarily neuronal (Chan & Lutfy, 2016). Expression of these different isoforms in neurons varies greatly depending on cell type and brain region, and different isoforms may vary in their acute sensitivities to opioids and their regulation following chronic opioid exposure (Ammer & Christ, 2002; Avidor-Reiss et al., 1997; Rivera & Gintzler, 1998)**.**

Chronic opioid exposure also alters function of the mitogen-activated protein kinase (MAPK) signaling cascades, which play a role in regulating gene transcription (Chen & Sommer, 2009). Of this family of proteins, the most relevant to MOR signaling appears to be extracellular signal-regulated protein kinase (ERK). Acutely, morphine increases levels of phosphorylated ERK in both cultured cells and rodents. By contrast, phosphorylated ERK is attenuated in cells pretreated with morphine (Bilecki et al., 2005). In rodents, however, chronic morphine treatment has been found to increase, decrease, or have no effect on phosphorylated ERK levels depending on brain region (Chen & Sommer, 2009). Pretreatment with ERK

inhibitors prior to chronic morphine administration attenuates opioid analgesic tolerance (Chen et al., 2008; Wang et al., 2009). Thus, it appears that ERK activity is important for the development of opioid analgesic tolerance in a region-specific manner.

Opioid-induced alterations in synaptic output can cause long-term changes in the signaling properties of individual neurons and neuronal networks. Activity-dependent strengthening and weakening of synapses, known as synaptic plasticity, is a critical process in learning and memory. This phenomenon is most heavily studied in the hippocampus, a region critical for learning and memory formation, but has been demonstrated at numerous synapses across the central nervous system (CNS), including in regions that are important sites of opioid action. Synaptic plasticity can arise from several different mechanisms affecting presynaptic neurotransmitter release, postsynaptic receptor makeup, or structural changes in the number of synapses. Many of the postsynaptic mechanisms drive insertion or removal of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors to alter the sensitivity of excitatory synapses to glutamate. The strengthening of synaptic connections, resulting from AMPA receptor insertion, is referred to as long-term potentiation (LTP), and the weakening of synapses, resulting from AMPA receptor removal, is referred to as long-term depression (LTD) (Citri & Malenka, 2008).

Acute and chronic opioid exposure can induce or alter these processes. In primary hippocampal neurons, chronic morphine treatment decreased the amplitude of miniature excitatory postsynaptic currents at glutamatergic synapses, indicative of a reduction in synaptic strength through postsynaptic mechanisms (Liao et al., 2005). Chronic morphine has also been shown to increase internalization of postsynaptic GluR1 AMPA receptor subunits at this synapse, suggesting a postsynaptic AMPA-receptor dependent mechanism of LTD (Kam et al.,

2010). Targeting the GluR1 subunit in these studies is important because changes in expression levels of these subunits are particularly relevant to reinforcement, reward, and withdrawal associated with drugs of abuse (Glass et al., 2005). Glutamatergic synapses that express N-methyl-D-aspartate (NMDA) receptors but lack functional AMPA receptors are referred to as silent synapses, as they cannot mediate glutamatergic neurotransmission. One study has shown that in the nucleus accumbens (NAc), chronic exposure to morphine can generate silent synapses in dopamine D2 receptor-expressing medium spiny neurons (MSNs, the output neurons of this region) through internalization of AMPA receptors at pre-existing synapses (Graziane et al., 2016). This finding suggests that chronic opioid exposure can not only weaken synaptic connections, but render them nonfunctional, through adaptations in AMPA receptor surface expression.

In contrast, acute and chronic morphine exposure have been demonstrated to enhance LTP in other brain regions. In dopamine neurons in the ventral tegmental area (VTA), a single systemic injection of morphine has been shown to increase the AMPA/NMDA ratio, a measure of LTP induction (Saal et al., 2003). In another study, acute morphine treatment increased the number of synapses with GluR1 AMPA receptor subunits, while chronic morphine increased both the number of GluR1-positive synapses and the number of surface GluR1 subunits at each GluR1-positive synapse in the VTA (Lane et al., 2008). This suggests both acute and chronic morphine exposure can induce LTP, but possibly through slightly different mechanisms. Dopamine signaling in the VTA plays a central role in reward and goal-directed behaviors and plasticity in this region is highly involved in addiction pathophysiology (Kauer, 2004). Increases in postsynaptic GluR1-containing AMPA receptors following chronic opioid treatment have also

been demonstrated in the basolateral amygdala (BLA) (Glass et al., 2005) and the NAc (Russell et al., 2016).

In order to understand how opioids alter postsynaptic AMPA receptor expression to alter synaptic efficacy, it is important to consider the physiological mechanisms through which AMPA receptors are inserted into and removed from synapses. NMDA receptor-dependent plasticity is one of the most important forms of synaptic plasticity involving changes in postsynaptic AMPA receptors. Mature excitatory synapses express both AMPA and NMDA receptors, two types of ionotropic glutamate receptors. NMDA receptors are blocked by Mg^{2+} ions at resting membrane potential and require membrane depolarization (through AMPA receptor activation) to release this blockade and allow current to pass. High-frequency stimulation of presynaptic terminals causes sufficient AMPA-mediated postsynaptic depolarization to activate NMDA receptors. Active NMDA receptors then allow an influx of Ca^{2+} into the cell, initiating cellular mechanisms that drive the insertion of new postsynaptic AMPA receptors, increasing sensitivity to glutamate and thereby strengthening the synapse. This is referred to as NMDA receptor-dependent LTP. In contrast, NMDA receptor-dependent LTD occurs following lower-frequency stimulation, where the postsynaptic membrane is not sufficiently depolarized to cause widespread release of the Mg^{2+} block from NMDA receptors. This drives internalization of postsynaptic AMPA receptors, decreasing glutamate sensitivity and weakening the synapse (Citri & Malenka, 2008).

NMDA receptor-dependent plasticity has been implicated in the development of opioid analgesic tolerance and dependence. It is well-established that co-administration of NMDA antagonists with opioids impairs the development of opioid analgesic tolerance but does not affect analgesia in already tolerant animals (Trujillo, 2000). Similarly, NMDA receptor

antagonism reduces morphine self-administration in drug-naïve mice (Semenova et al., 1999), and inhibits sensitization to the locomotor-stimulating effects of morphine, which is thought to correlate with addiction behavior humans (Jeziorski et al., 1994; Wolf & Jeziorski, 1993). Together, these studies suggest that NMDA receptors are an important mediator of synaptic plasticity that underlies opioid tolerance and addiction.

The mechanisms of opioid-induced plasticity described thus far have focused on postsynaptic changes in glutamate receptors, however synaptic plasticity can also be achieved through presynaptic mechanisms. Such adaptations drive an increase or decrease in neurotransmitter release. Presynaptic versus postsynaptic alterations in synaptic efficacy can be distinguished based on electrophysiological recordings of spontaneous excitatory and inhibitory currents (sEPSC and sIPSCs, respectively). A change in the frequency of sEPSCs and sIPSCs without a change in amplitude indicates a presynaptic mechanism. At medium spiny neurons (MSNs) in the NAc, chronic morphine was found to increase sEPSC frequency, but not amplitude. Furthermore, selective activation of BLA-NAc inputs revealed a decrease in the paired-pulse ratio in morphine treated mice, which correlates with an enhancement in presynaptic release probability. Together these results suggest chronic morphine causes facilitation of glutamatergic inputs to the NAc, and inputs from the BLA are particularly sensitive to morphine facilitation (Yuan et al., 2017). Presynaptic facilitation by chronic morphine has also been demonstrated at local GABAergic inputs in the VTA and NAc, and glutamatergic synapses the nucleus raphe magnus (Bie & Pan, 2005).

Finally, prolonged opioid exposure induces structural plasticity that may contribute to chronic opioid effects. Much work on this topic has focused on adaptations occurring within the NAc as it is considered a hub for mediating addiction-related behaviors (Thompson et al., 2021).

Repeated morphine administration decreases dendritic branching and spine density in NAc MSNs (Robinson & Kolb, 2004). Similar results have also been shown in the medial prefrontal cortex and hippocampus (Russo et al., 2010). Chronic morphine also increases the firing rate but decreases the soma size of VTA dopamine neurons, which innervate the NAc (Mazei-Robison et al., 2011). Opioid self-administration has been found to alter expression of genes that encode proteins involved in dendritic structure and axonal guidance in the NAc (Tapocik et al., 2013; Yuferov & Zhang, 2018). Opioid-mediated structural plasticity in MSNs is particularly relevant to this dissertation because MSNs are also the primary neurons within the dorsomedial striatum (DMS), and opioid-induced plasticity within this region is a major focus of the research that will be discussed in later chapters.

1.5 Presynaptic versus somatic MOR signaling

Our understanding of cellular MOR function comes primarily from studies conducted in heterologous expression systems, such as transfected cells, or from single-cell recordings at cell bodies of neurons in *ex vivo* brain slices. However, signaling at presynaptic terminals represents an important component of GPCR physiology. Somatic and presynaptic MORs decrease neuronal transmission through different mechanisms, so understanding the differences in how chronic opioid exposure alters the function of these receptors is critical to understanding the mechanisms driving opioid tolerance and dependence (Fig. 1.2). Somatic MORs cause cellular hyperpolarization, primarily through activation of G protein-coupled inwardly rectifying potassium (GIRK) channels, although they also couple to other effectors including AC, MAPK, and Ca^{2+} channels. In contrast, presynaptic MORs inhibit vesicular neurotransmitter release,

primarily through inhibition of voltage-gated calcium channels, although there is some overlap with somatic effectors and these mechanisms are not fully elucidated (Coutens & Ingram, 2023).

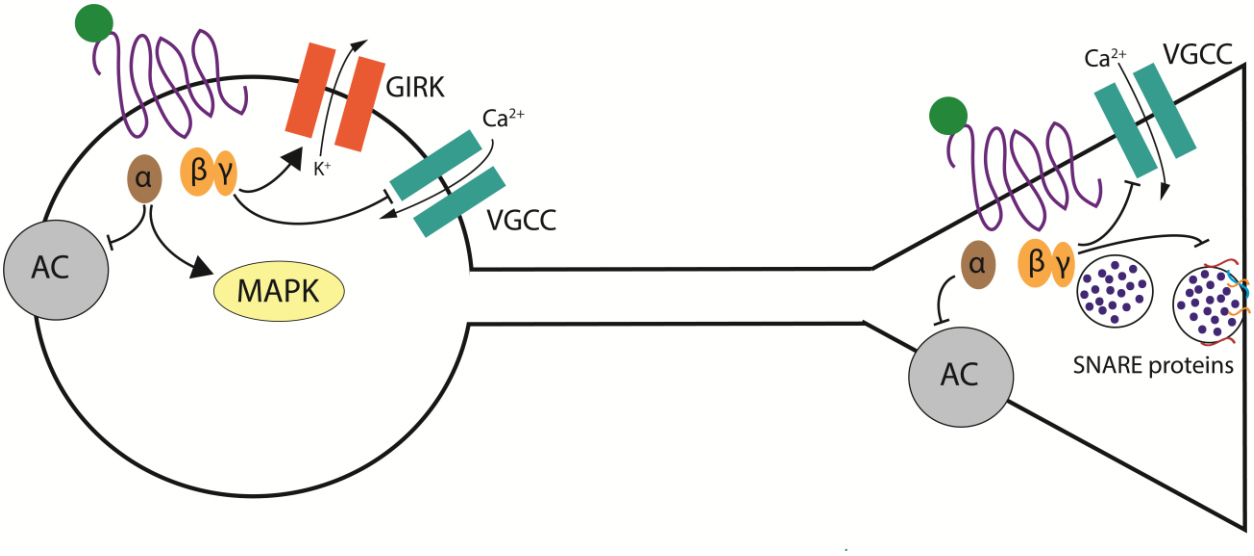

Figure 1.2. Presynaptic and somatic MORs inhibit synaptic transmission through different mechanisms. There is some overlap in MOR effectors in the presynaptic and somatic compartments. However, activation of somatic MORs results in cellular hyperpolarization, primarily through activation of potassium channels, while activation of presynaptic MORs results in decreased neurotransmitter release primarily through inhibition of Ca^{2+} signaling. AC: adenylyl cyclase; GIRK: G protein-coupled inwardly rectifying potassium channel; MAPK: mitogen-activated protein kinase; VGCC: voltage-gated calcium channel.

There is accumulating evidence that somatic and presynaptic MORs are regulated differently following acute and chronic opioid exposure, thus they may play unique roles in mediating opioid analgesic tolerance. For one, while somatic MORs rapidly desensitize upon

continuous stimulation by an agonist, presynaptic MORs are resistant to desensitization. At somatic receptors, desensitization in single cells is measured electrophysiologically as an acute decline from the peak agonist-induced outward current following continuous agonist stimulation, indicative of a gradual decrease in MOR-mediated activation of GIRK channels (Birdsong & Williams, 2020). The degree of desensitization is agonist- and concentration-dependent, but strongly desensitizing agonists like DAMGO at a saturating concentration have been observed to cause upwards of a 50% decrease from peak current after several minutes of agonist stimulation (Bailey et al., 2003; Dang & Williams, 2005; Virk & Williams, 2008). At presynaptic receptors, opioid-mediated presynaptic inhibition is quantified by evoking neurotransmitter release from opioid-sensitive terminals and measuring the amplitude of resulting postsynaptic currents. Studies have typically observed no change in the degree of inhibition of neurotransmitter release during continuous agonist stimulation, indicating a lack of desensitization (Blanchet & Lüscher, 2002; Fyfe et al., 2010; Lowe & Bailey, 2015; Pennock et al., 2012).

Second, recent key studies have uncovered important differences in the internalization and trafficking mechanisms of presynaptic MORs following activation compared with somatic receptors. In agreement with the canonical pathway, presynaptic receptors undergo agonist-induced internalization that is phosphorylation-dependent. However, internalization occurs almost entirely at the axon terminal as opposed to along the entire length of the axon. Agonist stimulation only induces depletion of ~17% of terminal surface MORs, compared to ~50% of somatic receptors. Presynaptic MORs were shown to be laterally mobile at the axon surface, such that following agonist activation receptors outside of a synapse can diffuse into the synapse, replacing internalized receptors (Jullié et al., 2020). This property may potentially explain the lack of desensitization at presynaptic MORs while providing a model for presynaptic tolerance,

especially considering a 70-80% loss in receptors is predicted to be required to observe a rightward shift in the concentration response following chronic morphine treatment (Christie et al., 1987). In this model, a gradual depletion of axonal surface receptors develops through repetitive rounds of endocytosis of synaptic receptors and translocation of new receptors into the synapse during prolonged agonist exposure (Jullié et al., 2022).

Finally, studies examining chronic opioid effects at presynaptic MORs have observed results that are not always consistent with those observed at somatic receptors. Single-cell recordings in live brain tissue have consistently demonstrated cellular tolerance (i.e. a decrease in opioid-mediated GIRK activation) at neuronal cell bodies in multiple brain regions including the LC and PAG (Adhikary & Williams, 2022; Bagley et al., 2005; Christie et al., 1987; Levitt & Williams, 2018). However, brain slice recordings examining presynaptic receptors have observed cellular tolerance (i.e. a decrease in opioid-mediated presynaptic inhibition) in some studies (Fyfe et al., 2010; Matsui et al., 2014), but cellular facilitation (i.e. in increase in opioid-mediated presynaptic inhibition) in others (Chieng & Williams, 1998; Hack et al., 2003; Ingram et al., 1998; Pennock et al., 2012). Based on this lack of agreement, it appears that the regulation of presynaptic MORs may be more complex than postsynaptic receptors. However, the behavioral relevance of presynaptic MOR facilitation is not clear, nor are the mechanisms driving this phenomenon. The data presented in this dissertation seek to add to our understanding of presynaptic versus postsynaptic adaptations in opioid signaling by examining the effects of chronic morphine exposure in presynaptic versus postsynaptic compartment of one neuronal population.

1.6 Chronic opioid effects *in vivo*

Opioid analgesic tolerance has been thoroughly characterized in mice, rats, and humans. Pharmacologically, tolerance is defined as a decrease in drug potency following prolonged drug exposure, indicated by a rightward shift in the dose-response curve. In humans, much of our understanding comes from clinical observations and studies performed in chronic pain patients (Collett, 1998). Patients taking opioids long-term often display dose escalation, sometimes exceeding 10-fold increases in daily dose to adequately manage pain (Buntin-Mushock et al., 2005). However, while there is an abundance of anecdotal and subjective reporting of opioid analgesic tolerance, quantifying the magnitude in human patients is difficult due to confounding factors such as disease progression and increasing pain severity, and dose escalation does not necessarily reflect pharmacological tolerance (Morgan & Christie, 2011).

In rodents, opioid analgesic tolerance has been demonstrated across a range of opioids, doses, and routes of administration, with each of these factors contributing to the magnitude of tolerance. Agonists with low intrinsic efficacy, such as morphine, have typically been observed to produce greater tolerance than agonists with high intrinsic efficacy, such as fentanyl and etorphine, when comparing equianalgesic doses (Pawar et al., 2007; Sosnowski & Yaksh, 1990; Stevens & Yaksh, 1989). However, the negative correlation between agonist intrinsic efficacy and magnitude of tolerance appears to hold up when agonists are continuously infused but not when they are administered intermittently (Duttaroy & Yoburn, 1995).

Behavioral testing in rodents allowed us to greatly improve our understanding of the neurobiological mechanisms governing opioid analgesic tolerance through genetic and pharmacological manipulations. While it has long been postulated that MOR phosphorylation and subsequent β-arrestin recruitment is the first step in cellular tolerance, the role of these

processes in mediating tolerance *in vivo* was first demonstrated following the development of β-arrestin2 knockout mice. These mice fail to develop opioid analgesic tolerance to morphine, although they still show signs of physical dependence (Bohn et al., 2000). Further supporting the role of these processes in opioid analgesic tolerance, knockin mice expressing phosphorylation-deficient MORs were shown to have greatly reduced morphine tolerance and no tolerance to fentanyl (Kliewer et al., 2019). The role of specific kinases in mediating opioid analgesic tolerance appears to be agonist-dependent and variable. One study using inhibitors of PKC and GRK found that acute PKC inhibition reversed tolerance to meperidine, morphine, and fentanyl, but not DAMGO, while GRK inhibition reversed tolerance only to DAMGO (Hull et al., 2010). Other studies have observed a lack of tolerance following co-administration of PKC inhibitors and morphine (Granados-Soto et al., 2000) or DAMGO (Narita et al., 1995). In transgenic mice lacking GRK3, opioid analgesic tolerance to fentanyl was greatly reduced, but tolerance to morphine was unaffected (Terman et al., 2004).

Numerous studies have also indicated a critical role of upregulation of the cAMP-PKA pathway in the development of opioid analgesic tolerance. Chronic morphine treatment *in vivo* increases PKA activity (Nestler & Tallman, 1988), and acute administration of PKA inhibitors in mice reverses tolerance to morphine (Bernstein & Welch, 1997; Javed et al., 2004; Smith et al., 2006). Treatment with PKA inhibitors has also been shown to decrease withdrawal behavior (Maldonado et al., 1995; Tokuyama et al., 1995), and treatment with activators of PKA enhance withdrawal behavior (Punch et al., 1997). Treating mice with an antisense oligodeoxynucleotide to knock down PKA expression completely blocked tolerance to morphine and partially blocked tolerance to etorphine (Shen et al., 2000). Because these studies primarily examined tolerance to

morphine, it is unclear how the role of PKA upregulation in tolerance may differ with high efficacy agonists that strongly promote receptor internalization.

While there are many studies on opioid analgesic tolerance, there are relatively few examining respiratory tolerance. Understanding the mechanisms and magnitude of respiratory tolerance in critically important considering respiratory depression and the resulting hypoxia are the primary cause of death in opioid overdose (White & Irvine, 1999). One study in opioid-dependent patients receiving medically-assisted heroin found that acute intravenous infusion of a maintenance dose was sufficient to induce respiratory depression, dropping blood oxygenation to 79% on average (Stoermer et al., 2003). These findings suggest that tolerance to respiratory depression does not develop as readily as tolerance to other properties of opioids. In mice and rhesus monkeys, direct comparisons between analgesic and respiratory tolerance have found that chronic treatment with opioids produces greater tolerance to analgesia than to respiratory depression (Brandt & France, 2000; Mohammed et al., 2013; Paronis & Woods, 1997). One study also found that morphine-treated mice had increased cAMP concentrations in the PAG (a major site mediating opioid analgesia), but not in the brainstem (where the breathing center is located), suggesting the potential involvement of cAMP in contributing to tolerance (Mohammed et al., 2013).

Paradoxically, repeated exposure to opioids and other drugs of abuse induces sensitization (an increase in response following repeated drug administration) to some opioid-mediated behaviors. In the context of drugs of abuse, behavioral sensitization typically refers to sensitization to the psychomotor-stimulating effects (Steketee & Kalivas, 2011). Behavioral sensitization to opioids has not been well-established in humans, but the effects observed in rodents are robust; a single opioid administration causes a marked increase in locomotor activity,

and this effect is exacerbated following repeated administration. There is interest in this phenomenon because it is thought to have relevance to the neurobiology underlying drug craving and addiction (Robinson & Berridge, 2000). Behavioral sensitization and addiction are both believed to be mediated through the same circuitry involving the NAc. In this model, the accumbens acts as an interface between the limbic system, which mediates goal-directed behaviors (such as drug-seeking), and the motor system, which mediates execution of these behaviors (Wolf, 2002).

1.7 Sex differences in opioid analgesia and tolerance

There is substantial evidence suggesting an influence of sex on the experience of pain, opioid analgesia, and opioid tolerance. Based on historical data, women report chronic pain at higher rates than men, and report greater levels of acute pain compared to men with the same diagnosis (Bartley & Fillingim, 2013). In human pain studies, women are more sensitive than men to experimentally induced pain by both subjective (i.e. self-reported measured of pain intensity) and objective (i.e. electromyographic responses, pupil dilation) measures (Paller et al., 2009). Women are also more likely than men to be prescribed opioids and at higher doses (Pisanu et al., 2019). While some human studies on analgesic properties of acutely administered opioids have found greater efficacy in women, others report no differences between men and women (Fillingim et al., 2009). It is important to note that in these human studies, the terms "men" and "women" reflect both gender and biological sex. There are social and cultural factors related to gender and sex that could contribute to differences in pain reporting and opioid prescribing, but research in animal models suggests the sex differences observed in humans are, at least in large part, biological in nature.

In rodents, the majority of studies have found that opioids have greater analgesic potency and efficacy in males than females (Averitt et al., 2019). This is consistently observed across pain type (thermal, mechanical, etc.) and agonist, although the magnitude of the difference seems to vary depending on these factors (Kest et al., 2000). Greater differences in analgesia are typically observed with lower efficacy agonists. One study examining the effect of agonist efficacy on opioid analgesia found morphine and etorphine (high efficacy agonists in assays of analgesia) were on average 2.5-fold more potent in male rats than females, but buprenorphine (a low efficacy agonist) was up to 13-fold more potent in males (Cook et al., 2000). Despite this, morphine is the most widely used agonist in studies of sex differences on opioid analgesia. Sex differences in opioid analgesia are likely not due to differences in pharmacokinetics considering that they persist even when drugs are delivered intracerebroventricularly or directly into brain regions involved in pain modulation (Kepler et al., 1989; Krzanowska & Bodnar, 1999). Furthermore, similar levels of morphine are observed in the brains of male and female rats following systemic morphine administration (Cicero et al., 1997).

Male and female rodents also appear to differ in their susceptibility to tolerance. First, males appear to develop tolerance to morphine to a greater extent than females across assays and dosing regimens (Kasson & George, 1984; Loyd et al., 2008; Mousavi et al., 2007; South et al., 2001). One early study demonstrated that 2 weeks of twice daily morphine injections produced a 6.9-fold rightward shift in the morphine ED_{50} in male rats but only a 3.7-fold shift in females in the hotplate assay (Craft et al., 1999). Males also develop tolerance more rapidly than females and recover from tolerance more rapidly following cessation of morphine administration (Barrett et al., 2001; Craft et al., 1999; Mousavi et al., 2007; South et al., 2001). However, a handful of studies have found no sex difference or a greater magnitude of tolerance in females (Holtman Jr

et al., 2004; Kest et al., 2000). The reasons behind these discrepancies are unclear, but one possibility is the influence of estrus phase on the development of tolerance.

To date, there are not many studies examining the role of estrous cycle and sex hormones on opioid tolerance, but the ones that exist suggest an influence of these hormones. In intact female rats, tolerance to a single morphine injection is most pronounced during the proestrus phase (Shekunova & Bespalov, 2004). Ovariectomized females do not become tolerant following a single morphine injection, but pretreatment with estradiol in ovariectomized females restores the induction of tolerance (Shekunova & Bespalov, 2006). However when tolerance is measured following 8 days of repeated morphine injections, ovariectomized females develop significant tolerance and to the same extent as gonadectomized males (Mousavi et al., 2007). Castrated male rats also develop tolerance more slowly than testosterone-pretreated females, suggesting a potential influence of male sex hormones as well (South et al., 2001).

In agreement with these behavioral studies, a growing body of evidence also suggests a physiological influence of sex and sex hormones on opioid signaling. MOR density in the hypothalamus varies with estrous phase (Maggi et al., 1993) and ovariectomy decreases MOR density (Joshi et al., 1993). As will be discussed in greater detail in the following section, the periaqueductal gray (PAG) is a key region in propagating pain signals and mediating opioid analgesia (Fields & Heinricher, 1985). Systemic morphine administration increases overall PAG neuronal activity equally in males and females, however PAG projections to the rostral ventromedial medulla (RVM) are preferentially activated in males (Loyd et al., 2007). Moreover, chronic morphine treatment resulted in decreased morphine-mediated activation of PAG-RVM neurons in males but not females (Loyd et al., 2008). As these projections are part of the descending pain modulatory pathway, the findings of this study could provide some insight

about the increased analgesic potency and tolerance liability in males. While there appears to be a clear interaction between sex hormones and the endogenous opioid system, sex differences in morphine binding affinity at MOR or morphine-mediated G protein activation have not been observed (Kepler et al., 1991; Peckham & Traynor, 2006).

1.8 Opioid modulation of CNS anatomy

Opioids exert a wide range of effects due to the broad distribution of MORs throughout the nervous system. The concentration of MORs in regions involved in pain processing make opioids highly effective analgesics. However, their therapeutic potential remains limited given the number of undesirable effects mediated through activity at MOR in other brain regions, such as those involved in reward, breathing, and gastrointestinal motility. To improve the safety profile of opioids, one major focus of the field is to better understand how opioids modulate different neural circuits to drive behavior, particularly those involved in pain transmission.

The experience of pain involves signaling across many distinct but interconnected circuits within the nervous system. Broadly, pain perception is mediated through the activity of ascending sensory and descending modulatory systems. Noxious stimuli activate nociceptors in peripheral tissues, which transmit pain signals to the spinal cord via the dorsal root ganglion, where the cell bodies of nociceptive neurons are located. This signal is then transmitted up the spinothalamic tract, and from the thalamus projects to multiple areas within the cortex, where sensory information is processed, as well as subcortical structures, such as the striatum and amygdala (Dubin & Patapoutian, 2010). Pain modulation is an adaptive mechanism that decreases pain sensation in the presence of noxious stimuli through top-down inhibition of the ascending pathway. The most well-studied descending pain modulatory circuit consists of

projections from the PAG to the spinal cord via the RVM. Electrical or chemical activation of this circuit elicits analgesia by inhibiting transmission within the ascending pathway at the level of the spinal cord (Lau & Vaughan, 2014).

MORs are present in nearly every brain region known to be involved in pain perception. Within the ascending pathway, opioid receptors on glutamatergic neurons suppress excitatory transmission that propagates pain signals to higher-order areas. Within the descending modulatory pathway, opioids are thought to produce analgesia by acting at GABAergic neurons to decrease tonic inhibition of PAG output neurons (i.e. disinhibition) (Corder et al., 2018). Microinjection of morphine directly into the PAG produces analgesia (Jacquet & Lajtha, 1974), as does chemogenetic inhibition of PAG GABAergic neurons or stimulation of glutamatergic neurons (Samineni et al., 2017), suggesting a critical role of the PAG in opioid modulation of pain. As such, how chronic opioid exposure alters signaling within the PAG to produce tolerance is an area of great interest. Microinjection of MOR antagonists directly into the PAG significantly attenuates tolerance, suggesting adaptations within this region are indeed important drivers of tolerance (Lane et al., 2005). The opposing effects of PAG glutamatergic and GABAergic transmission in pain signaling, along with the apparent modulation specifically of GABAergic transmission by opioids is an example of how opioid effects of overall circuitry depend on the types of neurons in which opioid receptors are expressed.

MORs are also highly expressed among circuits related to addiction, such as those involved in reward, motivation, and habit formation. The mesolimbic dopamine system is widely recognized to play a central role in addiction-like behaviors, and within this system, the VTA and NAc are enriched in MOR and enkephalins. Exogenous opioid administration enhances dopamine release in the NAc, at least in part through disinhibition of NAc-projecting

dopaminergic neurons in the VTA (Rysztak & Jutkiewicz, 2022). As discussed previously, chronic opioid exposure induces structural adaptations in NAc MSNs, and rats will self-administer morphine directly into the VTA but not the PAG, supporting a specific role of this pathway in opioid reward (Bozarth & Wise, 1984).

While opioid modulation of some behaviorally relevant pathways is relatively well-defined, it is important to understand how opioid signaling within different systems interacts to influence behavior. For example, while the analgesic and rewarding properties of opioids appear to be mediated through separate pathways, relief of pain itself is rewarding and may contribute to opioid reinforcement. Furthermore, the experience of pain is a complex phenomenon consisting of both sensory and affective dimensions. Whereas sensory pain refers to the physical intensity of a noxious stimulus, affective pain refers to the perceived unpleasantness of the stimulus. Sensory and affective pain are largely mediated through separate neural circuits, and opioids inhibit both dimensions (Corder et al., 2018). Low doses of morphine decrease subjective ratings of pain unpleasantness while higher doses decrease both pain intensity and subjective intensity, suggesting affective pain circuitry may be more sensitive to opioids (Price et al., 1985). Despite this, how opioids modulate affective pain is relatively understudied.

1.9 Opioid modulation of glutamatergic thalamo-cortico-striatal circuitry

The medial thalamus (MThal), anterior cingulate cortex (ACC), and DMS are three interconnected brain regions involved in affective pain processing. Chronic pain is associated with increased activity of the MThal (Whitt et al., 2013), and lesioning the MThal reduces hyperalgesia associated with neuropathic pain (Saade et al., 2007). The MThal is a major source of glutamatergic innervation of the ACC and DMS (Hunnicutt et al., 2016; Hunnicutt et al.,

2014). The ACC is activated by noxious stimuli and is tonically active during chronic pain (Johansen & Fields, 2004; Price, 2000). This region has long been thought to play a critical role in affective pain perception, as patients who received cingulotomies for chronic pain reported pain relief despite having intact sensation of pain intensity (Hurt & Ballantine Jr, 1974). In agreement with these observations, lesioning the ACC in rodents decreases pain-related aversive responses without affecting acute nociceptive responses (Johansen et al., 2001). Activation of MThal inputs to the ACC drives pain-related aversion, suggesting that these projections, specifically, are mediators of affective pain (Meda et al., 2019). The DMS receives strong glutamatergic innervation from both the MThal and ACC. Acute pain states have been shown to increase activity in the DMS and the magnitude of activity is positively correlated with subjective pain ratings (Scott et al., 2006). ACC inputs to the DMS are involved in pain modulation (Zhuang et al., 2021), and connectivity between these regions is altered in chronic pain states (Baliki et al., 2012).

MORs are expressed within the MThal, ACC, and DMS (Mansour et al., 1994; Pert et al., 1976). Infusion of MOR agonists directly into the MThal produces pain relief (Carr & Bak, 1988), into the ACC produces conditioned place preference in animals with chronic pain (Navratilova et al., 2015). While there is little evidence regarding a direct role of opioid signaling within the DMS and pain modulation, this region is heavily involved in reward processes, and pain relief is inherently rewarding. Moreover, the DMS is among the brain regions with the highest concentration of MORs and enkephalin (Koshimizu et al., 2008; Pert et al., 1976). Thus, the DMS may represent an important site of action for opioid modulation of affective pain, as well as a potential site where the mechanisms driving opioid analgesic and motivational effects overlap. Our lab has previously shown that MOR agonists inhibited MThal inputs to the ACC

and DMS but did not inhibit ACC inputs to the DMS, providing specificity to how opioids modulate synaptic transmission within this circuit (Birdsong et al., 2019). Given the functional connectivity between the MThal, DMS, and ACC, the central role of the ACC in pain-related aversion, and the relatively high levels of MOR expression, this thalamo-cortico-striatal circuit is likely an important site of action in mediating opioid analgesia and possibly addiction.

To date, few studies have examined how sustained opioid action within this circuit contributes to tolerance. Studies of how chronic opioid exposure alters synaptic transmission have typically focused on CNS anatomy involved in sensory pain processing and reward, such as the PAG-RVM pathway. Moreover, measuring opioid effects at specific projections of interest requires more sophisticated techniques than measuring opioid effects within a brain region more generally. However, our understanding of the neural basis for opioid tolerance and addiction will not be complete without determining the precise mechanisms by which chronic opioid exposure differentially alters opioid signaling within different opioid-sensitive circuits. Therefore, the goal of this dissertation is to characterize the effects of chronic morphine exposure on subsequent MOR signaling within the glutamatergic thalamo-cortico-striatal circuit and investigate the cellular and synaptic mechanisms underlying these effects. Chapter 2 will examine how chronic morphine exposure alters subsequent MOR signaling at MThal-DMS projections and MThal cell bodies, as well as investigate the role of MOR phosphorylation in mediating the observed effects. Chapter 3 will examine chronic morphine effects on MOR signaling at MThal-ACC projections and whether the balance of excitatory and inhibitory signaling in the ACC is altered by chronic morphine treatment. Chapter 4 will explore circuit-level adaptations that drive the chronic morphine effects observed within the MThal-DMS arm of this circuit. Finally, in chapter 5, I will

discuss how this work contributes to our overall understanding of opioid physiology, and how future studies can expand upon this work.

Chapter 2

Chronic Morphine Induces Adaptations in Opioid Receptor Signaling in a Thalamo-Striatal Circuit That Are Location-Dependent, Sex-Specific and Regulated by Mu-Opioid Receptor Phosphorylation[1]

2.1 Abstract

Chronic opioid exposure induces tolerance to the pain-relieving effects of opioids but sensitization to some other effects. While the occurrence of these adaptations is well-understood, the underlying cellular mechanisms are less clear. This study aimed to determine how chronic treatment with morphine, a prototypical opioid agonist, induced adaptations to subsequent morphine signaling in different subcellular contexts. Opioids acutely inhibit glutamatergic transmission from medial thalamic (MThal) inputs to the dorsomedial striatum (DMS) via activity at μ-opioid receptors (MORs). MORs are present in somatic and presynaptic compartments of MThal neurons terminating in the DMS. We investigated the effects of chronic morphine treatment on subsequent morphine signaling at MThal-DMS synapses and MThal cell bodies in male and female mice. Surprisingly, chronic morphine treatment increased subsequent morphine inhibition of MThal-DMS synaptic transmission (morphine facilitation) in male, but not female, mice. At MThal cell bodies, chronic morphine treatment decreased subsequent morphine activation of potassium conductance (morphine tolerance) in both male and female mice. In knockin mice expressing phosphorylation-deficient MORs, chronic morphine treatment

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resulted in tolerance to, rather than facilitation of, subsequent morphine signaling at MThal-DMS terminals, suggesting phosphorylation-deficiency unmasks adaptations that counter the facilitation observed at presynaptic terminals in wild-type mice. The results of this study suggest that the effects of chronic morphine exposure are not ubiquitous; rather adaptations in MOR function may be determined by multiple factors such as subcellular receptor distribution, influence of local circuitry and sex.

2.2 Introduction

Repeated exposure to opioids such as morphine results in tolerance to their pain-relieving properties, whereby increasing doses of drug are required to achieve the same effect (McQuay, 1999). Conversely, behavioral sensitization develops to other properties, notably conditioned reward and locomotor stimulation, whereby repeated exposure enhances drug response (Gaiardi et al., 1991; Lamb et al., 1991; Lett, 1989; Stewart & Badiani, 1993). While behavioral tolerance and sensitization are well-described, the underlying cellular adaptations are not. Defining these mechanisms is challenging given that different opioid responses to which tolerance or sensitization develop are primarily mediated through the same receptor, the μ-opioid receptor (MOR) (Matthes et al., 1996).

MORs are distributed throughout neurons, regulating neuronal excitability in somato-dendritic (somatic) regions and inhibiting neurotransmitter release in axonal (presynaptic) compartments. Previous work has established important differences in how presynaptic and somatic MOR signaling adapts to chronic opioid exposure. In the somatic compartment, chronic morphine generally results in reduced opioid efficacy, or tolerance, although the degree of tolerance can vary between brain regions (Bagley et al., 2005; Christie et al., 1987; Levitt &

Williams, 2018). It is becoming recognized that many aspects of cellular morphine tolerance in the somatic compartment are mediated by MOR phosphorylation, and loss of phosphorylation sites within the C-terminal tail of MOR attenuates somatic cellular tolerance. Within the presynaptic compartment, multiple adaptations to chronic opioid exposure have been observed; tolerance in some instances (Atwood et al., 2014; Fyfe et al., 2010; Matsui et al., 2014), and enhanced opioid efficacy, or facilitation, in others (Chieng & Williams, 1998; Hack et al., 2003; Ingram et al., 1998; Pennock et al., 2012). MOR phosphorylation also regulates presynaptic cellular tolerance in cultured striatal neurons (Jullié et al., 2022; Jullié et al., 2020). However, the role of phosphorylation in mediating presynaptic facilitation and tolerance in intact brain circuits is not established. Complicating the matter, studies investigating these differences have been done across species and brain regions making it difficult to generalize how a particular cell type or synapse will adapt to repeated opioid exposure. Differences in how male and female human patients and rodents respond to opioids are well documented; In rodents, MOR-selective opioids are generally more potent in males, but in humans are more potent in females (Craft, 2003). Numerous studies in rats have also demonstrated greater or more rapid morphine tolerance in males (Kest et al., 2000). Given these phenotypic differences, there may also be important sex differences in how opioids alter cellular signaling. However, the influence of sex on opioid-induced cellular adaptations is poorly understood.

Neurons in the medial thalamus (MThal), centered around the mediodorsal nucleus, provide an ideal system to investigate presynaptic and somatic adaptations to opioids. MThal neurons express relatively high levels of MOR and single thalamic neurons send broad axonal projections to both the striatum and many cortical areas. MThal projections provide a major source of glutamatergic innervation to the dorsomedial striatum (DMS) (Hunnicutt et al., 2016;

Hunnicutt et al., 2014). Signaling within the MThal and DMS is involved in numerous opioid-sensitive processes, including motivated learning, movement, and perception of pain affect (Balleine et al., 2007; Graybiel et al., 1994; Johansen et al., 2001; McDevitt et al., 2021; Navratilova et al., 2015; Peyron et al., 2000; Price, 2000; Vogt, 2015). Glutamatergic thalamic innervation of the DMS, specifically, has been implicated in learning processes, including reinforcement-based learning (Johnson et al., 2020; Kato et al., 2021). Because of their behavioral relevance and high levels of MOR expression, MThal-DMS projections serve as a relevant system to directly compare chronic morphine effects on subsequent morphine signaling within somatic and presynaptic compartments of the same neuronal population. The objective of the present study was to determine how chronic morphine exposure modulated subsequent morphine signaling within MThal axon terminals synapsing in the DMS and MThal neuronal cell bodies, and whether the observed effects were sex-specific. We further investigated the role of MOR phosphorylation in mediating these adaptations.

2.3 Materials and Methods

Drugs

Reagent	Source	Identifiers
Naloxone	Hello Bio	HB2451
Picrotoxin	Hello Bio	HB0506
Dizocilpine (MK-801)	Hello Bio	HB0004
Baclofen	Hello Bio	HB0953

Bestatin	Sigma Aldrich	B8385
Thiorphan	Sigma Aldrich	T6031
[met^5]enkephalin	Sigma Aldrich	M6638
ML-297	Tocris Bioscience	5380
Morphine sulfate	Sigma-Aldrich	1448005
Morphine sulfate	Spectrum Chemical	M1167
rAAV2-hsyn-hChR2(H134R)-EYFP-WPRE-PA	Gift of Karl Deisseroth produced by UNC vector core	Lot # AV4384H
Cholera Toxin Subunit B Alexa Fluor 488 Conjugate	ThermoFisher	C22841

Animals

All procedures were conducted in accordance with the National Institutes of Health guidelines and with approval from the Institutional Animal Care and Use Committee at the University of Michigan. Mice were maintained on a 12-hour light/dark cycle and given free access to food and water. C57Bl/6J mice were obtained from Jackson Laboratories, and MOR 10 S/T-A mice were created by Dr. Stefan Schulz (Kliewer et al., 2019). Mice were 4 to 8 weeks old at the time of viral injection and 6 to 10 weeks old at the time of brain slice preparation. Mice of both sexes were used.

Chronic opioid treatment

Morphine treated mice were implanted with an osmotic minipump (Alzet model 2001, Cupertino, CA) continuously releasing morphine (80 mg/kg/day) for 7 days prior to brain slice preparation. Drug concentrations were calculated based on the mean pump rate and mouse mass at the time of surgery to achieve the desired dose. Mice were anesthetized with isoflurane (4% induction, 2% maintenance), and an incision was made along the lower back. Pumps were inserted subcutaneously, and the incision was closed with surgical glue and wound clips. Pumps remained implanted until mice were euthanized for brain slice preparation. Brain slices were incubated in the absence of morphine for a minimum of one hour prior to experimentation to ensure no residual drug was present in the slices during the baseline recordings, representing a state of acute morphine withdrawal.

Stereotaxic injections

For evoked synaptic responses: Mice were injected bilaterally with an adeno-associated virus type 2 encoding channelrhodopsin-2 (ChR2) (AAV2-hsyn-ChR2(H134R)-EYFP) targeting MThal. Mice were anesthetized with isoflurane (4% induction, 2% maintenance) and placed on a stereotaxic frame (Kopf Instruments, Tujunga, CA). An incision was made along the scalp and holes drilled through the skull above the injection sites. A glass pipette filled with virus was inserted into the brain and lowered to the appropriate depth. 60-70 nL of virus was injected bilaterally into the medial thalamus (A/P: -1.2 mm, M/L: ±0.6 mm, D/V: 3.6 mm). Virus was delivered using a microinjector (Nanoject II, Drummond Scientific, Broomall, PA). For somatic recordings: Mice were injected bilaterally with choleratoxin subunit B conjugated to Alexa 488 (Ctx-488) (ThermoFisher, Waltham, MA) into DMS for retrograde labeling of DMS-projecting medial thalamic neurons. Injections were performed identically to viral injections, with the

exceptions that 130-140 nL were injected and the following stereotaxic coordinates were used for DMS: A/P +0.8, M/L ±1.2, D/V 3.6 mm.

Brain slice electrophysiology

Brain slices were prepared 2-3 weeks following injection of ChR2 or 1-2 weeks following injection of Ctx-488. Mice were deeply anesthetized with isoflurane and decapitated. Brains were removed and mounted for slicing with a vibratome (Model 7000 smz, Campden Instruments). During slicing brains were maintained at room temperature in carbogenated Krebs solution containing (in mM): 136 NaCl, 2.5 KCl, 1.2 $MgCl_2$-$6H_2O$, 2.4 $CaCl_2$-2 H_2O, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, 11.1 dextrose supplemented with 5 μM MK-801. Coronal sections (250-300 μM) containing the DMS or MThal were made and incubated in carbogenated Krebs supplemented with 10 μM MK-801 at 32°C for 30 minutes. Slices were then maintained at room temperature in carbogenated Krebs until used for recording. Only one cell was recorded from each slice to ensure baseline recordings were not contaminated from prior drug application. A maximum of 3 cells per animal were included in each data set to avoid oversampling.

For DMS recordings, borosilicate glass patch pipettes (Sutter Instruments) were pulled to a resistance of 2.0-4.0 MΩ and filled with a potassium gluconate-based internal solution (in mM: 110 potassium gluconate, 10 KCl, 15 NaCl, 1.5 $MgCl_2$, 10 HEPES, 1 EGTA, 2 Na ATP, 0.4 Na GTP, 7.8 Na_2 phosphocreatine). Slices were placed in the recording chamber and continuously perfused with carbogenated Krebs solution supplemented with 100 μM picrotoxin at 32-34°C. Striatal MSNs were identified based on cell morphology, resting membrane potential, and firing frequency (Kreitzer, 2009), and MSN subtype (D1- versus D2-expressing) was not distinguished. Whole-cell recordings were made in MSNs in voltage-clamp mode at -70 mV holding potential.

All drug solutions for DMS recordings were prepared in carbogenated Krebs solution supplemented with 100 μM picrotoxin.

For MThal recordings, patch pipettes were pulled to a resistance of 2.0-4.0 MΩ and filled with a potassium methanesulfonate-based internal solution containing (in mM): 115 potassium methane sulfonate, 10 KCl, 15 NaCl, 1.5 $MgCl_2$, 10 HEPES-K, 10 BAPTA 4K, 2 Na ATP, 0.4 Na GTP, 7.8 Na_2 phosphocreatine. Slices were placed in the recording chamber and continuously perfused with carbogenated Krebs solution at 32-34°C. Whole cell recordings were made in DMS-projecting thalamic neurons, identified based on cell morphology and the presence of Ctx-488 in the soma. Recordings of GIRK currents were made in voltage-clamp mode and cells were maintained at a holding potential of -60 mV. During recording, all solutions were supplemented with 10 μM ML-297 to enhance the size of the GIRK-mediated outward currents for quantification purposes.

Whole-cell recordings were made with a multiclamp 700B amplifier (Molecular Devices, San Jose, CA) digitized at 20 KHz (National Instruments BNC-2090A, Austin, TX). Synaptic recordings were acquired using Matlab Wavesurfer (Mathworks, Natick, MA)**.** Currents were evoked every 30 seconds by illuminating the field of view through the microscope objective (Olympus BX51W, Tokyo, Japan) using a TTL-controlled LED driver and a 470 nm LED (Thor Labs, Newton, NJ). LED stimulation duration was 1 ms and power output measured after the microscope objective ranged from 0.5-3 mW, adjusted to obtain consistent current amplitudes across cells. Somatic responses were recorded using LabChart (AD Instruments, Colorado Springs, CO) to passively record and measure drug-induced changes in holding current. For both presynaptic and somatic responses, series resistance was monitored throughout the recordings

and only recordings in which the series resistance remained <15 MΩ and did not change more than 18% were included.

Data analysis

For synaptic responses, raw data were analyzed using Matlab or Axograph. Peak current amplitude was calculated for each sweep after baseline subtraction, with baseline defined as the average holding current during the first 10 ms of each sweep, prior to optical stimulation. For each condition (baseline, drug, washout/reversal), baseline subtracted sweeps were averaged together, and peak current amplitude of the averaged trace was calculated. For the baseline condition, the first 2-4 sweeps were omitted from the average to allow the currents to stabilize. For the drug and washout/reversal conditions, the first 4-8 sweeps were omitted from the average to allow for equilibration of drug or washout of drug within the tissue. Average drug and washout/reversal current amplitudes were normalized to the average baseline current peak amplitude and plotted as % of baseline to analyze sensitivity of MThal terminals to opioid-mediated presynaptic inhibition. For somatic responses, raw data were analyzed using Axograph. Average holding current was calculated for each condition, and morphine-induced GIRK current was normalized to baclofen-induced GIRK conductance. Across all conditions, perfusion of 10 μM ML-297 alone induced an average current of 12.59 ± 1.96 pA (mean ± SEM) with no differences across sex or treatment. To account for a small change in holding current or GIRK current induced by ML-297 alone, a two-region sloping baseline subtraction was performed using Axograph, in which a line interpolated between the ML-297 baseline and naloxone reversal was subtracted from the trace. Cells in which morphine responses were not distinguishable from the ML-297 baseline were considered non-responders and excluded from

analysis. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Statistical comparisons were made using a t-test or one-way or 2-way ANOVA with Tukey's (one-way ANOVA) or Šidák's (2-way ANOVA) *post-hoc* analysis. Complete data sets for male and female mice were obtained and analyzed to examine sex as a factor for all experiments involving morphine. For all experiments, statistical significance was defined as $p<0.05$. For all comparisons, n (number of cells) and N (number of animals) are both reported.

2.4 Results

MOR agonists inhibit optically evoked MThal-DMS glutamate release.

Agonist-induced activation of MOR decreases the amplitude of optically-evoked excitatory postsynaptic currents (oEPSCs) in DMS medium spiny neurons (MSNs) via presynaptic inhibition (Adhikary, Jaeckel, et al., 2022; Atwood et al., 2014; Birdsong et al., 2019). We first demonstrated opioid-mediated inhibition of oEPSCs in MThal-DMS terminals was induced by opioid agonists morphine and [Met^5]-enkephalin (ME) by performing whole-cell recordings in voltage clamp mode in DMS MSNs following viral expression of ChR2 in MThal neurons (Fig. 2.1A, B) in male and female mice. After recording a stable baseline of oEPSCs, agonist was perfused onto the slices, followed by the opioid receptor antagonist naloxone or Krebs to reverse inhibition (Fig. 2.1C-E). Naloxone was perfused to reverse inhibition by morphine, and ME was reversed with Krebs due to ME readily washing out of the slice. Consistent with opioid mediated presynaptic inhibition of glutamate release from MThal terminals , perfusion of morphine (3 μM) caused a significant decrease in the mean amplitude of the MThal-DMS oEPSC relative to baseline, and this effect was largely reversed upon perfusion of naloxone (Fig. 2.1 C, E, F; $p < 0.0001$, main effect of condition, repeated measures one-way

ANOVA; baseline vs morphine: $p < 0.0001$; baseline vs naloxone: $p = 0.0091$; morphine vs naloxone: $p = 0.0052$, Tukey's multiple comparisons test). Like morphine, perfusion of ME (3 μM) also caused a significant decrease in oEPSC mean amplitude in a reversible manner (Fig. 2.1 D, E, G; $p = 0.0018$, main effect of condition, repeated measures one-way ANOVA; baseline vs ME: $p = 0.0082$; baseline vs washout: $p = 0.0615$; ME vs washout: $p = 0.0091$, Tukey's multiple comparisons test).

The inhibition of oEPSCs by 3 μM morphine was significantly less than inhibition induced by the same concentration of ME (Fig. 2.1H; 3 μM morphine: 72.26 ± 2.44% of baseline, N = 15, n = 21, 3 μM ME: 31.61 ± 5.38% of baseline, N = 10, n = 11, 3 μM morphine vs 3 μM ME: $p < 0.0001$, ordinary 2-way ANOVA with Šidák's multiple comparisons test). Likewise, our previous work has shown that perfusion of the MOR full agonist DAMGO inhibits MThal-DMS oEPSCs to 38.2 ± 6.1% of baseline (Birdsong et al., 2019). Inhibition of oEPSCs by 10 μM morphine was similar to inhibition by 3 μM morphine, indicating that 3 μM morphine is a saturating concentration (Fig. 2.1H; 3 μM morphine: 72.26 ± 2.44% of baseline; 10 μM morphine: 72.32 ± 4.24% of baseline; 3 μM morphine vs 10 μM morphine: $p > 0.9999$, ordinary 2-way ANOVA with Šidák's multiple comparisons test). Inhibition of oEPSCs by 3 μM ME and 10 μM ME were also similar, indicating that inhibition by ME is saturated at 3 μM (Fig. 2.1H; 3 μM ME: 31.61 ± 5.38% of baseline; 10 μM ME: 25.16 ± 4.20% of baseline; 3 μM ME vs 10 μM ME: $p = 0.8815$, ordinary 2-way ANOVA with Šidák's multiple comparisons test).

These results indicate that, at this synapse, morphine acts as a partial agonist for inhibition of MThal-DMS EPSCs. Morphine was selected for future experiments because, as a partial agonist, observable changes in the sensitivity of MORs are less likely to be occluded by receptor reserve than with a full agonist. Under these conditions, there were no statistically

significant differences in oEPSC inhibition by morphine between slices from untreated male and female mice (Fig. 2.1I; males: 74.70 ± 4.06% of baseline, N = 7, n = 10; females: 70.04 ± 2.85% of baseline, N = 9, n = 11; $t_{(19)} = 0.9533$, p = 0.3524, unpaired t-test).

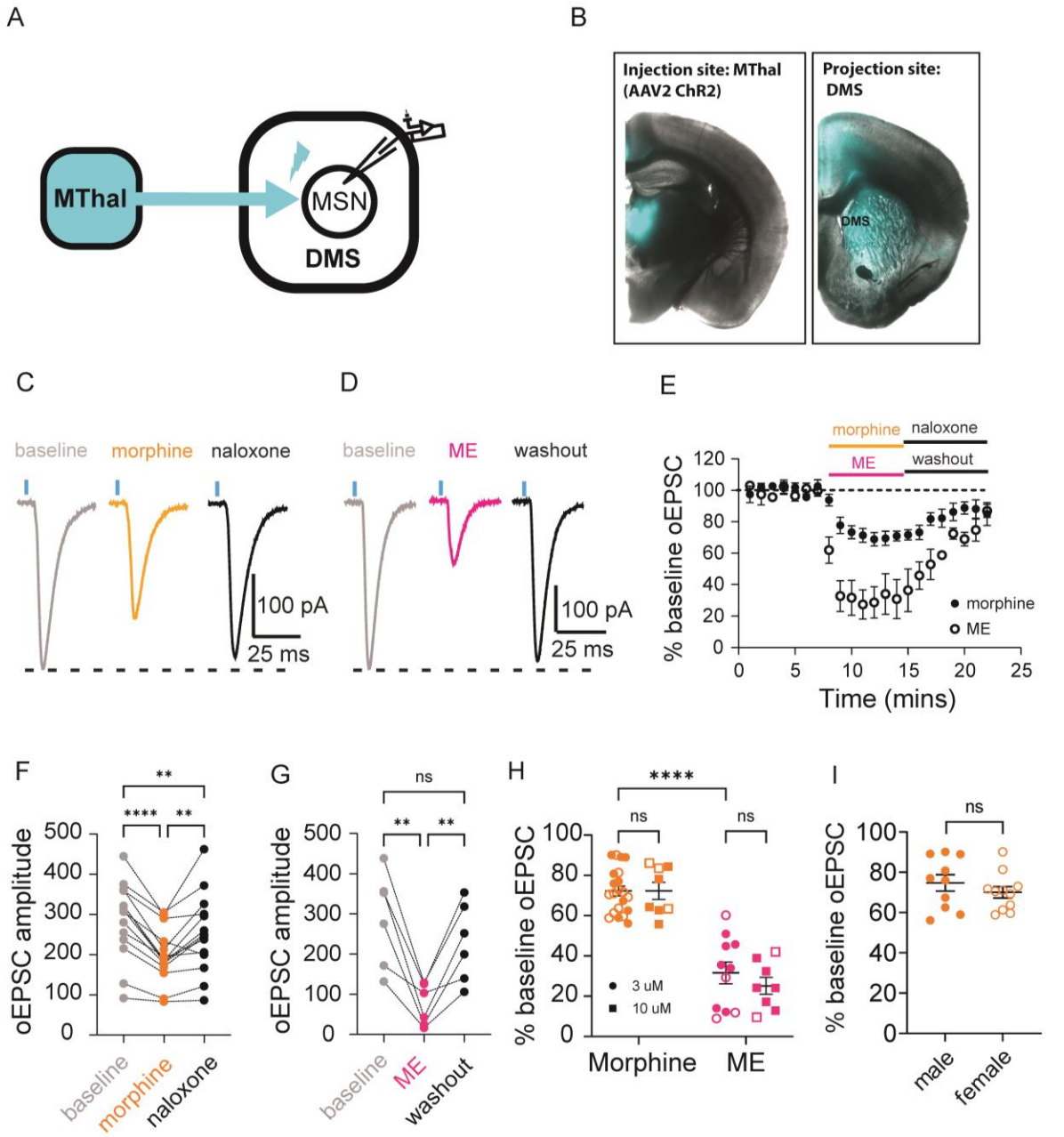

Figure 2.1. Mu-opioid receptor agonists inhibited glutamatergic MThal-DMS oESPCs. **A.** Schematic showing viral mediated expression of ChR2 in the medial thalamus (MThal) and axonal projections onto striatal MSNs. **B.** Examples of acute brain slices showing EYFP fluorescence in the injection site (MThal, left) and axon terminals in the DMS (right). **C.** Representative traces showing oEPSCs in an MSN evoked by 470 nm LED light pulses during baseline (gray), perfusion of morphine (3 μM, orange), and perfusion

of naloxone (1 μM, black). **D.** Representative traces showing oEPSCs in an MSN evoked by 470 nm LED light pulses during baseline (gray), perfusion of ME (3 μM, pink), and washout (black). **E.** Time course of normalized oEPSC amplitude during baseline, perfusion of morphine (3 μM) or ME (3 μM), followed by perfusion of naloxone (1 μM,) or washout (morphine: N = 12 (6 M 6 F), n = 14 (7 M, 7 F), ME: N = 6 (2 M, 4 F), n = 6 (2 M, 4 F)). **F.** Raw amplitudes of oEPSCs in individual striatal MSNs during baseline, perfusion of morphine (3 μM), and perfusion of naloxone (1 μM) (main effect of condition: $F_{(1.687, 21.93)}$ = 28.01, $p < 0.0001$, N = 12 (6 M, 6 F), n = 14 (7 M, 7 F), filled circles represent males, open circles represent females; repeated measures one-way ANOVA; baseline vs morphine: $p < 0.0001$; baseline vs naloxone: $p = 0.0091$; morphine vs naloxone: $p = 0.0052$, Tukey's multiple comparisons test). **G.** Raw amplitudes of oEPSCs in individual striatal MSNs during baseline, perfusion of ME (3 μM), and washout (main effect of condition: $F_{(1.259, 6.297)} = 24.17$, $p = 0.0018$, N = 6 (2 M, 4 F), n = 6 (2 M, 4 F), repeated measures one-way ANOVA; baseline vs ME: $p = 0.0082$; baseline vs washout: $p = 0.0615$; ME vs washout: $p = 0.0091$, Tukey's multiple comparisons test). **H.** Summary data comparing oEPSC inhibition following perfusion of 3 μM (circles) and 10 μM (squares) morphine (orange) and ME (pink) in male and female mice (3 μM morphine: 72.26 ± 2.44% of baseline, N = 16 (7 M, 9 F), n = 21 (10 M, 11 F), 10 μM morphine: 72.32 ± 4.24% of baseline, N = 7 (5 M, 2 F), n = 8 (5 M, 3 F), 3 μM ME: 31.61 ± 5.38% of baseline, N = 11 (7 M, 4 F), n = 11 (7 M, 4 F), 10 μM ME: 25.16 ± 4.20% of baseline, N = 6 (5 M, 1 F), n = 8 (6 M, 2 F), filled data points represent males, open data points represent females; main effect of concentration: $F_{(1,44)} = 0.6015$, $p = 0.4421$; main effect of agonist: $F_{(1,44)} = 113.8$, $p < 0.0001$; interaction: $F_{(1,44)} = 0.6253$, $p = 0.4333$, ordinary 2-way ANOVA; 3 μM morphine vs 10 μM morphine: $p > 0.9999$, 3 μM ME vs 10 μM ME: $p = 0.8815$, 3 μM morphine vs 3 μM ME: $p < 0.0001$, 10 μM morphine vs 10 μM ME: $p < 0.0001$, Šidák's multiple comparisons test). **I.** Summary data comparing oEPSC inhibition following perfusion of morphine (3 μM) in male versus female mice (males: 74.70 ± 4.06% of baseline, N = 7, n = 10; females: 70.04 ± 2.85% of baseline, N = 9, n = 11, $t_{(19)} = 0.9533$, $p = 0.3524$, unpaired t-test). Lines and error bars represent mean ± SEM. ****$p < 0.0001$.

Chronic morphine treatment increased morphine sensitivity at MThal-DMS terminals in male, but not female, mice

We next investigated whether exposing mice to chronic morphine altered the sensitivity of MThal-DMS oEPSCs to inhibition by a subsequent morphine challenge in a sex-dependent manner. Chronic morphine treatment was achieved through implantation of an osmotic minipump continuously releasing morphine (80 mg/kg/day) for 7 days prior to recording (Fig. 2.2I). To ensure no morphine from the minipump was present in the slices during the baseline recordings, slices were incubated in the absence of morphine for a minimum of one hour before performing electrophysiology recordings. After recording a stable baseline, morphine (3 μM) was perfused onto the slices, followed by naloxone (1 μM, Fig. 2.2A-F). Surprisingly, morphine caused greater inhibition of oEPSCs in morphine withdrawn slices from morphine-treated mice

compared to slices from drug-naïve mice in males. However, in slices from female mice, no differences were observed between morphine inhibition of oEPSCs in drug-naïve and chronically morphine treated mice (Fig. 2.2G; naïve male: 74.70 ± 4.06% of baseline; chronic morphine male: 54.76 ± 4.11% of baseline; naïve female: 70.04 ± 2.85% of baseline; chronic morphine female: 70.44 ± 2.82% of baseline; treatment x sex interaction: $p = 0.0052$; male naïve vs chronic morphine: $p = 0.0004$, female naïve vs chronic morphine: $p = 0.9957$, Šidák's multiple comparisons test). To ensure the effects observed in males was a result of morphine exposure rather than an effect associated with minipump implantation, recordings were performed in slices taken from a cohort of male mice implanted with saline-containing osmotic minipumps (Fig. 2.2H). Morphine inhibition of oEPSCs in slices from saline treated mice did not differ from naïve mice, but was significantly less than morphine inhibition in slices from chronic morphine treated mice, suggesting that facilitation of morphine inhibition was an effect of morphine exposure rather than surgical manipulation (saline treated: 69.10 ± 4.11% of baseline; $F(2,29) = 6.6910$, $p = 0.0035$, ordinary one-way ANOVA; naïve vs saline treated: $p = 0.6038$, saline treated vs chronic morphine treated: $p = 0.0413$, Tukey's multiple comparisons test). These results suggest that chronic morphine exposure resulted in facilitation of, rather than tolerance to, morphine inhibition of glutamatergic MThal-DMS oEPSCs and that this adaptation occurred in a sex-specific manner.

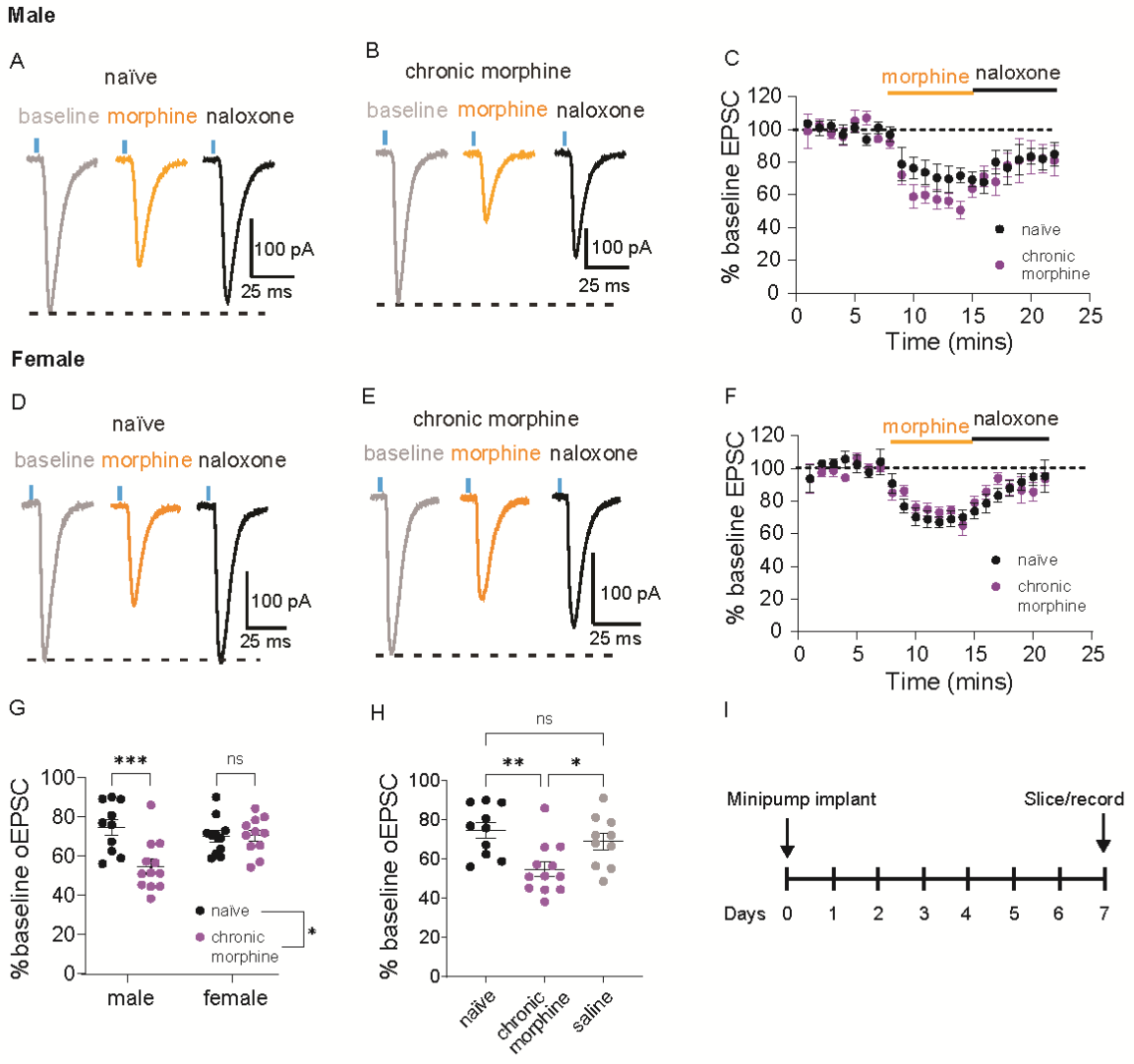

Figure 2.2. Chronic morphine treatment increased morphine sensitivity at MThal-DMS terminals in male, but not female, mice. **A, B.** Representative traces showing oEPSCs in MSNs during baseline (gray), following perfusion of morphine (3 μM, orange), and following perfusion of naloxone (1 μM, black) in drug-naïve male (A) and chronic morphine treated male (B) mice. **C.** Time course of normalized oEPSC amplitude during baseline, perfusion of morphine (3 μM), and perfusion of naloxone (1 μM) in drug-naïve (black) and chronically treated (purple) male mice (naïve: N = 5, n = 7, chronic morphine: N = 6, n = 9). **D, E.** Representative traces showing oEPSCs in MSNs during baseline (gray), following perfusion of morphine (3 μM, orange), and following perfusion of naloxone (1 μM, black) in drug-naïve female (**D**) and chronically treated female (**E**) mice. **F.** Time course of normalized oEPSC amplitude during baseline, perfusion of morphine (3 μM), and perfusion of naloxone (1 μM) for drug-naïve (black) and chronically treated (purple) female mice (naïve: N = 6, n = 7, chronic morphine: N = 6, n = 10). **G.** Summary of normalized oEPSC inhibition following perfusion of morphine in drug-naïve and chronically treated male and female mice (naïve male: 74.70 ± 4.064% of baseline; chronic morphine male: 54.76 ± 4.11% of baseline; naïve female: 70.04 ± 2.85% of baseline; chronic morphine female: 70.44 ± 2.82% of baseline; main effect of treatment: $F_{(1, 40)} = 8.048$, p = 0.0071; main effect of sex: $F_{(1,40)} = 2.524$, p = 0.1200; treatment x sex interaction: $F_{(1,40)} = 8.723$, p = 0.0052; N = 7-9, n = 10-12 for each group, ordinary 2-way ANOVA; male naïve vs chronic morphine: p = 0.0004, female naïve vs chronic morphine: p = 0.9957,

Šidák's multiple comparisons test). **H.** Summary of normalized oEPSC inhibition following perfusion of morphine in naïve, saline treated, and morphine treated male mice (untreated: 74.70 ± 4.064% of baseline; morphine treated: 54.76 ± 4.11% of baseline; saline treated: 69.10 ± 4.11% of baseline; $F_{(2,29)}$ = 6.6910, p = 0.0035, ordinary one-way ANOVA; naïve vs saline treated: p = 0.6038, saline treated vs chronic morphine: p = 0.0413, Tukey's multiple comparisons test). **I.** Schematic of chronic morphine treatment. Morphine (80 mg/kg/day) was continuously administered via osmotic minipump for 7 days prior to brain slice preparation and recording. Lines and error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

Chronic morphine treatment attenuated morphine-activated GIRK current amplitude at MThal cell bodies in male and female mice

We next investigated whether morphine treatment affected subsequent morphine signaling at MThal cell bodies in male and female mice. Within the somatodendritic compartment, activation of MOR can activate G protein-gated inward rectifying potassium (GIRK) channels. When measuring somatic GIRK conductance, chronic opioid exposure has been shown to induce varying degrees of tolerance (or decreased response amplitude) to morphine in a cell-type specific manner (Bagley et al., 2005; Christie et al., 1987; Levitt & Williams, 2018). Using exogenously expressed MOR, we have previously shown that MOR agonists can activate GIRK in MThal cell bodies and that this signaling desensitizes over time in MOR phosphorylation dependent manner (Birdsong et al., 2015). However, to our knowledge, chronic opioid effects at MThal cell bodies specifically have not yet been investigated.

To address this, the retrograde tracer Ctx-488 was injected into the DMS to fluorescently label DMS-projecting MThal neurons (Fig. 2.3A-C). Whole-cell voltage clamp recordings were made from identified Ctx-488-positive MThal neurons in acute brain slices prepared 1-2 weeks later. GIRK currents were activated by bath perfusion of the partial MOR agonist morphine (3 μM) and the $GABA_B$ receptor agonist baclofen (3 μM) (Fig. 2.3D-G). To compensate for varying degrees of GIRK expression between cells, the morphine response was normalized to the

baclofen response within each cell. 6 cells across sexes and treatment conditions (3 male naïve, 1 male chronic morphine, and 2 female chronic morphine) did not respond to morphine perfusion and were excluded from analysis. Comparing the effects of chronic morphine treatment and sex, we observed a main effect of treatment, where baclofen-normalized morphine responses were significantly smaller in slices from chronically treated mice. (Fig. 2.3H; male naïve: $I_{morphine}$ = 52.61 ± 4.22% of $I_{baclofen}$; male chronic morphine: $I_{morphine}$ = 36.30 ± 7.10% of $I_{baclofen}$; female naïve: $I_{morphine}$ = 45.85 ± 7.87% of $I_{baclofen}$; female chronic morphine: $I_{morphine}$ = 33.93 ± 4.37% of $I_{baclofen}$; main effect of treatment: p = 0.0266; main effect of sex: p = 0.4595; treatment x sex interaction: p = 0.7217, ordinary 2-way ANOVA). Pos-hoc analysis did not show a statistically significant effect of chronic morphine treatment in males or females (male naïve vs chronic morphine: p = 0.1296, female naïve vs chronic morphine: p = 0.3209, Šidák's multiple comparisons test). These results indicate that chronic morphine treatment induced small but significant tolerance to a subsequent morphine challenge at MThal cell bodies in both male and female mice.

Raw GIRK current amplitudes induced by perfusion of morphine were also examined. No significant effect of chronic morphine treatment was observed in raw GIRK currents induced by morphine in slices from male or female mice (Fig. 2.3I, I; male naïve: $I_{morphine}$ = 90.56 ± 8.67 pA; male chronic morphine: $I_{morphine}$ = 65.17 ± 12.14 pA; female naïve: $I_{morphine}$ = 81.75 ± 11.81 pA; female chronic morphine: $I_{morphine}$ = 66.50 ± 13.75 pA; main effect of treatment: p = 0.0920, ordinary 2-way ANOVA. However, the high degree of variability in raw current amplitude makes these findings difficult to interpret. Together, these results indicate that chronic morphine treatment induced small but significant tolerance to subsequent morphine signaling in the somatic compartment in MThal projection neurons in both male and female mice.

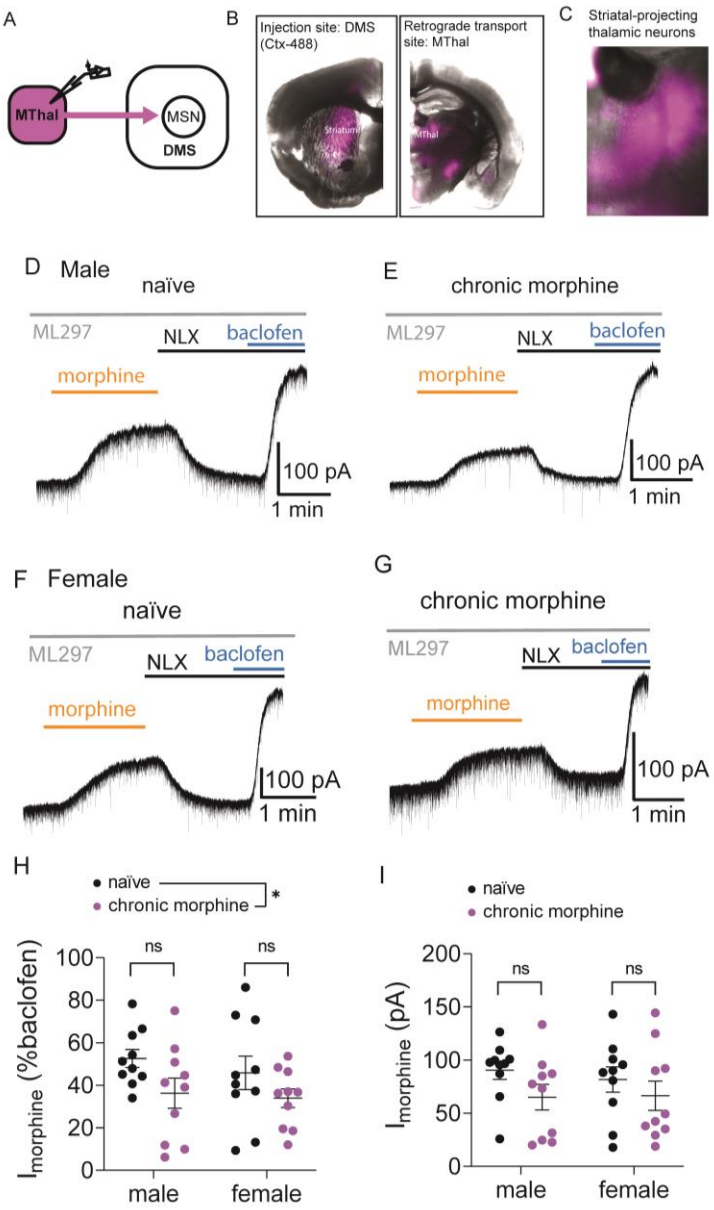

Figure 2.3. Chronic morphine treatment attenuated morphine-activated GIRK current amplitude at MThal cell bodies in male and female mice. **A.** Schematic showing retrograde labeling of MThal projection neurons following injection of Ctx-488 into the DMS. **B.** Examples of acute brain slices showing fluorescence in the injection site (DMS, left) and retrograde labeling site (MThal, right). **C.** Example of an acute brain slice at 5X magnification showing fluorescence in the cell bodies of individual DMS-projecting MThal neurons. **D, E.** Representative traces showing GIRK conductance at medial thalamic cell bodies following perfusion of morphine (3 µM), perfusion of naloxone (1 µM), and perfusion of baclofen (3 µM) in drug-naïve male (**D**) and chronically treated male (**E**) mice. **F, G.** Representative traces showing GIRK current at medial thalamic cell bodies following perfusion of morphine (3 µM), perfusion of naloxone (1 µM), and perfusion of baclofen (3 µM) in drug-naïve female (**F**) and chronically treated female (**G**) mice. **H.** Summary of morphine (3 µM)-induced GIRK currents normalized to baclofen-induced GIRK currents in drug-naïve and chronically treated male and female mice (naïve male: $I_{morphine}$ = 52.61 ± 4.22% of $I_{baclofen}$; chronic morphine male: $I_{morphine}$ = 36.30 ± 7.10% of $I_{baclofen}$; naïve

female: $I_{morphine}$ = 45.85 ± 7.87% of $I_{baclofen}$; chronic morphine female: $I_{morphine}$ = 33.93 ± 4.37% of $I_{baclofen}$; main effect of treatment: $F_{(1, 36)} = 5.342$, p = 0.0266; main effect of sex: $F_{(1, 36)} = 0.4595$, p = 0.4595; treatment x sex interaction: $F_{(1, 36)} = 0.1289$, p = 0.7217; N = 6-8, n = 10 per group, ordinary 2-way ANOVA; male naïve vs chronic morphine: p = 0.1296, female naïve vs chronic morphine: p = 0.3209, Šidák's multiple comparisons test). **I.** Summary of raw morphine (3 μM)-induced GIRK currents in drug-naïve and chronically treated male and female mice (naïve male: $I_{morphine}$ = 90.56 ± 8.66 pA; chronic morphine male: $I_{morphine}$ = 65.17 ± 12.14 pA; naïve female: $I_{morphine}$ = 81.75 ± 11.81 pA; chronic morphine female: $I_{morphine}$ = 66.50 ± 13.75 pA; main effect of treatment: $F_{(1, 36)} = 2.998$, p = 0.0920; main effect of sex: $F_{(1, 36)} = 0.1014$, p = 0.7520; treatment x sex interaction: $F_{(1, 36)} = 0.1870$, p = 0.6680; N = 6-8, n = 10 per group, ordinary 2-way ANOVA). Lines and error bars represent mean ± SEM. *p<0.05.

Mice lacking phosphorylation sites in the MOR C-terminus are more sensitive to morphine at MThal-DMS terminals and develop tolerance following chronic morphine treatment.

Receptor phosphorylation is a key regulator of MOR signaling. However, the role of phosphorylation in regulating MOR signaling in the presynaptic compartment is not well-established. Using a knockin mouse line in which mice express MORs with 10 serine (S) and threonine (T) to alanine (A) mutations in the MOR C-terminal tail (10 S/T-A; Fig. 2.4L, K) (Kliewer et al., 2019), we first determined whether loss of phosphorylation sites altered basal sensitivity to morphine at MThal-DMS terminals. Phosphorylation-deficient MORs have been shown to display reduced receptor internalization and desensitization, however, binding affinity, activation kinetics, and signaling through the G protein pathway is similar to WT receptors. MOR 10 S/T-A mice display enhanced opioid analgesia and reduced tolerance, further suggesting phosphorylation plays an important role in regulating opioid effects following acute and chronic exposure (Kliewer et al., 2019). To our knowledge, the effects of the 10 S/T-A mutations have not been characterized in presynaptic terminals. We first aimed to determine whether opioid-mediated inhibition of synaptic transmission was altered under baseline conditions in MOR 10 S/T-A male and female mice relative to WT mice. Inhibition of glutamate release from MThal-DMS terminals by perfusion of ME or morphine in slices from untreated 10 S/T-A mice was quantified and compared to WT mice (Fig. 2.4A-D, G).

Perfusion of ME (3 μM) reduced oEPSC amplitude to 30.82 ± 6.03% of baseline in slices from 10 S/T-A mice, similar to the 31.61 ± 5.38% of baseline observed in WT mice (Fig. 2.4B). This result suggests 10 S/T-A and WT mice have similar sensitivity to a full agonist at a saturating concentration in MThal-DMS terminals and is not surprising because receptor reserve has been previously demonstrated at presynaptic MORs (Fyfe et al., 2010; Jullié et al., 2022). Thus, we would not expect an increase in the number of functional membrane receptors to translate to an increase in maximum inhibition. In contrast to the effect of ME, morphine (3μM) inhibited oEPSC amplitude significantly more in 10 S/T-A mice than WT mice using an ordinary 2-way ANOVA (Fig. 2.4C). We observed a main effect of strain, with morphine perfusion causing greater oEPSC inhibition in 10 S/TA mice compared to WT, but no main effect of sex or strain x sex interaction (WT male: 74.70 ± 4.06% of baseline; 10 S/T-A male: 57.79 ± 4.49% of baseline; WT female: 70.04 ± 2.85% of baseline; 10 S/T-A female: 59.74 ± 6.35% of baseline; main effect of strain: $p = 0.0048$; main effect of sex: $p = 0.766$; strain x sex interaction: $p = 0.4717$, ordinary 2-way ANOVA). These results indicate that morphine is more efficacious at inhibiting oEPSCs in 10 S/T-A mice than WT mice, consistent with enhanced analgesic potency observed at the behavioral level (Kliewer et al., 2019). Post-hoc analysis revealed a significant difference between WT and 10 S/T-A mice in male, but not female mice, suggesting the overall effect is primarily driven by males (WT vs 10 S/T-A male: $p = 0.0244$; WT vs 10 S/T-A female: $p = 0.2210$; Šidák's multiple comparisons test).

We next determined whether the morphine facilitation observed at MThal-DMS synapses in WT mice was dependent on MOR phosphorylation by comparing morphine mediated inhibition of MThal-DMS oEPSCs in drug-naïve and morphine treated 10 S/T-A mice. Although facilitation was observed only in WT males, mice of both sexes were used to determine whether

any adaptations occur in females that may be unmasked in the phosphorylation-deficient MOR mice. While chronic morphine treatment enhanced inhibition of oEPSC amplitude by subsequent morphine in WT mice, we observed decreased morphine inhibition of oEPSC amplitude in slices from chronically morphine treated 10 S/T-A mice, indicated by a main effect of treatment (Fig. 2.4D-J; naïve male: 57.79 ± 4.49% of baseline; chronic morphine male: 70.03 ± 3.70% of baseline; naïve female: 59.74 ± 6.35% of baseline; chronic morphine female: 69.79 ± 2.46% of baseline; main effect of treatment: $p = 0.0149$; ordinary 2-way ANOVA); We did not observe a main effect of sex or a treatment x sex interaction. Post-hoc analysis did not reveal statistical significance in either sex separately (main effect of sex: $p = 0.8467$; treatment x sex interaction: $p = 0.8039$; ordinary 2-way ANOVA; male naïve vs chronic morphine: $p = 0.1018$; female naïve vs chronic morphine: $p = 0.2197$, Šidák's multiple comparisons test). These findings indicate a small but significant tolerance effect but the experiments were insufficiently powered to determine whether this tolerance was driven preferentially in one sex. When comparing WT and 10 S/T-A mice across treatment conditions in males only, we observed a treatment x strain interaction with naïve WT males being less sensitive to morphine than naïve 10 S/T-A males and chronically treated WT males being more sensitive to morphine than chronically treated 10 S/T-A males (Fig. 2.4K; WT naïve: 74.70 ± 4.06% of baseline; 10 S/T-A naïve: 57.79 ± 4.49% of baseline; WT chronic morphine: 54.76 ± 4.11% of baseline; 10 S/T-A chronic morphine: 70.03 ± 3.70% of baseline; treatment x sex interaction: $p = 0.0003$, ordinary 2-way ANOVA; WT vs 10 S/T-A naïve: $p = 0.0117$; WT vs 10 S/T-A chronic morphine: $p = 0.0190$, Šidák's multiple comparisons test). Together, the results of these experiments suggest that MOR C-terminal phosphorylation-deficient mice were initially more sensitive to morphine inhibition at MThal-DMS terminals but developed tolerance, rather than facilitation, to subsequent morphine

signaling following chronic morphine exposure. These changes resulted in a reversal in sensitivity to morphine inhibition following chronic morphine treatment (WT were more sensitive than 10 S/T-A) and suggest that loss of MOR phosphorylation sites did not simply mimic or occlude the effect of morphine treatment seen in WT male mice but may have unmasked counteradaptations leading to the development of tolerance.

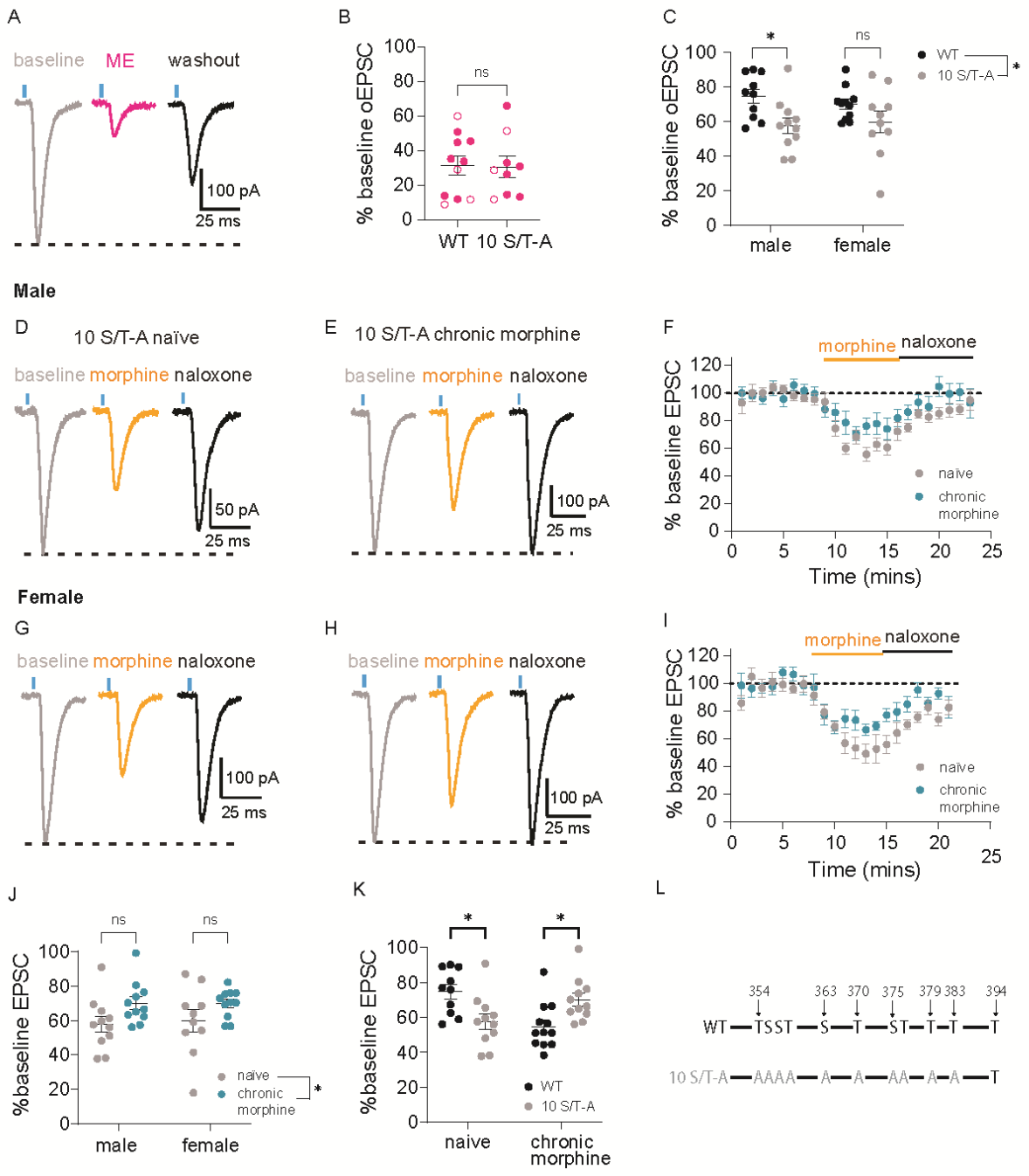

Figure 2.4. Mice lacking phosphorylation sites in the MOR C-terminus are more sensitive to morphine at MThal-DMS terminals and develop tolerance following chronic morphine treatment. **A.** Representative traces showing oEPSCs in DMS MSNs during baseline (gray), following perfusion of ME (3 μM, pink), and following washout (black) in drug-naïve MOR 10 S/T-A mice. **B.** Summary data comparing oEPSC inhibition following perfusion of ME (3 μM) in male and female WT and 10 S/T-A mice (WT: 31.61 ± 5.38% of baseline, N = 11 (7 M, 4 F), n = 11 (7 M, 4 F), 10 S/T-A: 30.83 ± 6.03% of baseline, N = 5 (3 M, 2 F), n = 9 (6 M, 3 F), $t_{(18)}$ = 0.09722, p = 0.9236, filled circles represent males, open circles represent females). **C.** Summary data comparing oEPSC inhibition following perfusion of morphine (3 μM) in male and female WT and 10 S/T-A mice (WT male: 74.70 ± 4.064% of baseline; 10 S/T-A male: 57.79 ±

4.49% of baseline; WT female: 70.04 ± 2.85% of baseline; 10 S/T-A female: 59.74 ± 6.35% of baseline; main effect of strain: $F_{(1, 38)} = 8.957$, p = 0.0048; main effect of sex: $F_{(1,38)} = 0.08938$, p = 0.7666; strain x sex interaction: $F_{(1,38)} = 0.5285$, p = 0.4717; N = 6-9, n = 10-11 for each group, ordinary 2-way ANOVA; male WT vs 10 S/T-A: p = 0.0244, female WT vs 10 S/T-A: p = 0.2210, Šidák's multiple comparisons test). **D, E.** Representative traces showing oEPSCs in DMS MSNs during baseline (gray), following perfusion of morphine (3 μM, orange), and following perfusion of naloxone (1 μM, black) in drug-naïve male (D) and chronically treated male (E) 10 S/T-A mice. **F.** Time course of normalized oEPSC amplitude in DMS MSNs during baseline, perfusion of morphine (3 μM), and perfusion of naloxone (1 μM) for drug-naïve male (grey) and chronically treated male (cyan) mice (naïve: N = 9, n = 11, chronic morphine: N = 5, n = 8). **G, H.** Representative traces showing oEPSCs in DMS MSNs during baseline (gray), following perfusion of morphine (3 μM, orange), and following perfusion of naloxone (1 μM, black) in drug-naïve female (G) and chronically treated female (H) 10 S/T-A mice. **I.** Time course of normalized oEPSC amplitude in DMS MSNs during baseline, perfusion of morphine (3 μM), and perfusion of naloxone (1 μM) for drug-naïve female (grey) and chronically treated female (cyan) mice (naïve: N = 5, n = 7, chronic morphine: N = 5, n = 7). **J.** Summary of normalized oEPSC inhibition following perfusion of morphine in drug-naïve and chronically treated male and female 10 S/T-A mice (naïve male: 57.79 ± 4.49% of baseline; chronic morphine male: 70.03 ± 3.70% of baseline; naïve female: 59.74 ± 6.35% of baseline; chronic morphine female: 69.79 ± 2.46% of baseline; main effect of treatment: $F_{(1, 39)} = 6.485$, p = 0.0149; main effect of sex: $F_{(1,39)} = 0.03786$, p = 0.8467; treatment x sex interaction: $F_{(1,38)} = 0.06250$, p = 0.8039; N = 5-9, n = 10-11 for each group, ordinary 2-way ANOVA; male naïve vs chronic morphine: p = 0.1018, female naïve vs chronic morphine: p = 0.197, Šidák's multiple comparisons test). **K.** Summary of normalized oEPSC inhibition following perfusion of morphine in drug-naive and chronically treated WT and 10 S/T-A male mice (main effect of strain, F(1, 40) = 0.04962; p = 0.8249; main effect of treatment, F(1, 40)= 0.8821; p = 0.3533; strain × treatment interaction, F(1, 40)= 15.88; p = 0.0003; ordinary 2-way ANOVA; WT vs 10 S/T-A naive, p = 0.0117; WT vs 10 S/T-A chronic morphine, p = 0.0190, Šidák's multiple-comparisons test). **L.** Schematic of MOR C-terminal phosphorylation site mutations in 10 S/T-A mice. Lines and error bars represent mean ± SEM. *p < 0.05.

2.5 Discussion

The present study provided a direct comparison of how chronic morphine treatment differentially alters morphine signaling at presynaptic and somatic subcellular compartments within the same neuronal population in a sex-specific manner. Seven days of continuous morphine exposure facilitated morphine responses at MThal-DMS terminals in male, but not female mice, but induced tolerance at MThal cell bodies in both sexes. In MOR phosphorylation-deficient mice, chronic morphine treatment induced tolerance, rather than facilitation, at MThal-DMS presynaptic terminals, indicating that receptor phosphorylation may regulate processes that drive facilitation. One caveat of our study is that because brain slices were maintained in the

absence of morphine, slices from chronically treated mice were in an acutely withdrawn state during baseline recordings. Chronic morphine induces both desensitization and tolerance in locus coeruleus neurons. Desensitization recovers in less than one hour, but tolerance to morphine persists beyond 6 hours (Levitt & Williams, 2012). Thus, conditions in our study represent an acutely withdrawn "tolerant", but not desensitized, state. In this withdrawn state, opioid signaling in presynaptic periaqueductal grey terminals has been shown to utilize additional adenylyl cyclase-dependent signaling pathways (Ingram et al., 1998). In this context, the loss of facilitation in phosphorylation-deficient MOR mice may suggest that receptor phosphorylation could regulate the ability of MOR to either induce adaptive responses or signal through this alternate withdrawn signaling pathway. However, the effects of morphine and the role of receptor phosphorylation in regulation of morphine effects may be different in opioid-maintained conditions.

Facilitation of morphine signaling at MThal-DMS presynaptic terminals

Previous studies have observed facilitation of opioid signaling at GABAergic terminals in various brain regions (Chieng & Williams, 1998; Hack et al., 2003; Ingram et al., 1998; Pennock et al., 2012), while others have observed tolerance (Fyfe et al., 2010; Matsui et al., 2014). At excitatory synapses in the striatum, a single exposure to oxycodone blocked the subsequent induction of long term depression by MOR agonists, possible evidence of opioid tolerance (Atwood et al., 2014). Studies which have described presynaptic facilitation have primarily attributed the effect to a compensatory upregulation of adenylyl cyclase (AC) that drives hyperexcitability of the terminals (Sharma et al., 1975). We hypothesized that, because AC upregulation is proposed to be blunted by MOR internalization (Finn & Whistler, 2001),

phosphorylation-deficient/ low internalizing 10 S/T-A mice might display increased morphine facilitation. Instead, chronic morphine treatment eliminated facilitation and induced tolerance in 10 S/T-A mice, suggesting that either AC upregulation may not mediate facilitation observed in our study, that receptor phosphorylation and receptor trafficking/ localization may be important for AC upregulation (Zhao et al., 2006), or that alternative mechanisms may be involved. Other possible mechanisms of presynaptic facilitation include increases in functional receptor number, increases in receptor-effector coupling efficiency or circuit-level changes in the strength of innervation of MOR-expressing thalamic inputs to DMS. It is also possible that, rather than blocking the process of facilitation, phosphorylation deficiency could mimic and, therefore, occlude morphine-induced facilitation. Alternatively, phosphorylation-deficiency may unmask other forms of morphine-induced tolerance that act in opposition to the morphine-induced facilitation. These competing mechanisms can be investigated in future studies.

From the data we cannot conclude that morphine facilitation at MThal-DMS terminals is driven by presynaptic, rather than postsynaptic, adaptations given that opioids have been shown to induce synaptic plasticity (Gerdeman et al., 2003). We have recently shown that morphine acting at postsynaptic sites can negatively modulate tonic adenosine signaling at glutamatergic presynaptic terminals in the DMS, suggesting that presynaptic effects on glutamate release can be influenced by postsynaptic adaptations (Adhikary, Jaeckel, et al., 2022). Precise differences in local circuitry such as these could provide insight as to why facilitation occurs at thalamic presynaptic terminals in DMS, while presynaptic tolerance has been shown within other circuits.

Sex differences in the development of morphine facilitation

Sex differences in the development of opioid analgesic tolerance are well known, with numerous studies showing greater tolerance in males than females (Bodnar & Kest, 2010), but the underlying physiological mechanisms are not yet clear. Female and castrated male rats developed tolerance more slowly than testosterone-pretreated females or intact males, suggesting that testosterone may influence the development of tolerance (South et al., 2001). Morphine tolerance has also been shown to develop in male and proestrus female rats, but not ovariectomized females or females in other estrous phases (Shekunova & Bespalov, 2004, 2006). Repeated morphine administration has been associated with a decrease in the number of PAG-RVM output neurons activated by morphine in male but not female rats, providing a neural correlate with sex differences in opioid tolerance (Loyd et al., 2008).

Many studies investigating chronic opioid effects on somatic or presynaptic effects of opioids either did not report sex differences or conducted experiments only in males. To our knowledge, this study is the first to describe sex-specific facilitation of opioid effects. However, from our data we cannot determine a mechanism driving the observed sex differences or whether facilitation at MThal-DMS terminals is not present in females at all, or if these effects are masked by additional counteradaptations that are not present in males.

Tolerance to morphine signaling at medial thalamic cell bodies

Cellular tolerance at somatic MORs induced by chronic morphine treatment has been observed in many brain regions including the locus coeruleus, Kölliker-Fuse neurons and PAG (Bagley et al., 2005; Christie et al., 1987; Levitt & Williams, 2018). In agreement with these studies, we observed cellular tolerance at MThal cell bodies following continuous morphine exposure in both males and females. To our knowledge, decreased coupling of opioids to GIRK

conductance in thalamic regions following chronic opioid exposure has not been previously demonstrated. Recently, GIRK currents induced by DAMGO in paraventricular thalamic (PVT) neurons were not found to be different between saline and morphine treated mice, however tolerance developed to the DAMGO-mediated inhibition of PVT neuron firing. (Hou et al., 2023). This lack of tolerance may be due to DAMGO being a full agonist, making detection of tolerance difficult, or the high variability of GIRK currents seen in both their study and ours. While the tolerance in our study was statistically significant, the magnitude of the effect was small. This may be due, in part, because morphine mediated GIRK current was small under physiological conditions, prompting the use of ML-297 to enhance the amplitude of the currents for quantification. This indicates that, at the soma, MOR expression may be low or GIRK coupling weak, making a decrease in receptor-effector coupling difficult to capture. Furthermore, we observed a high degree of variability in the amplitude of morphine-activated GIRK currents and several cells were excluded from analysis due to a lack of response to morphine. These results suggest that there may be heterogeneity in MOR expression patterns across MThal (and possibly PVT) neurons.

The role of MOR C-terminal phosphorylation in driving presynaptic opioid adaptations

Phosphorylation of serine and threonine residues in the C-terminal tail of MOR, promotes arrestin recruitment to MORs, uncoupling of associated G proteins, and receptor internalization. Deletion of phosphorylation sites in the C-terminal tail of MOR has been shown to reduce both acute desensitization and tolerance in the somatic compartment (Arttamangkul et al., 2018; Arttamangkul et al., 2019; Williams et al., 2013). Given the small effect of chronic morphine treatment on morphine signaling at MThal cell bodies and the large variability of morphine-

mediated responses, we did not investigate chronic morphine effects at MThal cell bodies in 10 S/T-A mice. However, based on the findings of previous studies, we would not expect tolerance to occur in phosphorylation-deficient mice.

To our knowledge, the role of MOR phosphorylation in mediating chronic opioid effects at presynaptic receptors has not previously been examined in brain slices. However, based on current knowledge of MOR regulation by phosphorylation, it is not clear why loss of MOR phosphorylation produced tolerance, whereas facilitation was observed in WT mice at MThal-DMS presynaptic terminals. In 10 S/T-A mice, behavioral tolerance to morphine is reduced, but not eliminated while tolerance to fentanyl is eliminated (Kliewer et al., 2019). Receptor phosphorylation, desensitization, and tolerance are agonist-dependent, and unlike other agonists, desensitization by morphine is modest and dependent on phosphorylation by protein kinase C (Dang & Christie, 2012). A recent study demonstrated that agonist-induced MOR phosphorylation can vary by brain region (Fritzwanker et al., 2023) methadone, elicited very little phosphorylation at residues S375, T376, and T379 in the striatum but robust phosphorylation in the medial habenula and spinal cord. It is possible that the facilitation observed in our study is driven by a unique phosphorylation pattern that is dependent on agonist and brain region, and loss of phosphorylation in 10 S/T-A mice unmasks underlying tolerance. Several kinases, including GRK2/3, protein kinase C, and c-Jun N-terminal kinase are involved in phosphorylation of the MOR C-terminal tail (Williams et al., 2001). It is not known which of the 10 mutated phosphorylation sites or kinases mediate the chronic opioid effects we observed. MOR phosphorylation patterns within the axonal compartment and variability between sexes are unknown making a determination of which phosphorylation sites are affected by morphine treatment difficult to predict.

This study highlights that the effects of chronic opioid treatment on MOR signaling are not ubiquitous, but may instead be specific to agonist, brain region, and subcellular location of the receptor. A better understanding of the precise mechanisms by which these adaptations occur is critical to the development of safer, more effective therapeutics for chronic and severe pain for both females and males.

Chapter 3

Chronic Morphine Treatment Induces Sex- and Synapse-Specific Cellular Tolerance at Thalamo-Cortical Mu-Opioid Receptor Signaling

3.1 Abstract

How cellular adaptations give rise to opioid analgesic tolerance to opioids like morphine is not well understood. For one, pain is a complex phenomenon comprised of both sensory and affective components, largely mediated through separate circuits. Glutamatergic projections from the medial thalamus (MThal) to the anterior cingulate cortex (ACC) are implicated in processing of affective pain, a relatively understudied component of the pain experience. The goal of this study was to determine the effects of chronic morphine exposure on mu-opioid receptor (MOR) signaling at MThal-ACC presynaptic terminals within the direct (excitatory) and indirect (feedforward inhibitory) pathways. Using whole-cell patch clamp electrophysiology and optogenetics to selectively target these projections, we measured morphine-mediated inhibition of optically evoked postsynaptic currents in ACC layer V pyramidal neurons in drug-naïve and chronically morphine treated mice. We found that chronic morphine treatment robustly attenuated morphine presynaptic inhibition within the feedforward inhibitory pathway in males, but not females, and mildly attenuated presynaptic inhibition within the excitatory pathway in both sexes. These effects were not observed in MOR phosphorylation-deficient mice. This study indicates that chronic morphine treatment induces cellular tolerance to morphine within a thalamo-cortical circuit relevant to pain and opioid analgesia. Furthermore, it suggests that this tolerance may be driven by MOR phosphorylation. Overall, the findings of this study improve

our understanding of how chronic opioid exposure alters cellular signaling in ways that may contribute to opioid analgesic tolerance.

3.2 Introduction

Opioids are the mainstay treatment for chronic and severe pain. However, their clinical utility is limited by their adverse side effects and high addiction liability. Moreover, tolerance readily develops to their analgesic, or pain-relieving properties, whereby increasing doses are required to achieve the desired effect following prolonged use. One major goal of opioid research is to develop opioids with a reduced propensity for tolerance. However, the receptor and cellular adaptations that underlie opioid analgesic tolerance are currently not well-understood.

Opioid effects are primarily mediated through activity at the mu-opioid receptor (MOR) (Matthes et al., 1996). One challenge in linking cellular adaptations to behavioral outcomes is that MOR activation affects a range of physiological processes through activation of many intracellular effectors. Of particular importance to this study, presynaptic MORs, located within axon terminals, appear to have different signaling and regulation from somatic MORs, located at cell bodies (Coutens & Ingram, 2023). Although presynaptic MOR signaling is physiologically important, opioid effects within this compartment are not as well-studied as their somatic counterparts. Depending on brain region, some studies investigating the effects of chronic opioid exposure on presynaptic MOR signaling have observed cellular tolerance, where opioid efficacy is reduced (Atwood et al., 2014; Fyfe et al., 2010; Matsui et al., 2014), while others have observed facilitation, where efficacy is enhanced (Chieng & Williams, 1998; Hack et al., 2003; Ingram et al., 1998; Pennock et al., 2012). We recently found that within neurons in the medial thalamus (MThal) projecting to the dorsomedial striatum (DMS), chronic morphine treatment

induces cellular tolerance at MThal cell bodies but facilitation at striatal-projecting MThal terminals (Jaeckel et al., 2024). These findings suggest that the effects of chronic opioid exposure are not ubiquitous, but rather depend on several factors including receptor subcellular location. MOR phosphorylation is thought to be a major driver of opioid analgesic tolerance by causing functional uncoupling and downregulation of receptors (Williams et al., 2013). However, the role of phosphorylation in MOR regulation differs between presynaptic and somatic MORs, as agonist-mediated receptor downregulation is greater at somas versus terminals, providing a potential explanation for the inconsistent effects of chronic opioid exposure at presynaptic MORs (Jullié et al., 2022; Jullié et al., 2020).

A second challenge in identifying opioid adaptations relevant to opioid analgesic tolerance is the complexity of pain signaling. The experience of pain is multidimensional, comprised of sensory pain, which describes the perceived intensity of a noxious stimulus, and affective pain, which describes the perceived unpleasantness of a noxious stimulus (Corder et al., 2018). Sensory and affective pain are largely mediated through separate neural circuits and may differ in their sensitivity to opioids. The anterior cingulate cortex (ACC) is heavily implicated in affective pain processing (Xiao & Zhang, 2018). Patients receiving cingulotomies for chronic pain reported pain relief despite having intact sensation of pain intensity (Hurt & Ballantine Jr, 1974), and lesioning the ACC in rodents decreases pain-related aversive responses without affecting acute nociceptive behavior (Johansen et al., 2001). MThal neurons strongly innervate the ACC and inactivation of these neurons greatly reduces ACC nociceptive responses (Dong et al., 1978; Hsu et al., 2000; Hsu & Shyu, 1997). Selective activation of MThal-ACC projections enhances pain-related aversion, suggesting a role of this pathway in mediating affective pain (Meda et al., 2019).

Opioid analgesia is mediated in part through signaling within the ACC (Navratilova et al., 2015). We have previously shown that MORs are functionally expressed presynaptically at both MThal-ACC direct (i.e. excitatory pathway) synapses onto pyramidal neurons and indirect (i.e. feedforward inhibitory pathway) synapses onto local inhibitory interneurons, providing potentially important sites of action for opioids to modulate affective pain (Arias-Hervert & Birdsong, 2024; Birdsong et al., 2019; Delevich et al., 2015). However, it is not known how opioid signaling at these terminals is altered by chronic opioid exposure. The goal of this study was to determine how chronic exposure to morphine, a prototypical and clinically relevant MOR agonist, alters MOR signaling at MThal-ACC terminals. We also examined the role of MOR phosphorylation in driving the observed effects given the general role of receptor phosphorylation in opioid tolerance. The results suggest that chronic morphine treatment induces cellular tolerance at Thal-ACC terminals that is sex-specific and dependent on MOR phosphorylation.

3.3 Materials and Methods

Drugs

Reagent	Source	Identifiers
CGP 55845	Tocris Bioscience	1248
Dizocilpine (MK-801)	Hello Bio	HB0004
Mecamylamine	Sigma-Aldrich	1376006
Morphine sulfate	Sigma-Aldrich	1448005

Morphine sulfate	Spectrum Chemical	M1167
MPEP	Tocris Bioscience	1212
Naloxone	Hello Bio	HB2451
Scopolamine	Sigma Aldrich	S0929
rAAV2-hsyn-hChR2(H134R)-EYFP-WPRE-PA	UNC Vector Core	N/A

Animals

All animal procedures were conducted in accordance with the National Institutes of Health guidelines and with approval from the Institutional Animal Care and Use Committee at the University of Michigan. Mice were given free access to food and water and maintained on a 12-hour light/dark cycle. C57Bl/6J mice were obtained from Jackson Laboratories, and MOR 10 S/T-A mice were gifted by Dr. Stefan Schulz. Mice were stereotaxically injected at 4 to 8 weeks of age and sacrificed for recording at 6 to 10 weeks of age. Mice of both sexes were used.

Chronic morphine treatment

Chronic morphine treatment was achieved through implantation of an osmotic minipump (Alzet model 2001, Cupertino, CA) continuously releasing morphine (80 mg/kg/day) for 7 days prior to experimentation. Drug concentrations were calculated based on the mean pump rate and mouse mass at the time of surgery to achieve the correct dose. Mice were anesthetized with isoflurane (4% induction, 2% maintenance), and an incision was made along the lower back. Pumps were inserted subcutaneously and the incision was closed with surgical glue and wound

clips. Pumps remained implanted until mice were euthanized for brain slice preparation. Brain slices were incubated in the absence of morphine for a minimum of one hour prior to experimentation to ensure no residual drug was present in the slices during the baseline recordings.

Stereotaxic injections

Mice were bilaterally injected using stereotaxic methods with an adeno-associated virus type 2 expressing channelrhodopsin-2 (AAV2-hsyn-ChR2(H134R)-EYFP; ChR2) targeting MThal. Mice were anesthetized with isoflurane (4% induction, 2% maintenance) and placed on a stereotaxic frame (Kopf Instruments, Tujunga, CA). An incision was made along the midline of the scalp and a craniotomy was performed on the skull above MThal. A glass pipette filled with virus was inserted into the brain and lowered to the appropriate depth. 60-70 nL of virus was injected bilaterally into MThal (A/P: -1.2 mm, M/L: ±0.6 mm, D/V: 3.6 mm) using a microinjector (Nanoject II, Drummond Scientific, Broomall, PA). Virus was injected over the course of 1-2 minutes and a 5-minute incubation was allowed before retracting the pipette to allow for perfusion into the tissue. Incisions were closed with surgical glue.

Brain slice electrophysiology

Brain slices were made 2-3 weeks following viral injection of ChR2. Mice were deeply anesthetized with isoflurane and decapitated. Brains were removed and mounted for slicing with a vibratome (Model 7000 smz, Campden Instruments). During slicing, brains were maintained in room temperature (20-22°C) carbogenated Krebs solution containing (in mM): 136 NaCl, 2.5 KCl, 1.2 $MgCl_2$-$6H_2O$, 2.4 $CaCl_2$-2 H_2O, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, 11.1 dextrose,

supplemented with 5 μM MK-801. Coronal sections (250-300 μM) containing the ACC were sliced and incubated in carbogenated Krebs solution supplemented with 10 μM MK-801 at 32°C for 30 minutes. Slices were then transferred to a room temperature carbogenated Krebs solution until used for recording.

For whole-cell patch clamp recordings, borosilicate glass patch pipettes were pulled to a resistance of 2.0-3.0 MΩ and filled with a low-chloride cesium gluconate-based internal solution containing (in mM): 135 cesium gluconate, 1 EGTA, 1.5 $MgCl_2$, 10 Na HEPES, 3 NaCl, 2 Na ATP, 0.4 Na GTP, 7.8 Na_2 phosphocreatine. Slices were placed in the recording chamber and continuously perfused with carbogenated Krebs solution supplemented with a 100 nM scopolamine, 1 μM mecamylamine, 100 nM MPEP, and 200 nM CGP55845. Whole-cell recordings were made in ACC layer V pyramidal neurons, identified based on cell morphology. Both optically evoked excitatory postsynaptic currents (oEPSCs) and inhibitory postsynaptic currents (oIPSCs) were recorded in voltage-clamp mode. Cells were maintained at a holding potential of -60 mV to record oEPSCs and +5 mV to record oIPSCs, the reversal potential for oIPSCs and oEPSCs, respectively. For each condition (baseline, morphine, naloxone), both oEPSCs and oIPSCs were recorded before moving to the next condition. Drug solutions were prepared in recoding Krebs solution and perfused into the recording chamber through a manifold.

Whole-cell recordings were made with a multiclamp 700B amplifier (Molecular Devices, San Jose, CA) digitized at 20 KHz (National Instruments BNC-2090A, Austin, TX). Matlab Wavesurfer (Mathworks, Natick, MA) was used for data acquisition**.** Postsynaptic currents were evoked every 30 seconds by illuminating the recording field through the microscope objective (Olympus BX51W, Tokyo, Japan) using a TTL-controlled LED driver and a 470 nm LED (Thor Labs, Newton, NJ). LED stimulation duration was 1 ms and power output measured after the

microscope objective ranged from 0.5-3 mW, adjusted to obtain consistent baseline current amplitudes across cells. Series resistance was monitored throughout the recordings and only recordings in which the series resistance remained <15 MΩ and did not change more than 18% were included in the analysis.

Data analysis

Raw data were analyzed using Axograph. For each condition (baseline, morphine, naloxone), baseline-subtracted sweeps were averaged together, and peak current amplitude was calculated from the averaged trace. For the baseline condition, the first 2-4 sweeps were omitted from the average to allow the currents to stabilize. For the morphine and naloxone conditions, the first 4-8 sweeps were omitted from the average to allow for equilibration of drug or washout of drug within the tissue. Average morphine and naloxone current amplitudes were normalized to the average baseline current peak amplitude and plotted as % of baseline to analyze sensitivity of MThal terminals to opioid-mediated presynaptic inhibition. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Statistical comparisons were made using a t-test (ratio or unpaired), one-way ANOVA, or 2-way ANOVA with Tukey's (one-way ANOVA) or Šidák's (2-way ANOVA) *post-hoc* analysis. Statistical analysis of E/I ratios was performed on $\log_2$-transformed data. For all comparisons, n (number of cells) and N (number of animals) are both reported.

3.4 Results

Morphine inhibits glutamate release from MThal-ACC terminals in the excitatory and inhibitory pathways

Within the ACC, glutamate release from thalamic afferents evokes direct excitatory synaptic responses onto layer V pyramidal neurons as well as indirect, polysynaptic inhibitory responses mediated through GABAergic interneurons (Delevich et al., 2015). We have previously shown that these projections express MOR, as evoked glutamate release is inhibited by MOR-selective agonists (Arias-Hervert & Birdsong, 2024; Birdsong et al., 2019). We first recapitulated these findings using morphine, a partial agonist at MOR. A partial agonist, rather than a full agonist, was selected for this study as observable decreases in morphine sensitivity following chronic morphine treatment were less likely to be occluded by receptor reserve (where a loss of functional receptors may not translate to a loss of drug effect) or a ceiling effect (where increases in sensitivity cannot be detected in the assay). Whole-cell patch clamp recordings were performed in ACC layer V pyramidal neurons in acute brain slices from mice expressing ChR2 in MThal neurons (Fig. 3.1A, B). Optically-evoked excitatory and inhibitory postsynaptic currents (oEPSCs and oIPSCs, respectively) were recorded to measure effects at direct and indirect MThal-ACC projections. After recording a stable baseline, morphine (3 μM) was bath-perfused onto the slices, followed by the opioid receptor antagonist naloxone (1 μM). Perfusion of morphine significantly inhibited MThal-ACC terminals within both the excitatory and inhibitory pathways, indicated by a reduction in peak amplitude of both oEPSCs and oIPSCs (Fig. 3.1C-E; oIPSC: N = 13, n = 20, $t_{(19)} = 5.023$, $p < 0.0001$, ratio paired t test; oEPSC: N = 13, n = 20, $t_{(19)} = 5.813$, $p < 0.0001$, ratio paired t test). In agreement with previous studies using other MOR agonists, these results demonstrated that morphine inhibits MThal-ACC glutamate release. While not statistically significant, there appeared to be a trend toward greater sensitivity of oIPSCs to morphine inhibition in males compared to females (Fig. 3.1F; males: 40.45 ± 9.31% of baseline; N = 6; n = 9; females: 62.34 ± 7.75% of baseline; N = 8; n = 11; $t_{(18)} = 1.824$,

p = 0.0849; unpaired t test). Inhibition of oEPSCs, however, was similar between males and females, indicating there may be sex differences in morphine-induced alterations in excitatory-inhibitory balance (Fig. 3.1G, males: 80.24 ± 6.40% of baseline; N = 6; n = 9; females: 72.22 ± 4.59% of baseline; N = 8; n = 11; $t_{(18)} = 1.043$, p = 0.3110; unpaired t test).

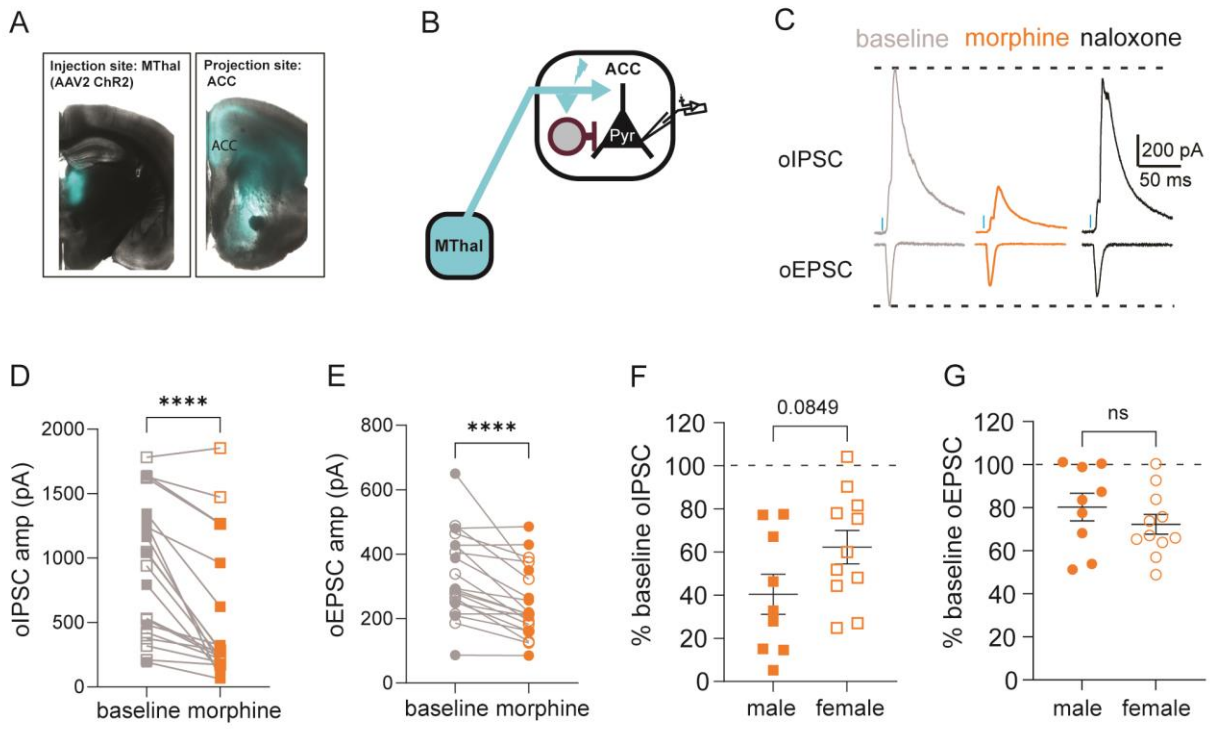

Figure 3.1. Morphine inhibited direct and feedforward inhibitory signaling at MThal-ACC terminals. **A.** Examples of acute brain slices showing viral expression of AAV2-ChR2 at the injection site (MThal) and axonal projections to the ACC. **B.** cartoon illustration depicting selective optogenetic activation of MThal-ACC terminals with patch clamp recordings performed in ACC layer V pyramidal neurons. **C.** Representative traces showing oIPSCs and oEPSCs during baseline conditions (gray), perfusion of morphine (3 μM, orange), and perfusion of naloxone (1 μM, black) in a cell recorded from a male mouse. **D, E.** Summary of baseline oIPSC (D) and oEPSC (E) amplitudes recorded from male (filled data points) and female (open data points) mice (N = 13, n = 20). **F, G.** Inhibition of oIPSCs (F) and oEPSCs (G) by perfusion of morphine recorded from male (filled data points) and female (open data points) mice (males: N = 6, n = 9; females: N = 8, n = 11). oIPSCs were inhibited to 40.45 ± 9.31% of baseline in males and 62.34 ± 7.75% of baseline in females. oEPSCs were inhibited to 80.24 ± 6.40% of baseline in males and 72.22 ± 4.59% of baseline in females. Connecting lines denote recordings from the same cell. Lines and error bars represent mean ± SEM. ****p<0.0001.

Morphine alters the excitatory to inhibitory balance at MThal-ACC terminals

We also examined how morphine acutely altered the balance between excitatory and inhibitory MThal glutamatergic innervation of ACC layer V pyramidal neurons in slices from male and female mice. Consistent with our previous work (Arias-Hervert & Birdsong, 2024), baseline excitatory and inhibitory currents differed significantly; oIPSCs were significantly larger than the corresponding oEPSCs in slices from both male and female mice (Fig. 3.2A, C, E; male oEPSC: 356.7 ± 55.7 pA; male oIPSC: 1018.0 ± 151.4 pA; female oEPSC: 314.9 ± 29.8 pA; female oIPSC: 854.7 ± 176.6 pA; N = 6-8, n = 9-11 for each group; main effect of current type: $F_{(1, 18)} = 25.60$, $p < 0.0001$; main effect of sex: $F_{(1, 18)} = 0.6492$, $p = 0.4309$; sex x current type interaction: $F_{(1, 18)} = 0.2623$, $p = 0.6148$; repeated measures 2-way ANOVA; male oEPSC vs oIPSC: $p = 0.0029$; female oEPSC vs oIPSC: $p = 0.0065$; Šidák's multiple comparisons test). Following bath perfusion of morphine, both oEPSCs and oIPSCs were significantly inhibited in males and females (Fig. 3.2B, D; male oEPSC: $t_{(8)} = 2.873$, $p = 0.0207$, N = 6, n = 9; male oIPSC: $t_{(8)} = 3.951$, $p = 0.0042$, N = 6, n = 9; female oEPSC: $t_{(10)} = 5.467$, $p = 0.0003$, N = 8, n = 11; female oIPSC: $t_{(10)} = 3.965$, $p = 0.0027$, N = 8, n = 11, ratio paired t tests) with oIPSCs in males being inhibited the most (Fig. 3.2F). We observed a main effect of current type, as well as an interaction between sex and current type (male oEPSC in morphine: 80.24 ± 6.40% of baseline; male oIPSC in morphine: 40.45 ± 9.31% of baseline; female oEPSC in morphine: 72.22 ± 4.59% of baseline; female oIPSC in morphine: 62.34 ± 7.75% of baseline; N = 6-8, n = 9-11 for each group; main effect of current type: $F_{(1, 18)} = 14.41$, $p = 0.0013$; main effect of sex: $F_{(1, 18)} = 0.8174$, $p = 0.3779$; sex x current type interaction: $F_{(1, 18)} = 5.227$, $p = 0.0346$). Post-hoc analysis revealed that morphine caused greater inhibition of oIPSCs than oEPSCs in males, but

not in females (male oEPSC vs oIPSC: p = 0.0013, female oEPSC vs oIPSC: p = 0.4744, Šidák's multiple comparisons test).

The excitation to inhibition (E/I) synaptic ratio is an indicator of the balance between excitatory and inhibitory drive, and disruption in this balance, particularly in the cortex, is thought to impair neurological function (Tao et al., 2014). Because morphine inhibited oIPSCs significantly more than oEPSCs in male mice, we hypothesized that morphine would alter the E/I balance. To assess whether the differences in morphine inhibition of oIPSCs and oEPSCs altered E/I balance, we compared the $\log_2$-transformed E/I ratios between baseline and morphine conditions in male and female mice (Fig. 3.2G). We observed a main effect of condition and a sex x condition interaction (N = 6-8, n = 9-11 for each group; main effect of condition: $F_{(1, 18)}$ = 13.99, p = 0.0015; main effect of sex: $F_{(1, 18)}$ = 0.1579, p = 0.6958; sex x condition interaction: $F_{(1, 18)}$ = 5.504, p = 0.0306; repeated measures 2-way ANOVA). Post-hoc analysis revealed that perfusion of morphine increased the E/I ratio in male, but not female, mice (male baseline vs morphine: p = 0.0013; female baseline vs morphine: p = 0.5270; Šidák's multiple comparisons test). Together, these results indicate that perfusion of morphine altered the balance of excitatory to inhibitory drive elicited by MThal-ACC inputs in males, but not females. Therefore, the net effect of morphine at these inputs appears to be a preferential disinhibition leading to increased relative excitatory drive.

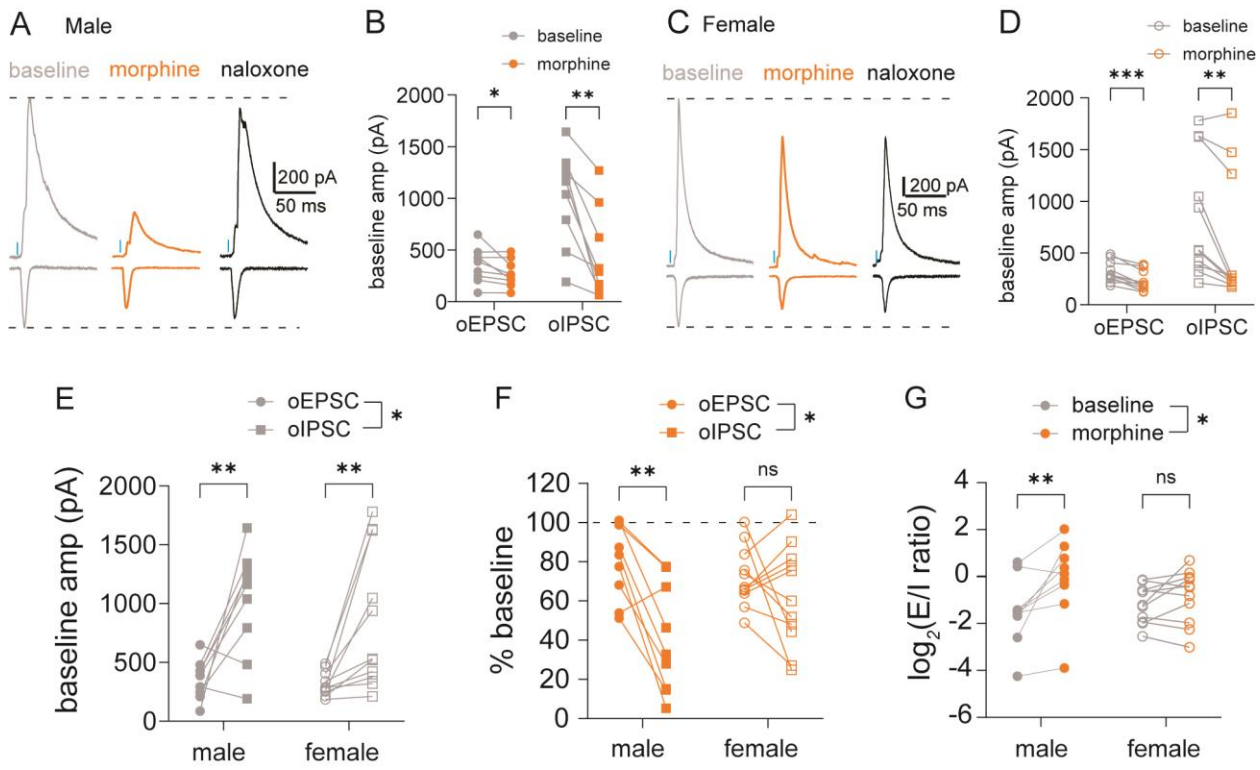

Figure 3.2. Morphine altered the excitatory to inhibitory synaptic balance at MThal-ACC projections. **A.** Representative traces showing oIPSCs and oEPSCs during baseline conditions (gray), perfusion of morphine (3 μM, orange), and perfusion of naloxone (1 μM, black) in a cell recorded from a drug-naïve male mouse. **B.** Summary of oEPSC and oIPSC raw amplitudes during baseline (gray) and perfusion of morphine (orange) in cells recorded from male mice (oEPSCs: $p = 0.0207$; oIPSCs: $p = 0.0042$). **C.** Representative traces showing oIPSCs and oEPSCs during baseline conditions (gray), perfusion of morphine (orange), and perfusion of naloxone (black) in a slice from a drug-naïve female mouse. Baseline amplitudes of oEPSCs and oIPSCs recorded from male (filled data points) and female (open data points) mice (males: N = 6, n = 9; females: N = 8, n = 11). **D.** Summary of oEPSC and oIPSC raw amplitudes during baseline (gray) and perfusion of morphine (orange) in cells recorded from female mice (oEPSCs: $p = 0.0003$; oIPSCs: $p = 0.0027$). **E.** Baseline amplitudes of oEPSCs and oIPSCs recorded from male (filled data points) and female (open data points) mice (males: N = 6, n = 9; females: N = 8, n = 11). **F.** Summary graph showing inhibition of oEPSCs and oIPSCs by perfusion of morphine in males (filled data points) and females (open data points). In males, oIPSCs were inhibited to 40.45 ± 9.31% of baseline and oEPSCs were inhibited to 80.24 ± 6.40% of baseline (N = 6, n = 9). In females, oIPSCs were inhibited to 62.34 ± 7.75% of baseline and oEPSCs were inhibited to 72.22 ± 4.59% of baseline (N = 8, n = 11). **G.** Summary of $\log_2$-transformed E/I ratios during baseline conditions and perfusion of morphine in male (filled data points) and female (open data points) mice (males: N = 6, n = 9; females: N = 8, n = 11). Connecting lines denote recordings from the same cell. *p<0.05, **p<0.01, ***p<0.001.

Chronic morphine treatment induced tolerance to morphine at excitatory and inhibitory pathway MThal-ACC terminals in a sex-dependent manner

We next investigated whether chronic morphine treatment in mice altered the sensitivity of MThal-ACC oIPSCs and oEPSCs to subsequent inhibition by perfusion of morphine. Mice were continuously exposed to morphine via implantation of an osmotic minipump releasing 80 mg/kg/day morphine for 7 days prior to recording (Fig. 3.3I) and patch clamp recordings were performed identically to those in slices from drug-naïve mice. Morphine inhibited oIPSCs significantly less in slices from morphine treated male mice than in slices from drug-naïve male mice, and this effect was not seen in females (Fig. 3.3A-E; male naïve: 40.45 ± 9.31% of baseline; male chronic morphine: 78.34 ± 8.48% of baseline; female naïve: 62.34 ± 7.75% of baseline; female chronic morphine: 71.78 ± 9.05% of baseline; N = 6-8, n = 9-11 for each group; main effect of treatment: $F_{(1, 35)} = 7.472$, $p = 0.0098$; main effect of sex: $F_{(1, 35)} = 0.7833$, $p = 0.3822$; treatment x sex interaction: $F_{(1, 35)} = 2.701$, $p = 0.1092$, ordinary 2-way ANOVA; male naïve vs chronic morphine: $p = 0.0103$; female naïve vs chronic morphine: $p = 0.6729$; Šidák's multiple comparisons test). These findings suggest robust tolerance of inhibitory pathway MThal-ACC terminals to morphine in males, but not females.

Chronic morphine treatment also decreased the amount of inhibition of oEPSCs following morphine perfusion in slices from morphine treated animals as a whole when compared to slices from naïve mice. In total, there was a main effect of morphine treatment without a main effect of sex or an interaction (Fig. 3.3A,B,D-F; male naïve: 80.24 ± 6.40% of baseline; male chronic morphine: 94.59 ± 5.99% of baseline; female naïve: 72.22 ± 4.59% of baseline; female chronic morphine: 83.51 ± 5.12% of baseline; N = 6-8, n = 9-11 for each group; main effect of treatment: $F_{(1, 35)} = 5.452$, $p = 0.0254$; main effect of sex: $F_{(1, 35)} = 3.025$, $p = 0.0908$; treatment x sex interaction: $F_{(1, 35)} = 0.7823$, $p = 0.7823$). However, unlike with oIPSCs, there was not a significant difference in morphine inhibition of oEPSCs between naïve and

morphine treatment groups in either males or females using post-hoc analysis (male naïve vs chronic morphine: $p = 0.1600$; female naïve vs chronic morphine: $p = 0.2593$; Šidák's multiple comparisons test). The lack of statistical significance in either sex individually is likely due to the relatively small effect size and high cell to cell variability of morphine inhibition of oEPSCs in naïve animals such that a reduction in morphine effect was difficult to observe without a very large sample size. Together, these results suggest that chronic morphine exposure induced tolerance to morphine inhibition of synaptic transmission within MThal-ACC circuitry in a sex-specific manner; dramatic tolerance was observed within polysynaptic inhibitory circuitry (oIPSCs) in males but not females while a small, but significant, tolerance was observed within MThal-ACC excitatory circuitry (oEPSCs) potentially in both males and females.

The dramatic tolerance to morphine inhibition of oIPSCs in male mice would suggest that morphine may lose its ability to alter E/I balance in morphine treated male mice. Whereas perfusion of morphine increased the E/I ratio in slices from drug-naïve male mice, in morphine treated mice, perfusion of morphine did not alter the E/I ratio in slices from males or females to a statistically significant extent (Fig. 3.3G; N = 6-8, n = 9-10 for each group; main effect of condition: $F_{(1, 17)} = 0.319$, $p = 0.0531$; main effect of sex: $F_{(1, 17)} = 0.07398$, $p = 0.7889$; sex x condition interaction: $F_{(1, 17)} = 0.002425$, $p = 0.3973$; repeated measures 2-way ANOVA). We also compared the E/I ratio during the morphine-free baseline condition between drug-naïve and chronically morphine treated male and female mice to determine whether chronic morphine treatment altered basal excitatory to inhibitory balance at MThal-ACC synapses. Neither a main effect of sex or morphine treatment, nor a sex x treatment interaction was observed (Fig. 3.3H; N = 6-8, n = 9-11 for each group; main effect of treatment: $F_{(1, 35)} = 1.179$, $p = 0.2850$; main effect of sex: $F_{(1, 35)} = 0.03806$, $p = 0.8464$; treatment x sex interaction: $F_{(1, 35)} = 0.5348$, $p = 0.5348$;

ordinary 2-way ANOVA). These findings indicate that although chronic morphine treatment induced tolerance to the effects of subsequent morphine on MThal-ACC E/I balance, basal E/I balance in the absence of drug was not affected.

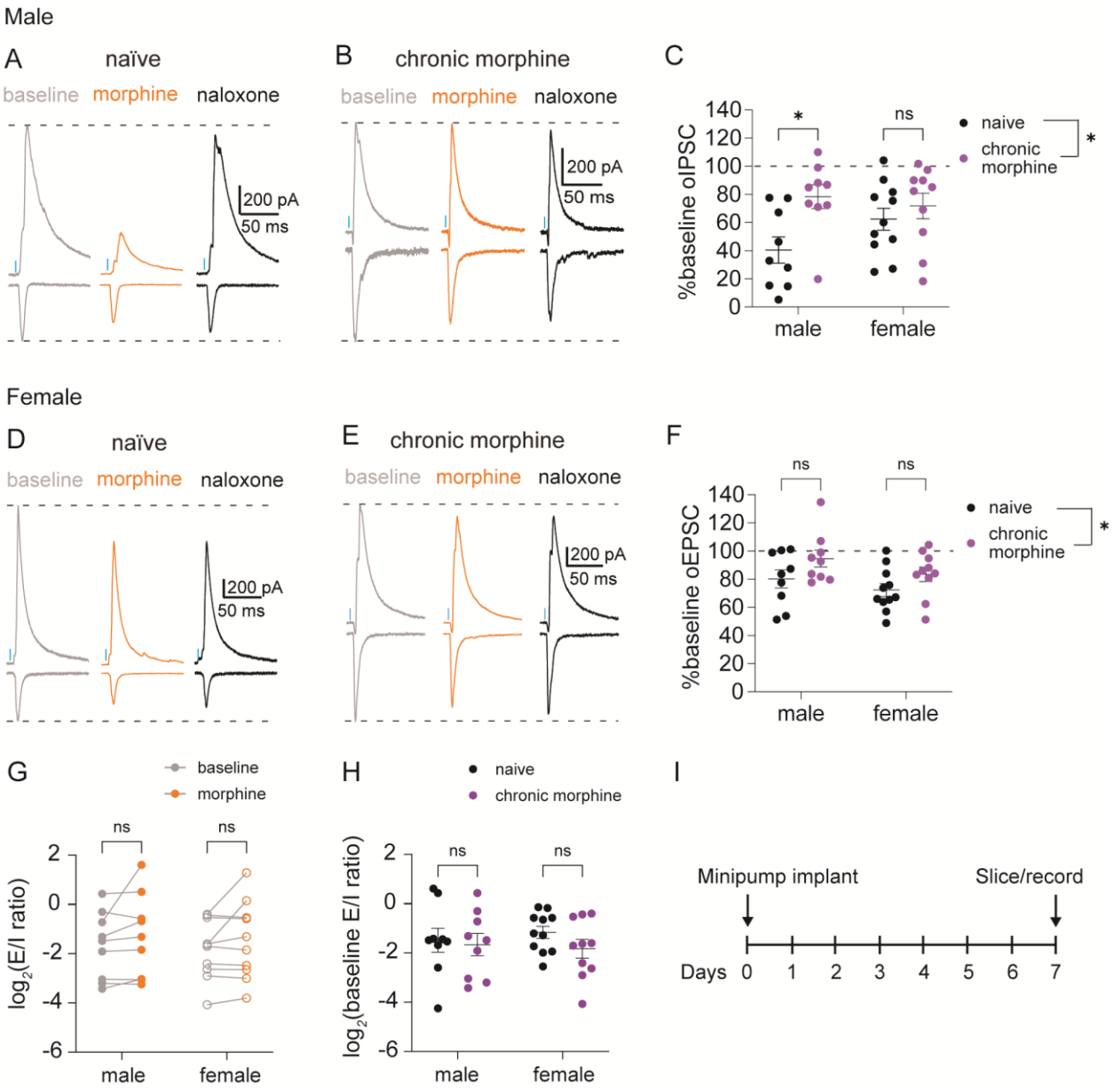

Figure 3.3. Chronic morphine treatment decreased the sensitivity of MThal-ACC terminals to subsequent morphine in a sex-specific manner. **A, B.** Representative traces showing oEPSCs and oIPSCs during baseline (gray), perfusion of morphine (3 μM, orange), and perfusion of naloxone (1 μM, black) in drug-naïve (A) and chronically treated (B) male mice. **C.** Summary of normalized oIPSC inhibition by perfusion of morphine in drug-naïve (black) and chronically treated (purple) male and female mice. In

males, morphine perfusion decreased the oIPSC amplitude to 40.45 ± 9.31% of baseline in drug-naïve mice and 78.34 ± 8.48% of baseline in chronically treated mice (drug-naïve: N = 6, n = 9; chronic morphine: N = 7, n = 9). In females, morphine perfusion decreased the oIPSC amplitude to 62.34 ± 7.75% of baseline in drug naïve mice and 71.78 ± 9.05% of baseline in chronically treated mice (drug-naïve: N = 8, n = 11; chronic morphine: N = 7, n = 10). **D, E.** Representative traces showing oEPSCs and oIPSCs in L5 pyramidal neurons during baseline (gray), perfusion of morphine (3 μM, orange), and perfusion of naloxone (1 μM, black) in drug-naïve (A) and chronically treated (B) female mice. **F.** Summary of normalized oEPSC inhibition by perfusion of morphine in drug-naïve (black) and chronically treated (purple) male and female mice. In males, morphine perfusion decreased the oEPSC amplitude to 80.24 ± 6.40% of baseline in drug-naïve mice and 94.59 ± 5.99% of baseline in chronically treated mice (drug-naïve: N = 6, n = 9; chronic morphine: N = 7, n = 9). In females, morphine perfusion decreased the oEPSC amplitude to 72.22 ± 4.59% of baseline in drug naïve mice and 83.51 ± 5.12% of baseline in chronically treated mice (drug-naïve: N = 8, n = 11; chronic morphine: N = 7, n = 10). **G.** Summary of log_2-transformed E/I ratios during baseline conditions and perfusion of morphine in male (filled data points) and female (open data points) chronically treated mice (males: N = 7, n = 9; females: N = 7, n = 10). Connecting lines denote baseline and morphine recordings from the same cell. **H.** Summary of log_2-transformed E/I ratios during the baseline condition in drug-naïve (black) and chronically treated (purple) male and female mice (male naïve: N = 6, n = 9; male chronic morphine: N = 7, n = 9; female naïve: N = 8, n = 11; female chronic morphine: N = 7, n = 10). **I.** Schematic showing chronic morphine treatment paradigm. Morphine (80 mg/kg/day) was continuously administered via osmotic minipump for 7 days prior to recordings. Lines and error bars represent mean ± SEM. *p<0.05.

Tolerance to morphine is eliminated in MOR C-terminal phosphorylation-deficient mice

Receptor phosphorylation is a critical regulator of MOR signaling and is highly implicated in tolerance at both the cellular and behavioral levels (Williams et al., 2013). Thus, we next determined whether MOR C-terminal phosphorylation plays a role in driving the tolerance at MThal-ACC terminals observed in this study. To investigate this, we measured morphine inhibition of MThal-ACC terminals in a knockin mouse line in which mice express MORs with serine or threonine to alanine mutations at 10 of 11 phosphorylation residues on the MOR C-terminal tail (MOR 10 S/T-A; Fig. 3.4C). In these experiments, only male MOR 10 S/T-A mice were used because the most robust tolerance effects in wild-type mice were observed in males. Whereas chronic morphine treatment induced tolerance to MThal-ACC oIPCS in wild-type mice, this effect was not observed in 10 S/T-A mice (Fig. 3.4A, B, E; WT naïve: 40.45 ± 9.31% of baseline; WT chronic morphine: 78.34 ± 8.48% of baseline; 10 S/T-A naïve: 59.20 ±

9.85% of baseline; 10 S/T-A chronic morphine: 61.62 ± 8.23% of baseline; N = 5-7, n = 9-10 for each group; main effect of strain: $F_{(1, 34)} = 0.01263$, p = 0.9112; main effect of treatment: $F_{(1, 34)} = 3.861$, p = 0.0322; strain x treatment interaction: $F_{(1, 34)} = 3.861$, p = 0.0576; ordinary 2-way ANOVA; WT naïve vs chronic morphine: p = 0.0132; 10 S/T-A naïve vs chronic morphine: p = 0.9765; Šidák's multiple comparisons test). Comparing the effects of chronic morphine treatment on MThal-ACC oEPSCs between WT and 10 S/T-A male mice, no main effect of strain or morphine treatment, nor an interaction was observed (Fig. 3.4A, B, F; WT naïve: 80.24 ± 6.40% of baseline; WT chronic morphine: 94.59 ± 5.99% of baseline; 10 S/T-A naïve: 94.24 ± 5.69% of baseline; 10 S/T-A chronic morphine: 97.85 ± 4.95% of baseline; N = 5-7, n = 9-10 for each group; main effect of strain: $F_{(1, 34)} = 2.249$, p = 0.1429; main effect of treatment: $F_{(1, 34)} = 2.437$, p = 0.1278; strain x treatment interaction: $F_{(1, 34)} = 0.8710$, p = 0.3573; ordinary 2-way ANOVA). However, comparing oEPSC amplitude between baseline conditions and during perfusion of morphine revealed no significant inhibition of oEPSCs by morphine in 10 S/T-A mice (Fig. 3.4D; 0.94 ± 0.06 times baseline, $t_{(9)} = 1.130$, p = 0.2875, N = 5, n = 10; ratio paired t-test) unlike the significant inhibition seen in WT males (Fig. 3.2B; p = 0.0207). Thus, the lack of a change in sensitivity of oEPSCs to morphine inhibition following chronic morphine treatment in 10 S/T-A mice is difficult to interpret. In drug-naïve 10 S/T-A mice, oIPSCs remained significantly inhibited by morphine perfusion (Fig. 3.4D; 0.59 ± 0.10 times baseline, $t_{(9)} = 2.887$, p = 0.0180, N = 5, n = 10; ratio paired t-test).

We next examined whether E/I balance at MThal-ACC projections is altered by chronic morphine treatment in 10 S/T-A male mice, as was observed with WT males. Comparing the effect of drug condition and morphine treatment on the $\log_2$-transformed E/I ratio, we observed a main effect of condition without a main effect of treatment or an interaction (Fig. 3.4G; N = 5-6,

n = 10 for each group; main effect of condition: $F_{(1, 18)} = 13.87$, p = 0.0016; main effect of treatment: $F_{(1, 18)} = 0.7626$, p = 0.3940; strain x treatment interaction: $F_{(1, 18)} = 0.0845$, p = 0.7746; repeated measures 2-way ANOVA). Pos-hoc analysis shows that perfusion of morphine increases the E/I ratio in drug-naïve 10 S/T-A males, but not in chronically treated 10 S/T-A males (naïve baseline vs morphine: p = 0.0217; chronic morphine baseline vs morphine: p = 0.0512; Šidák's multiple comparisons test). However, the effects of morphine perfusion on the E/I ratio in chronically treated mice are approaching statistical significance and these results are difficult to interpret given the large variability in the inhibition of oIPSCs and the lack of significant inhibition in the oEPSCs in 10 S/T-A mice. Like in WT male mice, chronic morphine treatment did not significantly alter the E/I ratio during the baseline condition (Fig. 3.4H; N = 5-7, n = 9-10 for each group; main effect of treatment: $F_{(1, 34)} = 0.1732$, p = 0.6899; main effect of strain: $F_{(1, 34)} = 2.912$, p = 0.0971; treatment x strain interaction: $F_{(1, 34)} = 0.7255$, p = 0.4003; ordinary 2-way ANOVA). Together, these results suggest MOR-phosphorylation-deficient mice do not develop tolerance to subsequent morphine signaling at MThal-ACC terminals. However, they also appear to have less sensitive and more variable responses to morphine perfusion.

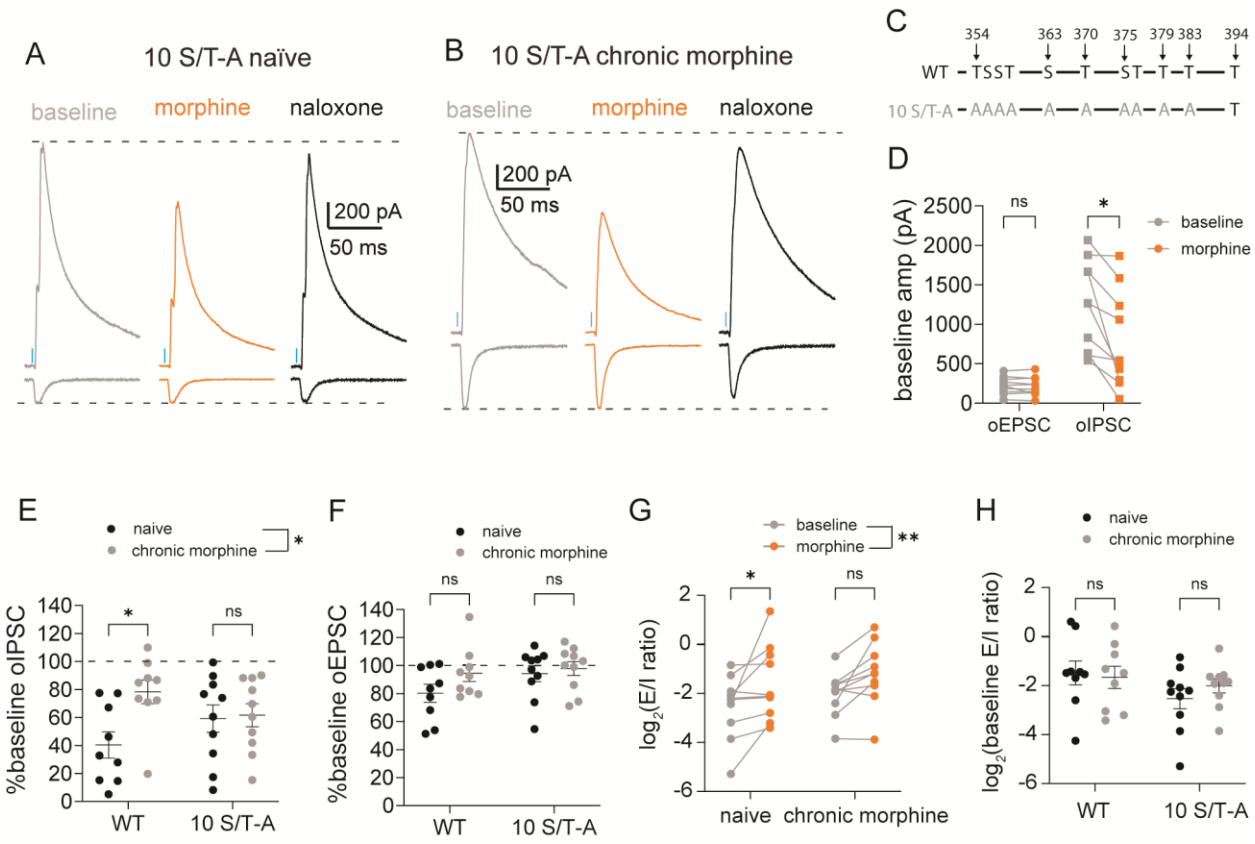

Figure 3.4. Morphine tolerance at MThal-ACC terminals was eliminated in MOR phosphorylation-deficient mice. **A, B.** Representative traces showing oEPSCs and oIPSCs during baseline (gray), perfusion of morphine (3 μM, orange), and perfusion of naloxone (1 μM, black) in drug-naïve (A) and chronically treated (B) male 10 S/T-A mice. **C.** Schematic representation of serine (S) and threonine (T) to alanine (A) phosphorylation site mutations on the MOR C-terminus in MOR 10 S/T-A mice. **D.** Summary of oEPSC and oIPSC raw amplitudes during baseline (gray) and perfusion of morphine (orange) in cells recorded from male 10 S/T-A mice (oEPSCs: $p = 0.2875$; oIPSCs: $p = 0.0180$). **E.** Summary of normalized oIPSC inhibition by perfusion of morphine in drug-naïve (black) and chronically treated (gray) male WT and 10 S/T-A mice. In WT males, morphine perfusion decreased the oIPSC amplitude to 40.45 ± 9.31% of baseline in drug-naïve mice and 78.34 ± 8.48% of baseline in chronically treated mice (drug-naïve: N = 6, n = 9; chronic morphine: N = 7, n = 9). In 10 S/T-A males, morphine perfusion decreased the oIPSC amplitude to 59.20 ± 9.85% of baseline in drug naïve mice and 61.62 ± 8.23% of baseline in chronically treated mice (drug-naïve: N = 6, n = 10; chronic morphine: N = 5, n = 10). **F.** Summary of normalized oEPSC inhibition by perfusion of morphine in drug-naïve (black) and chronically treated (gray) male WT and 10 S/T-A mice. In WT males, morphine perfusion decreased the oEPSC amplitude to 80.24 ± 6.40% of baseline in drug-naïve mice and 94.59 ± 5.99% of baseline in chronically treated mice (drug-naïve: N = 6, n = 9; chronic morphine: N = 7, n = 9). In 10 S/T-A males, morphine perfusion decreased the oEPSC amplitude to 94.24 ± 5.69% of baseline in drug naïve mice and 97.85 ± 4.95% of baseline in chronically treated mice (drug-naïve: N = 6, n = 10; chronic morphine: N = 5, n = 10). **G.** Summary of $\log_2$-transformed E/I ratios during baseline conditions and perfusion of morphine in drug-naïve and chronically treated 10 S/T-A mice (males: N = 7, n = 9; females: N = 7, n = 10). Connecting lines denote baseline and morphine recordings from the same cell. **H.** Summary of $\log_2$-transformed E/I ratios during the baseline condition in drug-naïve (black) and chronically treated (gray) WT and 10 S/T-A male mice (WT naïve: N = 6, n = 9; WT chronic morphine: N = 7, n = 9; 10 S/T-A

naïve: N = 6, n = 10; female chronic morphine: N = 5, n = 10). Lines and error bars represent mean ± SEM. *p<0.05, **p<0.01.

3.5 Discussion

The purpose of this study was to determine how chronic morphine treatment altered MThal innervation of the ACC, a region heavily involved in affective pain perception. We demonstrated that chronic morphine treatment resulted in tolerance to morphine-mediated inhibition of MThal-ACC glutamate release in a sex- and pathway-specific manner. In drug-naïve mice, morphine inhibited both excitatory (oEPSCs) and inhibitory (oIPSCs) MThal inputs to the ACC, but more strongly inhibited oIPSCs in males. Following chronic morphine treatment, we observed robust tolerance to morphine inhibition of oIPSCs in males but not females, and small but significant tolerance to inhibition of oEPSCs in both sexes. These chronic morphine effects were not observed in phosphorylation-deficient MOR 10 S/T-A mice, suggesting a role of receptor phosphorylation in mediating cellular tolerance within this circuit.

Morphine effects on excitation to inhibition balance in drug-naïve mice

In drug-naïve mice, we observed a clear sex difference in how morphine perfusion shifted the excitatory to inhibitory MThal-ACC synaptic balance; in males, morphine inhibited oIPSCs significantly more than oEPSCs, but in females oIPSC and oEPSCs were similarly inhibited. As a result, morphine perfusion had a net increase in excitatory drive from MThal onto ACC layer V pyramidal neurons (through preferential disinhibition) in males but did not alter E/I balance in females. MThal inputs to the ACC have been demonstrated to selectively synapse onto pyramidal cells and inhibitory parvalbumin (PV) interneurons while direct excitation of other interneurons was not observed, suggesting that feedforward inhibition is mediated predominantly

through PV interneurons (Delevich et al., 2015). We have previously shown that ACC PV interneurons do not express functionally meaningful levels of MOR, suggesting that morphine inhibition of the feedforward oIPSC is not mediated by direct inhibition of PV neurons and is, therefore, likely due to presynaptic inhibition of glutamate release MThal afferents.

If the inhibition of the oIPSC by morphine is due to inhibition of glutamate release from MThal inputs onto interneurons, the preferential inhibition of oIPSCs relative to oEPSCs by morphine may suggest that MOR is preferentially localized to MThal synapses onto inhibitory interneurons rather than onto principal pyramidal cells in the ACC. Alternatively, this may suggest that MThal inputs are exciting a population of MOR-sensitive interneurons that are distinct from the MOR-lacking PV interneurons we have previously described.

Morphine tolerance within the ACC

Despite the clear role of the ACC in affective pain perception, little is known about how chronic opioid exposure alters MOR signaling in this region to produce opioid analgesic tolerance. Here, we demonstrated that morphine-mediated inhibition of glutamate release at MThal-ACC terminals was attenuated following chronic morphine treatment, and this tolerance may be driven by phosphorylation of presynaptic MORs. Furthermore, while morphine perfusion caused a shift in E/I balance toward excitation in drug-naïve male mice, this E/I shift was abolished in morphine treated mice, representing an additional form of tolerance in this region. Given that these inputs are thought to transmit pain signals, adaptations here are potentially highly relevant to opioid analgesic tolerance. However, from our data we cannot say with certainty that the tolerance we observed, particularly in the feedforward inhibitory pathway is a presynaptic, rather than a postsynaptic, effect.

Cessation of opioid exposure in dependent subjects produces aversive withdrawal symptoms and pain hypersensitivity. Neuronal excitability within the ACC is increased during opioid withdrawal (Chu et al., 2015; Erdtmann-Vourliotis et al., 1998; Lowe et al., 2002) and appears to be driven by an increase in the intrinsic excitability of ACC layer V pyramidal neurons, without changes in spontaneous synaptic excitatory or inhibitory currents onto these neurons (McDevitt et al., 2021). This is in line with the lack of a difference in the baseline E/I ratio between drug-naïve and morphine treated mice in our study considering both E/I ratio and spontaneous excitatory to inhibitory synaptic transmission are indicators of synaptic balance. Electrophysiological measures of tolerance to morphine in brain slices have previously been demonstrated to persist beyond 6 hours (Levitt & Williams, 2012). In our study, slices were incubated in the absence of morphine prior to recording. Thus, in our system slices remained in a morphine tolerant but acutely withdrawn state during recordings, and while we are investigating chronic morphine-induced adaptations this factor must be taken into consideration.

It is not clear why, in our study, chronic morphine treatment induced tolerance at MThal-ACC oEPSCs in both sexes but tolerance at oIPSC only in males. Acutely, opioids are typically more potent in males than females (Averitt et al., 2019), and males develop greater and more rapid opioid analgesic tolerance (Bodnar & Kest, 2010). Given the relevance of MThal inputs to the ACC in pain signaling, the sex-dependent effect observed here may contribute, at least in part, to differences between males and females in opioid analgesia and tolerance. Alternatively, while not statistically significant, we observed a trend toward decreased morphine sensitivity of oIPSCs in females compared to males. However, in chronically treated mice inhibition of oIPSCs was similar between males and females. Thus, it is possible this lack of an effect in females is indicative of basal differences in morphine sensitivity rather than effects of chronic

morphine exposure. Repeating these studies using multiple doses/concentrations of morphine (both for chronic morphine treatment and as a challenge in slice after chronic morphine treatment) would help better understand the nature of the sex differences observed here.

Chronic morphine-induced adaptations within greater thalamo-cortico-striatal circuitry

In addition to the ACC, MThal neurons also project to the DMS. We previously found that at MThal-DMS projections, chronic morphine treatment induced facilitation of morphine signaling (i.e. an enhancement of morphine-mediated inhibition of oEPSCs) only in males, but cellular tolerance at MThal cell bodies in both sexes (Jaeckel et al., 2024). This demonstrated opposing effects of chronic opioid exposure depending on subcellular compartment (somatic versus presynaptic). These findings are relevant to the present study because single MThal neurons send opioid-sensitive axon collaterals to the DMS and ACC, thus presynaptic tolerance (At ACC terminals) and facilitation (at DMS terminals) are observed (Birdsong et al., 2019; Kuramoto et al., 2017). Together, these results suggest that even within different *presynaptic* compartments of a neuronal population, opposing effects of chronic morphine exposure can be observed. It is important to note, however, that the tolerance and/or facilitation observed in these studies may be driven by adaptations outside the presynaptic compartment, such as postsynaptic adaptations or changes within local circuitry.

Role of MOR phosphorylation in mediating tolerance to MThal-ACC MOR signaling

Following agonist activation, phosphorylation of the MOR C-terminus promotes β-arrestin recruitment, functionally uncouples receptors from their associated G proteins, and initiates receptor internalization (Williams et al., 2013). Cellular tolerance is typically attributed

to a decrease in functional MORs driven by receptors phosphorylation, and this has been demonstrated at presynaptic MORs (Jullié et al., 2022; Jullié et al., 2020). The lack of any tolerance in our study in 10 S/T-A mice suggests this process is responsible for the tolerance observed in wild-type mice. Previous studies have demonstrated that chronic morphine-induced cellular adaptations are eliminated in 10 S/T-A mice (Arttamangkul et al., 2018; Jaeckel et al., 2024), and the results of this study are in line with previous findings. One limitation of this study, however, is that the large variability in these data and small magnitude of morphine inhibition in drug-naïve 10 S/T-A mice would make the effect of chronic morphine difficult to observe. While oIPSCs were significantly inhibited by perfusion of morphine, oEPSCs were not, thus any tolerance would not be measurable. It is not clear why morphine sensitivity appeared to be decreased in 10 S/T-A mice relative to wild-type mice. Based on their lack of constitutive internalization and the increased sensitivity of these mice to opioid analgesia (Kliewer et al., 2019), we expected sensitivity of MThal-ACC terminals to morphine inhibition to be enhanced, rather than diminished. One possible explanation is that the trafficking mechanisms are different between 10 S/T-A and WT receptors such that fewer receptors are trafficked to these terminals. In our previous study we found drug-naïve 10 S/T-A mice were more sensitive to morphine inhibition at MThal-DMS terminals. Taken together with the results of the present study, these findings are in line with a mechanism by which trafficking deficiencies of 10 S/T-A receptors result in reduced trafficking to distal (i.e. MThal-ACC) axon terminals while accumulating at proximal (i.e. MThal-DMS) terminals.

Overall, this study improves our understanding of how chronic morphine exposure alters MOR signaling at presynaptic terminals within MThal-ACC projections, a site that may be relevant to pain signaling and opioid analgesia. It also suggests that the cellular mechanisms that

give rise to tolerance are complex and depend on many factors, such as sex, brain region, and receptor subcellular location.

Chapter 4

Differential Effects of Chronic Morphine Treatment on Mu-Opioid Receptor-Expressing and Mu-Opioid Receptor-Lacking Thalamo-Striatal Terminals

4.1 Abstract

Repeated morphine exposure drives tolerance, or diminished responses, to the analgesic effects of opioids but sensitization, or amplified responses, to some other effects such as reward and locomotor stimulation. These phenomena have been well characterized behaviorally, but the underlying mechanisms are not fully elucidated. Glutamatergic projections from the medial thalamus (MThal) to the dorsomedial striatum (DMS) are part of larger circuitry involved in pain and reward processing and express the mu-opioid receptor (MOR). We have previously shown that chronic morphine treatment induces cellular tolerance at MThal cell bodies (i.e. a reduction in opioid effects) but cellular facilitation at MThal-DMS terminals (i.e. an enhancement in opioid effects), suggesting that activity within these projections may be relevant to opioid analgesic tolerance and/or sensitization. The goal of this study was to further characterize chronic morphine effects within this circuitry by investigating whether MThal-DMS terminals consisted of both MOR-expressing and MOR-lacking subpopulations, and how chronic morphine treatment altered synaptic transmission within these subpopulations individually. We found that both MOR-expressing and MOR-lacking MThal neurons functionally innervated the DMS, and that chronic morphine treatment induced cellular tolerance at MOR-expressing terminals without altering MOR signaling at MOR-lacking terminals. We have previously shown that when glutamate release from MThal-DMS terminals is non-selectively evoked (regardless of MOR

expression), chronic morphine induced facilitation of, rather than tolerance to, subsequent morphine signaling. Taken together, these results suggest the existence of multiple, opposing adaptations that drive both tolerance and facilitation at different loci within this glutamatergic MThal-DMS circuitry.

4.2 Introduction

Repeated exposure to opioids like morphine produces tolerance to their analgesic properties, where pain-relieving effects are decreased over time. In contrast, opioids (and other drugs of abuse) also produce behavioral sensitization, where the locomotor-stimulating effects are enhanced over time (Stewart & Badiani, 1993). Opioid analgesic tolerance is generally thought to arise from phosphorylation of the mu-opioid receptor (MOR), the receptor through which opioids primarily mediate behavioral effects, resulting in receptor downregulation and functional uncoupling (Matthes et al., 1996; Williams et al., 2013). Behavioral sensitization is thought to reflect an enhancement in motivation to take drug by engaging the same circuitry, and involves adaptations in glutamatergic and dopaminergic systems involved in learning and motivational processes (Robinson & Berridge, 2000; Wolf, 2002). However, the precise mechanisms by which cellular adaptations induced by chronic opioid exposure give rise to tolerance and sensitization are complex and not fully elucidated. Moreover, given that pain relief is inherently rewarding, these opposing processes may not be completely separable.

The dorsomedial striatum (DMS) highly expresses MOR (Koshimizu et al., 2008; Pert et al., 1976), is activated during acute pain states (Scott et al., 2006), and mediates reward-based learning and goal-directed behavior, processes relevant to addiction (Balleine et al., 2007). It also receives strong glutamatergic inputs from the medial thalamus (MThal) and anterior cingulate

cortex (ACC), two interconnected regions involved in mediating the aversive component of pain (Fields, 2004; Price, 2000; Rainville et al., 1997). Thus, the DMS may represent a site of overlap in the processes mediating opioid analgesic tolerance and reward. Synaptic plasticity refers to activity-dependent strengthening or weakening of synapses (Citri & Malenka, 2008). Glutamatergic synaptic plasticity influences dopamine signaling in the DMS and within the striatum more broadly to mediate some forms of learning (Calabresi et al., 1997; Lovinger, 2010). A growing body of evidence suggests that opioids can induce or alter glutamate plasticity in the striatum, however the direction of plasticity is not consistent. For example, chronic morphine treatment in the nucleus accumbens has been shown to both increase postsynaptic GluR1-containing AMPA receptors (Russell et al., 2016) and generate silent synapses through the removal of AMPA receptors at pre-existing synapses (Graziane et al., 2016). One potential explanation for these discrepancies is that opioid modulation of striatal inputs is synapse specific. Glutamatergic striatal innervation most strongly arises from thalamic (~30% of total inputs) and cortical (~60% of inputs) regions (Guo et al., 2015; Huerta-Ocampo et al., 2014). Inputs from the MThal to the DMS are generally opioid-sensitive, while MThal-ACC inputs are not (Birdsong et al., 2019). Furthermore, it is not clear whether all MThal inputs are opioid sensitive given that glutamate release from these terminals is not completely inhibited by MOR full agonists (Birdsong et al., 2019; Jaeckel et al., 2024). Therefore, chronic opioid-induced synaptic adaptations may differ depending on the opioid sensitivity of the synapse.

We have previously shown that in male mice, chronic morphine treatment resulted in facilitation of subsequent morphine signaling at MThal-DMS presynaptic terminals, where morphine-mediated presynaptic inhibition of glutamate release was enhanced. (Jaeckel et al., 2024). However, this was a measure of postsynaptic responses summated from multiple MThal-

DMS synapses, and from these findings we could not determine whether the facilitation was driven by increased morphine efficacy at presynaptic MORs or some form of synaptic plasticity occurring elsewhere within this circuit. Morphine also did not elicit responses in all individual DMS-projecting MThal neurons within the somatic compartment, suggesting not all neurons in this population express MOR. Thus, differential synaptic adaptations may occur within MOR-expressing and MOR-lacking MThal-DMS terminals. The goal of the present study was to first determine whether MThal inputs to the DMS consisted of both MOR-expressing and MOR-lacking subpopulations. We next investigated whether innervation by both populations was functional and whether the previously observed facilitation persisted when MOR-expressing terminals were selectively targeted. Utilizing *Oprm1*-cre mice to selectively target the MOR-expressing neuronal subpopulation, we showed that chronic morphine treatment induced tolerance to subsequent morphine at these terminals. Together with our previous findings, these findings suggest the presence of both receptor-level adaptations driving tolerance as well as circuit-level counteradaptations that enhance morphine signaling at these terminals.

4.3 Materials and Methods

Drugs

Reagent	Source	Identifiers
Naloxone	Hello Bio	HB2451
Picrotoxin	Hello Bio	HB0506
DAMGO	Sigma-Aldrich	E7384
Dizocilpine (MK-801)	Hello Bio	HB0004

Morphine sulfate	Spectrum Chemical	M1167
rAAV2-hsyn-hChR2(H134R)-EYFP-WPRE-PA	UNC virus vector core	N/A
AAV5-EF1α-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA	Addgene	20298-AAV5
pAAV-Ef1a-DO-hChR2(H134R)-mCherry-WPRE-pA Packaged into AAV2	Addgene/ University of Michigan Vector Core	37082
AAV8-nEF-Coff/Fon-mCherry	Addgene	137144
AAV8-syn-Con/Fon-ChR2-EYFP	Addgene	55650

Animals

All animal procedures were conducted in accordance with the National Institutes of Health guidelines and with approval from the Institutional Animal Care and Use Committee at the University of Michigan. Male mice were used for all experiments. Mice were maintained on a 12-hour light/dark cycle and given free access to food and water. C57Bl/6J mice were obtained from Jackson Laboratories, and *Oprm1*-cre mice were gifted by Dr. Brigitte Kieffer (Bailly et al., 2020). Mice were aged 4 to 8 weeks at the time of viral injection and 6 to 10 weeks at the time of recording.

Chronic morphine treatment

Mice treated with chronic morphine (80 mg/kg/day) or saline were implanted with an osmotic minipump (Alzet model 2001, Cupertino, CA) continuously releasing solution for 7 days prior to experimentation. Morphine concentrations were calculated based on the mean pump rate and mouse mass at the time of surgery to achieve the correct dose. Mice were anesthetized with isoflurane (4% induction, 2% maintenance), and an incision was made along the lower back. Pumps were inserted subcutaneously and the incision was closed with surgical glue and wound clips. Pumps remained implanted until mice were euthanized for brain slice preparation. Brain slices were incubated in the absence of morphine for a minimum of one hour prior to experimentation to ensure no residual drug was present in the slices during the baseline recordings.

Stereotaxic viral injections

Mice were bilaterally injected with adeno-associated viruses encoding channelrhodopsin-2 targeting the MThal using stereotaxic methods. Mice were anesthetized with isoflurane (4% induction, 2% maintenance) and placed on a stereotaxic frame (Kopf Instruments, Tujunga, CA). An incision was made along the midline of the scalp and a craniotomy was performed on the skull above the MThal (A/P: -1.2 mm, M/L: ±0.6 mm, D/V: 3.6 mm). A glass pipette filled with virus was inserted into the brain and lowered to the appropriate depth. 60-90 nL of virus was injected using a microinjector (Nanoject II, Drummond Scientific, Broomall, PA). Virus was injected over the course of 1-2 minutes and a 5-minute incubation was allowed before retracting the pipette to allow for perfusion into the tissue. Incisions were closed with surgical glue. For nonselective targeting of MThal-DMS terminals, mice were injected with AAV2-hsyn-ChR2(H134R)-EYFP (ChR2). For selective targeting of MOR-expressing MThal-DMS

terminals, mice were injected with AAV5-EF1α-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA (DIO-ChR2). For selective targeting of MOR-lacking MThal-DMS terminals, mice were injected with AAV8-Ef1a-DO-hChR2(H134R)-mCherry-WPRE-pA (DO-ChR2).

Brain slice electrophysiology

Brain slice preparation was carried out as previously described (Jaeckel et al., 2024). 2-4 weeks following viral injection, mice were deeply anesthetized with isoflurane and decapitated. Brains were removed and mounted for slicing with a vibratome (Model 7000 smz, Campden Instruments). During slicing brains were maintained at room temperature in carbogenated Krebs solution containing (in mM): 136 NaCl, 2.5 KCl, 1.2 $MgCl_2$-$6H_2O$, 2.4 $CaCl_2$-2 H_2O, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, 11.1 dextrose supplemented with 5 μM MK-801. Coronal sections (250-300 μM) containing the DMS were made and incubated in carbogenated Krebs supplemented with 10 μM MK-801 at 32°C for 30 minutes. Slices were then maintained at room temperature in carbogenated Krebs until used for recording. Borosilicate glass patch pipettes (Sutter Instruments) were pulled to a resistance of 2.0-3.0 MΩ and filled with a potassium gluconate-based internal solution (in mM: 110 potassium gluconate, 10 KCl, 15 NaCl, 1.5 $MgCl_2$, 10 HEPES, 1 EGTA, 2 Na ATP, 0.4 Na GTP, 7.8 Na_2 phosphocreatine). Slices were placed in the recording chamber and continuously perfused with carbogenated Krebs solution supplemented with 100 μM picrotoxin at 32-34°C. Striatal MSNs were identified based on cell morphology, resting membrane potential, and firing frequency (Kreitzer et al., 2009). Whole-cell recordings were made in MSNs in voltage-clamp mode at -70 mV holding potential. All drug solutions were prepared in carbogenated Krebs solution supplemented with 100 μM picrotoxin.

Whole-cell recordings were made with a multiclamp 700B amplifier (Molecular Devices, San Jose, CA) digitized at 20 KHz (National Instruments BNC-2090A, Austin, TX). Recordings were acquired using Matlab Wavesurfer (Mathworks, Natick, MA)**.** Currents were evoked by illuminating the field of view through the microscope objective (Olympus BX51W, Tokyo, Japan) using a TTL-controlled LED driver and a 470 nm LED (Thor Labs, Newton, NJ). LED stimulation duration was 1 ms and power output measured after the microscope objective ranged from 0.5-3 mW, adjusted to obtain consistent current amplitudes across cells. Paired-pulse responses were evoked every 30 seconds, with a 50 ms inter-pulse interval, and 10-15 sweeps were recorded for each condition. Series resistance was monitored throughout the recordings and only recordings in which the series resistance remained <15 MΩ and did not change more than 18% were included.

Data analysis

Raw data were analyzed using Matlab. Peak current amplitude was calculated for each sweep after baseline subtraction, with baseline defined as the average holding current during the first 10 ms of each sweep, prior to optical stimulation. For each condition (baseline, drug, washout/reversal), baseline subtracted sweeps were averaged together, and peak current amplitude of the averaged trace was calculated. For the baseline condition, the first 2-4 sweeps were omitted from the average to allow the currents to stabilize. For the drug and washout/reversal conditions, the first 4-6 sweeps were omitted from the average to allow for equilibration of drug or washout of drug within the tissue. Average drug and washout/reversal current amplitudes were normalized to the average baseline current peak amplitude and plotted as % of baseline to analyze sensitivity of MThal terminals to opioid-mediated inhibition. For

paired-pulse analysis, paired-pulse ratio was calculated as the ratio of the peak amplitude of the second response to the peak amplitude of the first response. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Statistical comparisons were made using a ratio paired t-test, unpaired t-test, mixed-effects analysis with Tukey's multiple comparisons, or ordinary one-way ANOVA with Tukey's multiple comparisons. For all comparisons, statistical significance was defined as $p < 0.05$, and n (number of cells) and N (number of animals) are both reported.

4.4 Results

MOR-expressing and MOR-lacking MThal projection neurons functionally innervate the DMS

We have previously shown that glutamatergic MThal projection neurons functionally express MORs at terminals within the DMS, as agonist perfusion reduces the amplitude of optically evoked excitatory postsynaptic currents (oEPSCs) at MThal-DMS terminals (Birdsong et al., 2019; Jaeckel et al., 2024). However, based on the lack of responses to morphine at the cell body of some MThal neurons, we hypothesized that this population of neurons may be comprised of MOR-expressing and MOR-lacking subpopulations. Thus, we first injected *Oprm1*-Cre mice with a cre-on virus encoding EYFP and a cre-off virus encoding mCherry to visualize MOR-expressing and MOR-lacking MThal neurons. In the MThal, both EYFP-expressing (MOR-positive) and mCherry-expressing (MOR-negative) somas were present (Fig. 4.1A), and in the DMS, both EYFP-expressing and mCherry-expressing axon terminals were present (Fig. 4.1B). Consistent with our hypothesis, these findings suggest a heterogeneous

population of MOR-expressing and MOR-lacking MThal neurons that project to the DMS (Fig. 4.1C).

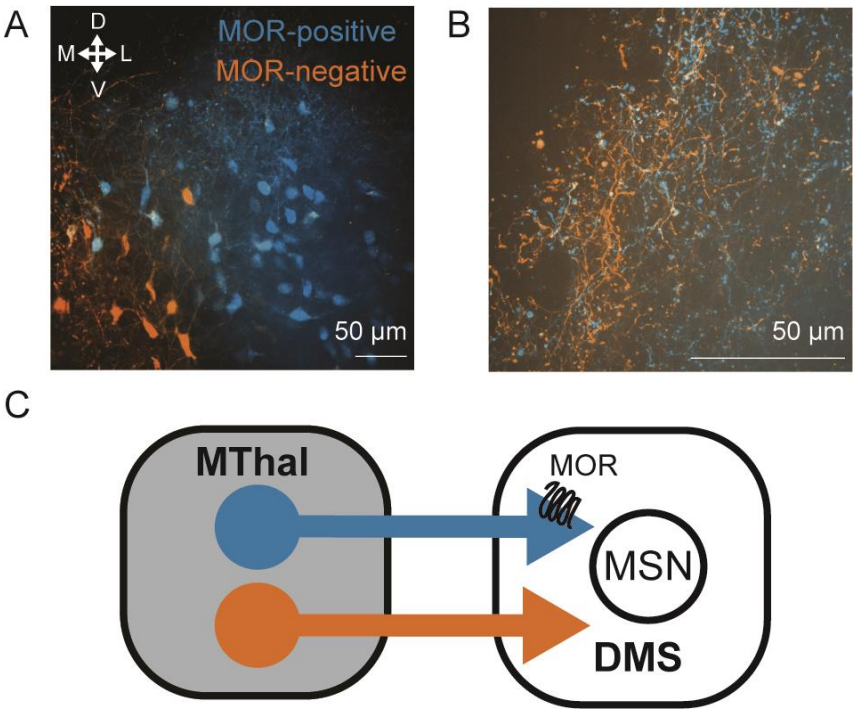

Figure 4.1. MThal inputs to the DMS were comprised of MOR-expressing and MOR-lacking neuronal projections. **A.** Fluorescent image of MThal in a brain section from an *Oprm1*-cre mouse in which cre-positive somas are labeled with GFP (blue) and cre-negative somas are labeled with mCherry (orange). **B.** Fluorescent image of DMS in a brain section from an *Oprm1*-cre mouse in which cre-positive terminals are labeled with GFP (blue) and cre-negative somas are labeled with mCherry (orange). **C.** Schematic representation of the proposed circuit, in which both MOR-expressing and MOR-lacking MThal projection neurons innervate MSNs within the DMS.

We next sought to determine whether projections from these two subpopulations functionally innervate the DMS. If MOR-expressing neurons are a subpopulation of MThal neurons that innervate the DMS, we predicted that opioid agonists would inhibit glutamate release to a greater extent from MOR-expressing MThal terminals than from MThal terminals as a whole. To address this, AAVs encoding the light-activated ion channel ChR2 expressed in a nonselective or cre-dependent manner were injected into the MThal of *Oprm1*-Cre mice and

whole-cell voltage clamp recordings were performed in DMS medium spiny neurons. To measure opioid-mediated inhibition of glutamate release, a stable baseline of oEPSCs was recorded, followed by perfusion of the partial MOR agonist morphine (3 μM) or the full agonist DAMGO (1 μM), and then perfused with the MOR antagonist naloxone (1 μM) to reverse the inhibition. When ChR2 was nonselectively expressed in MThal-DMS terminals regardless of the presence of cre (ChR2; Fig. 4.2A), perfusion of morphine decreased oEPSC amplitude to 67.66% of baseline, and this effect was largely reversed by perfusion of naloxone (Fig. 4.2B, C; baseline: 283.7 ± 22.6 pA, morphine: 191.4 ± 23.2 pA, naloxone: 246.7 ± 24.9 pA; $F_{(0.813,7.292)}$ = 22.55, p = 0.0025, n = 9-11, N = 7, mixed-effects analysis; baseline vs. morphine: p = 0.0010; baseline vs. naloxone: p = 0.0237; morphine vs. naloxone: p = 0.0054, Tukey's multiple comparisons test). Perfusion of DAMGO decreased oEPSC amplitude to 48% of baseline (Fig. 4.2D, E; baseline: 238.1 ± 28.1 pA, DAMGO: 120.0 ± 27.7 pA, naloxone: 204.8 ± 58.1 pA; $F_{(0.1260,0.8188)}$ = 24.08, p = 0.0778, n = 5-10, N = 5-8, mixed-effects analysis; baseline vs. DAMGO: p = 0.0003; baseline vs. naloxone: p = 0.0588; DAMGO vs. naloxone: p = 0.0753, Tukey's multiple comparisons test).

When MOR-expressing terminals were targeted using a virus that expresses ChR2 in cre-positive cells (DIO-ChR2; Fig. 4.2F), oEPSCs were inhibited by morphine to 36.31% of baseline (Fig. 4.2G, H; baseline: 301.4 ± 27.1 pA, morphine: 106.9 ± 13.2 pA, naloxone: 220.3 ± 24.0 pA; $F_{(1.796,17.06)}$ = 40.98, p < 0.0001, n = 9-12, N = 7-8; mixed-effects analysis; baseline vs. morphine: p < 0.0001; baseline vs. naloxone: p = 0.0208; morphine vs. naloxone: p = 0.0005, Tukey's multiple comparisons test) and DAMGO to 20% of baseline (Fig. 4.2 I, J; baseline: 280.7 ± 27.5 pA, DAMGO: 55.1 ± 14.1 pA, naloxone: 244.0 ± 18.9 pA; $F_{(1.936,17.42)}$ = 46.37, p <

0.0001, n = 9-11, N = 6-7, mixed-effects analysis; baseline vs. DAMGO: $p < 0.0001$; baseline vs. naloxone: $p = 0.2308$; DAMGO vs. naloxone: $p = 0.0002$, Tukey's multiple comparisons test).

Next, we measured opioid-mediated inhibition of MThal-DMS terminals when MOR-lacking terminals were targeted using a virus expressing ChR2 only in cre-negative cells (DO-ChR2; Fig. 4.2K). In brain slices from mice injected with DO-ChR2, perfusion of morphine did not inhibit MThal-DMS oEPSCs (Fig. 4.2L, M; baseline: 106.8 ± 14.6 pA, morphine: 102.2 ± 16.6 pA, naloxone: 102.7 ± 18.6 pA; $F_{(1.289,5.156)} = 1.786$, $p = 0.2464$, n = 7-9, N = 6, mixed-effects analysis). Due to the clear lack of inhibition by morphine, inhibition by DAMGO in mice injected with DO-ChR2 was not investigated.

A comparison of the magnitude of morphine-mediated inhibition of MThal-DMS oEPSCs in each condition indicated that inhibition was greater in slices from mice injected with DIO-ChR2 and lesser in slices from mice injected with DO-ChR2 relative to ChR2-injected mice (Fig. 4.2N; ChR2: 67.66 ± 7.44% of baseline, n = 11, N = 7; DIO-ChR2: 36.31 ± 4.29% of baseline, n = 12, N = 8; DO-ChR2: 95.42 ± 5.26% of baseline, n = 9, N = 6; $F_{(2,29)} = 25.16$, $p < 0.0001$, ordinary one-way ANOVA; DIO-ChR2 vs. ChR2: $p = 0.0013$, DO-ChR2 vs. ChR2: $p = 0.0081$, DIO-ChR2 vs. DO-ChR2: $p < 0.0001$, Tukey's multiple comparisons test). Similarly, DAMGO-mediated inhibition of MThal-DMS oEPSCs was greater in slices from mice injected with DIO-ChR2 relative to ChR2 (Fig. 4.2O; ChR2: 47.57 ± 6.55% of baseline, n = 10, N = 8; DIO-ChR2: 20.10 ± 4.99% of baseline, n = 11, N = 7; $t_{(19)} = 3.374$, $p = 0.0032$, unpaired t-test). Together, these results suggest that both MOR-expressing and MOR-lacking MThal projection neurons functionally innervate the DMS and we were able to isolate oEPSCs from these populations individually. However, when MThal-DMS terminals were non-selectively excited, glutamate release was evoked from a mixture of both populations and morphine generated an

intermediate amount of overall inhibition- strongly inhibiting MOR-expressing inputs while sparing MOR-lacking inputs.

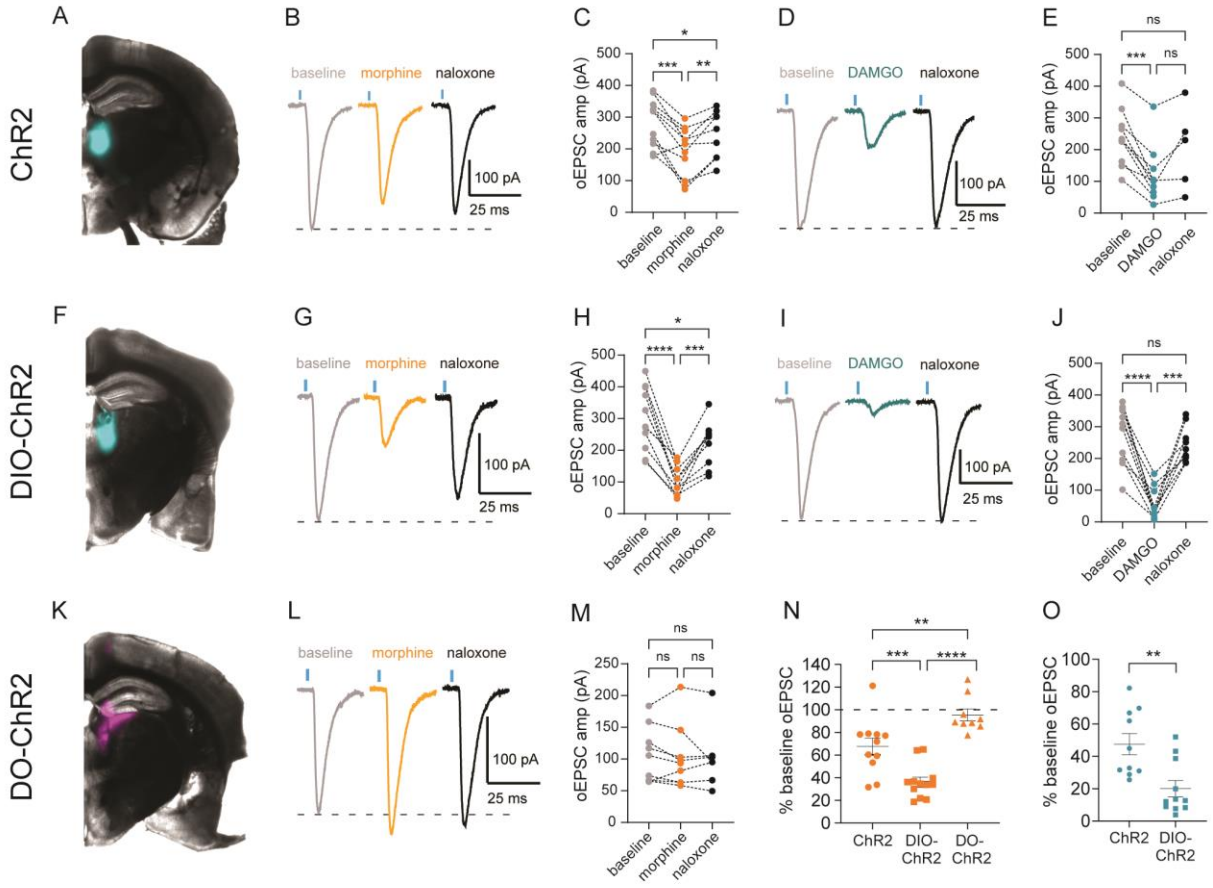

Figure 4.2. MThal innervation of the DMS from both MOR-expressing and MOR-lacking projections neurons was functional. **A.** Representative image of a brain slice from a mouse injected with ChR2 showing EYFP fluorescence in the injection site (MThal). **B.** Representative traces showing oEPSCs during baseline, morphine, and naloxone in an MSN following injection of ChR2. **C.** Summary of oEPSC raw amplitudes during baseline, morphine, and naloxone in MSNs following injection of ChR2 (baseline: 283.7 ± 22.6 pA, morphine: 191.4 ± 23.2 pA, naloxone: 246.7 ± 24.9 pA; baseline vs. morphine: $p = 0.0010$; baseline vs. naloxone: $p = 0.0237$; morphine vs. naloxone: $p = 0.0054$, mixed-effects analysis with Tukey's multiple comparisons test). **D.** Representative traces showing oEPSCs during baseline, DAMGO, and naloxone in an MSN following injection of ChR2. **E.** Summary of oEPSC raw amplitudes during baseline, DAMGO, and naloxone in MSNs following injection of ChR2 (baseline: 238.1 ± 28.1 pA, DAMGO: 120.0 ± 27.7 pA, naloxone: 204.8 ± 58.1 pA; baseline vs. DAMGO: $p = 0.0003$; baseline vs. naloxone: $p = 0.0588$; DAMGO vs. naloxone: $p = 0.0753$, mixed-effects analysis Tukey's multiple comparisons test). **F.** Representative image of a brain slice from a mouse injected with DIO-ChR2 showing EYFP fluorescence in MThal. **G.** Representative traces showing oEPSCs during baseline, morphine, and naloxone in an MSN following injection of DIO-ChR2. **H.** Summary of oEPSC raw amplitudes during baseline, morphine, and naloxone in MSNs following injection of DIO-ChR2 (baseline: 301.4 ± 27.1 pA, morphine: 106.9 ± 13.2 pA, naloxone: 220.3 ± 24.0 pA; baseline vs. morphine: $p < 0.0001$; baseline vs. naloxone: $p = 0.0208$; morphine vs. naloxone: $p = 0.0005$, mixed-

effects analysis with Tukey's multiple comparisons test). **I.** Representative traces showing oEPSCs during baseline, DAMGO, and naloxone in an MSN following injection of DIO-ChR2. **J.** Summary of oEPSC raw amplitudes during baseline, DAMGO, and naloxone in MSNs following injection of ChR2 (baseline: 280.7 ± 27.5 pA, DAMGO: 55.1 ± 14.1 pA, naloxone: 244.0 ± 18.9 pA; baseline vs. DAMGO: $p < 0.0001$; baseline vs. naloxone: $p = 0.2308$; DAMGO vs. naloxone: $p = 0.0002$, mixed-effects analysis with Tukey's multiple comparisons test). **K.** Representative image of a brain slice from a mouse injected with DO-ChR2 showing mCherry fluorescence in MThal. **L.** Representative traces showing oEPSCs during baseline, morphine, and naloxone in an MSN following injection of DO-ChR2. **M.** Summary of oEPSC raw amplitudes during baseline, morphine, and naloxone in MSNs following injection of DO-ChR2 (baseline: 106.8 ± 14.6 pA, morphine: 102.2 ± 16.6 pA, naloxone: 102.7 ± 18.6 pA; $p = 0.2464$, mixed-effects analysis). **N.** Mean inhibition of oEPSCs by perfusion of morphine in cells from mice injected with ChR2, DIO-ChR2, and DO-ChR2 (ChR2: 67.66 ± 7.44% of baseline; DIO-ChR2: 36.31 ± 4.29% of baseline; DO-ChR2: 95.42 ± 5.26% of baseline; DIO-ChR2 vs. ChR2: $p = 0.0013$, DO-ChR2 vs. ChR2: $p = 0.0081$, DIO-ChR2 vs. DO-ChR2: $p < 0.0001$, ordinary one-way ANOVA with Tukey's multiple comparisons test). **O.** Mean inhibition of oEPSCs by perfusion of DAMGO in cells from mice injected with ChR2 or DIO-ChR2 (ChR2: 47.57 ± 6.55% of baseline; DIO-ChR2: 20.10 ± 4.99% of baseline; $p = 0.0032$, unpaired t-test). Lines and error bars represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

MOR agonists alter presynaptic neurotransmitter release probability of MOR-expressing, but not MOR-lacking, MThal-DMS terminals

We next examined how perfusion of morphine and DAMGO altered the paired-pulse ratio (PPR) of oEPSCs, a reflection of presynaptic neurotransmitter release probability, at both MOR-expressing and MOR-lacking MThal-DMS terminals. Presynaptic release probability is determined by numerous factors at the presynaptic terminal, such as calcium dynamics, the number of available vesicles, and the number of functional release sites (Graziane & Dong, 2022). The PPR is inversely correlated with release probability and can be used to assess changes in this property. Given that MORs are inhibitory-coupled receptors and that we and others have demonstrated that this inhibition is due to presynaptic MORs (Adhikary, Jaeckel, et al., 2022; Atwood et al., 2014), we expected that agonist perfusion would decrease the probability of release, and thus increase the PPR, of oEPSCs elicited from stimulation of MOR-expressing MThal-DMS terminals. In mice injected with ChR2, neither morphine nor DAMGO altered the PPR (Fig. 4.3 A-D; morphine: 1.16 ± 0.08 times baseline, $t_{(8)} = 1.655$, $p = 0.1366$, $n = 9$, $N = 6$,

ratio paired t test; DAMGO: 1.03 ± 0.10 times baseline, $t_{(8)} = 0.0873$, $p = 0.9326$, n = 9, N = 8, ratio paired t test). In contrast, both morphine and DAMGO significantly increased the PPR in slices from DIO-ChR2-injected mice (Fig. 4.3E-H; morphine: 1.45 ± 0.15 times baseline, $t_{(11)} = 3.474$, $p = 0.0052$, n = 12, N = 10, ratio paired t test; DAMGO: 1.73 ± 0.31 times baseline, $t_{(10)} = 0.2.284$, $p = 0.0455$, n = 11, N = 7, ratio paired t test). Morphine perfusion had no effect on PPR in slices from DO-ChR2-injected mice (Fig. 4.3I, J; 1.02 ± 0.05 times baseline, $t_{(8)} = 0.2324$, $p = 0.8221$, n = 9, N = 6, ratio paired t test). Like what was observed with morphine-mediated inhibition, these results again suggest that both MOR-expressing and MOR-lacking MThal terminals innervate the DMS, and that perfusion of morphine affects only synapses in which MOR is expressed in the presynaptic terminal. PPR is commonly used to confirm a presynaptic site of drug action, and this further supports a presynaptic mechanism of opioid inhibition given that the PPR was unaffected at synapses in which MORs are not expressed.

Comparison of the baseline PPR between the three conditions revealed that oEPSCs in cells from DIO-ChR2-injected mice had a significantly higher PPR than those of ChR2-injected mice, and the PPR was significantly lower in cells from DO-injected mice (Fig. 4.3K; ChR2: 1.13 ± 0.06, DIO-ChR2: 1.35 ± 0.06, DO-ChR2: 0.82 ± 0.07; $F_{(2,70)} = 17.11$, $p < 0.0001$, n = 20-28, N = 7-11 for each group, ordinary one-way ANOVA; ChR2 vs DIO-ChR2: $p = 0.0328$, ChR2 vs DO-ChR2: $p = 0.0036$, DIO-ChR2 vs DO-ChR2: $p < 0.0001$, Tukey's multiple comparisons test). This suggests a difference in the release probability between MOR-expressing and MOR-lacking terminals under normal physiological conditions, thus there may be other important differences in signaling between these two neuronal populations in addition to the presence or absence of MORs. The depressing nature of the oEPSCs from stimulation of the opioid-insensitive MOR-lacking inputs can also explain why the PPR did not increase in the

presence of opioids when ChR2 was non-selectively expressed. While the PPR was increasing in the presence of morphine and DAMGO at facilitating MOR-expressing inputs, they were also getting smaller and, therefore, making up a smaller fraction of the non-selective oEPSC, while the depressing MOR-lacking terminals were making up a larger fraction of the overall non-selective oEPSC.

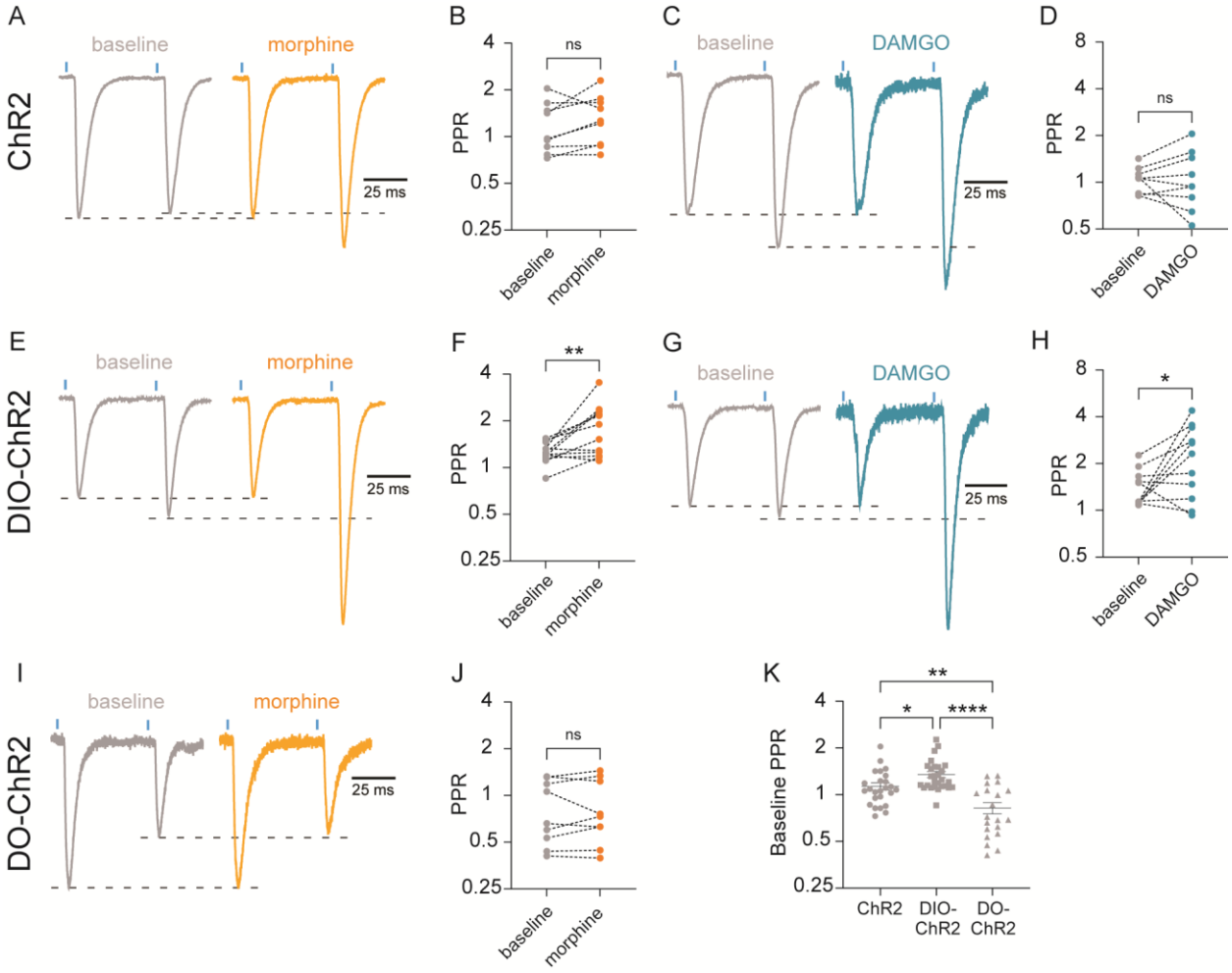

Figure 4.3. MOR agonists altered the paired-pulse ratio at MOR-expressing MThal-DMS terminals. **A.** Representative traces showing paired oEPSCs during baseline and morphine in an MSN following injection of ChR2. **B.** Summary of paired-pulse ratios during baseline and morphine in cells from mice injected with ChR2 (1.16 ± 0.08 times baseline, p = 0.1366, ratio paired t test). **C.** Representative traces showing paired oEPSCs during baseline and DAMGO in an MSN following injection of ChR2. **D.** Summary of paired-pulse ratios during baseline and DAMGO in cells from mice injected with ChR2 (1.03 ± 0.10 times baseline, p = 0.9326, ratio paired t test). **E.** Representative traces showing paired

oEPSCs during baseline and morphine in an MSN following injection of DIO-ChR2. **F.** Summary of paired-pulse ratios during baseline and morphine in cells from mice injected with DIO-ChR2 (1.45 ± 0.15 times baseline, p = 0.0052, ratio paired t test). **G.** Representative traces showing paired oEPSCs during baseline and DAMGO in an MSN following injection of DIO-ChR2. **H.** Summary of paired-pulse ratios during baseline and DAMGO in cells from mice injected with DIO-ChR2 (1.73 ± 0.31 times baseline, p = 0.0455, ratio paired t test). **I.** Representative traces showing paired oEPSCs during baseline and morphine in an MSN following injection of DIO-ChR2. **J.** Summary of paired-pulse ratios during baseline and morphine in cells from mice injected with DO-ChR2 (1.02 ± 0.05 times baseline, p = 0.8221, ratio paired t test). **K.** Mean baseline paired-pulse ratio in cells from mice injected with ChR2, DIO-ChR2, and DO-ChR2 (ChR2: 1.13 ± 0.06, DIO-ChR2: 1.35 ± 0.06, DO-ChR2: 0.82 ± 0.07; ChR2 vs DIO-ChR2: p = 0.0328, ChR2 vs DO-ChR2: p = 0.0036, DIO-ChR2 vs DO-ChR2: p < 0.0001, ordinary one-way ANOVA with Tukey's multiple comparisons test). Lines and error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001.

Chronic morphine treatment decreases the sensitivity of MOR-expressing MThal-DMS terminals to subsequent morphine inhibition

We have previously shown that in male mice, chronic morphine treatment induced facilitation of morphine signaling (i.e. the sensitivity of the terminals from mice previously exposed to morphine to subsequent inhibition by morphine was increased) when glutamate released was evoked non-selectively from MThal-DMS terminals (Jaeckel et al., 2024). However, from the results of this study we could not determine whether this effect was driven by adaptations induced at MORs present at presynaptic terminals (increased morphine sensitivity in MOR-expressing neurons) or other circuit-level adaptations (increased contribution of MOR-expressing MThal neurons to the overall oEPSC). Thus, we sought to determine whether facilitation following chronic morphine treatment was still observed when MOR-expressing terminals were selectively targeted. *Oprm1*-cre mice injected with either ChR2 or DIO-ChR2 were implanted subcutaneously with osmotic minipumps continuously releasing morphine (80 mg/kg/day) or saline 7 days prior to recordings (Fig. 4.4A) and morphine-mediated inhibition of MThal-DMS oEPSCs was quantified. In mice injected with ChR2, inhibition of oEPSCs by subsequent perfusion of morphine did not significantly differ between saline and chronic

morphine treated mice (Fig. 4.4B-D; saline: 65.40 ± 5.21% of baseline, n = 14, N = 5; chronic morphine: 61.43 ± 4.36% of baseline, n = 14, N = 6; $t_{(26)} = 0.5846$, p = 0.5638, unpaired t test). However, in mice injected with DIO-ChR2, inhibition of oEPSCs by subsequent morphine was significantly decreased in chronic morphine treated mice compared to saline controls (Fig. 4.4E-G; saline: 33.19 ± 3.08% of baseline, n = 12, N = 4; chronic morphine: 46.79 ± 3.62% of baseline, n = 12, N = 6; $t_{(22)} = 2.864$, p = 0.0090, unpaired t test). In contrast to our previous study examining MThal-DMS terminals without regard to the presence or absence of presynaptic MORs, these results indicate that at MOR-expressing MThal-DMS terminals, chronic morphine exposure resulted in tolerance to subsequent morphine signaling.

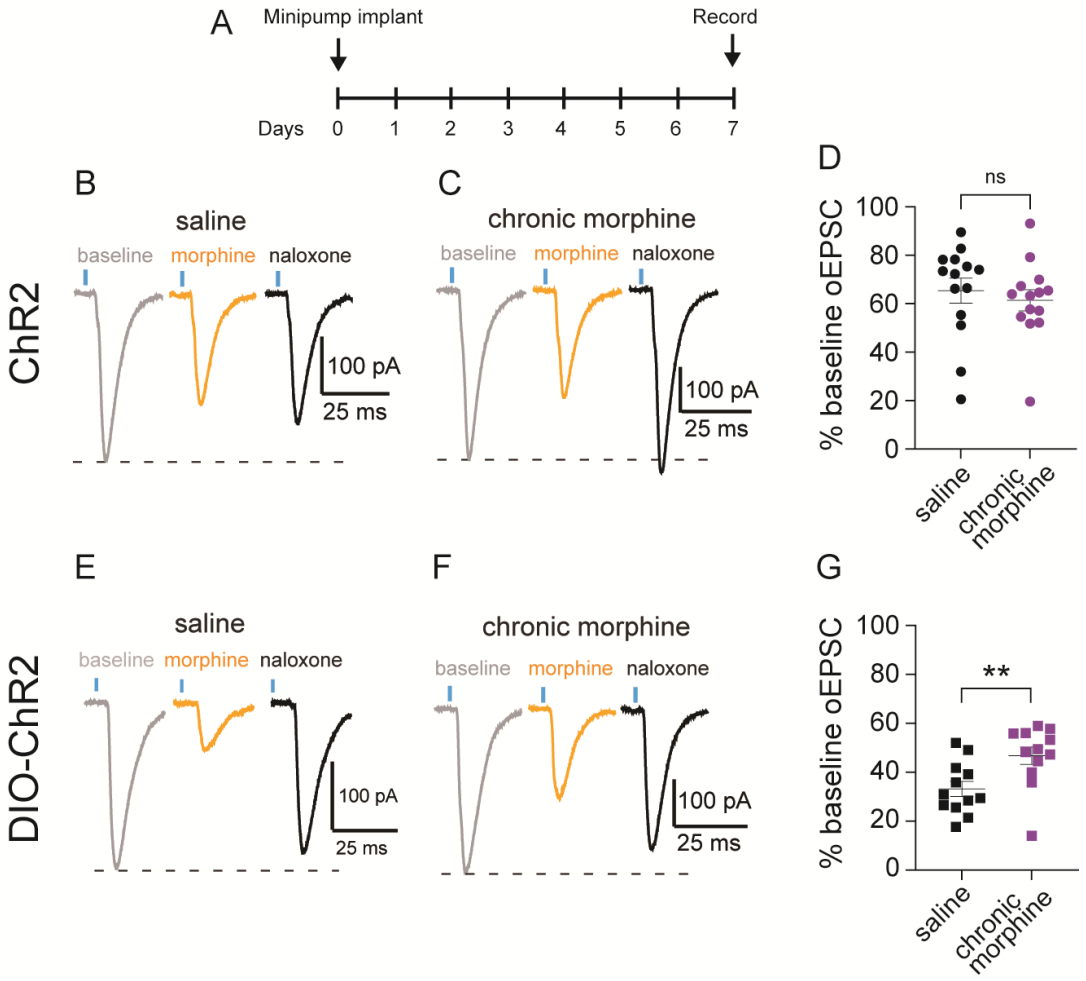

Figure 4.4. Chronic morphine treatment induced tolerance at MOR-expressing MThal-DMS terminals. **A.** Schematic of timeline for saline or chronic morphine treatment. **B, C.** Representative traces showing oEPSCs during baseline, morphine, and naloxone in an MSN following injection of ChR2 and saline **(B)** or chronic morphine **(C)** treatment. **D.** Mean inhibition of oEPSCs by perfusion of morphine in cells from mice saline- or chronic morphine-treated mice injected with ChR2 (saline: 65.40 ± 5.21% of baseline; chronic morphine: 61.43 ± 4.36% of baseline, p = 0.5638, unpaired t test). **E, F.** Representative traces showing oEPSCs during baseline, morphine, and naloxone in an MSN following injection of DIO-ChR2 and saline **(B)** or chronic morphine **(C)** treatment. **G.** Mean inhibition of oEPSCs by perfusion of morphine in cells from mice saline- or chronic morphine-treated mice injected with DIO-ChR2 (saline: 33.19 ± 3.08% of baseline, chronic morphine: 46.79 ± 3.62% of baseline, p = 0.0090, unpaired t test). Lines and error bars represent mean ± SEM. **p < 0.01.

The contrast between morphine tolerance seen at MOR-expressing MThal-DMS synapses of these *Oprm1*-cre mice and the facilitation to morphine seen in our previous could be resolved if there were two opposing mechanisms that occur in response to morphine treatment; the first,

cellular tolerance to the effect of morphine at MOR-expressing terminals, combined with an increased relative contribution of MOR-expressing terminals to the non-selective oEPSC due to some form of plasticity within this circuit. To address whether chronic morphine treatment may induce presynaptic plasticity, we assessed whether chronic morphine treatment altered the baseline PPR and whether chronic morphine treatment altered the effect of subsequent morphine on changing the PPR. We did not observe any significant differences in baseline PPR between the saline and morphine treated conditions in either ChR2- or DIO-ChR2-injected mice (Fig. 4.5A, C, E, F, H, J; ChR2 saline: 1.31 ± 0.08, n = 19, N = 5; ChR2 chronic morphine: 1.29 ± 0.07, n = 22, N = 7; $t_{(39)} = 0.2596$, p = 0.7965, unpaired t test; DIO-ChR2 saline: 1.49 ± 0.10, n = 16, N = 4; DIO-ChR2 chronic morphine: 1.45 ± 0.11, n = 23, N = 8; $t_{(37)} = 0.3000$, p = 0.7659, unpaired t test).

Like in untreated mice, perfusion of morphine did not alter the PPR in saline treated mice injected with ChR2 **(**Fig. 4.5A, B; 1.28 ± 0.18 times baseline, $t_{(13)} = 1.661$, p = 0.1206, n = 14, N = 5, ratio paired t test). However, morphine perfusion significantly increased the PPR in chronic morphine treated mice, indicative of an overall decrease in release probability in the presence of morphine (Fig. 4.5C, D; 1.40 ± 0.14 times baseline, $t_{(14)} = 3.36$, p = 0.0046, n = 14, N = 6, ratio paired t test). It was possible this was driven by a change in the number of MOR-expressing relative to MOR-lacking MThal-DMS terminals following chronic morphine treatment. Consistent with untreated mice, morphine perfusion increased the PPR in saline treated mice injected with DIO-ChR2 **(**Fig. 4.5F, G; 1.53 ± 0.13 times baseline, $t_{(11)} = 5.157$, p = 0.0003, n = 12, N = 4, ratio paired t test). This morphine-mediated increase in the PPR persisted in chronic morphine treated mice, although, consistent with cellular tolerance to morphine, the PPR change

appeared less statistically robust than in saline treated mice **(**Fig. 4.5H, I; 1.42 ± 0.16 times baseline, $t_{(11)}$ = 2.96, p = 0.013, n = 12, N = 6, ratio paired t test).

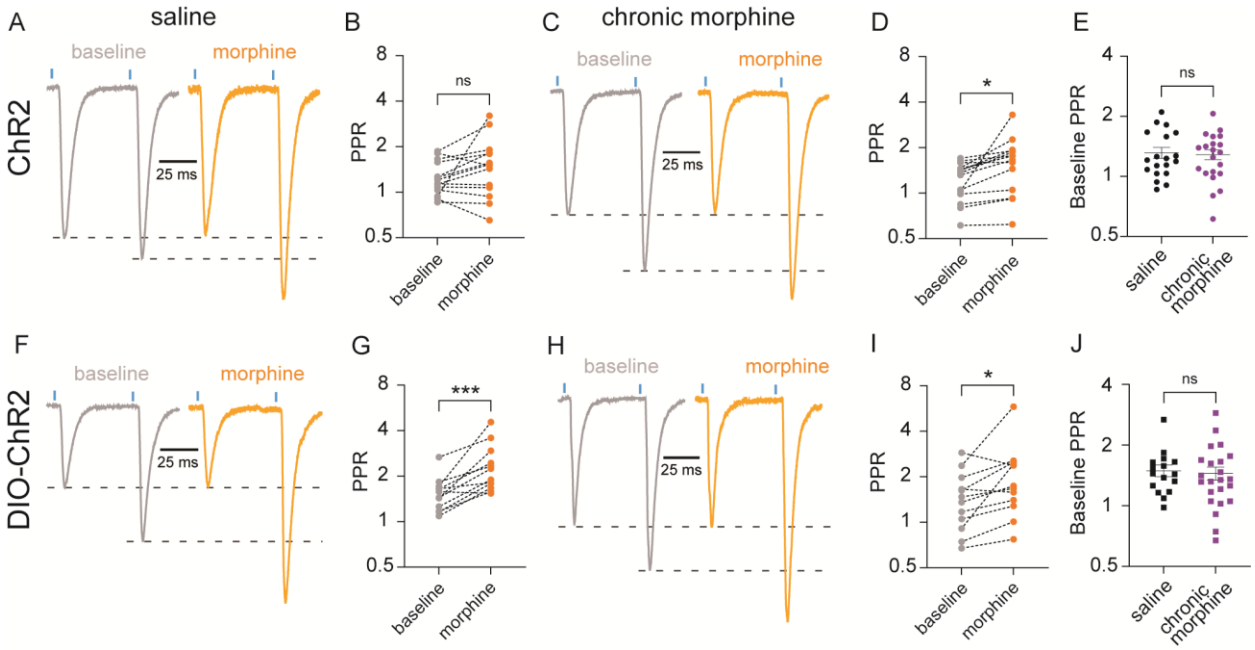

Figure 4.5. Chronic morphine treatment did not alter paired-pulse ratio at MOR-expressing MThal-DMS terminals. **A.** Representative traces showing paired oEPSCs during baseline and morphine in an MSN following injection of ChR2 in saline-treated mice. **B.** Summary of paired-pulse ratios during baseline and morphine in cells from saline-treated mice injected with ChR2 (1.28 ± 0.18 times baseline, p = 0.1206, ratio paired t test). **C.** Representative traces showing paired oEPSCs during baseline and morphine in an MSN following injection of ChR2 in chronic morphine-treated mice. **D.** Summary of paired-pulse ratios during baseline and morphine in cells from chronic morphine-treated mice injected with ChR2 (1.40 ± 0.14 times baseline, p = 0.0046, ratio paired t test). **E.** Mean baseline paired-pulse ratios in saline and chronic-morphine treated mice injected with ChR2 (saline: 1.31 ± 0.08, chronic morphine: 1.29 ± 0.07, p = 0.7965, unpaired t test). **F.** Representative traces showing paired oEPSCs during baseline and morphine in an MSN following injection of DIO-ChR2 in saline-treated mice. **G.** Summary of paired-pulse ratios during baseline and morphine in cells from saline-treated mice injected with DIO-ChR2 (1.53 ± 0.13 times baseline, p = 0.0003, ratio paired t test). **H.** Representative traces showing paired oEPSCs during baseline and morphine in an MSN following injection of DIO-ChR2 in chronic morphine-treated mice. **I.** Summary of paired-pulse ratios during baseline and morphine in cells from chronic morphine-treated mice injected with DIO-ChR2 (1.42 ± 0.16 times baseline, p = 0.013, ratio paired t test). **J.** Mean baseline paired-pulse ratios in saline and chronic-morphine treated mice injected with DIO-ChR2 (saline: 1.49 ± 0.10, chronic morphine: 1.45 ± 0.11, p = 0.7659, unpaired t test). Lines and error bars represent mean ± SEM. *p < 0.05, ***p < 0.001.

Chronic morphine treatment does not alter subsequent morphine signaling at MOR-lacking MThal-DMS terminals

It is possible that the facilitation observed previously could be due to previously MOR-lacking terminals becoming MOR-sensitive or from decreased glutamate release from MOR-lacking MThal-DMS terminals of morphine treated mice. To address these possibilities, we next investigated whether chronic morphine exposure altered signaling at MOR-lacking MThal-DMS terminals. In previous experiments, morphine sensitivity was not different between saline treated and untreated mice, thus chronically morphine treated mice here were compared to untreated mice to reduce the need for an additional manipulation. Like in untreated mice injected with DO-ChR2, perfusion of morphine did not significantly inhibit MThal-DMS oEPSCs in mice chronically treated with morphine (Fig. 4.6A, B; baseline: 203.7 ± 72.34 pA, morphine: 194.3 ± 70.77 pA, naloxone: 200.3 ± 70.75 pA; $F_{(1.289,5.156)} = 1.786$, p = 0.2464, n = 5, N = 4, repeated measures one-way ANOVA). Thus, there were no differences in morphine inhibition of oEPSCs between untreated and chronic morphine treated mice (Fig. 4.6C; untreated: 95.42 ± 5.26% of baseline, n = 9, N = 6; chronic morphine: 95.44 ± 20.5% of baseline, n = 5, N = 4; $t_{(12)} = 0.0028$, p = 0.9978, unpaired t test). Raw oEPSC baseline amplitudes did not differ between untreated and chronic morphine treated mice, suggesting the strength of these inputs was not altered by chronic morphine treatment (Fig. 4.6D; untreated: 75.6 ± 11.9 pA, n = 36, N = 6; chronic morphine: 96.2 ± 21.2 pA, n = 36, N = 5; $t_{(70)} = 0.8452$, p = 0.4009, unpaired t test). Perfusion of morphine also did not alter the PPR in chronically treated mice (Fig. 4.6E, F; 1.06 ± 0.06 times baseline, $t_{(4)} = 0.4768$, p = 0.4768, n = 5, N = 4, ratio paired t test). We also did not observe any difference in the baseline PPR between untreated and morphine treated mice (Fig. 4.6G; untreated: 0.82 ± 0.07, n = 20, N = 7; chronic morphine: 0.79 ± 0.05, n = 14, N = 4; $t_{(32)}$ =

0.3272, p = 0.7456, unpaired t test). These results suggest a lack of presynaptic adaptations occurring at MOR-lacking MThal-DMS terminals following chronic morphine treatment.

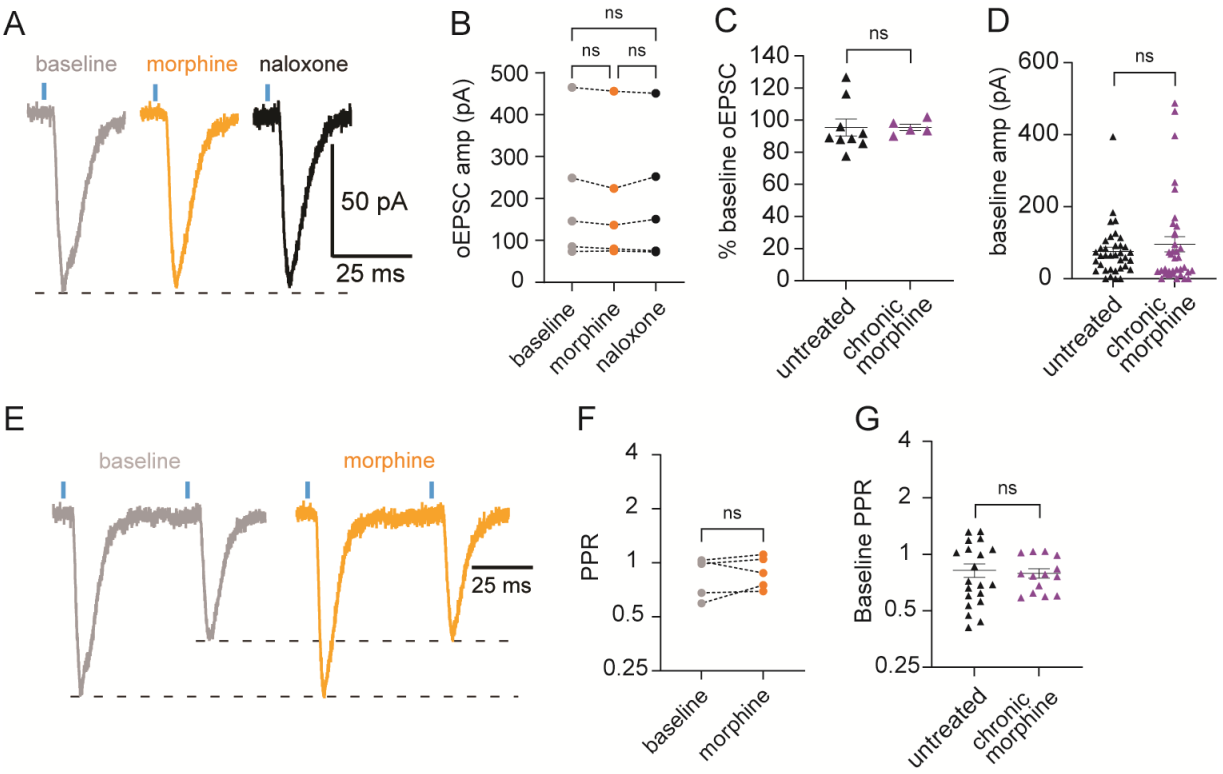

Figure 4.6. Chronic morphine did not alter signaling at MOR-lacking MThal-DMS terminals. **A.** Representative traces showing oEPSCs during baseline, morphine, and naloxone in an MSN following injection of DO-ChR2 and chronic morphine treatment. **B.** Summary of oEPSC raw amplitudes during baseline, morphine, and naloxone in MSNs following injection of ChR2 in chronic morphine-treated mice (baseline: 203.7 ± 72.34 pA, morphine: 194.3 ± 70.77 pA, naloxone: 200.3 ± 70.75 pA; p = 0.2464, repeated measures one-way ANOVA). **C.** Mean inhibition of oEPSCs by perfusion of morphine in cells from untreated or chronic morphine-treated mice injected with DO-ChR2 (untreated: 95.42 ± 5.26% of baseline, chronic morphine: 95.44 ± 20.5% of baseline, p = 0.9978, unpaired t test). **D.** Mean baseline raw oEPSC amplitudes in untreated and chronic morphine treated mice injected with DO-ChR2. **E.** Representative traces showing paired oEPSCs during baseline and morphine in an MSN following injection of DO-ChR2 and chronic morphine treatment. **F.** Summary of paired-pulse ratios during baseline and morphine in cells from chronic morphine-treated mice injected with DO-ChR2 (1.06 ± 0.06 times baseline, p = 0.4768, ratio paired t test). **G.** Mean baseline paired-pulse ratios in untreated and chronic-morphine treated mice injected with DO-ChR2 (untreated: 0.82 ± 0.07, chronic morphine: 0.79 ± 0.05, p = 0.7456, unpaired t test). Lines and error bars represent mean ± SEM.

4.5 Discussion

The goal of this study was to improve our understanding of the mechanisms through which chronic morphine exposure alters MOR signaling at MThal inputs to the DMS. Here, we demonstrated that both MOR-expressing and MOR-lacking MThal projection neurons functionally innervated the DMS. Chronic morphine treatment induced tolerance to subsequent morphine at MOR-expressing terminals but did not significantly alter baseline or morphine-mediated changes in PPR. At MOR-lacking terminals, chronic morphine treatment did not alter the opioid sensitivity or PPR of synaptic transmission.

To determine whether MOR-expressing and MOR-lacking MThal neurons targeted the DMS, we selectively expressed ChR2 in either MOR-expressing and MOR-lacking terminals in *Oprm1-cre* mice using viruses dependent on the presence or absence or cre. Compared to nonselective activation of MThal terminals, opioid-mediated inhibition was enhanced when cre-positive (i.e. MOR-expressing) terminals were selectively excited and abolished when cre-negative (i.e. MOR-lacking) terminals were selectively excited. Thus, when ChR2 was expressed in a nonselective manner at MThal-DMS terminals (i.e. regardless of MOR expression), we observed an intermediate amount of inhibition, suggesting glutamate release was evoked from both neuronal subpopulations. Given the magnitude of the differences in sensitivity of MThal-DMS glutamate release to morphine-mediated inhibition in the three conditions, it appears as though MOR-expressing and MOR-lacking neurons both made up a substantial portion of DMS-projecting MThal neurons. In the majority of cells recorded from mice injected with DIO-ChR2, perfusion of DAMGO, a full agonist, reduced oEPSCs to <20% of baseline, suggesting that MOR-expressing terminals were nearly completely inhibited by activation of MOR with a full agonist. In contrast, DAMGO inhibited oEPSCs to approximately 50% of baseline when ChR2

was constitutively expressed, suggesting that about 60-70% of the oESPC was provided by MOR-expressing MThal terminals while 30-40% may be provided by MOR-lacking terminals. From the fluorescent images of viral injection sites, it appears as though the pattern of expression differed when MOR-expressing versus MOR-lacking terminals were targeted, with greater fluorescence in the ventral portion of MThal with DIO-ChR2 and greater fluorescence in the dorsal portion of MThal with DO-ChR2. Thus, there may be regional heterogeneity in MOR expression within the MThal.

The morphine-mediated decrease in the PPR observed at MOR-expressing terminals supports that oEPSC inhibition was due to a presynaptic effect. This is in agreement with previous literature showing a lack of opioid-mediated inhibition of MThal-DMS oEPSCs in mice lacking presynaptic MORs at these synapses (Adhikary, Jaeckel, et al., 2022) and a DAMGO-mediated increase in PPR at the same synapses (Atwood et al., 2014). Unexpectedly, there were differences in the baseline PPR between MOR-expressing and MOR-lacking terminals. The oEPSCs from MOR-expressing terminals displayed paired pulse facilitation (suggestive of a relatively low release probability) while oEPSCs from MOR-lacking terminals showed paired pulse depression (suggesting higher release probability).

In slices from mice constitutively expressing ChR2, morphine did not significantly increase the PPR as would be expected from presynaptic inhibition of transmitter release. It is possible that this resulted from the depressing nature of MOR-lacking terminals. Application of morphine inhibited glutamate release from facilitating, MOR-expressing inputs, while depressing, MOR-lacking inputs were unaffected. Thus, the morphine PPR may have remained unchanged given that the terminals from which evoked glutamate release would be expected to demonstrate an increase in PPR were also making up a smaller proportion of the

overall oEPSC. One caveat of this study was that the viruses encoding DIO-ChR2 and DO-ChR2 utilized different serotypes and appeared to have different levels of expression, so we cannot rule out the possibility that the differences in baseline PPR between oEPSCs from the two MThal subpopulations were due to these differences in experimental conditions. However, the PPR within each subpopulation was similar between cells with a range of oEPSC amplitudes, suggesting expression level likely did not contribute to differences in PPR. This is further supported by the PPR of oEPSCs from non-selectively targeted MThal neurons being intermediate between the PPR of the MOR-expressing and MOR-lacking subpopulations.

Some studies examining chronic opioid effects at presynaptic terminals within various brain regions have demonstrated facilitation, where opioid sensitivity is enhanced (Chieng & Williams, 1998; Hack et al., 2003; Ingram et al., 1998; Pennock et al., 2012). In contrast, other studies have demonstrated tolerance (Fyfe et al., 2010; Lowe & Bailey, 2015; Matsui et al., 2014), including one study demonstrating tolerance to oxycodone at thalamic inputs to the dorsal striatum (Atwood et al., 2014) The mechanisms underlying these opposing effects are not clear, although facilitation is often attributed to an upregulation of adenylyl cyclase and tolerance is thought to arise from a canonical uncoupling and downregulation of MORs. In cultured striatal neurons, pre-exposure to DAMGO induced presynaptic opioid tolerance resulting from GRK-mediated phosphorylation and downregulation of MOR (Jullié et al., 2022). We have previously shown that when MThal-DMS terminals were targeted regardless of MOR expression, chronic morphine treatment resulted in facilitation of, rather than tolerance to, morphine signaling (Jaeckel et al., 2024). The motivation for the present study was to expand on this work by uncovering the mechanisms underlying this facilitation; thus, the results presented here were surprising considering they appeared to contradict our previous findings. One interpretation to

address this discrepancy is the presence of multiple opposing adaptations induced by chronic morphine exposure. At MOR-expressing terminals, chronic morphine may drive receptor- or cellular-level adaptations that decrease the sensitivity of these terminals to morphine (such as receptor downregulation or reduced receptor-effector coupling), while simultaneously driving circuit-level plasticity that increases the relative contribution of MOR-expressing terminals to overall glutamatergic MThal-DMS innervation.

Circuit level plasticity could be attributed to any combination of pre- or postsynaptic adaptations in synaptic transmission from MOR-expressing or MOR-lacking MThal neurons. Because baseline PPRs of the oEPSCs were not altered following chronic morphine treatment in either MThal subpopulation, plasticity was probably not due to changes in presynaptic compartments. One possible adaptation that would increase the relative contribution of glutamate release from MOR-expressing MThal neurons to the overall oEPSCs is the induction of silent synapses selectively at MOR-lacking terminals. In the nucleus accumbens, chronic morphine has been shown to induce silent synapses at D2-expressing MSNs through internalization of AMPA receptors at previously existing synapses (Graziane et al., 2016). If silent synapses were generated at MOR-lacking terminals through a similar mechanism, we would expect a decrease in the baseline oEPSC amplitude in morphine treated mice injected with DO-ChR2. We did not observe this, however in our experiments we did not distinguish between D1- and D2-expressing MSNs, nor did we account for variability in viral expression levels between mice. Thus, comparing raw baseline oEPSC amplitude between treatment conditions was not a reliable measure.

Other potential circuit-level adaptations include an increase in synaptic strength at MOR-expressing terminals and/or decrease in synaptic strength, but not silencing, at MOR-lacking

terminals. This could be investigated by measuring the AMPA:NMDA ratio in saline versus chronic morphine treated mice selectively at MOR-expressing and MOR-lacking terminals. Future studies examining whether MThal-DMS synaptic plasticity is specific to D1- or D2-expressing MSNs would provide a deeper understanding of how chronic morphine-induced adaptations within these projections alter overall striatal function, as D1- and D2-expressing MSNs mediate opposing effects on striatal output and behavior (Kravitz et al., 2012; Lobo & Nestler, 2011). Chronic morphine-induced increases in relative strength of MOR-expressing to MOR-lacking inputs could also explain why morphine perfusion increased the PPR in chronic morphine treated, but not saline treated, mice injected with ChR2 given that morphine perfusion increases the PPR at MOR-expressing terminals.

Our justification for using only male mice in this study was that facilitation at MThal-DMS terminals was observed previously only in males. However, given the results of the present study and the interpretation that chronic morphine exposure induces opposing receptor/cellular- and circuit-level adaptations, repeating these experiments in female mice would be valuable in determining which effects are sex-specific. It is possible that when MThal-DMS terminals are non-selectively activated, opposing chronic morphine-induced adaptations in females are equal in magnitude, thus no apparent changes in subsequent morphine signaling are observed. Alternatively, both receptor/cellular and circuit-level adaptations may be absent in females. It is unclear why male *Oprm1-cre* mice injected with ChR2 in this study did not develop morphine facilitation following chronic morphine treatment, as was previously observed in male wild-type mice injected with ChR2. One possibility is variability in MOR expression levels within MThal-DMS terminals in the *Oprm1*-cre mice due to the insertion of a T2A-EGFP-Cre at the end of exon 4 of the *Oprm1* gene (Bailly et al., 2020). This is consistent with the highly variable degree

of oEPSC inhibition by morphine between cells in naïve and saline MOR-cre mice that we did not observe in WT mice in our previous study. However, we interpreted the tolerance at MOR-expressing MThal-DMS terminals in *Oprm1*-cre mice to be physiologically relevant despite the lack of an effect in *Oprm1*-cre mice constitutively expressing ChR2.

Overall, the findings presented in this study demonstrate the synapse-specificity with which opioids modulate glutamatergic MThal-DMS signaling. Chronic morphine exposure appears to induce simultaneous, opposing adaptations at MOR-expressing terminals and within the circuit at large. Understanding the precise mechanisms driving these adaptations will improve our understanding of how prolonged opioid exposure influences opioid-mediated behaviors.

Chapter 5
Discussion

5.1 Summary, significance, and overall interpretations

The overarching goal of this dissertation was to determine how chronic morphine exposure differentially alters subsequent MOR signaling within different subcellular compartments of glutamatergic thalamo-cortico-striatal circuitry and the underlying cellular adaptations that drive these effects. Despite the relevance of this circuitry to both pain and reward processing, acute and chronic opioid effects here are not well-studied. Furthermore, the high levels of MOR expression and broad axonal projections of MThal neurons provide a system in which chronic opioid effects between different subcellular compartments of one neuronal population can be dissected and studied. Overall, this work contributes to our understanding of the intricacies with which chronic opioid exposure alters CNS physiology and the findings presented here can be used to guide future studies examining how receptor-, cellular-, and circuit- level adaptations give rise to behavioral outcomes.

Given the role of thalamo-cortico-striatal circuitry in pain processing and the tolerance that develops to the analgesic effects of opioids, we initially hypothesized that chronic morphine treatment would induce cellular tolerance at each subcellular locus we investigated. We also hypothesized that the effects of chronic morphine treatment would not be present in MOR phosphorylation-deficient 10 S/T-A mice given the role of this process in regulating signaling through receptor internalization and mediating opioid analgesic tolerance (Kliewer et al., 2019; Williams et al., 2013). In chapter 2, we demonstrated that chronic morphine treatment induced

cellular tolerance (i.e. decreased responses to subsequent morphine) at MThal cell bodies in males and females but facilitation (i.e. enhanced responses to subsequent morphine) at MThal-DMS terminals in males, with no chronic morphine-induced changes in females. In chapter 3, we demonstrated that chronic morphine treatment induced cellular tolerance at MThal-ACC terminals within the excitatory pathway in both males and females but tolerance at terminals within the feedforward inhibitory pathway only in males. Our observations at MThal cell bodies and MThal-ACC projections were generally in line with our initial hypothesis. This contrasts with the facilitated morphine effects observed at MThal-DMS synapses in Chapter 2, which did not support this hypothesis.

From the results we could not definitively determine whether the facilitation observed at MThal-DMS terminals was due to receptor-, cellular-, or circuit-level adaptations. To help clarify where, specifically, adaptations within this circuit occurred to drive overall facilitation, in chapter 4 we asked whether both MOR-expressing and MOR-lacking subpopulations of MThal neurons innervated the DMS and, if so, whether facilitation persisted when MOR-expressing terminals were selectively excited. If facilitation was driven by an enhancement in presynaptic MOR signaling or baseline presynaptic excitability (for example, through an upregulation of adenylyl cyclase), we would expect to observe more robust facilitation when measuring morphine effects selectively at MOR-expressing terminals. In contrast, if facilitation was driven by adaptations within the greater circuit (for example, an increase in the relative strength of MOR-expressing inputs compared to MOR-lacking inputs), we would not expect to observe facilitation when MOR-expressing terminals were selectively targeted. To gain insight about where adaptations occurred within the circuit to drive facilitation, we utilized *Oprm*-cre mice to selectively target either MOR-expressing or MOR-lacking MThal-DMS terminals. Surprisingly,

in contrast to the facilitation previously observed when MThal-DMS terminals are nonselectively excited, we found that chronic morphine induces tolerance, rather than facilitation, at MOR-expressing terminals while sparing MOR-lacking terminals. Taken together, these findings suggest two simultaneous processes are occurring: 1) Receptor- and/or cellular-level tolerance, and 2) circuit-level counteradaptations that enhance MOR signaling.

Blocking MOR phosphorylation has been shown to prevent cellular tolerance in other systems, likely by decreasing agonist-stimulation receptor internalization (Arttamangkul et al., 2018; Birdsong et al., 2015; Leff et al., 2020; Lowe et al., 2015). Previous studies which have found presynaptic facilitation have typically attributed these effects to an upregulation of AC resulting from persistent inhibition by morphine (Chieng & Williams, 1998; Hack et al., 2003; Ingram et al., 1998). Due to the lack of phosphorylation-mediated receptor uncoupling and internalization, we would expect AC upregulation to be exacerbated following chronic morphine treatment in 10 S/T-A mice in our studies. Thus, if AC upregulation was the mechanism underlying facilitation at MThal-DMS terminals, we would expect to see more robust facilitation in 10 S/T-A mice resulting from greater AC upregulation. In this context, the observation of MThal-DMS tolerance in 10 S/T-A mice is another indication that the adaptations driving facilitation in wild-type mice were not occurring at presynaptic MORs or morphine-sensitive terminals. Given the relatively small magnitude of tolerance at MThal cell bodies in wild-type mice, we did not investigate chronic opioid effects in 10 S/T-A mice at this locus, but we would expect tolerance here to be eliminated as well given that mice expressing phosphorylation-deficient receptors do not develop somatic tolerance within other neuronal populations (Arttamangkul et al., 2018; Arttamangkul et al., 2019).

5.2 Sex differences in the effects of chronic morphine on MThal-DMS and MThal-ACC MOR signaling

Sex differences in opioid analgesia and tolerance are relatively well-established in rodents and humans, and the neurobiology underlying these differences is a focus of some opioid research (Averitt et al., 2019; Kest et al., 2000). However, previous electrophysiological studies investigating acute and chronic opioid effects typically have only used male mice or used both sexes but not reported statistical comparisons between sexes. In our studies, we found morphine facilitation at MThal-DMS terminals and tolerance within the excitatory MThal-ACC circuit in males, but not females. Given the lack of sex differences in the literature, the sex-specific effects observed in our study were unexpected, particularly the complete lack of MThal-DMS facilitation in female mice considering the robustness of this effect in males. These effects add a layer of complexity to the interpretation of our data, and further investigation is required to uncover the physiology underlying these differences.

Because morphine facilitation at MThal-DMS terminals only occurred in males in our initial study in chapter 2, we did not include females in our experiments in chapter 4 testing whether facilitation persists when MOR-expressing terminals are selectively targeted. However, considering the observation that at these MOR-expressing terminals, chronic morphine treatment induces tolerance, rather than facilitation, performing these experiments in females is of interest, as it is possible the lack of facilitation observed in chapter 2 results from adaptations that are equal in magnitude but opposite in direction (i.e. adaptations that drive the same degree of tolerance and facilitation). Observing tolerance in females at MOR-expressing terminals would indicate this may be the case and would warrant further investigation of chronic morphine-induced adaptations at these terminals. Alternatively, a lack of tolerance would indicate that no

adaptations in either direction occur in females here. However, this seems less likely given that female 10 S/T-A mice develop a small degree of tolerance, thus the balance between adaptations and counteradaptations appear shifted in these mice. There may be differences in MOR expression in thalamic neurons that would make facilitation here more difficult to observe. For example, given that tolerance occurred at MOR-expressing MThal-DMS terminals, if a greater proportion of the total MThal population consisted of MOR-expressing, compared to MOR-lacking neurons, we would expect tolerance to be more difficult to observe. However, this would not account for the decreased baseline morphine sensitivity, nor the lack of tolerance at MThal-ACC terminals, within the inhibitory pathway in females.

It is possible that the susceptibility of these terminals to morphine tolerance and/or facilitation is influenced by gonadal hormones and estrous phase. For MThal-DMS recordings, we recorded postsynaptically from MSNs, the output neurons of this region. These neurons express membrane-associated estrogen receptors (Almey et al., 2015) and there is a growing body of literature to suggest MSN intrinsic and synaptic properties vary throughout the estrous cycle. For example, during the proestrus and estrus phases (the phases associated with increased estradiol levels), spontaneous excitatory synaptic inputs are enhanced, and intrinsic excitability is decreased compared to males and diestrus female rats (Proaño et al., 2018). It should be noted, however, that most of these studies were conducted in the nucleus accumbens, with few studies examining differences in the properties of DMS MSNs across estrous cycle. Given that we cannot rule out postsynaptic adaptations as the driving mechanisms underlying facilitation, MSN properties such as these could contribute to the lack of morphine facilitation at MThal-DMS terminals in females observed in our study.

The sex-specific effects of chronic morphine treatment at MThal-ACC terminals are more difficult to interpret. Morphine tolerance was observed in both sexes at oEPSCs but only in males at oIPSCs, resulting in a sex-dependent effect of morphine on ACC E/I balance. Females have been reported to express higher levels of MOR and have a greater proportion of MOR-expressing neurons than males in the ACC, but the distribution across neuronal subtypes appear similar between sexes (Zamfir et al., 2023; Zubieta et al., 1999). Thus, the lack of tolerance in females may suggest a loss of functional receptors that is not sufficient to produce a physiological change in morphine sensitivity. Another explanation is that, like our proposed mechanism at MThal-DMS terminals, tolerance at MThal-ACC oIPSCs is not observed in females due to equal and opposite counteradaptations occurring elsewhere in the circuit. Glutamatergic synaptic plasticity has been demonstrated within the ACC (Bliss et al., 2016), although this has not been specifically demonstrated in the context of chronic opioid exposure. One caveat is that oIPSCs appeared less sensitive to morphine inhibition in drug-naïve females than males (although this effect did not reach statistical significance), which could provide an alternative explanation for the lack of a tolerance effect in females at oIPSCs in our experiments.

Ovarian hormones and estrous cycle also appear to play a role in behavioral opioid tolerance. In intact female rats, one study found opioid analgesic tolerance to a morphine injection was most pronounced during the proestrus phase, suggesting an influence of estrous cycle on tolerance development behaviorally in females (Shekunova & Bespalov, 2004). One limitation of our study is that we did not track estrous cycle in our study; thus, we cannot determine if estrous cycle influenced MThal-DMS or MThal-ACC morphine sensitivity in drug-naïve or chronically treated mice. Understanding the role of gonadal hormones in chronic morphine effects within our circuit or interest could be addressed in a couple of ways. First,

morphine minipumps could be implanted during certain estrous phases to determine whether the timing of morphine treatment relative to estrous phase plays a role in the induction of tolerance and/or facilitation. Additionally, chronic morphine effects at MThal-DMS and MThal-ACC terminals could be measured in gonadectomized male and female mice to gain a more complete understanding of the role of these hormones in the effects observed in this study. If tolerance and/or facilitation, observed in intact males but not females in our study, are no longer present in castrated males, this would suggest a requirement of testosterone. Conversely, if the effects are present in ovariectomized females, this would suggest a protective role of ovarian hormones. Overall, the findings presented in this work highlight the importance of considering sex as a biological variable in animal research.

5.3 The role of MOR phosphorylation in mediating chronic morphine effects within thalamo-cortico-striatal circuitry

Our motivation to investigate MOR phosphorylation as a mediator of chronic morphine-induced adaptations at MThal presynaptic terminals was that this process is considered a critical mediator of receptor tolerance and hypothesized to drive opioid analgesic tolerance behaviorally. Following agonist activation, MOR C-terminal phosphorylation recruits β-arrestin, uncouples receptors from associated G proteins, and causes initiates receptor internalization (Williams et al., 2013). In 10 S/T-A mice, which possess phosphorylation-deficient receptors, morphine analgesic tolerance is attenuated while and fentanyl tolerance is eliminated (Kliewer et al., 2019). However, given this role of phosphorylation, it is puzzling why chronic morphine-induced facilitation at MThal-DMS terminals was eliminated in 10 S/T-A mice, as we expected it only to eliminate cellular tolerance (as was observed at MThal-ACC terminals).

Interestingly, the increased morphine sensitivity at MThal-DMS terminals but decreased sensitivity at MThal-ACC terminals in drug-naïve 10 S/T-A mice resembled the effects of chronic morphine treatment in WT male mice. This suggests that some chronic morphine-induced alterations in MOR signaling in morphine-treated WT mice may be present in drug-naive10 S/T-A mice. For example, in the DMS, the increased opioid sensitivity of MORs in 10 S/T-A mice could result in circuit-level plasticity occurring from endogenous opioid signaling in the absence of chronic morphine treatment. Likewise, endogenous opioid signaling at MThal-ACC terminals could drive adaptations that decrease the sensitivity of the terminals to opioid-mediated inhibition of glutamate release.

The observation that drug-naïve 10 S/T-A mice were more sensitive to morphine inhibition of MThal-DMS synapses but appeared less sensitive at MThal-ACC synapses may also indicate deficiencies of these receptors in axonal trafficking. MThal-DMS terminals are more proximal to the MThal soma and MThal-ACC terminals are more distal; thus, receptors must be trafficked from the soma, through the DMS, and further along the axon to reach MThal-ACC terminals. Thus, another potential explanation for the differences in baseline morphine sensitivity at MThal-DMS versus MThal-ACC terminals in these mice is that, because 10 S/T-A receptors have impaired trafficking properties, 10 S/T-A receptors may be less densely expressed at the distal ACC terminals compared to WT receptors, while accumulating more proximally along the axon (such as at collateral projections to the DMS). While phosphorylation is known to play a role in trafficking membrane receptors (both pre- and postsynaptically) through the endocytic pathway, this process may also be involved in trafficking receptors out to the presynaptic terminal.

Some GPCRs, such as the cannabinoid type 1 (CB1) and serotonin $5HT_{1B}$ receptors, are preferentially expressed within axon terminals compared to the soma (Engel et al., 1986; Irving et al., 2000). Two proposed models for how these receptors maintain their axonal polarity involve endocytosis. One is through transcytosis, a process whereby receptors are first expressed at the somatodendritic membrane, then rapidly internalized through constitutive endocytosis, and transported to the axon terminal through endosomes. The other is through nonpolarized delivery to the plasma membrane, followed by compartment-specific endocytosis to remove improperly sorted receptors (Winckler & Mellman, 2010). Inhibiting endocytosis shifts the surface expression of $5HT_{1B}$ (Carrel et al., 2011) and CB1 (Leterrier et al., 2006) receptors from the axonal to somatodendritic compartments, suggesting that endocytosis is necessary for the expression of GPCRs within axon terminals. MORs display measurable amounts of basal activity in the absence of agonist (Burford et al., 2000; Connor & Traynor, 2010) and presumably undergo some level of endogenous opioid activation under normal physiological conditions *in vivo*. While MORs are functionally expressed in both somatodendritic and presynaptic compartments rather than axonally polarized, it is conceivable that constitutive endocytosis is a mechanism through which MORs are delivered to presynaptic terminals and that this process is disrupted in mice with phosphorylation site mutations.

Microfluidic culture chambers allow for the separation of somatic and axonal compartments of cultured neurons via a barrier and can be used to investigate the distribution of receptors within the axonal compartment. This technology could be utilized to investigate differences in axonal trafficking of WT and 10 S/T-A receptors. To better understand whether the differences in morphine sensitivity observed at MThal-DMS and MThal-ACC terminals in 10 S/T-A mice can be attributed to trafficking deficiencies, primary thalamic neurons from WT and

10 S/T-A mice could be cultured and grown in a microfluidic chamber. Axonal trafficking would be assessed by quantifying the number of fluorescently labeled receptors at different distances from the soma. If 10 S/T-A receptors exhibit trafficking deficiencies, then receptors would be expected to accumulate closer to the soma relative to WT receptors.

5.4 Chronic morphine-induced synaptic plasticity within the DMS

In light of what we demonstrated in chapter 2, the results presented in chapter 4 suggest the presence of multiple adaptations in MThal-DMS MOR signaling following chronic morphine treatment. On one hand, chronic morphine treatment drives tolerance at MOR-expressing MThal-DMS synapses. This could be due to receptor-level changes, such as downregulation or decreased receptor-effector coupling, or cellular level changes, such as altered downstream signaling pathways. On the other hand, chronic morphine treatment also drives adaptations resulting in overall facilitation elsewhere within the circuit. We propose that these adaptations increase the relative strength of innervation of MOR-expressing to MOR-lacking MThal-DMS inputs. This would drive overall facilitation when MThal inputs are nonselectively excited because a greater proportion would be inhibited by morphine, thus resulting in greater inhibition of oEPSCs in morphine treated mice. From our data we cannot determine the nature or location of these adaptations but there are multiple possibilities.

One possibility is that chronic morphine treatment drives adaptations at MOR-lacking terminals that decrease synaptic efficacy within this subpopulation. Chronic morphine treatment has been shown to generate silent synapses (at which presynaptic neurotransmitter release does not elicit a postsynaptic response) at MSNs within the nucleus accumbens through the removal of AMPA receptors at pre-existing synapses (Graziane et al., 2016). Silent synapse generation

through this mechanism specifically at MOR-lacking MThal-DMS terminals would be consistent with our proposed model, as it would result in a proportionally greater number of functional MOR-expressing synapses in chronically treated mice. Proportional decreases in innervation by MOR-lacking terminals could also be achieved through weakening, but not silencing, of the synapses through a similar downregulation of postsynaptic AMPA receptors, as has been shown in other brain regions (Kam et al., 2010). If a weakening or silencing of MOR-lacking synapses was responsible for the facilitation observed when terminals were excited non-selectively, then we would expect smaller baseline response amplitudes in chronic morphine treated mice at MOR-lacking terminals. In our experiments we did not observe any changes in baseline oEPSC amplitudes between untreated and chronically treated mice, however we cannot rule this out as a possibility given that differences in response amplitudes were not a reliable measure in our study. This is because we did not control for variability in viral expression due to factors such as injection quality or the number of incubation days between viral injection and recording. Additionally, we initially targeted responsive cells rather than remaining agnostic as to whether cells responded. Thirdly, we did not distinguish between D1- and D2-expressing MSNs, which may differ in how they develop LTD following chronic morphine exposure (Graziane et al., 2016). Thus, to accurately measure changes in response amplitudes between treatment conditions these factors would need to be better controlled for. Alternatively, because we predict these adaptations would be mediated through changes in postsynaptic AMPA receptor expression, a more reliable method of investigating this is to measure the baseline postsynaptic AMPA:NMDA ratio or the amplitude of evoked quantal oEPSCs from MOR-lacking synapses in saline and chronic morphine treated mice. A decrease in either of these measures would indicate a synaptic weakening.

Another possibility is that chronic morphine treatment drives adaptations to increase synaptic efficacy within the MOR-expressing subpopulation. One way this could be achieved is through a presynaptic enhancement in neurotransmitter release probability, such as in the case of AC upregulation (Kaneko & Takahashi, 2004). However, as previously discussed, we do not believe this is the case given that in 10 S/T-A mice, facilitation was not enhanced when MThal-DMS terminals were non-selectively activated, and that tolerance, rather than facilitation, occurred when MOR-expressing inputs were targeted. Additionally, chronic morphine treatment did not alter the baseline PPR at MOR-expressing terminals, suggesting that release probability here was unaltered. Increased synaptic efficacy could also be achieved at MOR-expressing terminals through a postsynaptic increase in AMPA receptors. This would be more in line with our findings overall. An increase in synaptic efficacy through this mechanism, with no change or a decrease in synaptic efficacy at MOR-lacking terminals would be expected to result in facilitation overall (because innervation by MOR-sensitive terminals would be proportionally greater) but would not be expected to alter sensitivity specifically at MOR-expressing terminals. Like at MOR-lacking terminals, this could be tested by measuring the AMPA to NMDA ratio selectively at MOR-expressing terminals, with an increase in this ratio indicating an AMPA receptor-dependent synaptic strengthening.

5.5 Agonist- and dosing-specificity of chronic opioid effects on thalamo-cortico-striatal MOR signaling

At the behavioral level, studies have typically found opioid analgesic tolerance to develop more robustly with low efficacy agonists (Madia et al., 2009; Pawar et al., 2007; Sosnowski & Yaksh, 1990). However, these studies have the caveat that tolerance is typically

tested for using the same agonist used for chronic treatment, and the relationship between agonist efficacy and the magnitude of cross-tolerance may differ given that shifts in the dose-response are easier to observe with partial agonists. Chronic treatment with fentanyl produces greater cellular tolerance to subsequent morphine than what is seen following chronic treatment with morphine (Adhikary, Koita, et al., 2022; Levitt & Williams, 2012). Given the apparent agonist-specificity of cellular and opioid analgesic tolerance, there may be differences in the magnitude of tolerance and/or facilitation induced by different agonists within the thalamo-cortico-striatal circuit investigated in this work. Thus, future studies investigating the effects of chronic treatment with various agonists within this circuit would provide insight as to whether the presynaptic and circuit-level adaptations are also agonist-specific. Considering fentanyl and its analogs are highly abused and oxycodone is widely used in the clinic, these agonists are highly relevant to clinical and illicit opioid use.

Previous studies have also demonstrated that cellular adaptations at the soma and opioid analgesic tolerance induced by chronic opioid exposure are agonist specific. In rat locus coeruleus neurons, continuous infusion of oxycodone does not induce cellular tolerance (measured by a reduction in morphine-activated potassium conductance), while morphine induces an intermediate amount of tolerance and fentanyl induces robust tolerance (Adhikary, Koita, et al., 2022; Levitt & Williams, 2012). Treatment with morphine and oxycodone, but not buprenorphine or fentanyl, results in an inability of GRK2/3 inhibitors to block acute desensitization of the somatostatin receptor, a nonopioid GPCR. This suggests opioid agonist-specific regulation of kinase signaling in addition to the well-characterized differences in receptor-level adaptations.

The development of tolerance and sensitization is also highly dependent on whether drugs are continuously or intermittently administered. Continuously administered opioids produce greater opioid analgesic tolerance than intermittently administered opioids at comparable doses, and this correlation holds up for low and high efficacy agonists (Dighe et al., 2009; Kumar et al., 2008; Madia et al., 2009). In contrast, locomotor sensitization develops more robustly following intermittent administration (Le Marec et al., 2011; Post, 1980; Vanderschuren et al., 1997). Withdrawal severity is correlated with the magnitude of locomotor sensitization, suggesting these periods of withdrawal may precede the development of sensitization (Rothwell et al., 2010). In humans, illicit opioid use is intermittent, cycling between periods of drug use and abstinence/withdrawal, thus an intermittent model of chronic treatment would be more relevant drug-taking in humans.

We have conducted preliminary studies examining how agonist and dosing regimen affects the facilitation observed at MThal-DMS terminals in wild-type mice. First, we measured morphine-mediated inhibition of MThal-DMS terminals following continuous infusion of fentanyl. Unlike morphine, mice chronically treated with fentanyl did not show increased inhibition of MThal-DMS terminals by subsequent morphine perfusion relative to saline-treated mice (Fig 5.1A**;** saline: 69.10 ± 4.11% of baseline; chronic fentanyl: 63.15 ± 4.06% of baseline; n = 10 for each group; $t_{(18)} = 1.030$, p = 0.3167, unpaired t test). Given our interpretation that at these terminals, both tolerance and facilitation are simultaneously occurring (but in varying magnitudes), the apparent lack of facilitation may result from increased fentanyl-induced tolerance at MOR-expressing terminals. This would be in line with the previous findings in LC neurons that chronic fentanyl produces greater morphine tolerance than chronic morphine (Adhikary, Koita, et al., 2022; Levitt & Williams, 2012). Thus, the balance between tolerance

and facilitation could be shifted such that no facilitation nor tolerance is observed. This hypothesis could be tested by measuring morphine-mediated inhibition of MOR-expressing MThal-DMS oEPSCs following chronic treatment with fentanyl. Observing greater tolerance following chronic fentanyl treatment compared to chronic morphine treatment would support this hypothesis. On the other hand, observing no tolerance following chronic fentanyl treatment would indicate that neither adaptation occurs with fentanyl. These experiments were conducted only in male mice, but females should also be included in the dataset to determine if any potential effects are sex-dependent.

Second, we measured morphine-mediated inhibition of MThal-DMS oEPSCs following chronic morphine treatment using an intermittent dosing regimen of seven days of twice daily injections escalating from 10-50 mg/kg/injection. Mice receiving intermittent morphine treatment showed increased inhibition of MThal-DMS terminals by subsequent morphine perfusion relative to untreated mice (Fig 5.1B; untreated: 72.26 ± 2.44% of baseline, n = 21; intermittent morphine: 58.23 ± 4.28% of baseline, n = 7; $t_{(26)} = 2.87$, p = 0.008, unpaired t test). Mice of both sexes were included in this dataset and 5/7 data points were collected from morphine treated female mice, suggesting a potential overall facilitation effect in both males and females, unlike the sex-dependent effect observed with continuous morphine treatment. One explanation we proposed as to why females did not show facilitation was that both receptor/cellular tolerance and circuit-level facilitation occurred in females but were equal in magnitude. In this case, the presence of facilitation in females with intermittent treatment might indicate that this dosing regimen produces greater facilitation in both sexes compared to continuous treatment. This hypothesis would align with behavioral studies showing opioid analgesic tolerance is more pronounced with continuous exposure but locomotor sensitization is

more pronounced with intermittent exposure (Dighe et al., 2009; Le Marec et al., 2011; Rothwell et al., 2010). To further explore this question, complete datasets should be generated with intermittent morphine treatment in both sexes and with nonselective activation or MThal-DMS terminals and selective activation of MOR-expressing terminals. If, in females, intermittent morphine induces facilitation at nonselective terminals but little/no tolerance at MOR-expressing terminals, this would support our proposed mechanism that the overall effects of chronic opioid treatment within this circuitry result from a balance of both receptor/cellular tolerance and circuit-level facilitation. Additionally, saline-injected, rather than untreated, mice should be used as a proper control.

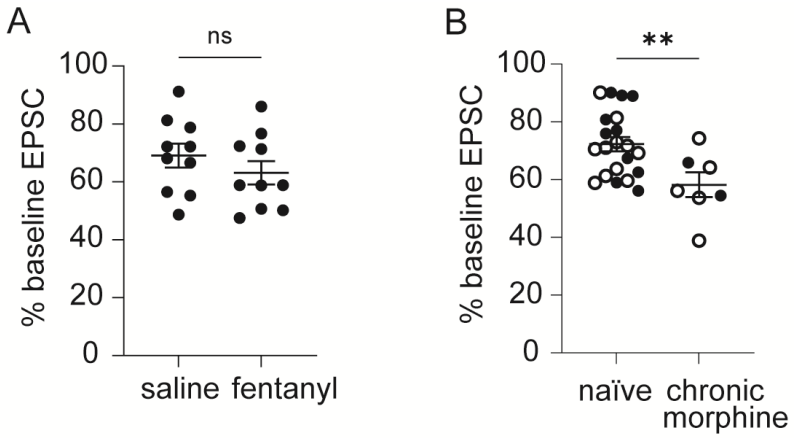

Figure 5.1. Agonist- and dosing-specificity of MThal-DMS facilitation. **A.** Mean inhibition of MThal-DMS EPSCs by perfusion of morphine in cells from mice treated with saline or chronic fentanyl (2 mg/kg/day) for seven days via continuous infusion. **B**. Mean inhibition of MThal-DMS EPSCs by perfusion of morphine in cells from untreated mice or mice treated with intermittent morphine (twice-daily injections for seven days escalating from 10-50 mg/kg/injection). Closed circles denote cells recorded from males and open circles denote cells recorded from females. Lines and error bars represent mean ± SEM. **p<0.01.

5.6 Final thoughts

Taken together, the data presented in this dissertation suggest that within glutamatergic thalamo-cortico-striatal circuitry, chronic opioid exposure can induce multiple simultaneous, yet opposing adaptations that alter MOR signaling. The data presented here are unique in that they dissect the effects of chronic opioid exposure on subsequent MOR signaling within different subcellular compartments of one neuronal population, highlighting the complexity with which opioids alter physiology within the CNS. The behavioral effects of opioid use (both acutely and chronically) can be thought of as the outcome of the sum of the receptor- and cellular-level effects throughout the entire nervous system, and this work provides one piece of the puzzle as to how opioids ultimately produce their effects. Given the role of the brain regions studied here in mediating both pain and addiction processes, this work provides novel insight into how different neural systems can be targeted to enhance or diminish certain opioid effects to manipulate behavioral outcomes. Future studies could build on this work by correlating opioid signaling within specific loci of this circuit to opioid-mediated behaviors such as analgesia, locomotor stimulation, or place preference. The ubiquity with which opioids act in the brain is one of the challenges associated with developing opioids that are safer and more effective than what is currently available. However, the need for these therapeutics is greater than ever given the state of the opioid crisis. Targeting MORs within certain pathways while sparing others is one potential avenue in which novel opioids could preferentially mediate analgesia with fewer adverse effects, and this work may help identify potential neuronal populations or projections to target.

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