ADAPTATIONS FOLLOWING CHRONIC OPIOID TREATMENT IN THE PRESYNPATIC AND POSTSYNAPTIC COMPARTMENTS OF NEURONS

By

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CERTIFICATE OF APPROVAL

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ABSTRACT

Opioids such as morphine, oxycodone, and fentanyl effectively relieve acute and post-operative pain, but long-term use can lead to tolerance. Chronic opioid use leads to cellular and circuit level adaptations that mediate tolerance, but how different opioids can differentially regulate the activity of the μ opioid receptor (MOR) and downstream effectors is not known. Additionally, MORs have different regulatory mechanisms based on whether they are in the somatodendritic or presynaptic terminal compartment. The mechanisms that mediate tolerance in the presynaptic compartment are also unknown.

The goal of this dissertation is to advance our understanding of tolerance induced by opioids in both the somatodendritic and presynaptic terminal compartments. It is known that chronic morphine treatment inhibits MOR mediated effects and also disrupts kinases that mediate acute desensitization. Though morphine causes very little acute desensitization, it is still able to induce profound behavioral and cellular tolerance. In contrast, fentanyl causes robust acute desensitization, but the development of behavioral tolerance is slower than that caused by morphine and oxycodone. Therefore, the work presented here compared cellular tolerance induced by oxycodone and fentanyl. Both the regulation of MORs and changes to LC neuron kinase regulation was examined. In slices from untreated animals GRK2/3 inhibits phosphorylation and therefore desensitization of the MORs. The results reveal that chronic oxycodone treatment does not impact MOR regulation but, similar to morphine, alters kinase regulation of somatostatin receptors. In contrast, chronic fentanyl treatment induces profound downregulation of MORs but does not affect GRK2/3 activity. Lastly, the changes to kinase regulation by morphine and oxycodone was due to sustained signaling of MORs from the plasma membrane.

The regulation of MORs in the presynaptic terminal compartment was assessed in thalamic terminals synapsing into medium spiny neurons (MSNs) in the dorsal medial striatum. Activation of MORs in these terminals leads to inhibition of glutamate release. It is known that MORs' ability to inhibit neurotransmitter release does not acutely desensitize. But how these receptors adapt after chronic treatment is largely known. There is also evidence for interaction between MORs and the adenosine system in the striatum, where after chronic treatment, there is increased adenosine release. But how these two systems interact acutely is also unknown. The work presented here first examined how MORs modulate adenosine levels in the striatum. The results show that activation of MORs acutely inhibits adenosine release through their action at the postsynaptic D_1R positive MSNs. The mechanism behind this inhibition is through inhibition of adenyl cyclase activity, and consequently decreased cAMP levels.

However, though MORs acutely regulated adenosine activity, there was no change in adenosine levels after chronic treatment. Additionally, instead of decreasing MOR signaling, chronic morphine treatment facilitated MOR signaling. These results demonstrate how the regulation of somatodendritic and terminal MORs differ after chronic treatment.

In conclusion, it is clear that tolerance at the cellular level is different for different opioid agonists, and for MORs in different compartment of the neuron. There is no one key regulatory process that predicts the level of tolerance after chronic treatment but all adaptations start at the level of the MORs, and thus its regulation must be studied.

Chapter 1 INTRODUCTION

Opioids are powerful analgesics that reduce pain, but long-term use leads to tolerance and dependence, limiting their clinical utility. Opioids mediate both their euphoric and analgesic effects through their action at the μ-opioid receptor (MOR) (Sora et al. 1997; Matthes et al. 1996a)(Sora et al. 1997; Matthes et al. 1996a). Chronic treatment with opioids results in continuous activation of the MORs resulting in changes at both the receptor level and also in downstream signaling molecules. These adaptations include both 1) a decrease in receptor-effector coupling and 2) changes to downstream second messenger systems that can counteract the persistent activation of MORs. Regulation of MORs and cellular mechanisms that mediate these adaptations are thought to be precursors to developing tolerance. However, despite numerous studies identifying crucial mechanisms that contribute to opioid tolerance, no single regulatory mechanism that governs tolerance in both neurons and in intact animals has been identified.

Opioid tolerance is a multifaceted process that involves both individual neurons that contain MORs and neuronal circuits that undergo adaptations following continuous MOR activation. However, given that the most proximal event is agonist binding to the receptor, the mechanisms underlying regulation of MOR itself after chronic treatment is critical to study. Regulation of MORs in the presynaptic region of the neuron to modulate neurotransmitter release is vastly different from the mechanisms regulating MORs in the somatodendritic compartment (Blanchet and Lüscher 2002a; Fyfe et al. 2010a; Pennock and Hentges 2011; Jullié et al. 2020a). Additionally, recent evidence suggests that

chronic opioid treatment can not only lead to homologous adaptations to the MORs, but also heterologous adaptations to other $G_{i/o}$ coupled GPCRs (Leff, Arttamangkul, and Williams 2020a). And lastly, different opioid agonists with varying potencies and efficacies exert their effects on the MOR activation and regulation, and thus tolerance, differentially (Virk and Williams 2008; Quillinan et al. 2011). Therefore, the focus of this dissertation is to examine the mechanisms that mediate tolerance after chronic treatment in both somatodendritic and presynaptic compartments. Specifically, tolerance induced by five clinically relevant and frequently prescribed opioid agonists is examined in the somatodendritic compartment in the locus coeruleus. Next, both direct effects on the presynaptic MORs and indirect effects through second messengers to regulate synaptic transmission by acute and chronic morphine treatment in the striatum is also described.

This introduction will begin with summarizing the current state of opioid crisis facing the United States. Next, mechanisms that mediate opioid regulation will be summarized, followed by a summary of recent advances in discovering mechanisms that mediate cellular tolerance, and how cellular changes can be contextualized with whole animal tolerance.

Opioid Crisis and Addiction

The first mention of opioid as analgesics was in the Odyssey when Helen, the daughter of Zeus, gave opium to Telemachus and his friends to alleviate their suffering over Odysseus' absence. The Sumerians and the Mesopotamians used poppies and opium in

the early 300 B.C., and when trading expanded, Arab traders brought opium to India and China, and eventually, between tenth and 13th century, opium made its way to Europe (Brownstein 1993). With opium came abuse and tolerance. In 1806, Friedrich Wilhelm Adam Serturner extracted the active ingredient in opium and named it morphine. Starting in the 1850s, morphine was used as adjunct to general anesthetics for surgical procedures, but was soon to be found to have high abuse liability. The search for nonaddictive analgesics started towards the end of the century, and heroin was synthesized as a safer alternative (Cox 2020). The search for compounds that provide effective analgesia with a reduced side-effect profile continues to this day, but with limited success. Consequently, there are now numerous opioid agonists with varying potencies and differing pharmacodynamics that are regularly used in clinical settings.

Opioids continue to be the most effective analgesics to mediate acute pain and are widely used as adjunct to anesthetics in surgical procedures and to manage post-operative pain. However, though millions of Americans suffer from chronic pain – persistent pain lasting more than 6 months – opioids do not mitigate chronic pain and can contribute to hyperalgesia. The American Pain Society in 1995 declared pain as the fifth vital sign to improve the treatment outcomes for patients with pain (Max et al. 1995). Coinciding with this new guideline, between 1997 and 2002, the use of oxycodone, fentanyl, and morphine increased by 403%, 227%, and 73% respectively (Gilson et al. 2004). However, even with an increase in opioid prescription, chronic pain continues to be under treated. An unforeseen consequence of this increased opioid use has now led to an opioid epidemic. Currently, the United States is battling an opioid crisis, with more than 130

people dying every day after overdosing on opioids (Dowell, Haegerich, and Chou 2016). Up to 30 percent of patients prescribed opioids for chronic pain misuse them and 12 percent develop and opioid use disorder (Dowell, Haegerich, and Chou 2016). Opioids are highly addictive, and as a chronic, relapsing disorder, opioid addiction not only affects the individual patient, but also has significant public health and economic costs. In addition to mortality, opioid abuse costs $78.5 billion annually, with over one third of this amount spent in increased health care and substance abuse treatment costs (Florence et al. 2016). Opioid agonist therapy with either methadone, buprenorphine, or buprenorphine/naloxone combination (Suboxone) is currently the most effective treatment modality for opioid dependence (Mattick et al. 2014). Agonist therapy helps alleviate the withdrawal symptoms associated with cessation of opioid use by occupying the MORs. Long-term treatment with these agonists has proven effective, safe, and has led to improved health outcomes (Joranson et al. 2000). Though dependence to opioids might not cease, these treatment modalities lead to enriched overall functioning.

Tolerance is defined as an increase in the dose of opioids required to maintain the same therapeutic analgesia. Long-term opioid use can also lead to dependence, whereas abrupt cessation from drug use results in severe physiological and psychological withdrawal symptoms. However, opioid addiction is a complex disease that not only involves tolerance, but also other symptoms that lead to recurring substance use despite the negative consequences to one's life. Therefore, understanding the mechanisms behind tolerance is only a starting point to appreciate the pathogenesis of opioid use disorder and understand the complexity of addiction as a brain disease.

Structure and Function of Opioid Receptors

Structure

The first demonstration of the presence of opioid receptors in the nervous system was done independently in 1973 by the research groups of Solomon Snyder in Baltimore, Eric Simon in New York, and Lars Terenius in Sweden (Cox 2020). The cloning of the MOR, δ-opioid receptor (DOR), K-opioid receptor (KOR), and the orphanin FQ/nociception receptor (OFQ) in the 1990s marks another key milestone in understanding the structure and function of these receptors. All four receptors are class A (Rhodopsin) family of $G_{i/o}$ coupled GPCRs with seven transmembrane helical domains with the N-terminus on the extracellular side and C-terminus on the intracellular side. However, because the MOR mediates analgesia, tolerance, and reward, the focus will be on this receptor (Sora et al. 1997; Matthes et al. 1996a). MORs are encoded by a single structural gene (*OPRM1*), but there is evidence for alternatively sliced variants of the mRNA resulting in polymorphisms. The roles of these variants are minimally known with several having no known cellular activity, while some underlie different behaviors, such as tolerance and MOR's ability to internalize. The X-ray crystal structure of MOR in its inactive conformation bound to its antagonist (Manglik et al. 2012) and in its active conformation bound to DAMGO has been reported (Koehl et al. 2018). Compared to most other GPCRs with buried binding pocket, MOR seems to have a large solvent-exposed pocket. Additionally, DAMGO occupies the ligand pocket with its N-terminus interacting with conserved receptor residues, and its C-terminus engaging regions important for opioid-ligand selectivity. Lastly, similar to other active GPCRs, agonist bound MOR causes an outward shift of transmembrane helix (TM) 6 (Huang et al. 2015).

Function

The four major family of endogenous opioid ligands are β-endorphins, enkephalins, dynorphins, and nociceptin. These peptides are exclusively packaged in dense core vesicles in the cell body of neurons and are transported to axon terminals. These peptides are originally cleaved from a parent molecule (eg., propremelanocortin or preproenkephalins), and exert their function on respective opioid receptor subtypes. MORs are activated by both enkephalins and β-endorphins. Functionally, activation of the MORs leads to analgesia and reward through its action at multiple sites in the central and peripheral nervous system. Activation of MORs inhibits the release of substance P from sensory neurons in the dorsal horn of the spinal cord and thus decreases the transmission of painful stimuli to the brain (Corder et al. 2018). Additionally, activation of MORs in the brainstem modulates nociceptive activity in the descending inhibitory pathways in the dorsal horn (Al-Rodhan, Yaksh, and Kelly 1992; Rossi, Pasternak, and Bodnar 1994). And lastly, recent evidence suggests that opioids also mitigate the affective component of pain processing through their actions in the forebrain (Price et al. 1985; Remeniuk et al. 2015).

Acute activation of MORs also leads to increased locomotor activity, respiratory depression, pruritus, xerostomia, constipation, and immunosuppression (Inturrisi and Jamison 2002; Streicher and Bilsky 2018; Volkow and McLellan 2016). These latter effects of opioids are usually debilitating for patients, and even though analgesic tolerance develops after long-term use, there is minimal development of tolerance to the undesirable side effects of opioid agonists. Mechanism underlying the differences in

MOR activation and resistance to tolerance in the gastrointestinal and respiratory track is an area of active research (Levitt and Williams 2018).

MOR Signaling and Regulation

Signaling

Agonist binding leads to a conformational change in the MOR leading to its activation. MORs are coupled to pertussis toxin-sensitive $G_{i/o}$ proteins, and once activated, allow for heterotrimeric G-proteins to bind and engage subsequent effectors. Activation of G-proteins requires GDP to be exchanged for GTP on the α subunit, which causes the $\beta\gamma$ subunit to dissociate. The α subunit inhibits adenylyl cyclase (Ingram and Williams 1994; Taddese, Nah, and McCleskey 1995), while the $\beta\gamma$ subunit inhibits voltage gated Ca^{2+} channels (Connor, Borgland, and Christie 1999), inhibits neurotransmitter release (Paton 1957), and activates G protein-coupled inwardly rectifying potassium (GIRK) channels (John T. Williams, Egan, and North 1982). The combination of these effects ultimately leads to hyperpolarization of the cell and a decrease in neuronal excitability.

Desensitization

Acute desensitization, or the decrease in the signaling response of the MOR in the continuous presence of the agonist, involves multiple complex steps. Within seconds to minutes of agonist binding, the C-terminus tail of the MOR is phosphorylated. The leading kinases involved in the phosphorylation of MORs are G protein-coupled receptor

kinases (GRK 2/3, Fig, 1A). There is substantial evidence for the role of phosphorylation in inducing MOR desensitization and trafficking (Birdsong et al. 2015; Yousuf et al. 2015; Miess et al. 2018; A. Kliewer et al. 2019a; Arttamangkul, Leff, et al. 2019). When phosphorylation by GRK2/3 is blocked pharmacologically (Leff, Arttamangkul, and Williams 2020a), or when the phosphorylation sites are mutated to alanine (Birdsong et al. 2015; Yousuf et al. 2015; A. Kliewer et al. 2019a), the acute decline in signaling by saturating concentration of agonist is blocked.

However, it is important to note that agonists with varying efficacy can induce phosphorylation, and consequently desensitization, differentially (Johnson et al. 2006a). Peptide agonists like ME and DAMGO induce approximately 50 percent decline from peak current (Harris and Williams 1991; Fiorillo and Williams 1996a; Alvarez et al. 2002; Bailey et al. 2003), while alkaloid agonists like morphine induce 10-35 percent decline (Dang and Williams 2005), and oxycodone causes no desensitization (Virk and Williams 2008). Additionally, recovery of functional receptors after desensitization is also agonist dependent, with ME, DAMGO, fentanyl, and etorphine causing significant desensitization and dramatic inhibition of MOR-mediated current following desensitization, morphine causing intermediate amount of desensitization and reduced recovery, and oxycodone causing no desensitization of MOR mediated current (Virk and Williams 2008). Therefore, acute desensitization of MOR is both phosphorylation and agonist dependent.

The degree of desensitization of the MORs also depends on receptor expression levels. Decreasing receptor reserve using irreversible antagonists increases the amount of acute desensitization and shifts dose-response curves. The locus coeruleus in particular has high receptor reserve, so it is important to note that degree of desensitization and regulation of MORs might be vastly different in areas that lack spare receptors.

It is important to note that alternative mechanisms of desensitization for MOR have been described, particularly those not involving the GRKs. MORs can be phosphorylated by cyclic AMP dependent protein A (PKA), protein kinase C (PKC), calcium calmodulin kinase (CaMKII), mitogen activated protein kinase (MAPK), and extracellular signaling-related kinase 1/2 (ERK 1/2) (Cai et al. 1996; Harada et al. 1990; Schmidt et al. 2000; Terwilliger et al. 1994). Morphine in particular has been shown to be phosphorylated by PKA rather than GRK2/3, and in the absence of overexpression of GRK2/3, morphine is poor at engaging GRK2/3 for MOR phosphorylation (Johnson and North 1992; Bailey et al. 2009).

Rapid acute desensitization also temporally overlaps with receptor internalization and shares common mechanisms. Phosphorylation of the MOR also leads to an increase in the receptor's affinity for β-arrestin, a scaffolding protein that mediates clathrin-induced receptor internalization (Gurevich and Gurevich 2018; 2019b). Arrestin binding simultaneously prevents additional G-protein activation through steric hinderance, leading to desensitization. These two processes act redundantly to ensure that opioid

signaling from the plasma membrane gets terminated by a) physically removing the receptors from cell surface and b) by effectively uncoupling the effectors from the GPCR. Therefore, the overlap in the molecular events leading to both desensitization and internalization does not allow for complete separation of these events in rat brain slices. However, even though desensitization and internalization share common mechanisms, they are separate processes. In primary cultures from LC neurons, blocking MOR internalization did not affect desensitization or recovery from desensitization (Arttamangkul et al. 2006; Dang et al. 2011; Johnson et al. 2006b).

The mechanisms described above have been studied extensively for the MORs in the somatodendritic compartment of neurons. However, there are important functional differences between MORs in this compartment and presynaptic compartment: namely, MORs ability to inhibit neurotransmitter release does not acutely desensitize (Blanchet and Lüscher 2002b; Fyfe et al. 2010b; Pennock, Dicken, and Hentges 2012a). The mechanisms underlying this lack of desensitization are not fully known, but recent work using single particle tracking has demonstrated that presynaptic MORs are phosphorylated and internalized, but get rapidly replaced at sites of transmitter release by lateral diffusion from an extrasynaptic axonal reserve of receptors that are protected from the phosphorylation-endocytosis machinery (Jullié et al. 2020b).

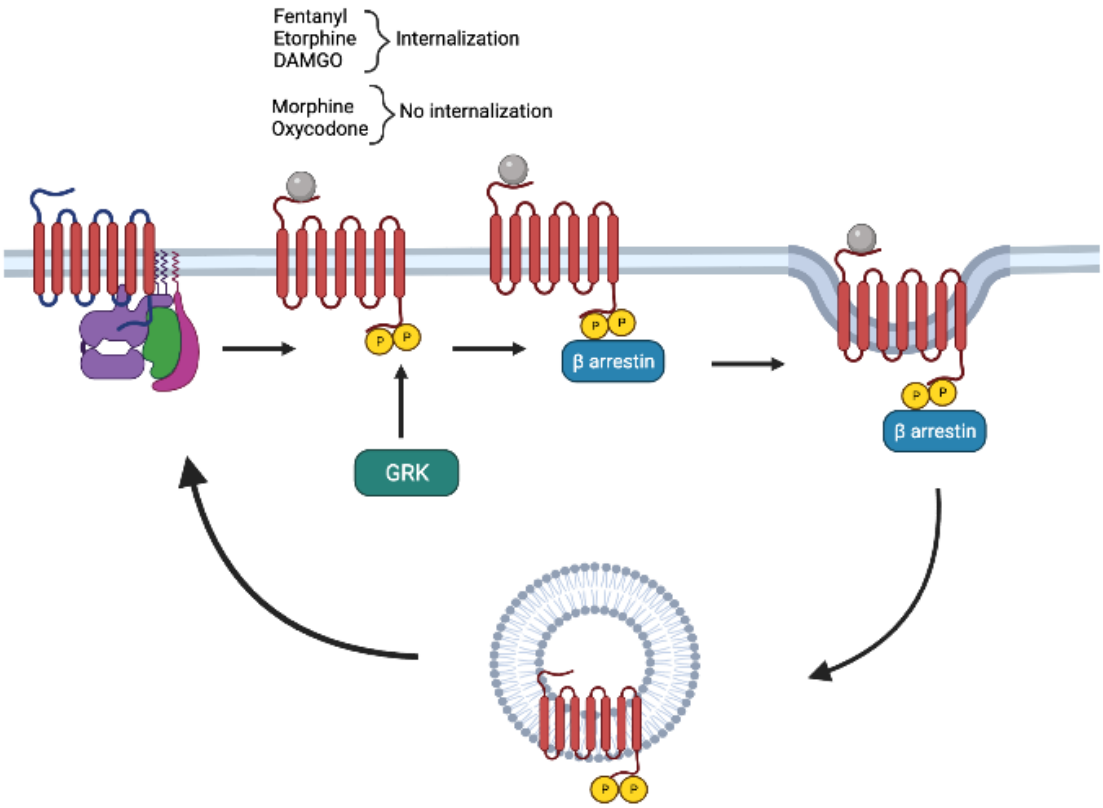

Fig 1.1 Illustration depicting regulation of MORs upon agonist binding.

Without agonist binding, MOR is in its inactivated GDP bound state with Galpha, beta, and gamma subunit bound to the receptor. MOR Agonist binding leads to phosphorylation of the c-terminus tail of MOR by GRK2/3 kinase. Phosphorylation of MOR increases its affinity for β -arrestin, a scaffolding protein that mediates clathrin induced endocytosis of the receptor. The ability to recruit β- arrestin and induce internalization is agonist dependent, with fentanyl, etorphine, and DAMGO causing robust internalization and morphine and oxycodone causing little to no internalization.

MOR Trafficking and Compartmentalization

As previously mentioned, agonist-induced downregulation of opioid receptors requires phosphorylation by GRKs and engagement of β-arrestins, leading to clathrin and dynamin induced endocytosis (von Zastrow, Keith, and Evans 1993; von Zastrow et al. 1994). Internalized MOR can be recycled back to the plasma membrane or tagged for degradation. The sorting of the receptors is highly selective and relies on specific sequence in the C-terminus tail (Tanowitz and Von Zastrow 2003). Classically, these endocytosed receptors were thought to be in their inactive state, but recent evidence suggests that opioid receptors, through the engagement of β-arrestin, can continue signaling from the internal membrane compartments (Stoeber et al. 2018). Though this continued signaling was observed for DORs, there is the potential for MORs to also engage with G-proteins from endosomes. Additionally, distinct agonists of the MOR also vary in their ability to induce internalization, though these differences have primarily been studied in heterologous systems. ME and DAMGO are highly internalizing agonists and can do so within minutes, while morphine fails to cause internalization even after a long incubation period (Arden et al. 1995; Keith et al. 1996; Sternini et al. 1996; Keith et al. 1998; Abbadie and Pasternak 2001). But, there is evidence for morphine-induced MOR internalization in systems with overexpressed GRK2 or overexpressed β-arrestin 2 or 3 (Whistler and Von Zastrow 1998b; Zhang et al. 1998).

Studies involving agonist-induced internalization of MOR rank morphine, buprenorphine, and oxycodone as least capable, fentanyl and methadone as moderately capable, and DAMGO and ME as the most capable (Keith et al. 1996; Alvarez et al. 2002b; Borgland

et al. 2003; Koch et al. 2005; Arttamangkul et al. 2008). The functional relevance of MOR internalization and continued signaling post internalization is not fully known. Additionally, compartment specific signaling also raises the possibility of differential signaling efficacy based on the receptor location. Recent advances in genetically encoded biosensors has allowed for high spatiotemporal resolution to be able to detect receptor signals in specific subcellular compartments, like the plasma membrane, Golgi and the endosome (Gondin et al. 2019; Metz et al. 2019; Tobin et al. 2019). These compartments differ in their molecular environment and can affect signaling and opioid efficacy and selectivity differently (Lobingier et al. 2017). In fact, conformational specificity of opioid receptors based on their subcellular location has recently been demonstrated for DORs (Kunselman et al. 2021).

Biased Signaling and Allosteric Modulators

Furthermore, ligand bias, or functional selectivity, is now a widespread concept in GPCR pharmacology. Biased signaling refers to the ability of different ligands to differentially engage downstream effectors, while acting on the same receptor (Kenakin 1995; Kenakin and Miller 2010). In relation to the MORs, the utilization of the β-arrestin 2 knock out animals were initially characterized to dissociate the analgesic effects of opioid agonists from their undesirable side effects such as respiratory depression and constipation (Bohn et al. 1999). This finding led to the hypothesis that decreased arrestin recruitment by G-protein biased ligands can reduce the side effects of opioid analgesics. However, recent studies using both phosphorylation deficient animals (A. Kliewer et al. 2019a) and β-arrestin 2 knock out animals (Andrea Kliewer et al. 2020) failed to replicate the original

findings. Therefore, though there is a consensus that different opioid ligands can differentially modulate downstream effectors, this modulation having completely dissociable effects on therapeutic analgesia vs undesirable side effects remains controversial.

Additionally, positive allosteric modulators (PAMs) of the MORs have now been developed for potential pain management with reduced side-effect profiles. The theory behind utilizing PAMs is, because modulation of opioid signals only occurs when an orthosteric ligand is bound, PAMs may induce less receptor downregulation or other compensatory adaptive mechanisms that develop with exogenous orthosteric agonists (Burford et al. 2013). This hypothesis was recently tested in a proof of principle study where BMS-986122 was able to have antinociceptive effects when administered *in vivo* and had reduced side effect profile like constipation and locomotor sensitization (Kandasamy et al. 2021). A critical assessment of these new compounds with more follow-up studies focusing on chronic treatment is warranted.

Cellular Mechanisms Mediating Tolerance

The hallmark of cellular tolerance is the compensatory upregulation of adenyl cyclase after repeated agonist exposure. This upregulation of adenyl cyclase was first demonstrated in neuroblastoma and glioma hybrid cell lines following morphine treatment. This cyclase upregulation was defined as a tolerant state because acute activation of MOR leads to downregulation of adenyl cyclase. The functional

consequence of cyclase supersensitivity was overproduction of cAMP when morphine was removed (Sharma, Klee, and Nirenberg 1975a). Furthermore, the role of MORs themselves was investigated in guinea pig ileum after chronic treatment with morphine causing a reduction in MOR receptor reserve (Chavkin and Goldstein 1984). This finding was later extended to the LC, showing that chronic treatment with morphine causes a rightward shift of the concentration-response curve of ME and morphine (Christie, Williams, and North 1987a). Together, these cellular adaptations provide a mechanism of cellular tolerance that have been established in neuronal tissues.

Additionally, remarkable progress has happened in the last two decades in discovering the mechanisms mediating tolerance after chronic opioid treatment in the somatodendritic compartment of the neurons. The relevant ones pertaining to this thesis will be discussed below. First, compared to untreated animals, acute decline of peak current by ME and morphine was facilitated and recovery from desensitization was inhibited in rats treated with morphine (Dang and Williams 2004a; 2005). The enhancement of desensitization suggests that after chronic treatment a subsequent desensitizing stimulus causes a greater uncoupling of MORs from its effectors compared to untreated animals. Second, chronic treatment of rats with methadone also led to increased desensitization and shifted the concentration-response curve of ME twofold, but did not alter the recovery from desensitization (Quillinan et al. 2011), suggesting that agonist efficacy might play a role in mediating cellular tolerance after chronic treatment. Third, when cellular tolerance after chronic morphine treatment was measured by creating concentration-response curve to morphine, morphine's efficacy was reduced and *in vivo* desensitization was induced

(Levitt and Williams 2012a). The interpretation from these observations is that after chronic morphine treatment, there is a greater uncoupling of MOR from its effector, leading to higher desensitization and tolerance. Fourth, chronic morphine treatment resulted in an upregulation of PKC and JNK, resulting in these kinases contributing to desensitization of MOR and the somatostatin (SST) receptor (Leff, Arttamangkul, and Williams 2020a). Fifth, the mechanisms mediating tolerance after chronic morphine treatment are phosphorylation dependent, as tolerance is greatly reduced in rats that express total phosphorylation deficient (TPD) MORs (Arttamangkul et al. 2018). And lastly, mice that also lack phosphorylation sites on MORs and are chronically treated with either morphine or fentanyl have diminished antinociceptive tolerance compared to WT mice (Kliewer et al. 2019b).

The adaptations in the presynaptic terminals of the neuron after chronic treatment uses the cAMP/ PKA pathways as substrates. In cell lines, after chronic opioid treatment, there is an up-regulation of adenylyl cyclase activity to maintain homeostatic levels of cAMP (Avidor-Reiss et al. 1995; Sharma, Klee, and Nirenberg 1975a; Terwilliger et al. 1991). Acute withdrawal following chronic treatment then unmasks these adaptations leading to an increased in cAMP levels and PKA activity (Nestler and Tallman 1988). These adaptations lead to tolerance, and can lead to cellular withdrawal symptoms such as increased transmitter release from presynaptic terminals (Bonci and Williams 1996a; 1997; Chieng and Williams 1998a). Additionally, there is evidence of enhanced opioid efficacy, and not opioid tolerance, following chronic morphine treatment, suggesting that

mechanisms mediating adaptations in the presynaptic terminals might be vastly different from those in the somatodendritic compartments (Ingram et al. 1998a).

Tolerance in Whole Animals

It has been well-established that different agonists induce *in vivo* antinociceptive tolerance differentially. Analgesic efficacy in whole animal is determined by measuring the antinociceptive effect of an opioid after pretreatment with an irreversible antagonist of MORs (eg, β-CNA) to reduce the number of receptors. High-efficacy agonists require fewer receptors to produce antinociception, whereas maximal antinociceptive response for low-efficacy agonists will be reduced after irreversible block (Kumar et al. 2008; Sirohi et al. 2009; Madia et al. 2009). These studies and others found that fentanyl has the greatest relative efficacy, followed by etorphine, methadone and morphine, hydromorphone, oxycodone and lastly hydrocodone. Relative efficacy also correlated with analgesic tolerance with low-efficacy agonists like morphine and oxycodone inducing higher tolerance and high-efficacy agonists like etorphine and fentanyl inducing tolerance to a lower degree (Walker and Young 2001; Grecksch et al. 2006; Pawar et al. 2007; Kumar et al. 2008). Additionally, it has also been shown that high dose etorphine, but not morphine or oxycodone, causes substantial upregulation of dynamin-2, leading to downregulation of MORs (Pawar et al. 2007).

However, how different agonists mediate regulation of MORs after chronic treatment, and therefore, cellular tolerance is not fully understood. It is also not known if it is agonist efficacy or some other regulatory property of an agonist that plays a role in mediating cellular tolerance. For example, morphine is able to induce *in vivo* tolerance, but is relatively inefficient in inducing desensitization and internalization *in vitro*. However, morphine-bound MOR is capable of being phosphorylated, and therefore can remain desensitized in the plasma membrane (Koch et al. 2001; Zhang et al. 1998; Koch et al. 2005). Therefore, one theory of tolerance postulates that the lack of internalization, and consequently reduced resensitization, contributes to tolerance. However, a second theory states that lack of internalization by morphine leads to continuous and persistent signaling by MORs, resulting in counterregulatory adaptations; and these adaptations lead to high level of tolerance induced by morphine (Whistler and Von Zastrow 1998a; Whistler et al. 1999). It is likely that both persistent signaling and decoupling of MORs from its effectors are contributing to a tolerant state.

Somatostatin Receptors

The locus coeruleus contains somatostatin receptors (SSTRs, SSTR2) that also couple to GIRK channels (Fiorillo and Williams 1996b). Similar to MORs, supersaturating concentration of agonist induces phosphorylation and β arrestin mediated acute desensitization of these receptors (Günther et al. 2018). Truncation of the phosphorylation sites on the C-terminus domain of SSTR resulted in decreased acute desensitization further supporting that desensitization of SSTR is phosphorylation dependent (Cole and Schindler 2000). Similar to MORs, there is also evidence for GRK

2/3 mediated phosphorylation of the intracellular region of these receptors (Günther et al. 2018).

Heterologous Adaptations

Opioid induced acute desensitization in the LC has been shown to be primarily homologous, meaning that desensitization of one GPCR does not affect signaling of another GPCR on the same cell (Bailey et al. 2003; Bailey et al. 2009; Dang et al. 2011; Harris and Williams 1991). Loss of function following chronic opioid treatment is also restricted to MORs and not to other GPCRs that couple to the same effectors (Bailey et al. 2009; Christie, Williams, and North 1987; Connor, Borgland, and Christie 1999), suggesting that decrease in potassium conductance is due to MOR desensitization and not due to effects at the GIRK channels. However, multiple $G_{i/o}$ coupled GPCR in the LC share signaling components, and there has been evidence for heterologous desensitization to the α_2 adrenergic receptor after MOR activation in mouse LC (Dang, Chieng, and Christie 2012). Furthermore, heterologous desensitization to the α_2 adrenergic receptor was also shown in rats less than 20 days old in the LC (Llorente et al. 2012). And lastly, it was recently shown that after chronic morphine treatment, adaptations that disrupt the kinase regulation of MOR desensitization also induce heterologous effects at the regulation of SST receptors (Leff, Arttamangkul, and Williams 2020b).

Locus Coeruleus

The locus coeruleus is a brainstem structure located in the pons and is the source of noradrenaline release in the brain. Located medial to the fourth ventricle, it consists of neurons that contain melanin granules, giving it its signature blue color. These neurons serve as a useful model to study the action of the MORs, as MORs are homogenously expressed in all of these neurons. Additionally, neurons in the locus coeruleus do not express DORs or KORs. There are widespread efferent projections from the locus coeruleus to multiple brain areas, regulating arousal, stress, and emotion. The dendrites of locus coeruleus neurons are arborized mostly in the rostral-caudal plane and define the pericoerulear region (Ishimatsu and Williams 1996). The locus coeruleus neurons are also electrotonically coupled and tonically fire in awake behaving animals (Aston-Jones and Bloom 1981; Ishimatsu and Williams 1996). In relation to opioid tolerance, LC has been implicated in mediating symptoms of withdrawal by increasing its firing rate (Aghajanian 1978).

Striatum

The striatum is one of the primary input nuclei of the basal ganglia. It receives glutamate inputs from the cortex as well as the thalamus, and dopaminergic inputs from the substantia nigra compacta and the ventral tegmental area of the midbrain (Kincaid, Zheng, and Wilson 1998; Bolam et al. 2000; Smith et al. 2014). The GABAergic medium spiny neurons (MSNs) are principal cells of the striatum and can be divided into two

different subtypes – D_1 receptor containing neurons and D_2 receptor containing neurons. These neurons have anatomical outputs that are also functionally distinct with D_1R expressing neurons projecting to the globus pallidus internus and substantia nigra reticulata, and the D_2R containing neurons projecting to the globus pallidus externus, which in turn sends inhibitory projections to the subthalamic nuclei (Smith et al. 1998). Additionally, the striatum can also be divided topographically between the ventral striatum (nucleus accumbens) and the dorsal striatum (Ragsdale and Graybiel 1990; Nicola 2007). These areas differ in both the afferent fibers from which they receive inputs, and also the efferent brain regions to which they send inhibitory signals. This thesis primarily focuses on the dorsal medial striatum because the thalamic terminals in this area is highly sensitive to opioids (Birdsong et al. 2019a). The dorsal medial striatum is primarily involved in goal-directed learning and the opioid modulation of this brain region by both exogenous application and endogenous release is critically important to study (Balleine, Delgado, and Hikosaka 2007).

Opioids and Adenosine

As a Gi/o coupled GPCR, acute activation of MORs lead to a decrease in adenyl cyclase activity, leading to a reduced cAMP concentration in neurons (Ingram and Williams 1994; Taddese, Nah, and McCleskey 1995). Previous studies have shown that upon chronic activation MORs, homeostatic adaptions lead to a hypertrophied adenyl cyclase system that increases the concentration of cAMP (Bonci and Williams 1996a; Chieng and Williams 1998b; Sharma, Klee, and Nirenberg 1975b; Matsui et al. 2014a). It has been previously shown that cAMP can be transported or is diffused onto the extracellular space and metabolized to AMP by

phosphodiesterase in the hippocampus (Dunwiddie, Diao, and Proctor 1997). AMP can then be converted to adenosine by ectonucleotidases (Brundege et al. 1997a). Once in the extracellular space, adenosine activates presynaptic terminal A_1 receptors to further inhibit neurotransmitter release (Fredholm and Dunwiddie 1988). However, both the acute regulation of adenosine and its regulation after chronic opioid treatment can be cell-type and synapse specific. Previous studies examining basal adenosine release in naïve animals found robust tone in the nucleus accumbens core but not in the shell in the rat striatum (Brundege and Williams 2002a). Additionally, there was no change in adenosine tone after chronic morphine treatment in the MSNs of the striatum, but an increase in tone in cholinergic interneurons in the striatum (Chieng and Williams 1998b). Chronic morphine treatment has also been shown to increase adenosine sensitivity in the nucleus accumbens (Brundege and Williams 2002c). In whole animals, adenosine A_1 receptor agonists have been shown to be analgesic (Herrick-Davis et al. 1989; Sawynok 1998). Additionally, morphine withdrawal symptoms were alleviated in genetically altered mice lacking basal adenosine tone or after agonism of A_1 receptor (Wu et al. 2013).

Thesis Statement and Course of Dissertation

The development of tolerance is a critical first step in the pathogenesis of opioid use disorder and can potentially lead to addiction. Tolerance can happen directly within the receptor leading to reduced receptor-effector coupling, or among the second messengers leading to altered signaling and regulatory mechanisms. The goal of this dissertation is to characterize both of these adaptations that lead to tolerance after long-term opioid use in both the somatodendritic and presynaptic compartment of neurons.

The work presented in this dissertation was collected through whole-cell patch clamp recordings from locus coeruleus neurons in acute horizontal rat brain slices and striatal medium spiny neurons in acute coronal mouse brain slices. Chapter 3 focuses on tolerance induced by chronic treatment with clinically relevant and frequently used opioid agonists, with results suggesting that agonists with varying efficacy mediate cellular tolerance uniquely. Chapter 3 also describes heterologous adaptations to the somatostatin receptor due to GRK2/3 disruption after chronic treatment with different opioid agonists and the mechanism underlying this disruption. Because MORs are differentially regulated in the presynaptic compartment of the neuron. Chapters 4 and 5 uses an optogenetic approach to examine the presynaptic MORs in the striatum. Chapter 4 explores the relationship between MORs and adenosine in mediating glutamate release in the striatum. Chapter 5 describes the regulation of presynaptic MORs after chronic morphine treatment.

Chapter 2 MATERIALS AND METHODS

Animals

All animals were maintained and sacrificed according to the approved protocols at Oregon Health and Science University. For Chapter 3, adult male and female rats, both wildtype Sprague-Dawley and μ-opioid receptor knockout (MOR KO) rats on a Sprague-Dawley background, with ages between 5 – 7 weeks were used. Wildtype Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). A pair of MOR-knockout Sprague-Dawley rats with ZFN target site (GCTGTCTGCCACCCAgtcaaaGCCCTGGATTTC within exon 2) were generated by Horizon (St. Louis, MO) and received as F3 generation. The animals were bred and raised in house for two more generations before use in the experiments. The gene deletion was confirmed by genotyping using the primer 5'CATATTCACCCTCTGCACCA3'. For Chapters 4 and 5, male and female WT C57BL/6J mice, FloxedMor mice ($Oprm1^{fl/fl}$, JAX stock #030074), $Vglut_2$:cre mice (JAX stock #016963), A_{2A}:cre mice (MMRC stock #036158-UCD), and D_1:cre mice (MMRC stock #030989- UCD) were used. Animals with *cre* recombinase were crossed with the FloxedMor animals to selectively knock out the MORs from specific cell-types. All mice used were between 8 and 10 weeks old. All mice were bred in house and maintained on a C57BL/6J background. FloxedMor and $Vglut_2$:cre mice were purchased from the Jackson Laboratory. $A_{2:}$cre mice maintained on a C57Bl/6 background were kindly provided by Dr. Tianyi Mao. D_1:cre mice maintained on a C57Bl/6 background were kindly provided by Dr. Christopher Ford (University of Colorado, Aurora, CO).

Chronic drug treatment

For Chapter 3, rats (5 – 6 weeks old) were treated with one of the following drugs continuously released from osmotic pumps: morphine sulfate, oxycodone hydrochloride, fentanyl hydrochloride, and buprenorphine hydrochloride. Osmotic pumps (2ML, Alzet, Cupertino, CA) were filled with the required concentration of the drug in water to deliver the following concentrations of: morphine sulfate 80 mg/kg/day, oxycodone 30 mg/kg/day or 80 mg/kg/day, or fentanyl 1.5 mg/kg/day or 2.8 mg/kg/ day, and buprenorphine 10 mg/kg/ day. For rats treated with fentanyl and oxycodone, intraperitoneal injection of oxycodone (5 mg/kg/day) fentanyl (0.3 mg/kg/day) for two days was given to induce tolerance and reduce the likelihood of death before chronic treatment. Osmotic pumps were implanted subcutaneously in the mid-scapular region of rats maintained on isoflurane anesthesia and remained in the animals until they were used for experiments 6 or 7 days later. For chapter 5, mice were treated with chronic morphine sulfate (80 mg/kg/day) using the same osmotic minipump system.

Electrophysiology

Brain slice preparation

Horizontal rat locus coeruleus slices (260 μm; Chapter 3) or coronal mouse striatal slices (242 μm; Chapters 4 and 5) slices were made in warm physiologically equivalent solution (modified Krebs buffer, see Recipes) with 10 μM MK-801. Slices were incubated at 30-

32 ºC in vials with 95/5% O2/CO2 saline with 1 μM MK-801 for at least 30 mins, before recordings. Rat LC slices were maintained in 30-32 ºC while mouse striatal slices were maintained at room temperature. Once slices were mounted on a recording chamber attached to an upright microscope (Olympus), they were maintained at 33- 37 ºC and perfused at a rate of 4.0 ml/min with modified Krebs buffer. Using infrared illumination, LC (Chapter 3), and dorsal medial striatum (Chapter 4 and 5) was identified visually, under 5x magnification. The LC was identified based on its proximity to the fourth ventricle and dorsal medial striatum was identified using its proximity to the lateral ventricles.

Electrophysiological recordings and analysis

Whole-cell recordings from LC neurons for chapters 3 were obtained with glass electrodes (1.75-2.5 MΩ) and a potassium-based internal solution containing BAPTA (10 mM; see Recipes). The cells were voltage-clamped at -60 mV with an Axopatch 200B amplifier (Axon Instruments). LC neurons were identified using their size, the presence of spontaneous action potentials, and the presence of both MOR and α_2 mediated potassium conductance. Immediately after gaining access to the cell, membrane capacitance, series resistance, and input resistance were measured with the application of square test pulses (+5 mV for 50 ms) averaged before computation using Axograph X (Axon Instruments, version 1.5.4). Only recordings where the series resistance remained < 14 MΩ were used.

Whole-cell recording from striatal medium spiny neurons (MSNs) for chapters 4 and 5 were obtained with glass electrodes (2.8-3.5 MΩ) and a gluconate-based internal solution containing EGTA (see Recipes). The cells were voltage-clamped at -75 mV with an Axopatch 200B amplifier (Axon Instruments). MSNs were identified using by their hyperpolarized resting membrane potential, low input resistance and a long delay to the initial spike. Similar to the LC neurons, immediately after gaining access to the cell, membrane capacitance, series resistance, and input resistance were measured with the application of square test pulses (+5 mV for 50 ms) averaged before computation using Axograph X (Axon Instruments, version 1.5.4). Only recordings where the series resistance remained < 18 MΩ, or did not change by more than 20 percent throughout the course of the experiment were used. Glutamate release was evoked using 470 nm LED light (0.5 to 1 ms). AMPA mediated current was pharmacologically isolated by the following receptor blockers in the perfusate (in μM): 0.2 $GABA_B$-receptor antagonist CGP 55845, 10 $GABA_A$-receptor antagonist picrotoxin, 1 nicotinic acetylcholine receptor antagonist mecamylamine, 0.1 muscarinic acetylcholine receptor antagonist scopolamine and 0.3 metabotropic glutamate receptor five antagonist MPEP.

Drug Perfusions and Incubations

For LC neurons in chapter 3, agonists, [Met5] enkephalin, somatostatin, and UK14304, and clonidine, and antagonists, idazoxan, and naloxone were applied via bath perfusion at a rate of about 2 mL/minute. For experiments using GRK2/3 inhibitor, compound 101,

slices were incubated in 30 μM for at least one-hour prior to recording. Compound 101 was also included in the perfusate (1μM) and drug perfusion solution (10 μM).

For MSNs in Chapters 4 and 5, agonists, [Met5] enkephalin, morphine, cyclopentyladenosine (CPA), U69, and antagonists, naloxone and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were applied via bath perfusion at a rate of about 2 mL/minute. For experiments using D_1 (SKF81297) and A_{2A} (CGS21680) receptor agonists and phosphodiesterase inhibitor (R0-230853), slices were incubated in the respective drugs for at least one hour prior to recording. These drugs were also included in the perfusate.

Measuring desensitization and cellular tolerance

The LC contains multiple $G_{i/o}$ coupled GPCRs that couple to G protein-coupled inwardly rectifying potassium channels (GIRKs). Therefore, GIRK induced outward current was used as a marker of activation for MORs, α_2 adrenergic receptors, and SST receptors (Fig 2.1). Acute desensitization of SST receptor was measured as the decline in the peak current during the continuous application of a supersaturating concentration of agonist (10 μM SST, 10 min). This decrease in the peak is referred to as acute desensitization (Fig 2.2). Desensitization of MORs was also measured by a brief application of saturating concentration of ME (10 μM, 2 mins), followed by a saturating concentration of either morphine or oxycodone agonist (10 μM). Current induced by morphine or oxycodone was normalized to current induced by saturating concertation of UK (3 μM), as peak UK current is unaffected after chronic treatment (Harris and Williams 1991a; Levitt and

Williams 2012a) In oxycodone treated animals, *in vivo* desensitization was measured by maintaining slices in oxycodone (1 μM) and revealing the oxycodone current by application of opioid antagonist naloxone (1 μM). Tolerance induced by chronic oxycodone treatment was assessed by creating concentration response curves to ME and oxycodone in naïve and treated animals. Furthermore, current induced by an EC50 concentration of 1 μM morphine was also used to measure tolerance as partial agonists are more sensitive to changes in receptor-effector coupling (Christie, Williams, and North 1987b). Tolerance induced by chronic fentanyl treatment was assessed by creating concentration response curves to ME and by measuring current induced by morphine (1 uM). Concentration response curves for fentanyl was not created due to the possibility of pre-desensitization in the rising phase of the current, occluding the maximum peak.

In the presynaptic compartment, inhibition of glutamate release was used as a marker for MOR activation (Williams, Christie, and Manzoni 2001). Unlike the somatodendritic MORs in the LC, presynaptic MORs can continue to inhibit transmitter release during the continuous application of supersaturating concentration of agonist (Blanchet and Lüscher 2002b; Fyfe et al. 2010b; Pennock, Dicken, and Hentges 2012a), suggesting that presynaptic MORs do not acutely desensitize. Desensitization after chronic morphine treatment was measured like described above, with application of naloxone (1 μM) in slices maintained in circulating concentration of the morphine. Tolerance after chronic treatment was measured by examining the adenosine inhibition of glutamate release via DPCPX (Chieng and Williams 1998a; Matsui and Williams 2011).

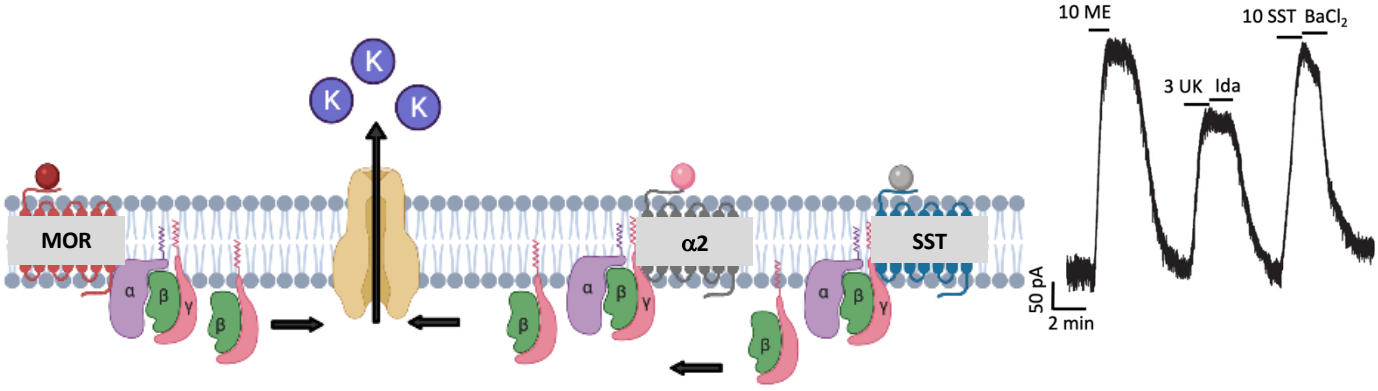

Fig 2.1 Illustration and electrophysiological recordings of receptors present in LC neurons

MOR, α2 adrenergic receptor, and SST receptor all couple to the same GIRK channel in a single LC neuron. Therefore, current induced by opioids were normalized to current induced by a saturating concentration of UK as a control agonist.

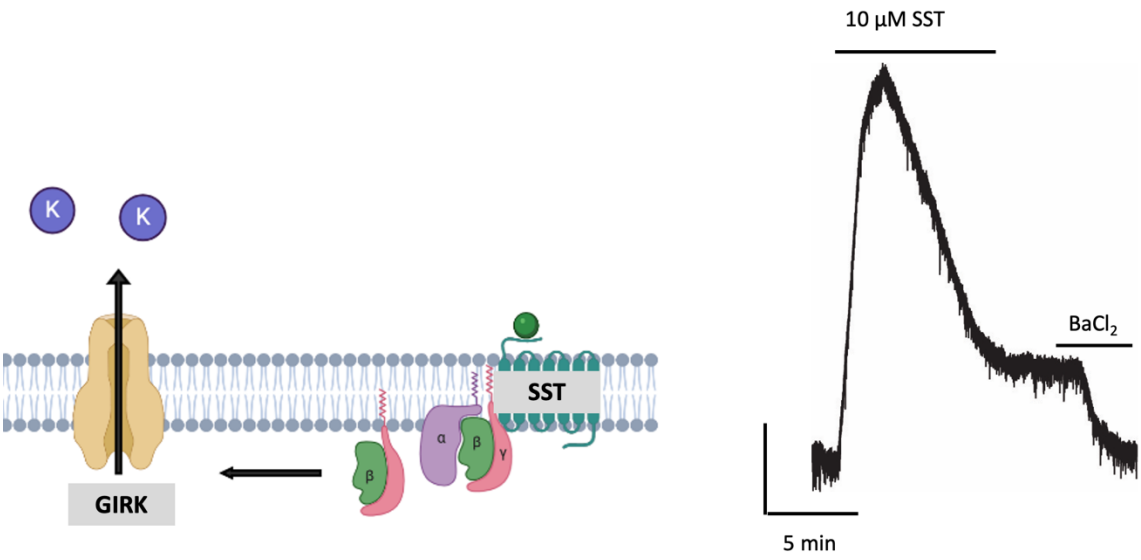

Fig 2.2. Illustration and electrophysiological recordings of SST receptor desensitization

Prolonged exposure to SST causes a decline in current induced by the agonist, a phenomenon called acute desensitization. This acute desensitization is demonstrated in the trace to the right as the current decreases even though the agonist is present. The remaining current is reversed by barium, a non-specific potassium channel blocker.

Concentration-response Curves

For Chapter 3, concentration-response curve for ME, and oxycodone in both naïve animals and chronically treated animals was created by measuring the outward current produced by various concentration of the agonists. Only one concentration of agonist was tested per slice. Because MORs and α_2-adrenergic receptors activate the same G protein-coupled inwardly rectifying potassium (GIRK) channels (North and Williams 1985), opioid induced current was normalized to the current induced by saturating concentration of α_2 agonist (3 μM).

Stereotaxic Virus Injections

For Chapters 3, MOR KO rats (P24-30) were anesthetized with isoflurane and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, with custom modifications. Adeno associated virus type 2 (AAV2) encoding total phosphorylation-deficient (TPD, AAV2-CAG-SS-GFP-MOR-TPD-WPRE-SV40pA) mutant MORs were injected into the LC. 200 nL of virus (2.06 x 1013 vg/ml) was injected at 0.1 μl/min, bilaterally in the LC (AP: -9.72 mm, ML: ±1.25 mm, DV: -6.95 mm, from bregma) using a computer controlled stereotaxic frame (Neurostar, Tubingen, Germany) (Arttamangkul et al. 2018). Viruses were obtained from Virovek (Hayward, CA). Electrophysiology experiments were carried out 2-4 weeks following injection. Infected neurons were identified in the slice by visualization of GFP.

For Chapters 4 and 5, mice were anesthetized with isoflurane and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, with custom modifications) for microinjections of recombinant adeno-associated virus type 2 (AAV2) to express channelrhodopsin. A glass pipette filled with 40 nL of virus was injected into the medial thalamus (Nanoject II, Drummond Scientific, Broomall, PA; BCJ: custom-built injector based on a MO-10, Narishige, Amityville, NY), unilaterally (M/L: +/-0.55, A/P: −1.2, D/V: -3.4). Electrophysiology experiments were done 2-3 weeks after viral injections. Expression of channelrhodopsin in thalamic terminals was checked in slice by visualization of GFP.

Pharmacology

Drug	Mechanism of Action	Abbreviation	Obtained from
[Met]^5 enkephalin	MOR / DOR agonist	ME	Sigma-Aldrich (St Louis, MO)
Somatostatin	SSTR agonist	SST	ProSpec (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel)
UK14304 tartate	α_2-adrenergic receptor agonist	UK	Tocris (Bio-Techne Corp., Minneapolis, MN)
Idazoxan	α_2--adrenergic receptor antagonist	Ida	Sigma-Aldrich
Morphine	MOR agonist	-	National Institute on Drug Abuse (Baltimore, MD)

Compound 101	Inhibits GRK2/3	CMPD 101	Hello Bio
Naloxone	Non-specific opioid antagonist	-	Abcam (Cambridge, MA)
Bestatin	Peptidase inhibitor	-	Sigma-Aldrich (St. Louis, MO)
Thiorphan	Peptidase inhibitor	-	Sigma-Aldrich (St. Louis, MO)
Dizocilpine maleate	NMDAR antagonist	MK-801	Abcam (Cambridge, MA)
Fentanyl	MOR agonist	-	National Institute on Drug Abuse (Baltimore, MD)
Oxycodone	MOR agonist		National Institute on Drug Abuse (Baltimore, MD)
Buprenorphine	MOR agonist	-	National Institute on Drug Abuse (Baltimore, MD)
8-Cyclopentyl-1,3-dipropylxanthine	A_1R antagonist	DPCPX	Tocris Bioscience (Ellisville, MO)
Cyclopentyladenosine	A_1R agonist	CPA	Sigma (St. Louis, MO)

Adenosine	Non-specific adenosine receptor agonist	-	Sigma (St. Louis, MO)
CGS21680	$A_{2A}R$ agonist	-	Tocris Bioscience (Ellisville, MO
SKF81297	D_1R agonist	-	Tocris Bioscience (Ellisville, MO
U-69,593	KOR partial agonist	U-69	
CGP 55845	$GABA_B$ antagonist	-	Tocris (Minneapolis, MN)
Picrotoxin	$GABA_A$ antagonist	-	
Mecamylamine	Nicotinic acetylcholine receptor antagonist	-	
MPEP	Metabotropic glutamate receptor 5 negative allosteric modulator	-	Tocris (Minneapolis, MN)
Scopolamine	Muscarinic acetylcholine receptor antagonist	-	

Data Computation and Analysis

Data analysis was performed using GraphPad Prism 8 (GraphPad Software, version 8.0, San Diego, CA) based on number of technical replicates (number of cells). Values are presented as average ± SEM. For Chapter 3, statistical comparisons were made using

one-way or two-way ANOVA, along with multiple comparison adjusted Tukey's post hoc tests, as appropriate. For Chapters 4 and 5, statistical comparisons were made using paired t-test or one-way ANOVA, along with multiple comparison adjusted Tukey's post hoc tests, as appropriate. For all experiments, a difference of $p < 0.05$ was considered significant.

Chapter 3 Opioid and Cellular Tolerance

Preface

This chapter describes how chronic opioid treatment with agonists of varying efficacy differentially regulates both the MORs and the SSTRs. It uncovers how partial agonists morphine and oxycodone alters kinase regulation of the LC neurons such that desensitization of SSTR is affected. In contrast, full agonist fentanyl causes profound receptor downregulation but does alter kinase activity. Additionally, when MOR knock out animals were injected with total phosphorylation deficient (TPD) receptors and treated with fentanyl, changes to kinase regulation could be induced. This result suggests that sustained and persistent signaling by partial agonists lead to alteration of kinase activity, which in turn leads to disruption of SSTR desensitization.

I conducted this study under the mentorship and assistance of Dr. John T. Williams. I designed and performed the experiments, analyzed and illustrated data, and prepared the manuscript.

In the context of this dissertation, this chapter directly investigates how MORs are regulated after chronic opioid treatment. Specifically, it explores how different opioid agonists can disrupt downstream kinases and have heterologous adaptation to other GPCRs. Lastly, it sheds light on how this adaptation requires persistent signaling from MORs from the plasma membrane.

Abstract

Chronic treatment of animals with morphine results in a long-lasting cellular tolerance in neurons of the locus coeruleus (LC) and alters the kinase regulation of μ opioid (MORs) and somatostatin (SST) receptors. Studies to date, however, have centered almost exclusively on the treatment of animals with morphine. This study examined the development of tolerance and regulation of kinase activity in rat LC neurons after chronic treatment of animals with oxycodone and fentanyl. No measure of opioid tolerance was detected in experiments with slices from oxycodone treated animals, but, as observed following treatment with morphine, blockade of the G protein Receptor Kinase 2/3 (GRK2/3), with compound 101, no longer inhibited desensitization of SST receptors. Chronic fentanyl treatment induced significant opioid tolerance, but unlike experiments from oxycodone and morphine treated animals, desensitization of the somatostatin receptor was blocked by compound 101. When total phosphorylation deficient (TPD) MORs were expressed in MOR knockout animals treated with fentanyl, compound 101 no longer blocked desensitization of the somatostatin receptor. The results suggest that sustained opioid receptor signaling initiates the process that results in altered kinase regulation of opioid and SST receptors that underlie acute desensitization.

Introduction

Opioids are widely used for pain management and they exert both their analgesic and rewarding effects through the activation of the MORs (Matthes et al. 1996b). Agonist-bound MORs undergo regulatory processes that include desensitization and internalization that are thought to be proximal to the development of cellular tolerance. Both desensitization and internalization of MORs are agonist specific. High efficacy ligands like [MET]^{5}enkephalin (ME) and fentanyl cause robust desensitization and receptor internalization while low efficacy agonists morphine and oxycodone cause less desensitization and internalization, but may induce counter regulatory and homeostatic adaptations without causing desensitization and internalization (Harris and Williams 1991b; Fiorillo and Williams 1996b; Alvarez et al. 2002a; Bailey et al. 2003; Dang and Williams 2004b; E. A. Johnson et al. 2006b; Virk and Williams 2008). Consequently, long-term treatment with agonists of different efficacy could result in unique adaptations at the cellular level, leading to differences in cellular tolerance.

Cellular tolerance is defined by a loss of receptor function after long-term agonist exposure. This loss of function can result from a decrease in receptor/effector coupling, internalization, or down regulation. Chronic morphine treatment results in both desensitization of MORs and a long-lasting reduction in MOR efficacy that persists even in the absence of morphine (Bailey et al. 2009; Levitt and Williams 2012a). Additionally, chronic morphine treatment alters the kinase regulation of both the MORs and the SST receptors. In untreated animals, inhibition of GRK2/3 with compound 101 inhibits SST receptor desensitization; however, after chronic morphine treatment, the inhibition of

GRK2/3 as well as inhibitors of protein kinase C (PKC) and c-Jun kinase (JNK) were required to prevent desensitization of SST receptors (Leff, Arttamangkul, and Williams 2020b). These findings suggest that chronic morphine treatment alters kinase regulation of LC neurons such that additional kinases are upregulated to induce desensitization of both MORs and SST receptors. The mechanism that underlies the altered kinase regulation is not known.

The present study treated animals for 6-7 days with one of several opioid agonists that had varying efficacy to investigate the role of efficacy in the development of cellular tolerance and kinase dependent regulation of desensitization. Cellular tolerance was examined using whole-cell voltage-clamp recordings from LC neurons. The results demonstrate that chronic treatment with oxycodone did not induce cellular tolerance to opioids, whereas in slices from animals treated with fentanyl the concentration response curve to ME was shifted to the right and peak current induced by morphine was decreased. The mechanism behind altered kinase regulation induced by chronic opioid treatment was examined by testing the ability of the GRK2/3 inhibitor compound 101 to block desensitization of the SST receptor. The results demonstrate that chronic treatment with both morphine and oxycodone, but not fentanyl or buprenorphine, altered the kinase regulation of SST receptor desensitization. Finally, when signaling induced by fentanyl was sustained using fentanyl treated MOR knockout animals expressing the total phosphorylation deficient (TPD) MORs, compound 101 no longer blocked desensitization induced by somatostatin. Thus, not only was there an agonist selective action on the development of cellular tolerance and the induction of an altered kinase

regulation of desensitization, but there was a distinct difference in the ability of agonists to affect the two measures.

Materials and Method

Drugs

Morphine sulfate, Oxycodone, Fentanyl, and Buprenorphine were obtained from the National Institute on Drug Abuse, Neuroscience Center (Bethesda, MD). [Met]5 enkephalin (ME), bestatin, thiorphan, and idazoxan were from Sigma-Aldrich (St. Louis, MO). Somatostatin was from ProSpec (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel). MK-801 (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten5,10-imine maleate) and compound 101 (CMP101, 3-[(4-methyl-5- pyridin-4-yl-1,2,4-triazol-3-yl)methylamino]-N-[[2-(trifluoromethyl) phenyl]methyl]benzamide hydrochloride) were purchased from Hello Bio (Princeton, NJ); UK14304 tartate (5-Bromo6-(2-imidazolin-2-ylamino)quinoxaline) was from Tocris (Bio-Techne Corp., Minneapolis, MN).). Potassium methanesulfonate was acquired from Alfa Aesar (Ward Hill, MA). Somatostatin, ME, UK, and idazoxan were dissolved in water, diluted to the appropriate concentration in ACSF, and applied by superfusion. CMP101 was first dissolved DMSO (10% of final volume), sonicated, and then brought to its final volume with 20% (2-Hydroxypropyl)-b-cyclo-dextrin (Sigma-Aldrich) and sonicated again to create a 10mM solution. Slices were incubated in CMP101 (30 mM) diluted in ACSF for at least 1 hour prior to recording, and CMP101 included in the bath (1 mM) and drug solutions (10 mM).

Animals

Adult rats of both sexes were used with ages between 5 and 8 weeks for all experiments. Wild-type Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA), MOR-knockout Sprague-Dawley rats were used as described in Arttamangkul et al. (2019). All animal experiments were conducted in accordance with the National Institutes of Health guidelines and with approval from the Institutional Animal Care and Use Committee of the Oregon Health & Science University (Portland, OR).

Viral Injections

MOR-knockout animals (P24-30) were anesthetized with isoflurane and placed in a stereotaxic frame for microinjection of adenoassociated virus type 2 (AAV2) encoding virally expressed total phosphorylation-deficient MORs (TPD, AAV2- CAG-SS-GFP-MOR-TPD-WPRE-SV40pA) in the LC. A total of 200 nl of virus was injected at 0.1 ml/min bilaterally in the LC (anteroposterior: -9.72 mm, mediolateral: +/-1.25 mm, dorsoventral: -6.95 mm, from bregma) using a computer-controlled stereotaxic frame (Neurostar, Tubingen, Germany). Both viruses were obtained from Virovek (Hayward, CA). Electrophysiology experiments were carried out 2 weeks following injection.

Chronic Opioid Treatment

Rats were treated with either morphine sulfate, oxycodone, fentanyl, buprenorphine, or clonidine continuously released from osmotic pumps as described previously (Quillinan et al., 2011). Osmotic pumps (2ML1; Alzet, Cupertino, CA) were filled with the required

concentration of each drug in water to deliver, 80 mg/kg/day morphine, 30 mg/kg/day oxycodone, or 1.5 mg/kg/day fentanyl. Each pump has a 2-ml reservoir that releases 10 ml/h for up to 7 days. Briefly, rats were anesthetized with isoflurane, and an incision was made in the midscapular region for subcutaneous implantation of osmotic pumps. Pumps remained until animals were used for experiments 6 or 7 days later. Rats receiving oxycodone and fentanyl were first given intraperitoneal injection of 5 mg/kg (oxycodone) or 0.5 mg/kg (fentanyl) twice daily for two days. On day three, they were implanted with minipump.

Tissue Preparation

Acute brain slice perpetration was performed as previously described (cite). Briefly, rats were deeply anesthetized and euthanized by cardiac percussion. Brains were removed, blocked, and mounted in a vibratome chamber (VT 1200S; Leica, Nussloch, Germany). Horizontal LC slices (260 uM) were prepared in warm (34°C) ACSF containing (in millimolars) 126 NaCl, 2.5 KCl, 1.2 MgCl2, 2.6 CaCl2, 1.2 NaH2PO4, 21.4 NAHCO3, and 11 D-glucose with +MK-801 (10 mM). Slices were allowed to recover in warm ACSF containing +MK-801 (10 mM) for at least 30 minutes and then stored in glass vials with warm (34°C) oxygenated (95% O2/ 5% CO2) ACSF until used.

Electrophysiology

Slices were hemisected and then transferred to the recording chamber, which was continuously superfused with 34°C carbogenated ACSF at 1.5–2 ml/min. Whole-cell recordings from LC neurons were obtained with an Axopatch 200B amplifier (Axon

Instruments) in voltage-clamp mode holding potential (V_{hold} = -55 mV). Recording pipettes (World Precision Instruments, Saratosa, FL) with a resistance of 1.0–1.5 MW were filled with an internal solution of (in millimolars) 115 potassium methanesulfonate or potassium methyl sulfate, 20 KCl, 1.5 MgCl2, 5 HEPES(K), 10 BAPTA, 2 Mg-ATP, 0.2 NaGTP, pH 7.4, and 275–280 mOsM. Liquid junction potential (10 mV) was not corrected. Data were filtered at 10 kHz and collected at 20 kHz with AxogrpahX or 400 Hz with PowerLab (Chart version 5.4.2; AD Instruments, Colorado Springs, CO). Only recordings in which the series resistance remained < 14 MW were included).

Data Analysis

For all conditions, animals were used to obtain at least six technical replicates per group; if more than six could be analyzed, all were included. Analysis was performed by using GraphPad Prism 9 (GraphPad Software, version 8.0d; San Diego, CA) based on number of technical replicates (number of slices). Values are presented as average +/-SEM. Statistical comparisons were made using T-test, one-way or two-way ANOVA as well as multiple comparison adjusted Tukey's post hoc tests, as appropriate. For all experiments, $P < 0.05$ was used to describe significance.

Results

Tolerance induced by chronic opioid treatment

Chronic treatment of rats with morphine has been shown to induce a twofold rightward shift of the concentration–response curve to the full MOR agonist DAMGO (D-Ala^2-N-Me-Phe^4-glycol^5-enkephalin) and a decrease in the maximum current induced by the partial agonist, normorphine in locus coeruleus neurons (Christie, Williams, and North

1987b). The present results aimed to determine the degree of tolerance induced by chronic treatment with two agonists with varying efficacy, the partial agonist oxycodone and the potent agonist, fentanyl. Rats were implanted with osmotic pumps that continuously released oxycodone (30 mg/kg/day) or fentanyl (1.5 mg/kg/day). Brain slices were prepared in opioid free solution and washed for 90 min before making whole cell recordings.

Tolerance measured with ME.

Concentration-response curves were created by measuring the outward current induced by ME in brain slices obtained from untreated, oxycodone, and fentanyl treated animals. The current induced by ME was normalized to that induced by a saturating concentration of the α_2-adrenergic receptor agonist, UK14304 (3 μM). In slices from opioid naïve rats, a saturating concentration of ME caused an outward current that was 128.9 ± 1.7 % of the current produced by UK14304 (Fig 1A, C). The concentration of ME that produced a half maximal outward current (EC50) in slices from control was 179 nM (N = 3, 4 cells per concentration from 4 male and 5 female; Fig. 3.1A, C). In slices from oxycodone-treated rats, the concentration response curve to ME was not different from that obtained in naive animals (EC50 166 nM; N = 3, 5 cells per concentration from 6 male and 8 female; Fig 1C). In slices taken from fentanyl treated animals, however, there was a twofold rightward shift in ME concentration–response curve (EC50 518 nM; N = 3, 5 cells per concentration from 6 male and 8 female; Fig 3.1B, C). The maximum current induced by ME was not different in slices from untreated, oxycodone treated, and fentanyl treated animals (128.9 ± 1.7 %, 116.19 ± 3.0 %, and 135 ± 3.0 %, respectively). By this measure,

the results indicated that treatment with fentanyl but not oxycodone resulted in tolerance to ME dependent activation of MORs in the LC. As previously reported for morphine treated animals, the current induced by UK14304 (3 μM) was the same among the treatment groups (untreated: 216.1 ± 18.0 pA; oxycodone treated: 224.0 ± 13.8 pA; fentanyl treated: 202.9 ± 10.8 pA).

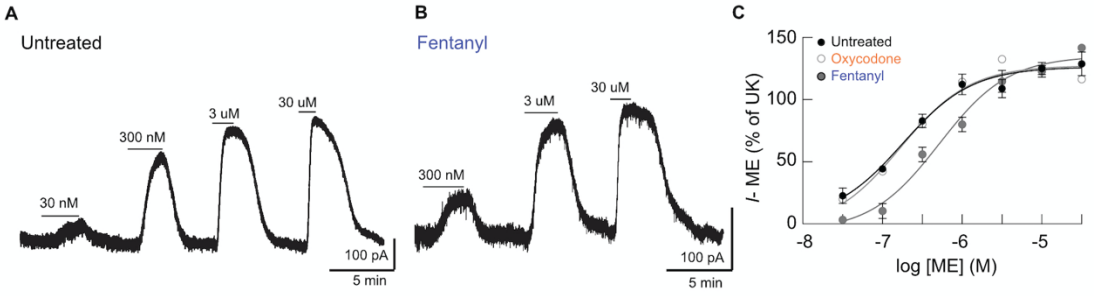

Figure 3.1. Cellular tolerance induced by chronic treatment with fentanyl but not oxycodone

(A) Representative trace of current induced by different concentrations of ME in a slice from an untreated animal

(B) Representative trace of current induced by different concentration of ME in a slice from a fentanyl treated animal

(C) Summary data of concentration response curve of ME normalized to UK current in slices from untreated animals (black circles), oxycodone treated animals (white circles), and fentanyl treated animals (gray circles). Chronic fentanyl treated causes a twofold rightward shift in concentration response curve to ME.

Oxycodone treatment - Tolerance measured with oxycodone.

It is possible that tolerance to ME was not observed following oxycodone treatment due to the high efficacy of ME or a potential agonist specific action. For example, following chronic morphine treatment, the peak current induced by morphine was decreased by approximately 30% (Levitt and Williams 2012a). The interpretation was that tolerance induced by chronic morphine treatment using the partial agonist, morphine, was a more sensitive assay. Thus concentration-response curves to oxycodone were constructed in slices from untreated or oxycodone treated animals. The oxycodone concentration-response curves were created by normalizing the peak outward current amplitude induced by oxycodone to the current induced by a saturating concentration of UK14304 (3 μM). A single concentration of oxycodone was tested in each slice. In slices from untreated animals, a saturating concentration of oxycodone caused an outward current that was 108 ± 2.0 % of the current produced by UK14304, compared to 128.9 ± 1.7 % for ME, demonstrating that oxycodone is a partial agonist in this assay. The EC50 of oxycodone was 2.5 μM (N = 3, 5 cells per concentration from 4 male and 6 female; Fig. 3.2A, D). In slices taken from oxycodone-treated animals (OTA), the concentration response to oxycodone was unchanged (saturating current 97 ± 7.0 % of UK14304, EC50 4.5 μM; n = 3, 4 cells per concentration from 3 males and 6 female; Fig 3.2B, D). Oxycodone treatment did not alter the average current induced by UK14304 (untreated: 242.2 ± 44.8 pA, OTA: 248.4 ± 26.4 pA). Thus, chronic treatment with oxycodone did not alter the sensitivity of MORs to either a subsequent challenge with ME or oxycodone suggesting that there was minimal cellular tolerance induced by 6-7 days of treatment with oxycodone.

In addition to long-lasting cellular tolerance induced by chronic morphine treatment, there is a short-lasting morphine-induced desensitization found in slices from morphine treated animals were maintained in a morphine solution (Bailey, Llorente, et al. 2009; Levitt and Williams 2012a). To determine whether chronic oxycodone treatment could induce this short-term desensitization LC brain slices were prepared and maintained in a solution containing oxycodone (1 μM). Recordings were made in the continued presence of oxycodone, and the opioid antagonist naloxone (1 μM) was applied to determine the steady-state oxycodone current (N = 8 cells from 1 male and 2 female; Fig 3.2C, D). The amplitude of this oxycodone-mediated current was not different from the current produced by oxycodone (1 μM) in slices from oxycodone treated animals that were prepared and maintained in drug free solution or in slices from untreated animals (OTA: Oxycodone 34.5 ± 4.0 % of UK14304; OTA, wash: 34.9 ± 3.5% of UK14304; untreated: 34.9 ± 4.3 % of UK14304; Figure 1G).

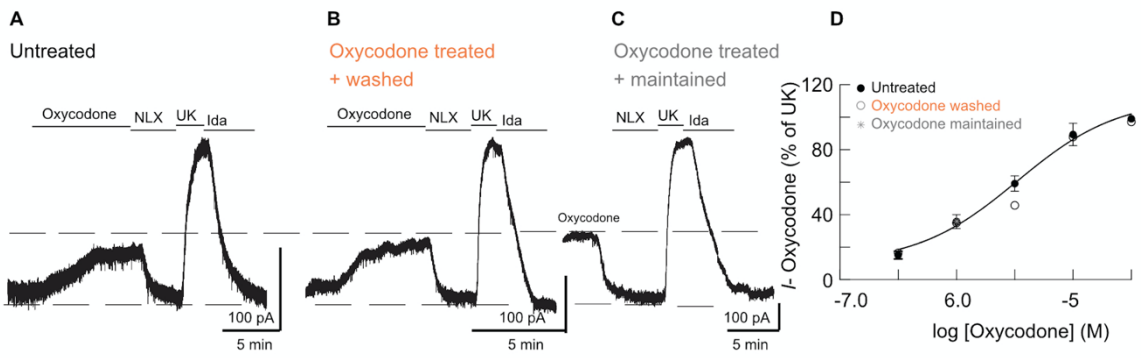

Figure 3.2. Chronic oxycodone does not change the sensitivity of MORs to oxycodone

(A) Representative trace of current induced by oxycodone (1 μM), reversal with naloxone (1 μM), followed by UK14304 (3 μM), and reversal by idaxozan (1 μM) in a slice from an untreated animal.

(B) Representative trace of current induced by oxycodone (1 μM), reversal with naloxone (1 μM), followed by UK14304 (3 μM), and reversal by idaxozan (1 μM) in a slice from an oxycodone treated animal.

(C) Representative trace of a slice from an oxycodone treated animal, maintained in oxycodone. Opioid induced current was revealed after antagonism by naloxone (1 μM), followed by UK14304 (3 μM), and reversal by idaxozan (1 μM).

(D) Summary data of concentration response curve of oxycodone normalized to UK current in slices from untreated animals (black circles), oxycodone treated animals (white circles), and oxycodone treated animal maintained in oxycodone (gray star). There was no change in current induced by oxycodone after chronic oxycodone treatment across all doses.

Oxycodone treatment - Tolerance measured with morphine.

As a partial agonist, the current induce by morphine has been used as more sensitive assay to detect tolerance than higher-efficacy agonists (Christie, Williams, and North 1987b). Therefore, the outward current induced by morphine (1 μM) was examined in slices from animals treated with oxycodone or fentanyl. In slices from untreated animals, morphine (1 μM) induced an outward current that was 66.7 ± 4.3 % of that produced by UK14304 (N = 11 cells from 3 male and 3 female; Fig. 3.3A, D). In slices from oxycodone-treated animals, morphine (1 μM) induced outward current was not different as in slices from untreated animals (54.7 ± 3.4%, $p > 0.05$, one-way ANOVA; N = 7 cells from 3 male and 2 female; Fig 3.2B, D). Thus, chronic treatment with oxycodone did not change the sensitivity of MORs to morphine. In slices from fentanyl treated animals, however, the morphine induced current was reduced to 18.9 ± 4.4% of the current induced by UK14304 ($p < .001$, one-way ANOVA; N = 10 cells from 4 male and 3 female; Fig. 3.3C, D). Thus, like the rightward shift in the concentration response curve to ME after treatment with fentanyl, there was a robust decrease in the current induced by morphine.

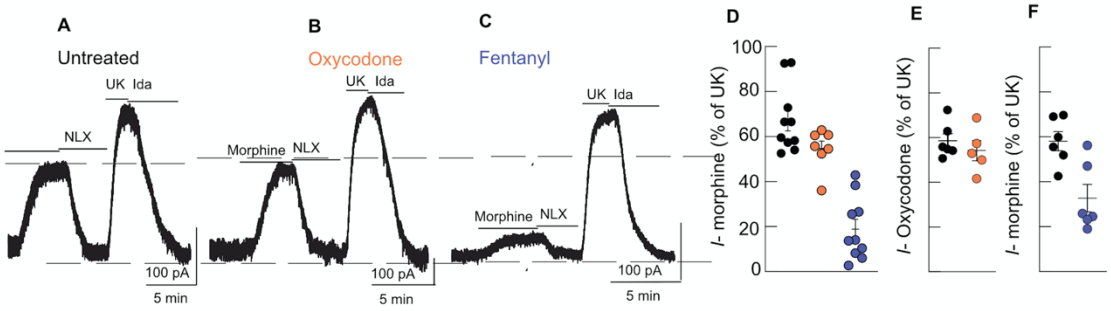

Figure 3.3. Chronic fentanyl treatment, but not oxycodone treatment, reduces current induced by morphine and increases MOR desensitization.

(A) Representative trace of current induced by morphine (1 μM), reversal with naloxone (1 μM), followed by UK14304 (3 μM), and reversal by idaxozan (1 μM) in a slice from an untreated animal.

(B) Representative trace of current induced by morphine (1 μM), reversal with naloxone (1 μM), followed by UK14304 (3 μM), and reversal by idaxozan (1 μM) in a slice from an oxycodone treated animal.

(C) Representative trace of current induced by morphine (1 μM), reversal with naloxone (1 μM), followed by UK14304 (3 μM), and reversal by idaxozan (1 μM) in a slice from a fentanyl treated animal.

(D) Summary data of current induced by morphine in slices from naïve animals (black), oxycodone treated animals (orange), and fentanyl treated animal (blue). Chronic fentanyl treatment significantly reduced the current induced fentanyl.

(E) Summary data of desensitization of oxycodone current induced by a brief application of ME in a slice from a naïve animal (black circle), and in a slice from an oxycodone treated animal (orange circle). There was no difference in desensitization induced by ME between groups.

(F) Summary data of desensitization of morphine current induced by a brief application of ME in a slice from a naïve animal (black circle), and in a slice from a fentanyl treated animal (blue circle). There was significantly higher desensitization induced by ME (lower morphine current) after chronic fentanyl treatment.

Increased acute ME induced desensitization after chronic opioid treatment.

A brief application of a saturating concentration of ME (10 μM, 2 min) is enough to desensitize MORs measure using a subsequent application of morphine (Dang and Williams 2004b). The present experiments examined the current induced by oxycodone (10 μM) or morphine (10 μM) in slices from oxycodone or fentanyl treated animals following application of saturating concentration of ME. Following washout of ME (10 μM, 2 min), oxycodone (10 μM) or morphine (10 μM) were applied followed by reversal of the current with naloxone (1 μM). Once the current reached baseline, UK14304 (3 μM) was applied and the morphine or oxycodone induced current was normalized to the current induced by UK14304. In slices from untreated animals, the current induced by oxycodone (10 μM) after ME application was 58.5± 3.23 % of UK14304 (N = 6 cells from 3 male and 2 female; Fig 3.3E, F) and the current induced by morphine (10 μM) after ME application was 58.1± 4.6% of UK14304 (N = 6 cells from 3 male and 2 female rats; Fig.3.3 E, F). There was no difference in the degree of desensitization induced by

ME between slices from untreated or oxycodone treated animals (OTA: 54.0 ± 4.6% of UK14304; n = 5 cells from 1 male and 3 female; $p > 0.05$, unpaired t-test; Fig 3.3E). However, in slices from fentanyl treated animals, the morphine induced current was decreased to 32.7 ± 6. 2% of UK14304 following application of ME (N = 6 cells from 3 male and 2 female; $p < 0.01$, unpaired t-test; Fig. 3.3F). Therefore, chronic fentanyl treatment, but not chronic oxycodone treatment, increased ME induced desensitization. Thus, as determined by several measures, the results indicate that the degree of tolerance induced by chronic oxycodone and fentanyl treatment differ substantially.

Agonist specific downstream adaptations.

Somatostatin (SST) activates the same potassium conductance as opioids in LC neurons (Fiorillo and Williams 1996b). The current induced by somatostatin (10 μM, 10 min) peaks and rapidly declines undergoing robust acute desensitization. Like MORs, SST receptors are known to be phosphorylated by GRK2/3 (Günther et al. 2018) and inhibition of GRK2/3 with compound 101 decreased SST receptor desensitization (Leff, Arttamangkul, and Williams 2020b). In slices taken from animals chronically treated with morphine, compound 101 alone was no longer sufficient to block acute SST induced desensitization (Leff, Arttamangkul, and Williams 2020b). Instead, the combination of kinase inhibitors, acting at GRK2/3, PKC and JNK were required to block SST induced desensitization. Thus, chronic morphine treatment altered the kinase dependence of GPCRs in LC neurons.

In the present study, animals were treated with morphine, oxycodone, fentanyl, and buprenorphine to investigate the agonist specific regulation of LC kinase signaling using

the desensitization of the somatostatin receptor. The current induced by SST (10 μM) declined to 30.2 ± 4.5% of the peak after 10 mins (N = 14 cells from 5 male and 7 female; Fig 3.4A, D) and in slices with compound 101 the decline was reduced to 63.7 ± 2.0% of the peak (N = 11 cells from 5 male and 5 female; Fig 3.4A, D). Thus, the inhibition of GRK 2/3 alone is sufficient to decrease acute SST-induced desensitization in untreated animals. (Fig 3.4A, D; $p < 0.01$, unpaired t-test). As previously reported, in slices from morphine treated animals, the decline in the current induced by somatostatin was insensitive to compound 101 (SST morphine treated without 101: 35.8 ± 3.0 % percent of peak, N = 7 cells from and 3 male and 3 female; SST morphine treated in compound 101: 33.2 ± 4.6 %, N = 7 cells from 3 male and 3 female; Fig.3.4B, D; $p > 0.05$, unpaired t-test). When MOR KO animals were treated with morphine, similar to untreated WT animals, compound 101 was still able to inhibit SST receptor desensitization, indicating that the change in kinase regulation was dependent on the activation of MORs (SST morphine treated without 101: 43.6 ± 1.5 % percent of peak, N = 6 cells from and 2 male and 3 female; SST morphine treated in compound 101: 67.42 ± 1.4 %, N = 6 cells from 2 male and 3 female; Fig S3). Similar to morphine treated animals, compound 101 alone did not block the SST induced desensitization in slices from oxycodone treated animals (SST oxycodone treated without 101: 39.3 ± 5.9 %; SST oxycodone treated in compound 101: 38.1 ±6.3 % Fig. 3.4C, D, $p > 0.05$, unpaired t-test). However, in slices from buprenorphine animals, similar to untreated animals, compound 101 inhibited SST receptor desensitization (SST buprenorphine treated without 101: 30.5 ± 5.3 %, n = 9 cells from 3 male and 3 female; SST buprenorphine

treated in compound 101: 64.3 ± 2.1, n = 13 cells in 5 male and 4 female; Fig. 3.4D; p > 0.05, unpaired t-test). The same result was obtained using slices from fentanyl treated animals. Compound 101 inhibited SST receptor desensitzzation (SST fentanyl treated without 101: 27.5 ± 4.5 %, n = 9 cells from 4 male and 3 female; SST fentanyl treated in compound 101: 67.1 ± 4.6, n = 8 cells in 3 male and 6 female; Fig. 3.2C, D; p > 0.05, unpaired t-test). Therefore, the opioid-induced regulation of LC kinase signaling after chronic treatment with morphine and oxycodone is agonist specific. These agonists have moderate efficacy and are inefficient at the induction of receptor internalization. However, how is it that chronic treatment with buprenorphine, a weak partial agonist, and chronic treatment with fentanyl, a potent agonist that does induce receptor internalization, result in no change in kinase regulation?

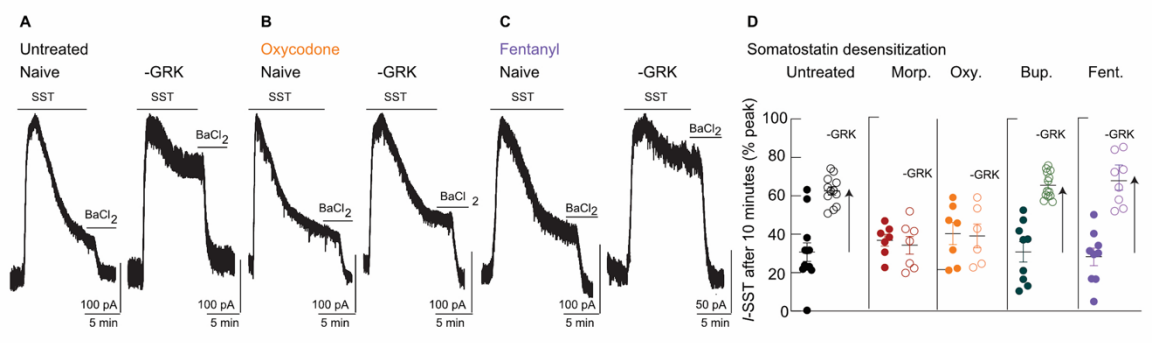

Fig 3.4 Agonist specific disruption of GRK2/3 kinase after chronic treatment with opioids

(A) Representative trace of desensitization induced by a saturating concentration of SST (10 µM, 10 minutes) in a slice from an untreated animal without compound 101 (left). Representative trace of inhibition of desensitization induced by a saturating concentration of SST (10 µM, 10 minutes) in a slice from an untreated animal with compound 101 (right).

(B) Representative trace of desensitization induced by a saturating concentration of SST (10 μM,10 mins) in a slice from an oxycodone treated animals without compound 101 (left). Representative trace of lack inhibition of desensitization induced by a saturating concentration of SST (10 μM, 10 minutes) in a slice from an oxycodone animal with compound 101 (right).

(C) Representative trace of desensitization induced by a saturating concentration of SST (10 μM, 10 minutes) in a slice from an oxycodone treated animal without compound 101 (left). Representative trace of desensitization induced by a saturating concentration of SST (10 μM, 10 minutes) in a slice from an oxycodone treated animal with compound 101 (right).

(D) Summary data comparing SST receptor desensitization in untreated, morphine treated, oxycodone treated, buprenorphine treated, and fentanyl treated animals with and without compound 101. Chronic morphine and oxycodone, but not buprenorphine or fentanyl disrupts the activity GRK2/3.

Sustained signaling of MORs and GRK2/3 dependent SST receptor desensitization

The opioid agonist dependent changes to LC kinase activity measured with the desensitization induced by SST could result from at least two mechanisms: (1) continued opioid receptor activation or (2) opioid receptor internalization. Although the ability of the different agonists to maintain signaling, induce desensitization and/or promote receptor internalization differ, none are completely biased. However, these processes can be separated on their reliance upon MOR receptor phosphorylation. Mutation of phosphorylation sites of the c-terminal tail of MOR reduces MOR desensitization and internalization without attenuating G-protein mediated signaling (Arttamangkul et al. 2018). To address these potential mechanisms of alteration in kinase activity following

chronic opioids, phosphorylation-deficient MORs (TPD-MOR) were expressed in the LC of MOR knockout rats. TPD-MOR expressing MOR knockout animals were treated with fentanyl and the action of compound 101 on SST receptor desensitization was assessed. In untreated MOR KO animals expressing the TPD-MOR, as in WT animals, the SST induced desensitization was robust and sensitive to compound 101 (SST TPD untreated treated without 101: percent peak 26.5 ± 4.1 %, N = 7 cells from 2 male and 3 female; SST TPD untreated in compound 101: 67.0 ± 2.6, N = 5 cells in 2 male and 1 female; Fig. 3.5A, C; $p > 0.05$, unpaired t-test; Fig.3 A, C). In fentanyl treated animals, however, the somatostatin receptor desensitization was insensitive to compound 101 (SST TPD fentanyl treated without 101: percent peak 29.6 ± 4.6 %, N = 6 cells from 3 male and 1 female; SST TPD fentanyl in compound 101: 31.5 ± 6.8, N = 7 cells in 2 male and 2 female; Fig. 3.5B, C; $p < 0.05$, unpaired t-test). This result is the same as the results obtained using WT animals treated with morphine or oxycodone. Thus, sustained signaling of MORs was sufficient to alter the kinase regulation that modulated SST receptor desensitization and suggests that desensitization and/or internalization may prevent these adaptations. Taken together the results indicate that treatment with fentanyl induces cellular tolerance and reduced signaling such that downstream adaptive mechanisms underlying the altered kinase regulation are not engaged.

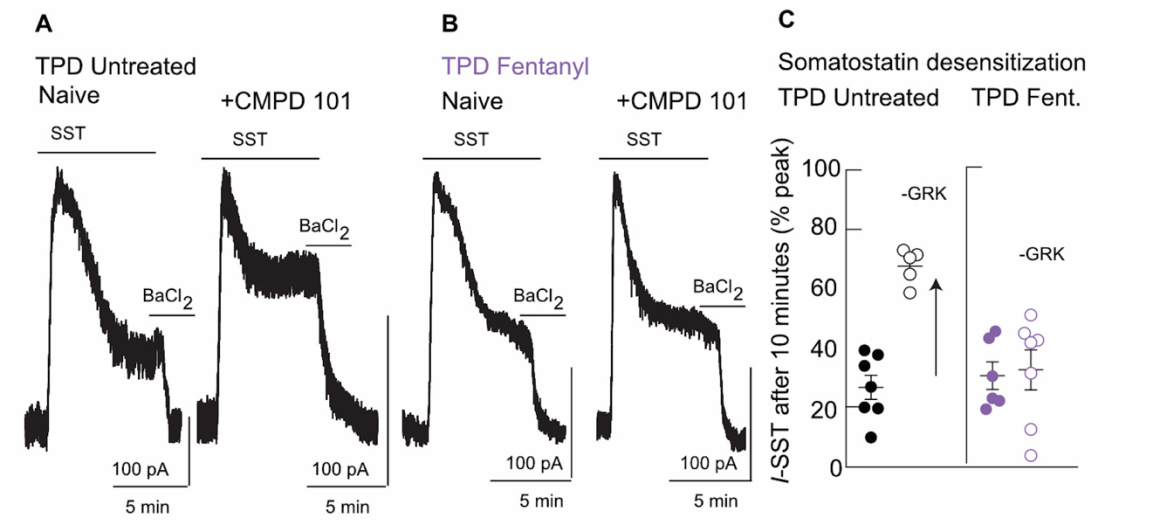

Fig. 3.5 Sustained signaling by MORs drive kinase upregulation

(A) Representative trace of desensitization induced by a saturating concentration of SST (10 µM, 10 minutes) in a slice from an untreated MOR KO animal injected with TPD receptor, without compound 101 (left). Representative trace of inhibition of desensitization induced by a saturating concentration of SST (10 µM, 10 minutes) in a slice from an untreated MOR KO animal injected with TPD receptor with compound 101 (right).

(B) Representative trace of desensitization induced by a saturating concentration of SST (10 µM, 10 minutes) in a slice from a fentanyl treated MOR KO animal injected with TPD receptor, without compound 101 (left). Representative trace of desensitization induced by a saturating concentration of SST (10 µM, 10 minutes) in a slice from a fentanyl treated MOR KO animal injected with TPD receptor with compound 101 (right).

(C) Summary data comparing SST receptor desensitization in untreated MOR KO animal injected with TPD with and without compound 101 (left panel). In slices from these animals, compound 101 inhibits SST receptor desensitization. Summary data comparing SST receptor desensitization in fentanyl treated MOR KO animal injected with TPD with and without compound 101 (left panel). In slices from these animals, compound 101 does not inhibit SST receptor desensitization.

Discussion

The present study found that chronic treatment of rats with different opioid agonists had markedly different actions that were dependent on the agonist. Both the degree of tolerance measured by MOR activation of potassium conductance and the GRK2/3 dependent desensitization of a non-opioid receptor were affected differently depending on the agonist treatment. Chronic treatment with oxycodone did not induce desensitization or measures of tolerance, but resulted in a decrease in the ability of the GRK2/3 inhibitor compound 101 to block acute desensitization of the somatostatin receptor. Chronic treatment with fentanyl induced robust tolerance and increased MOR desensitization, but had no effect on the ability of compound 101 to block desensitization of the somatostatin receptor. Previous work has shown that with viral expression of TPD-MORs in MOR knockout rats, acute desensitization, trafficking and tolerance are drastically decreased (Arttamangkul et al. 2018). In this study, treatment of MOR knockout animals expressing the TPD receptor with fentanyl resulted in a disruption of inhibition of GRK2/3 to induce SST receptor desensitization. This observation is the opposite of that seen in fentanyl treated wild type animals and is the same as in animals

treated with oxycodone and morphine. Taken together, the results suggest that sustained signaling from the plasma membrane is the underlying process that initiates the altered LC kinase signaling.

Agonist specific regulation after chronic treatment

Chronic opioid treatment results in a variable amount of tolerance that is dependent on agonist efficacy, the measure of tolerance and details of the treatment protocol (reviwed Morgan and Christie 2011; reviewed Williams et al. 2013). When measuring the antinociceptive action of opioids using the tail flick assay, chronic treatment with oxycodone or etorphine administered with osmotic mini pumps induced tolerance to oxycodone more quickly than that to etorphine (Pawar et al. 2007; Madia et al. 2009). Similar results were obtained in animals treated with a variety of opioids, where morphine and oxycodone resulted in a larger shift in the dose response curve than fentanyl measured also using a tail flick assay (He et al. 2021). The conclusion was the analgesic tolerance induced by high efficacy agonists developed slower than with treatment with lower efficacy agonists. Using a paw withdrawal assay, however, chronic treatment with fentanyl induced a larger rightward shift in the dose response than morphine (Kliewer et al. 2019a).

A significant component of the analgesic tolerance induced by chronic opioid treatment results from homeostatic compensatory mechanisms (reviwed Christie 2008; reviwed Williams et al. 2013). These compensatory mechanisms underlie acute withdrawal that is induced by treatment with naloxone. That the compensatory mechanisms are more

prevalent with the use of agonists that are less effective at the induction of desensitization or tolerance was suggested using experiments with wild type animals and knockin animals expressing the total phosphorylation deficient MOR (Kliewer et al. 2019b). Thus, compensatory mechanisms likely underlie difference in measures of tolerance in vivo and at the cellular level.

Agonist regulation of kinase dependent desensitization

The interaction of arrestin and MOR has received considerable interest as one mechanism that underlies biased signaling. Phosphorylation of the receptor is the first and necessary step in the recruitment of arrestin to MOR (reviwed Gillis et al. 2020). The GRK/GPCR interaction is transient, is dependent on agonist/receptor association and phosphorylation of the receptor at multiple sites, most likely requires multiple binding interactions between GRK and the receptor (Gurevich and Gurevich 2019a). The inability of morphine and oxycodone to recruit arrestin is certainly dependent on the lack of receptor phosphorylation induced by morphine and oxycodone (reviwed -- Gillis et al. 2020). In fact, with the over expression of GRK2, the recruitment of arrestin by all agonists is augmented (Gillis et al. 2020). Thus, the modulation of GRK2/3 activity will have a dramatic action on desensitization as seen in the present work that may be dependent on the recruitment of arrestin.

Persistent signaling gates adaptive mechanisms

Although chronic oxycodone did not induce cellular tolerance, the continued activation of the receptor resulted in a downstream alteration of kinase activity. The combination of

kinase inhibitors that acted on PKC and JNK and GRK2/3 were required to block desensitization of the somatostatin receptor after chronic morphine treatment (Leff, Arttamangkul, and Williams 2020b). Given that both morphine and oxycodone do not effectively recruit GRK2/3, the fact that other kinases are upregulated after chronic treatment is consistent with work indicating that morphine induces receptor phosphorylation by PKC (Bailey, Kelly, and Henderson 2004; Johnson et al. 2006b; C. P. Bailey et al. 2009; Levitt and Williams 2012a). This modulation is surely dependent on persistent activation of MORs, as TPD-MOR after treatment with fentanyl also alters the kinase signaling in the LC. Thus, agonist efficacy alone does not account for the downstream adaptation kinase activity.

Buprenorphine is a weak partial agonist of the MORs that activates the receptors to induce a hyperpolarization of LC neurons (Virk et al. 2009). Although buprenorphine is poor at the recruitment of GRK, as morphine and oxycodone, it has much lower intrinsic efficacy. It appears that while the outcomes of chronic treatment with buprenorphine and fentanyl are the same, it is likely that this results from different mechanisms. The low intrinsic efficacy of buprenorphine results in a low level of receptor activation that does not effectively result in downstream regulation of kinases. In that way it may be similar to results obtained with fentanyl treated animals where the receptor signaling is compromised by the development of tolerance.

Although inhibition of GRK2/3 alone in slices from morphine animals is not sufficient to block desensitization of the somatostatin receptor, the inhibition of a series of other

kinase inhibitors in addition to GRK2/3 was necessary to block desensitization (Leff, Arttamangkul, and Williams 2020b). Thus GRK2/3 remained active but inhibition of it alone was ineffective in blocking desensitization following an apparent up regulation of PKC and JNK. The change in kinase regulation was dependent on an increase in the ability of other kinases to phosphorylate both the MORs and the SST receptor. In our hands inhibition of PKC had little or no effect on desensitization of MORs in untreated animals (Arttamangkul, Birdsong, and Williams 2015; Levitt and Williams 2012a); however there was a component of short-term tolerance that was sensitive to inhibition of PKC in slices from morphine treated animals (Bailey et al. 2009; Levitt and Williams 2012a). In addition, the inhibition of JNK resulted in a block in acute desensitization at the spinal level (Melief et al. 2010). Thus, there is evidence that multiple kinases can underlie acute MOR desensitization. However, neither PKC or JNK alone had an effect in MOR or SST receptor desensitization from untreated or morphine treated animals (Leff, Arttamangkul, and Williams 2020b). Therefore, the conclusion of the present work is that following treatment of animals with low-efficacy opioids, multiple kinases are engaged to mediate acute desensitization of MORs and other GPCRs, and the engagement of these kinases is dependent on sustained signaling.

Summary

The present work distinguishes both receptor-level regulation and a downstream adaptation induced by opioid agonists with varying efficacies after chronic treatment. Chronic oxycodone treatment did not induce tolerance, unlike the profound tolerance induced by chronic treatment with fentanyl. Alternatively, chronic oxycodone treatment,

but not chronic fentanyl treatment, induced a decrease in the ability of GRK2/3 to prevent acute desensitization, and this adaptation included the desensitization of the SST receptor. When internalization was blocked and animals were treated with fentanyl, sustained signaling promoted the altered kinase regulation of somatostatin receptors. The results suggest that a) agonists with different efficacy differentially regulate MOR and thus cellular tolerance and b) though inhibiting phosphorylation may reduce one measure of tolerance, persistent signaling induced by these receptors can lead to downstream adaptations like upregulation of PKC and JNK that promote other measures of cellular and systemic tolerance that may underlie opioid withdrawal. The consequence is that signaling at the cellular level following chronic opioid treatment affects desensitization of GPCRs beyond the opioid receptors.

Chapter 4 Opioid and Adenosine

Preface

This chapter describes how MORs regulate the release of adenosine in a thalamo-striatal circuit in the dorsal medial striatum. It uncovers a novel role of opioids in acutely inhibiting adenosine accumulation and subsequent adenosine receptor signaling in the striatum by inhibiting the production of cAMP. Additionally, this work discovered that resting endogenous adenosine is released by D_1, but not D_2 receptor positive MSNs, suggesting that opioid signaling and manipulation of D_1R-expressing MSN cAMP activity can broadly affect striatal function and behavior.

I conducted this study under the mentorship and assistance of Dr. William T. Birdsong, and Dr. John T. Williams. I designed and performed the experiments, analyzed and illustrated data, and prepared the manuscript.

In the context of this dissertation, this chapter is a direct comparison of the role that MORs play in the presynaptic terminal of the neuron as opposed to the somatodendritic compartment. There are stark differences the regulation of the MORs and the functional consequences of the acute activation these receptors in these two compartments. This chapter sheds light on how opioids can acutely alter signaling of other neuromodulators and influences synaptic transmission in the striatum.

μ opioid receptors acutely regulate adenosine signaling in a thalamo-striatal circuit

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Abstract

Endogenous adenosine plays a crucial role in maintaining energy homeostasis and adenosine levels are tightly regulated across neural circuits. In the dorsal medial striatum (DMS) adenosine inhibits neurotransmitter release, but the source and mechanism underlying its accumulation are largely unknown. Opioids also inhibit neurotransmitter release in the DMS and influences adenosine accumulation after prolonged exposure. However, how these two neurotransmitter systems interact acutely is also largely unknown. This study demonstrates that activation of μ opioid receptors (MORs), but not δ opioid receptors (DORs) or κ opioid receptors (KORs), inhibits tonic activation of adenosine A_1Rs via a cyclic adenosine monophosphate (cAMP) dependent mechanism in both male and female mice. Further, selectively knocking-out MORs from presynaptic terminals and postsynaptic medium spiny neurons (MSNs) revealed that activation of MORs on D_1R positive MSNs, but not D_2R positive MSNs, is necessary to inhibit tonic adenosine signaling on presynaptic terminals. Given the role of D_1R positive MSNs in movement and motivated behaviors, these findings reveal a novel mechanism by which these neurons regulate their own synaptic inputs.

Introduction

Opioids such as morphine acutely mediate analgesia and long-term use leads to dependence and potentially addiction. The thalamus and dorsal medial striatum are important for regulating opioid dependence and modulating goal-directed behavior respectively (Balleine, Delgado, and Hikosaka 2007; Zhu et al. 2016). Opioids are known to inhibit both excitatory input to the striatum, and local GABA release within the striatal micro-circuitry (Atwood, Kupferschmidt, and Lovinger 2014; Banghart et al. 2015a; Birdsong et al. 2019b). Additionally, agonists selective to the adenosine A_1Rs also inhibit glutamate release in the striatum (Brundege and Williams 2002b) and influence striatal dynamics. Thus, understanding the role of opioid receptors and A_1Rs in modulating excitatory inputs to the striatum, and the potential interaction between these receptors, is important to understand how multiple neurotransmitter systems influence striatal activity.

Morphine binding to MOR activates $G_{i/o}$ heterotrimeric G-proteins to inhibit adenyl cyclase (AC) and consequently decreases cAMP levels (Heijna et al. 1992; Izenwasser, Buzas, and Cox 1993). Acutely, this inhibition of cAMP, along with other effectors, ultimately inhibits neuronal activity and neurotransmitter release. Similarly, activation of the A_1Rs also inhibits AC, and under basal conditions there is a resting extracellular adenosine tone in the striatum. This resting adenosine tone can tonically activate A_1Rs, inhibiting neurotransmitter release (Brundege and Williams 2002b). The fact that adenosine and opioids both act through the same effector systems suggests that these two neurotransmitters can influence each other's signaling. But neither the role of opioids in

modulating resting adenosine levels nor the source of this resting adenosine tone is known.

Agonists selective to MOR, but not DOR, potently inhibit glutamate release from thalamus onto the striatum (Birdsong et al. 2019b; Muñoz, Haggerty, and Atwood 2020), but the role of KORs in this circuit has not been examined. However, there is evidence that MOR, DOR and KOR are widely expressed throughout the striatum (Al-Hasani et al. 2015; Banghart et al. 2015; Massaly et al. 2019; Mansour et al. 1994; Muschamp and Carlezon 2013; Nestler and Carlezon 2006) and have been shown to inhibit neurotransmitter release in the striatum to varying degrees in a synapse-specific manner (Tejeda et al. 2017). Therefore, although all three subtypes of opioid receptors are present in the striatum, they potentially modulate the activity of the striatum and interact with A_1R signaling uniquely.

The present study examines the functional interaction between opioid receptors and adenosine signaling, the mechanism underlying extracellular adenosine accumulation, and the source of adenosine release in the striatum using a combination of brain slice electrophysiology, pharmacology, optogenetics, and genetic manipulation of MOR expression in mice. Optically-induced excitatory post synaptic current (oEPSCs) were recorded in striatal medium spiny neurons following optical excitation of channelrhodopsin-expressing medial thalamic axon terminals in the dorsomedial striatum. The facilitation of oEPSC amplitude by the A_1R antagonist 8-cyclopentyl-1,3-

dipropylxanthine (DPCPX) was used to measure tonic A_1R activation and as a proxy for extracellular adenosine accumulation. The results show (1) morphine inhibits tonic adenosine accumulation by inhibiting cAMP, (2) this inhibition is specific to MOR agonists and not DOR or KOR agonists, and (3) MOR regulation of dorsomedial striatal adenosine levels requires MOR expression on D_1R positive MSNs.

Materials and Method

Animals

Male and female C57BL/6J mice (8–10 weeks old) were bred in house and were housed under a 12-hr-light/dark cycle. Food and water were available *ad libitum*. Mice with exons 2 and 3 of the oprm1 gene flanked by the LoxP cassette (FloxedMor; *Oprm1*$^{fl/fl}$; JAX stock #030074), with a genetic background of 75:25% of C57BL/6J were provided by Dr. Brigitte L. Kieffer. $Vglut_2$- *cre* mice (*Slc17a6*$^{tm2(cre)Lowl}$; JAX stock #016963) were purchased from the Jackson Laboratory. The two mice were crossed to generate FloxMor-$Vglut_2$-*cre* mice that lack MORs in presynaptic terminals. A_{2A}-*cre* mice (*Tg(Adora2a-cre)KG139Gsat*; MMRC stock #036158-UCD) were provided by Dr. Tianyi Mao and were crossed with FloxedMor mice to generate FloxedMor-A_{2A}-*cre* mice lacking MORs in D_2 positive MSNs. D_1-*cre* mice (*Tg(Drd1-cre)EY262Gsat*; MMRC stock #030989- UCD) were provided by Dr. Christopher Ford and were crossed with FloxedMOR mice to generate FloxedMOR-D_1-*cre* mice lacking MORs in D_1 positive MSNs. All animal experiments were conducted in accordance with the National Institutes of Health guidelines and with approval from the Institutional Animal Care and Use Committee of the Oregon Health & Science University (Portland, OR).

Viral injection

Mice were anesthetized with isoflurane and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, with custom modifications) for microinjections of recombinant adeno-associated virus (AAV2-syn-CsChR-GFP) to express channelrhodopsin. A glass pipette filled with 40 nL of virus was injected into the medial thalamus (Nanoject II, Drummond Scientific, Broomall, PA; BCJ: custom-built injector based on a MO-10, Narishige, Amityville, NY). Injection coordinates for MD are in mm for medial/lateral (M/L), anterior/posterior from bregma (A/P), and dorsal/ventral from the top of the skull directly over the target area: M/L: +/-0.55, A/P: −1.2, D/V: -3.4. Electrophysiology experiments were done 2-3 weeks after viral injections.

Drugs

Morphine sulfate was obtained from the National Institute on Drug Abuse (Baltimore, MD). Naloxone and dizocilpine maleate (MK801) were from Abcam (Cambridge, MA). 8-Cyclopentyl-1,3-dipropylxanthine DPCPX, CGS21680, SKF81297, Mecamylamine, CGP 55845, and MPEP were from Tocris Bioscience (Ellisville, MO). Scopolamine, Adenosine, [Met^5] Enkephalin (ME), Bestatin, Thiorphan, and R0-20-1724 were from Sigma Aldrich (St. Louis, MO). Picrotoxin was from Hello Bio. ME, Morphine, Adenosine, Naloxone, MPEP, Scopolamine, and Mecamylamine were dissolved in water, diluted in artificial cerebrospinal fluid (ACSF) and applied by bath superperfusion. Bath perfusion of ME was with bestatin (10 μM) and thiorphan (1 μM) to limit breakdown of

ME. Picrotoxin was directly dissolved in ACSF. DPCPX, CGS21980, SKF81297 and R0-230853 were dissolved in dimethyl sulfoxide (DMSO), diluted in ACSF and applied during incubation and by bath superperfusion.

Tissue Preparation

Acute brain slice preparation was performed as previously described (Birdsong et al. 2019b). Briefly, mice were deeply anesthetized and euthanized using isoflurane. Brains were removed, blocked, and mounted in a vibratome chamber (VT 1200S; Leica, Nussloch, Germany). Coronal slices (242 μM) were prepared in warm (34°C) ACSF containing (in millimolars) 126 NaCl, 2.5 KCl, 1.2 MgCl2, 2.6 CaCl2, 1.2 NaH2PO4, 21.4 NAHCO3, and 11 D-glucose with +MK-801 (10 mM). Slices were allowed to recover in warm ACSF containing +MK-801 (10 μM) for at least 30 minutes and then stored in glass vials at room temperature with oxygenated (95% O2/ 5% CO2) ACSF until used.

Brain slice electrophysiology

Slices were hemisected and then transferred to the recording chamber, which was continuously superfused with 34°C carbogenated ACSF at 1.5–2 ml/min with (in μM): 0.2 $GABA_B$-receptor antagonist CGP 55845, 10 $GABA_A$-receptor antagonist picrotoxin, one nicotinic acetylcholine receptor antagonist mecamylamine, 0.1 muscarinic acetylcholine receptor antagonist scopolamine and 0.3 metabotropic glutamate receptor five antagonist MPEP. Whole-cell recordings from medium spiny neurons (MSNs) in the

dorsal medial striatum were obtained with an Axopatch 200A amplifier (Axon Instruments) in voltage-clamp mode, holding potential (V_{hold} = -75 mV). Recording pipettes (Sutter Instruments, Novato, CA) with a resistance of 2.8–3.5 MΩ were filled with an internal solution of (in millimolars) 110 potassium gluconate, 10 KCl, 15 NaCl, 1.5 $MgCl_2$, 10 HEPES, 1 EGTA, 2 Na_2ATP, 0.3 Na_2GTP, 7.8 phosphocreatine; pH 7.35–7.40 ~280 mOsm). Data were filtered at 10 kHz and collected at 20 kHz with AxographX. Only recordings in which the series resistance remained < 18 MΩ or changed by less than 20 percent throughout the experiment were included. A TTL-controlled LED driver and 470 nm LED (Thorlabs, Newton, NJ) was used to illuminate the slice through the microscope objective directly over the recorded cell with ~1 mW of power for 0.5 ms or 1 ms.

Electrophysiology data analysis

Data were analyzed in Axograph. Peak current amplitude was calculated relative to mean current during 50 ms baseline prior to the stimulus. Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA). For the time course and summary data, baseline oEPSCs were normalized to oEPSCs amplitudes three to four minutes prior to baseline (prebaseline condition, not shown). All other conditions were normalized to oEPSC amplitudes three to four minutes before drug application. Summary data were presented as the averages of six to 10 trials beginning three to four minutes after drug application after steady state was achieved. For all conditions, mice were used to obtain at least five technical replicates per group; if more than six could be

analyzed, all were included. Values are presented as average +/-SEM. Statistical analysis was performed on normalized data. Statistical comparisons were made with paired ratio T-test, one-way repeated measures ANOVA, or one-way ANOVA, followed by multiple comparison adjusted Tukey's post hoc tests. For all experiments, $P < 0.05$ was used to describe statistical significance.

Results

Thalamo-striatal glutamate release is sensitive to both opioid and adenosine agonists

Adeno-associated virus (AAV) type 2 encoding channelrhodopsin was microinjected into the medial thalamus, and whole-cell voltage-clamp recordings were made from medium spiny neurons in the dorsal medial striatum (DMS) (Fig 4.1A). Striatal MSNs were identified by their hyperpolarized resting membrane potential, low input resistance and a long delay to the initial spike (Kreitzer 2009). Glutamate release was evoked by optical stimulation with 470-nm light, and AMPA receptor-mediated excitatory postsynaptic currents (oEPSCs) were pharmacologically isolated and recorded as previously described (Birdsong et al. 2019b). After a stable baseline of oEPSCs was established, the partial agonist morphine (1 μM) was superperfused, followed by the antagonist naloxone (1 μM) (Fig 4.1C, E). The inhibition by morphine was determined by averaging the oEPSC three to five minutes after drug perfusion and normalizing the response to the average of oEPCS three to five minutes before drug perfusion. Morphine decreased the amplitude of the oEPSCs and this inhibition was reversed by naloxone (Fig 4.1C, E, F; morphine: 0.80 ± 0.05 fraction of baseline, $p = 0.0002$; naloxone: 0.98 ± 0.01 fraction of baseline, p =

0.002, n = 8 cells, 4 mice, $F_{(2, 14)} = 17.29$, one-way repeated measures ANOVA, Tukey's multiple comparisons test). In separate cells, an A_1R agonist cyclopentyladenosine (CPA, 1 μM) was superperfused, followed by antagonist DPCPX, 200 nM), (Fig D, E). CPA decreased the amplitude of the oEPSCs and this inhibition was reversed by DPCPX (Fig 4.1D, E, G; CPA 0.37 ± 0.04 fraction of baseline, $p < 0.0001$; DPCPX: 1.3 ± 0.08 fraction of baseline, $p < 0.0001$ n = 7 cells, 6 mice, $F_{(2, 12)} = 87.95$, one-way repeated measures ANOVA, Tukey's multiple comparisons test). Additionally, DPCPX caused a significant over-reversal of the amplitude of the oEPSCs (Fig 1D, E, G), suggesting tonic inhibition of glutamate release by activation of A_1Rs that was blocked by DPCPX. Thus, glutamate release in the thalamo-striatal synapses is regulated by both MORs and A_1 receptors, and there is an additional tonic activation of A_1Rs by endogenous adenosine.

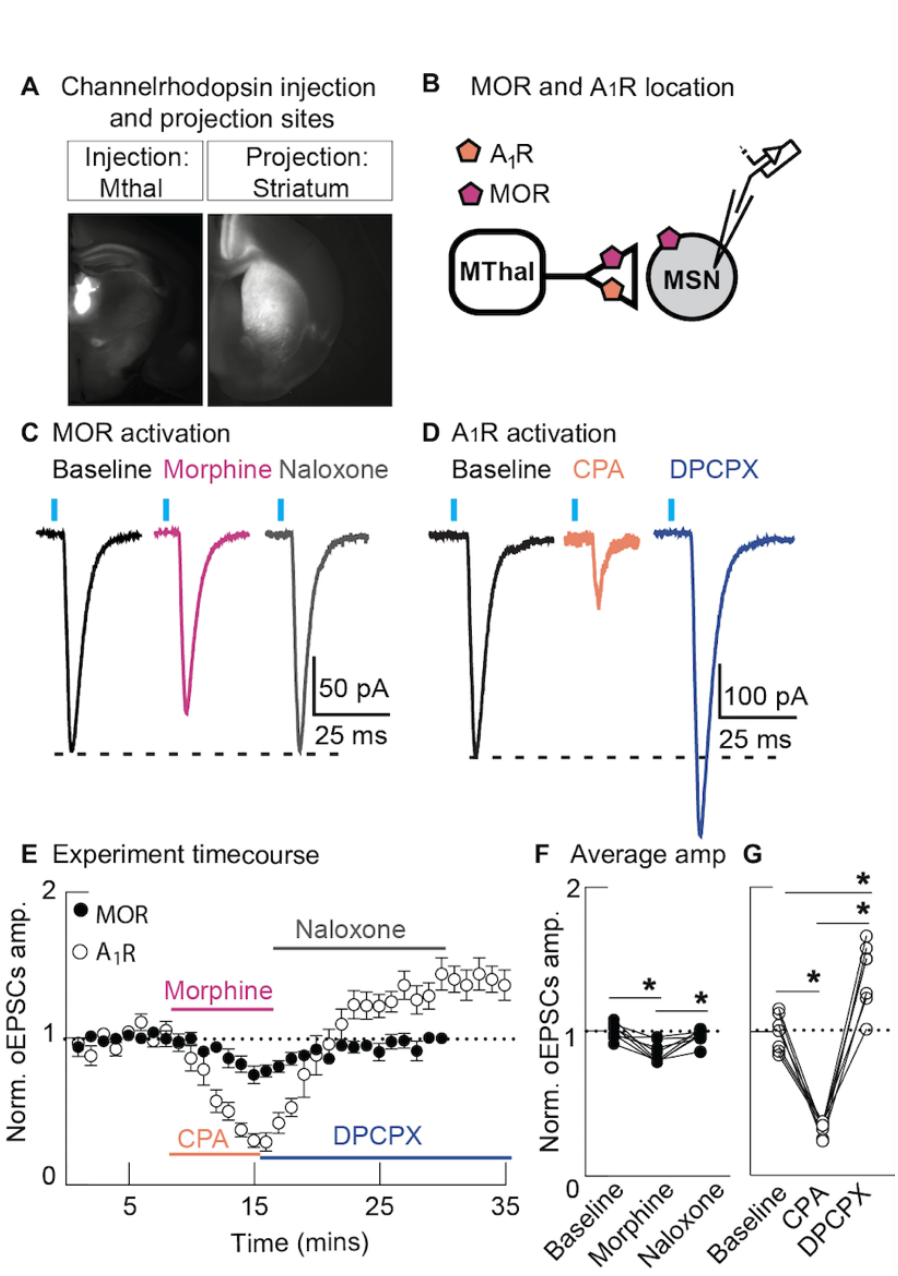


Figure 4.1. Activation of both μ opioid receptor and adenosine A_1 receptor leads to inhibition of thalamo-striatal oEPSCs.

(A) An acute mouse brain slice example of overlaid brightfield and epifluorescent images showing the viral injection site (Mthal; left) and the axonal projections (Striatum; right).

(B) Schematic showing the locations of both A_1Rs and MORs in the thalamo-striatal synapse.

(C) Representative oEPSCs evoked by 470 nm light (black label), inhibition of oEPSC amplitude by morphine (1 μM; pink label), and reversal by naloxone (1μM; gray label).

(D) Representative oEPSCs evoked by 470 nm light (black label), inhibition of oEPSC amplitude by CPA (1 μM; orange label), and over reversal by DPCPX (200 nM; blue label).

(E) Plot of the time course of normalized oEPSC amplitude for cells treated with morphine, followed by naloxone (dark circles; n = 8 cells, 4 mice), and for cells treated with CPA, followed by DPCPX (clear circles; n = 7 cells, 6 mice).

(F) Mean summary data of normalized oEPSC amplitude in baseline condition, after morphine perfusion, followed by naloxone (morphine: 0.80 ± 0.05 fraction of baseline, $p = 0.0002$; naloxone: 0.98 ± 0.01 fraction of baseline, $p = 0.002$, n = 8 cells, 4 mice, $F_{(2, 14)} = 17.29$, one-way repeated measures ANOVA, Tukey's multiple comparisons test).

(G) Mean summary data of normalized oEPSC amplitude in baseline condition, after CPA perfusion, followed by DPCPX (CPA 0.37 ± 0.04 fraction of baseline, $p < 0.0001$; DPCPX: 1.3 ± 0.08 fraction of baseline, $p < 0.0001$ n = 7 cells, 6 mice, $F_{(2, 12)} = 87.95$, one-way repeated measures ANOVA, Tukey's multiple comparisons test). Line and error bars represent mean ± SEM; * denotes statistical significance.

μ opioid receptor regulation of tonic adenosine A_1 receptor activation

Since both MORs and A_1Rs are coupled to $G_{i/o}$ G-proteins and both are present in thalamic terminals, functional interaction between the two receptors in regulating glutamate release was examined. oEPSCs were evoked as described above and DPCPX (200 nM) was superperfused to measure the effect of tonic A_1R activation. DPCPX increased oEPSC amplitude (Fig 4.2A, C, D; DPCPX: 1.3 ± 0.06 fraction of baseline, p = 0.0003, n = 10 cells, 7 mice, t(9) = 5.752, ratio paired T-test). In separate cells, morphine (1 μM) was superperfused, followed by DPCPX. Morphine reduced the amplitude of oEPSCs (Fig 2B, C, E,) as expected. However, in the presence of morphine, DPCPX did not increase oEPSC amplitude (Fig 4.2B, C, E; morphine 0.78 ± 0.03 fraction of baseline, p = 0.0011; DPCPX: 0.77 ± 0.05 fraction of baseline p = 0.04, and 0.99 ± 0.06 fraction of morphine, p = 0.84, n = 6 cells, 4 mice, $F_{(2, 10)}$ = 14.00, one-way repeated measures ANOVA, Tukey's multiple comparisons test), suggesting that morphine inhibited the tonic activation of A_1Rs. To determine whether morphine inhibited the tonic A_1R activation through MOR activation, or a non-specific morphine effect, global MOR knockout (KO) mice were used. Similar to WT mice, DPCPX increased the oEPSC amplitude in slices from these mice (Fig 4.2F, H, I; DPCPX: 1.37 ± 0.05 fraction of baseline, p = 0.0001, n = 8 cells, 5 mice, t(7) = 8.273, ratio paired T-test). In contrast to WT mice, morphine did not reduce the amplitude of oEPSCs (Fig 4.2G, H, J: morphine: 1.0 ± 0.05 fraction of baseline, p = 0.9863, n = 6 cells, 3 mice) in slices from MOR KO mice. Further, in the presence of morphine, DPCPX increased oEPSC amplitude (Fig 4.2G, H, J; DPCPX: 1.4 ± 0.07 fraction of baseline, p = 0.0006, 1.3 ± 0.09 fraction

morphine, p = 0.0008, n = 6 cells, 3 mice, $F_{(2, 12)} = 17.46$, one-way repeated measures ANOVA, Tukey's multiple comparisons test). There was no difference in the increase in oEPSC amplitude between control slices and slices in morphine, suggesting that MORs are required for morphine to modulate tonic adenosine levels. Therefore, morphine inhibits the tonic activation of A_1Rs in the thalamo-striatal circuit by activating MORs.

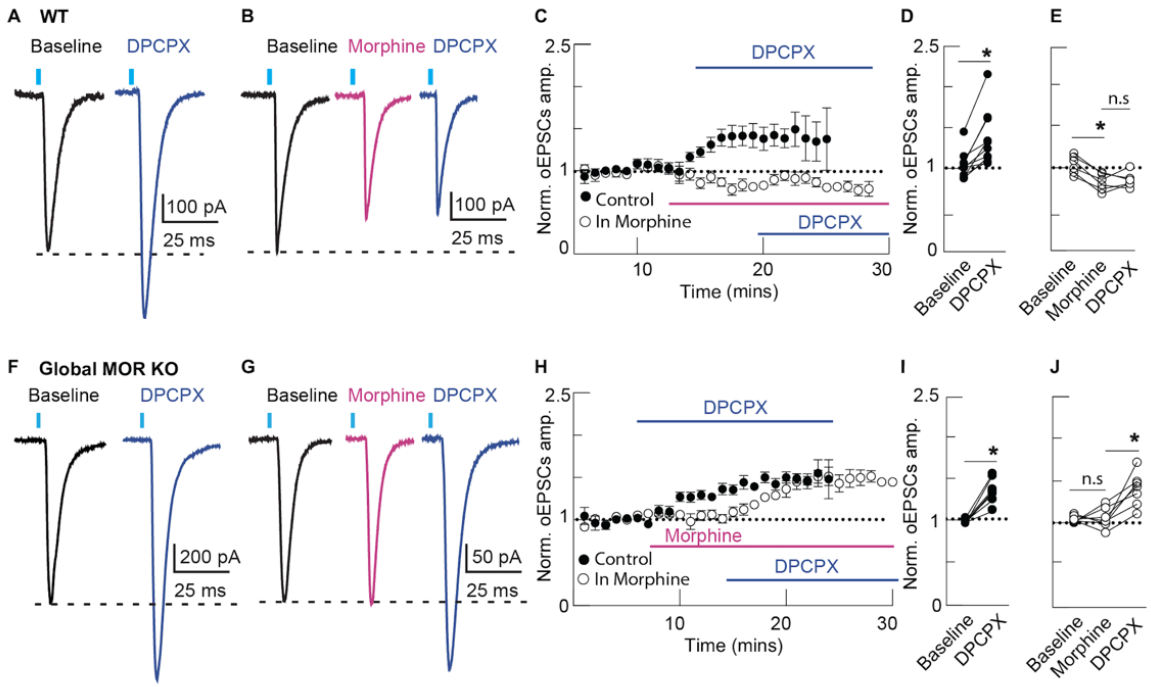


Figure 4.2. Morphine inhibits adenosine tone in the thalamo-striatal synapse by activating MORs.

(A) Representative traces of oEPSCs evoked by 470 nm light (black label) and facilitation of oEPSC amplitude by DPCPX (200 nM; blue label).

(B) Representative traces of oEPSCs evoked by 470 nm light (black label), inhibition of oEPSC amplitude by morphine (1 μM; pink label), and lack of facilitation by DPCPX (200 nM; blue label).

(C) Plot of the time course of normalized oEPSC amplitude for cells superperfused with DPCPX (dark circles; n = 10 cells, 7 mice), and for cells superperfused with morphine and then DPCPX (clear circle; n = 6 cells, 4mice).

(D) Mean summary data of normalized oEPSC amplitude in control and after DPCPX (1.3 ± 0.06 fraction of baseline, p = 0.0003, n = 10 cells, 7 mice, t(9) = 5.752, ratio paired T-test).

(E) Mean summary data of normalized oEPSC amplitude in control, after morphine superperfusion, and after DPCPX superperfusion. Morphine significantly inhibited oEPSC amplitude (morphine 0.78 ± 0.03 fraction of baseline, p = 0.0011; DPCPX: 0.77 ± 0.05 fraction of baseline p = 0.04, and 0.99 ± 0.06 fraction of morphine, p = 0.84, n = 6 cells, 4 mice, $F_{(2, 10)} = 14.00$, one-way repeated measures ANOVA, Tukey's multiple comparisons test).

(F) Representative traces of oEPSCs evoked by 470 nm light (black label) and facilitation of oEPSC amplitude by DPCPX (200 nM; blue label), in slices from global MOR knock-out mice.

(G) Representative traces of oEPSCs evoked by 470 nm light (black label), lack of inhibition by morphine (1 μM; pink label), and facilitation of oEPSC amplitude by DPCPX (200 nM; blue label), in slices from global MOR knock-out mice.

(H) Plot of the time course of normalized oEPSC amplitude for cells superperfused with DPCPX (dark circles; n = 8 cells, 5 mice), and for cells superperfused with morphine and then DPCPX (clear circle; n = 6 cells, 3 mice).

(I) Mean summary data of normalized oEPSC amplitude in control and after DPCPX (1.37 ± 0.05 fraction of baseline, p = 0.0001, n = 8 cells, 5 mice, t(7) = 8.273, ratio paired T-test).

(J) Mean summary data of normalized oEPSC amplitude in control, after morphine superperfusion, and after DPCPX superperfusion. Morphine did not inhibit oEPSC amplitude (morphine: 1.0 ± 0.05 fraction of baseline, p = 0.9863) and there was facilitation by DPCPX in the presence of morphine (1.4 ± 0.07 fraction of baseline, p = 0.0006, 1.3 ± 0.09 fraction morphine, p = 0.0008, n = 6 cells, 3 mice, $F_{(2, 12)} = 17.46$, one-way repeated measures ANOVA, Tukey's multiple comparisons test). Line and error bars represent mean ± SEM; * denotes statistical significance; ns denotes not significant.

Tonic endogenous activation of A_1Rs is regulated by cAMP levels

MOR is a $G_{i/o}$-coupled GPCR that can inhibit adenylyl cyclase so it is possible that morphine decreases tonic adenosine levels by preventing cAMP production and its subsequent metabolism to adenosine. Therefore, the role of cAMP metabolism on A_1R-mediated inhibition of glutamate release was examined. Slices were pretreated with phosphodiesterase (PDE) inhibitor, R0-20-1724 (400 μM) for at least an hour to block metabolism of cAMP. R0-20-1724 (400 μM) was also in the perfusate throughout the course of the experiment. In the presence of R0-20-1724, unlike in control slices, DPCPX (200 nM) did not cause an increase in oEPSC amplitude (Fig 4.3A, C, D; DPCPX 1.47 ± 0.13, p = 0.03, n = 6 cells, 4 mice, in control; Fig 4.3B, C, E; DPCPX 0.96 ± 0.1 fraction of baseline, p = 0.789, n = 6 cells, 4 mice, in R0-201724, $F_{(3,17)} = 13.51$, one-way repeated measures ANOVA, Tukey's multiple comparisons test), suggesting that inhibiting the metabolism of cAMP, and thus the conversion of cAMP to adenosine, blocked the tonic activation of A_1Rs. Exogenous application of adenosine (100 μM) in the either the presence or the absence of R0-20-1724 decreased the oEPSC amplitude, which was reversed by a washout (Fig 4.3A, B, C, D, E; adenosine 0.52 ± 0.08 fraction of baseline, p = 0.0001; washout: 0.87 ± 0.06 of baseline, p = 0.0001, n = 6 cells, 4 mice in R0-201724; adenosine 0.44 fraction ± 0.07 of baseline, p = 0.0094; washout: 1.0 ± 0.05 of baseline, p = 0.999, n = 6 cells, 4 mice, in control, $F_{(3, 16)} = 36.72$, one-way repeated measures ANOVA, Tukey's multiple comparisons test), demonstrating that R0-20-1724 is not directly antagonizing the ability of adenosine to inhibit glutamate release via A_1Rs.

In order to examine if endogenous adenosine levels could be increased by increasing cAMP concentration, G_s coupled GPCRs in both D_1R- and D_2R-positive MSNs were pharmacologically activated. Slices were preincubated in D_1R specific agonist SKF89217 (1 μM) for at least an hour, with the drug in the perfusate throughout the course of the experiment. DPCPX (200 nM) caused an increase in oEPSC amplitude (Fig 4.3G, H, I; DPCPX 1.6 ± 0.11 fraction of baseline, n = 7 cells, 5 mice). The increase in amplitude induced by DPCPX was significantly higher in slices treated with SKF89217 ($p = 0.003$, $F_{(5, 32)} = 32.24$, one-way ANOVA, Tukey's multiple comparisons test) compared to control slices. Next, slices were incubated in A_{2A}R agonist, CGS21680 (1 μM) for at least an hour, with the drug in the perfusate throughout the course of the experiment. A_{2A}Rs co-localize with D_2R positive MSNs only (Bogenpohl et al. 2012; Fink et al. 1992; Severino et al. 2020). DPCPX (200 nM) increased oEPSC amplitude (Fig 4.3H, I, J; DPCPX 1.76 ± 0.10 fraction of baseline, n = 6 cells, 4 mice). The increase in amplitude induced by DPCPX was also significantly higher in slices treated with CGS21680 ($p < 0.0001$, $F_{(5, 32)} = 32.24$, one-way ANOVA, Tukey's multiple comparisons test) compared to control slices. There was no difference in oEPSC amplitude after DPCPX superperfusion between slices incubated in SKF89217 and CGS21680 (one-way ANOVA, Tukey's multiple comparisons test). Thus, basal endogenous adenosine levels are affected by cAMP concentration, and activation of MORs by morphine appears to inhibit cAMP accumulation, consequently decreasing adenosine levels.

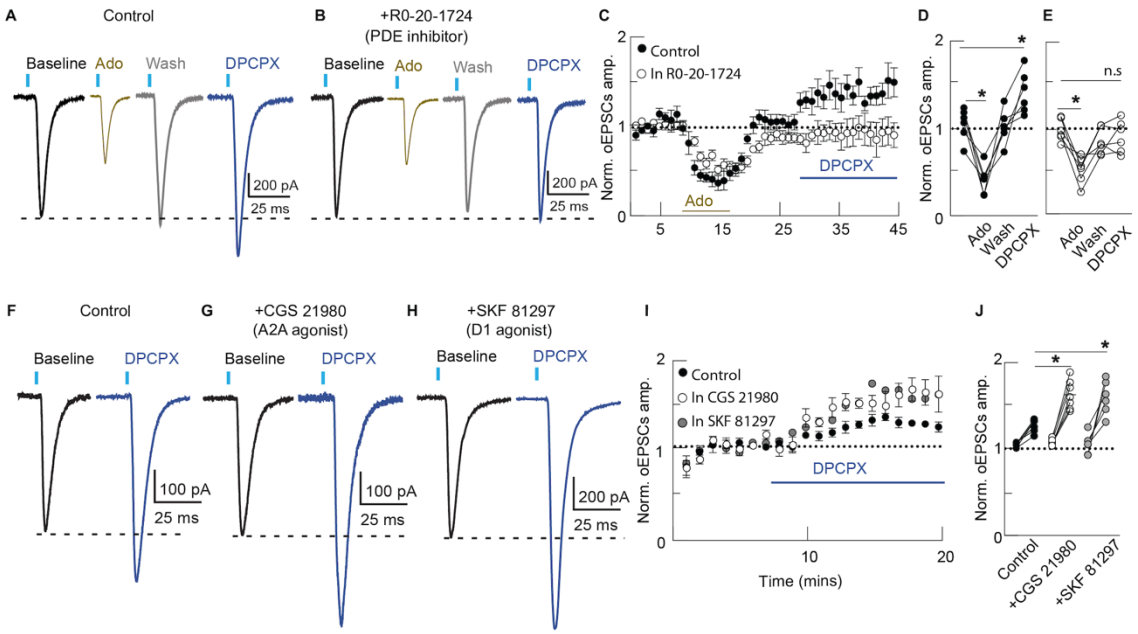


Figure 4.3. Morphine inhibits adenosine signaling via a cAMP dependent mechanism.

(A) Representative traces of oEPSCs evoked by 470 nm light (black label), inhibition of oEPSC amplitude by adenosine (100 μM; yellow label), washout of adenosine (gray label), and facilitation of oEPSC by DPCPX (200 nM; blue label) in naïve conditions.

(B) Representative traces of oEPSCs evoked by 470 nm light (black label), inhibition of oEPSC amplitude by adenosine (100 μM; yellow label), washout of adenosine (gray label), and lack of facilitation of oEPSC by DPCPX (200 nM; blue label) in slices preincubated in R0-20-1724.

(C) Plot of the time course of normalized oEPSC amplitude for cells superperfused with adenosine followed by washout and then DPCPX in naïve slices (dark circles, n = 6 cells, 4 Mice) and in slices preincubated in R0-20-1724 (n = 6 cells, 4 mice).

(D) Mean summary data of normalized oEPSC amplitude for naïve slices in baseline condition, after adenosine superperfusion, followed by a washout and then DPCPX. Adenosine significantly reduced oEPSC amplitude in naïve slices and DPCPX significantly facilitated oEPSC in these slices (adenosine 0.44 fraction ± 0.07 of baseline, p = 0.0094; washout: 1.0 ± 0.05 of baseline, p = 0.999, n = 6 cells, 4 mice, $F_{(3,17)}$ = 13.51, repeated measures ANOVA, Tukey's multiple comparisons test).

(E) Mean summary data of normalized oEPSC amplitude for slices incubated in R0-20-1724 in baseline condition, after adenosine superperfusion, followed by a washout and then DPCPX. Adenosine significantly reduced oEPSC amplitude in these slices (adenosine 0.52 ± 0.08 fraction of baseline, p = 0.0001; washout: 0.87 ± 0.06 of baseline, p = 0.0001, n = 6 cells, 4 mice, $F_{(3,17)} = 13.51$, repeated measures ANOVA, Tukey's multiple comparisons test), but DPCPX did not significantly facilitate oEPSC in these slices (DPCPX: 0.96 ± 0.10 fraction of baseline, p = 0.8755, repeated measures ANOVA, Tukey's multiple comparisons test).

(F) Representative traces of oEPSCs evoked by 470 nm light (black label) and facilitation of oEPSC amplitude by DPCPX (200 nM; blue label), in control slices.

(G) Representative traces of oEPSCs evoked by 470 nm light (black label) and facilitation of oEPSC amplitude by DPCPX (200 nM; blue label), in slices preincubated in CGS21980.

(H) Representative traces of oEPSCs evoked by 470 nm light (black label) and facilitation of oEPSC amplitude by DPCPX (200 nM; blue label), in slices preincubated in SKF81290.

(I) Plot of the time course of normalized oEPSC amplitude for cells superperfused with DPCPX in control condition (dark circles; n = 7 cells, 5 mice), for cells preincubated in SKF81290 (clear circles; n = 7 cells, 6 mice) and for cells preincubated in CGS21980 (gray circles; n = 6 cells, 4 mice).

(J) Mean summary data of normalized oEPSC amplitude in control, in slices preincubated in SKF81297, and CGS21980. The increase in amplitude induced by DPCPX was significantly higher in slices treated with SKF89217 (DPCPX 1.6 ± 0.11 fraction of baseline, p = 0.003,) and in slices treated with CGS21680 ($p < 0.001$, $F_{(5, 32)} = 32.24$, one-way ANOVA, Tukey's multiple comparisons test) compared to control slices. Line and error bars represent mean ± SEM; * denotes statistical significance.

Inhibition of cAMP by activation of MORs is reversible

The time-dependence of cAMP inhibition by MOR activation was examined next. [Met^5] enkephalin (ME; 1 μM) was used instead of morphine, as ME washes from brain slices. oEPSCs were induced as previously described and ME (1 μM) was superperfused. Like morphine, ME inhibited oEPSCs and DPCPX failed to facilitate oEPSCs in the presence of ME (Fig 4A, B, C; ME 0.67 ± 0.03 fraction of baseline, p = 0.0002; DPCPX 0.56 ± 0.03 fraction of baseline, p = < 0.0001; DPCPX 0.84 ± 0.05 fraction of ME, p = 0.2867, n = 6 cells, 4 mice, $F_{(3, 15)} = 78.77$, repeated measures one-way ANOVA, Tukey's multiple comparisons test). ME washed out of the slices in approximately five minutes. Following ME washout, there was an over-reversal of oEPSC in the presence of DPCPX (DPCPX 1.37 ± 0.07 fraction of baseline, p = <0.0001, repeated measures one-way ANOVA, Tukey's multiple comparisons test) which reached steady-state approximately in seven minutes, suggesting that inhibition of cAMP, and therefore inhibition of tonic adenosine levels, is acutely reversible.

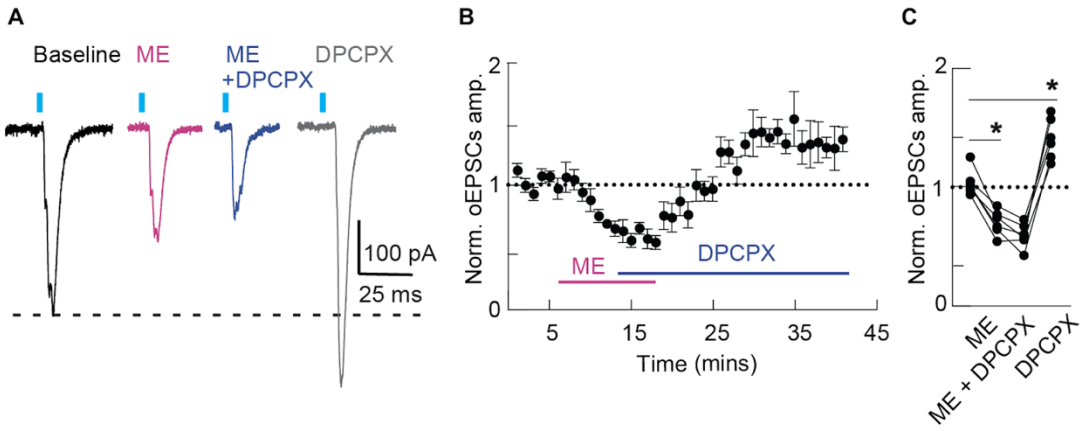


Fig 4.4 Inhibition of adenosine signaling by opioids is reversible.

(A) Representative traces of oEPSCs evoked by 470 nm light (black label), inhibition of oEPSC amplitude by ME (1 μM; pink label), lack of facilitation by DPCPX (200 nM; blue label), and an over-reversal of oEPSC after ME washout (gray label).

(B) Plot of the time course of normalized oEPSC amplitude for cells superperfused with ME, followed by DPCPX in the presence of ME, and then a washout of ME, but not DPCPX (n = 6 cells, 4 mice).

(C) Mean summary data of normalized oEPSC amplitude in baseline condition, after ME superperfusion, followed by DPCPX, and a washout of ME, but not DPCPX (ME 0.67 ± 0.03 fraction of baseline, p = 0.0002; DPCPX 0.56 ± 0.03 fraction of baseline, p = < 0.0001; DPCPX 0.84 ± 0.05 fraction of ME, p = 0.2867, n = 6 cells, 4 mice, $F_{(3, 15)} = 78.77$, repeated measures one-way ANOVA, Tukey's multiple comparisons test). Line and error bars represent mean ± SEM; * denotes statistical significance; ns denotes not significant.

Delta and kappa opioid receptors do not regulate tonic activation of adenosine A_1 receptors

Next, the effect on tonic activation of A_1Rs by delta opioid receptor (DOR) and kappa opioid receptor (KOR) activation were examined. oEPSCs were evoked as described above and the DOR selective agonist deltorphin (300 nM) was superperfused, followed by DPCPX (200 nM). Unlike morphine, deltorphin did not reduce the amplitude of oEPSCs and, in the presence of deltorphin, DPCPX increased oEPSC amplitude (Fig 4.5A, C, D; deltorphin 1.0 ± 0.04 fraction of baseline, p = 0.90; DPCPX: 1.42 ± 0.07 fraction of baseline, p = 0.0002, and 1.40 ± fraction of deltorphin, p = 0.0004, n = 6 cells, 3 mice, $F_{(2, 10)} = 24.60$, repeated measures one-way ANOVA, Tukey's multiple comparisons test), suggesting that DOR activation does not affect the tonic activation of A_1Rs. Next, in separate cells, the KOR selective agonist U69,593 (1 μM) was superperfused, followed by DPCPX (200 nM). Similar to deltorphin, U69,593 did not inhibit oEPSC, and in the presence of U69,593 DPCPX increased oEPSC amplitude (Fig 4.5B, C, E; U69,593 1.02 ± 0.06 fraction of baseline p = 0.9670; DPCPX: 1.6 ± 0.09 fraction of baseline, p = 0.0051, and 1.6 ± 0.13 fraction of U69,593, p = 0.0035, n = 6 cells, 4 mice, $F_{(2, 10)} = 12.24$, repeated measures one-way ANOVA, Tukey's multiple comparisons test), suggesting that KOR activation, like DOR activation, did not inhibit glutamate release from thalamic terminals or affect the tonic activation of A_1Rs. Hence, both the direct inhibition of glutamate release from thalamic afferents and the inhibition of tonic activation of A_1Rs seems to be agonist specific, both inhibited only by MOR agonists and not DOR or KOR agonists.

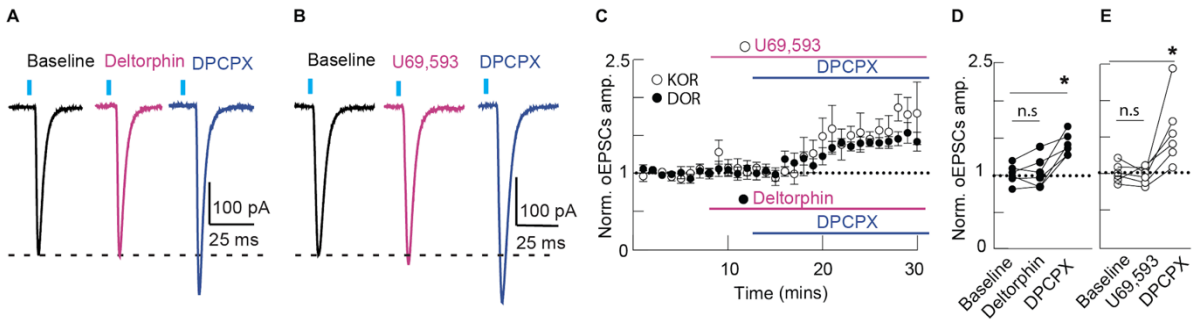


Fig 4.5. DORs and KORs do not mediate inhibition of adenosine signaling

(A) Representative traces of oEPSCs evoked by 470 nm light (black label), lack of inhibition of oEPSC amplitude by deltorphin (300 nM; pink label), and facilitation by DPCPX (200 nM; blue label).

(B) Representative traces of oEPSCs evoked by 470 nm light (black label), lack of inhibition of oEPSC amplitude by U69,593 (1 μM; pink label), and facilitation by DPCPX (200 nM; blue label).

(C) Plot of the time course of normalized oEPSC amplitude for cells superperfused with deltorphin (black circles), followed by DPCPX (n = 6 cells, 3 Mice), and for cells superperfused with U69 (clear circles), followed by DPCPX (n = 6 cells, 4 Mice).

(D) Mean summary data of normalized oEPSC amplitude in baseline condition, after deltorphin superperfusion, followed by DPCPX (deltorphin 1.0 ± 0.04 fraction of baseline, p = 0.90; DPCPX: 1.42 ± 0.07 fraction of baseline, p = 0.0002, and 1.40 ± fraction of deltorphin, p = 0.0004, n = 6 cells, 3 mice, $F_{(2, 10)} = 24.60$, repeated measures one-way ANOVA, Tukey's multiple comparisons test).

(E) Mean summary data of normalized oEPSC amplitude in baseline condition, after U69,593 superperfusion, followed by DPCPX (U69,593 1.02 ± 0.06 fraction of baseline p = 0.9670; DPCPX: 1.6 ± 0.09 fraction of baseline, p = 0.0051, and 1.6 ± 0.13 fraction of U69,593, p = 0.0035, n = 6 cells, 4 mice, $F_{(2, 10)} = 12.24$, repeated measures one-way ANOVA, Tukey's multiple comparisons test). Line and error bars represent mean ± SEM; * denotes statistical significance; ns denotes not significant.

Presynaptic effects of MOR agonists in the thalamo-striatal circuit

Because the inhibition of tonic adenosine release by opioids was selectively mediated by MORs, and since these receptors are expressed in the thalamic terminals, and both the D_1R-positive and D_2R-positive MSNs, the location of acute action of MOR agonist was investigated. FloxedMOR (*Oprm1*$^{fl/fl}$) mice and Vglut$_2$:*cre* mice were crossed to generate mice lacking MORs from Vglut$_2$ -expressing presynaptic terminals (*Oprm1*$^{fl/fl}$, Vglut$_2$-*cre* $^{+/-}$) (Vong et al. 2011). FloxedMOR homozygous littermates that did not express Vglut$_2$:*cre* were used as controls (*Oprm1*$^{fl/fl}$, Vglut$_2$-*cre* $^{-/-}$). oEPSCs were evoked as previously described. Superperfusion of the MOR agonist DAMGO (1 μM) decreased the amplitude of the oEPSCs, and this inhibition was reversed by the antagonist naloxone (1 μM) (Fig 6A, C, D; DAMGO 0.39 ± 0.05 fraction of baseline, $p < 0.0001$; naloxone: 0.80 ± 0.06 of baseline, n = 8 cells, 4 mice, $F_{(2, 14)} = 29.9$, repeated measures one-way ANOVA, Tukey's multiple comparisons test), in control mice. In FloxedMOR-Vglut$_2$-*cre* mice lacking MORs in the presynaptic terminals, DAMGO did not inhibit the amplitude of the oEPSCs (Fig 4B, C, E; DAMGO: 0.99 ± 0.02 fraction of baseline, $p = 0.6023$; naloxone: 0.92 ± 0.05 fraction of baseline, $p = 0.14$, n = 7 cells, 6 mice, $F_{(2, 12)} = 2.08$, repeated measures one-way ANOVA, Tukey's multiple comparisons test), suggesting that opioid action on the thalamo-striatal glutamate release is presynaptic and demonstrating the effectiveness of cre-dependent knockout in these animals.

Next, in order to examine if adenosine was released from presynaptic terminals, DPCPX (200 nM) was superperfused. DPCPX increased the amplitude of the oEPSCs in the presynaptic MOR KO mice (Fig 6F, H, I; DPCPX: 1.3 ± 0.07 fraction of baseline, p = 0.0012, n = 8 cells, 4 mice, t(7) = 5.225, ratio paired T-test). In separate cells, morphine (1 μM) was superperfused, followed by DPCPX. As expected, morphine did not reduce the amplitude of oEPSCs, however, in the presence of morphine, DPCPX did not increase the amplitude of oEPSCs (Fig 6G, H, J; morphine 1.0 ± 0.07 fraction of baseline, p = 0.9935; DPCPX: 1.0 ± 0.07 fraction of baseline, p = 0.9119, and 1.0 ± 0.09 fraction of morphine, p = 0.9513, n = 6 cells, 4 mice, $F_{(2, 10)} = 0.09141$, repeated measures one-way ANOVA, Tukey's multiple comparisons test), suggesting that morphine still inhibited the tonic activation of A_1Rs, even in mice lacking presynaptic MORs. Therefore, even though opioids presynaptically inhibit glutamate release from the thalamic terminals, the presynaptic MORs do not regulate extracellular adenosine accumulation, and subsequent tonic activation of the A_1Rs in this circuit.

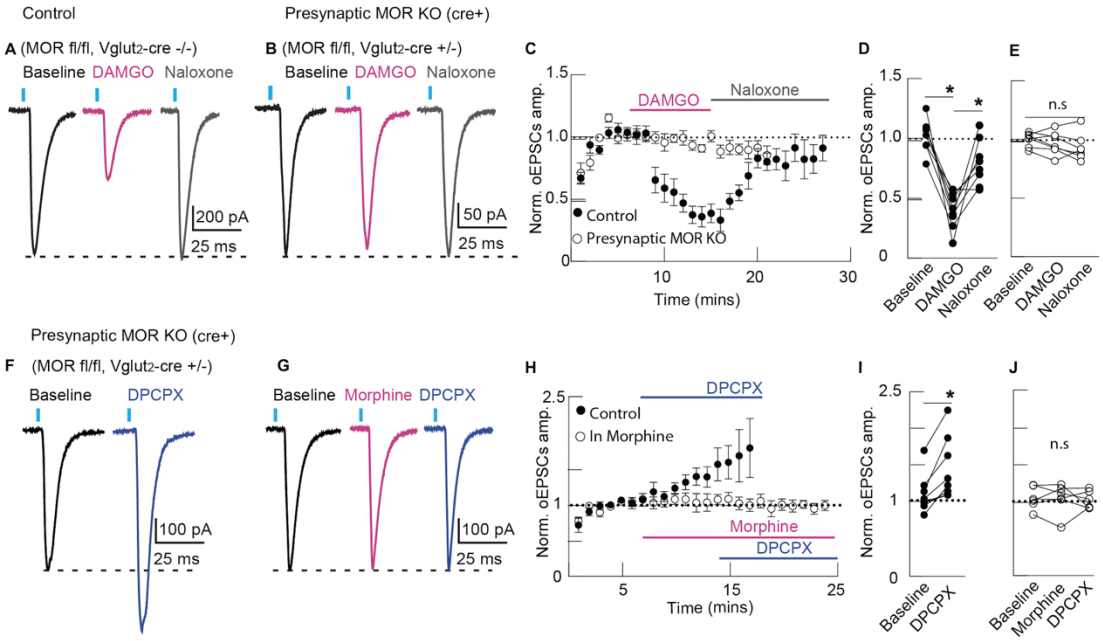


Figure 4.6. Presynaptic MORs suppress excitatory thalamic inputs, but do not regulate tonic A_1R activation.

(A) Representative traces of oEPSCs evoked by 470 nm light (black label), inhibition of oEPSC amplitude by DAMGO (1 μM; pink label), and reversal by naloxone (1 μM; gray label) in control mice expressing MORs in presynaptic terminals.

(B) Representative traces of oEPSCs evoked by 470 nm light (black label), lack of inhibition of oEPSC amplitude by DAMGO (1 μM; pink label), and no effect of naloxone (1 μM; gray label) in mice lacking MORs in presynaptic terminals.

(C) Plot of the time course of normalized oEPSC amplitude for cells superperfused with DAMGO followed by naloxone in control mice (dark circles, n = 8 cells, 4 mice) and in mice lacking MORs in presynaptic terminals (clear circles, n = 7 cells, 6 mice).

(D) Mean summary data of normalized oEPSC amplitude in for control mice in baseline condition, after DAMGO superperfusion, followed by naloxone (DAMGO 0.39 ± 0.05 fraction of baseline, $p < 0.0001$; naloxone: 0.80 ± 0.06 of baseline, n = 8 cells, 4 mice, $F_{(2, 14)} = 29.9$, repeated measures one-way ANOVA, Tukey's multiple comparisons test).

(E) Mean summary data of normalized oEPSC amplitude for presynaptic MOR KO mice in baseline condition, after DAMGO perfusion, followed by Naloxone (DAMGO: 0.99 ± 0.02 fraction of baseline, $p = 0.6023$; naloxone: 0.92 ± 0.05 fraction of baseline, $p = 0.14$, n = 7 cells, 6 mice, $F_{(2, 12)} = 2.08$, repeated measures one-way ANOVA, Tukey's multiple comparisons test). Line and error bars represent mean ± SEM; * denotes statistical significance; ns denotes not significant.

(F) Representative traces of oEPSCs evoked by 470 nm light (black label) and facilitation of oEPSC amplitude by DPCPX (200 nM; blue label) in mice lacking MORs in presynaptic terminals.

(G) Representative traces of oEPSCs evoked by 470 nm light (black label), lack of inhibition of oEPSC amplitude by morphine (1 μM; pink label), and lack of facilitation by DPCPX (200 nM; blue label).

(H) Plot of the time course of normalized oEPSC amplitude for cells superperfused with DPCPX (dark circles; n = 8 cells, 4 mice), and for cells superperfused with morphine and then DPCPX (clear circle; n = 6 cells, 4 mice).

(I) Mean summary data of normalized oEPSC amplitude in control and after DPCPX (1.3 ± 0.07 fraction of baseline, $p = 0.0012$, n = 8 cells, 4 mice, $t(7) = 5.225$, ratio paired T-test).

(J) Mean summary data of normalized oEPSC amplitude in control, after morphine superperfusion, followed by DPCPX. Morphine did not inhibit oEPSC amplitude (morphine 1.0 ± 0.07 fraction of baseline, $p = 0.9935$), and there was no facilitation by DPCPX in the presence of morphine (1.0 ± 0.07 fraction of baseline, $p = 0.9119$, and 1.0 ± 0.09 fraction of morphine, $p = 0.9513$, n = 6 cells, 4 mice, $F_{(2, 10)} = 0.09141$, repeated measures one-way ANOVA, Tukey's multiple comparisons test). Line and error bars represent mean ± SEM; * denotes statistical significance; ns denotes not significant.

μ opioid receptor sensitive adenosine release is regulated by D_1 receptor-expressing MSNs, not D_2 receptor-expressing MSNs.

MORs are expressed in both D_1 and D_2 receptor expressing MSNs (Cui et al. 2014; Oude Ophuis et al. 2014), and activation of D_1 and A_{2A} receptors, presumably in D_1 and D_2 receptor expressing MSNs, increased tonic adenosine inhibition of A_1Rs (Fig 3B), suggesting that MSNs are the potential source of extracellular adenosine. Therefore, MORs were selectively knocked-out in these cells. FloxedMOR mice and A_{2A}:*cre* mice were crossed to generate mice lacking MORs from D_2R expressing MSNs (*Oprm1*$^{fl/fl}$, A_{2A}-*cre* $^{+/-}$) (Gong et al. 2007). oEPSCs were evoked as previously described, and DPCPX increased the amplitude of the oEPSCs (Fig 7A, C, D; DPCPX: 1.3 ± 0.05 fraction of baseline, p = 0.0049, n = 6 cells, 4 mice, t(5) = 4.787, ratio paired T-test). In separate cells, morphine (1 μM) was superperfused, followed by DPCPX. Morphine reduced the amplitude of oEPSCs and, in the presence of morphine, DPCPX did not increase the amplitude of oEPSCs (Fig 7B, C, F; morphine 0.76 ± 0.03 fraction of baseline, p = 0.0001; DPCPX: 0.75 ± 0.02 fraction of baseline, and 0.99 ± 0.04 fraction of morphine, p = 0.9969, n = 6 cells, 4 mice, $F_{(2, 10)}$ = 30.38, repeated measures one-way ANOVA, Tukey's multiple comparisons test), suggesting that morphine inhibited the tonic activation of A_1Rs in mice lacking MORs in D_2R-expressing MSNs. Next, FloxedMOR mice and D_1: *cre* mice were crossed to generate mice lacking MORs from D_1R expressing MSNs (*Oprm1*$^{fl/fl}$, D_1-*cre*$^{+/-}$) (Gong et al. 2007). DPCPX (200 nM) increased the amplitude of the oEPSCs (Fig 7F, H, I; DPCPX; 1.4 ± 0.09 fraction of baseline, p = 0.006, n = 5 cells, 3 mice, t(4) = 5.253, ratio paired T-test). In seperate cells, morphine (1 μM) reduced the amplitude of oEPSCs and, in the presence of morphine,

unlike in WT mice, DPCPX increased the amplitude of oEPSCs (Fig 7G, H, J; morphine 0.72 ± 0.04 fraction of baseline, p = 0.013; DPCPX: 1.14 ± 0.06 fraction of baseline, p = 0.06 and 1.51 ± 0.08 fraction of morphine, p = 0.0001, 5 cells, 3 mice, $F_{(3, 12)} = 25.36$, repeated measures one-way ANOVA, Tukey's multiple comparisons test). Next, MOR antagonist naloxone caused an over-reversal of oEPSC, or an increase in oEPSC amplitude beyond reversal of opioid current, compared to baseline (Fig 7G, H, J; naloxone 1.30 ± 0.03 fraction of baseline, p = 0.004), suggesting that in mice lacking MOR in D_1R positive MSNs, morphine could no longer inhibit tonic A_{1R} activation. Surprisingly, mice lacking MORs in only one copy of the D_1R gene (FloxedMOR +/-, D_1-cre +/-), also showed similar results. In these mice, DPCPX (200 nM) increased the amplitude of the oEPSCs as well (Fig 7F, H, I; DPCPX; 1.38 ± .22 fraction of baseline, p < .001, n = 6 cells, 5 mice, t(5) = 4.466, ratio paired T-test). In separate cells, morphine (1 μM) reduced the amplitude of oEPSCs and, in the presence of morphine, unlike in WT mice, DPCPX increased the amplitude of oEPSCs (Fig 7G, H, J; morphine .70 ± .05 fraction of baseline, p = .0003; DPCPX: .91 ± .06 fraction of baseline, p = .39, and 1.34 ± .06 fraction of morphine, p = .0086, 6 cells, 4 mice, $F_{(3, 15)} = 27.12$, repeated measures one-way ANOVA, Tukey's multiple comparisons test). Next, MOR antagonist naloxone caused an over-reversal of oEPSC compared to baseline (Fig 7G, H, J; naloxone 1.2 ± .05 fraction of baseline, p = 0.0224), suggesting that a partial deletion of MORs from D_1R expressing MSNs was sufficient to eliminate the inhibition of tonic A_1R signaling. This effect is probably because both copies of MORs are required to suppress the release of adenosine. Combined, these results demonstrate that morphine-mediated adenosine release in the thalamo-striatal circuit comes from D_1R positive MSNs.

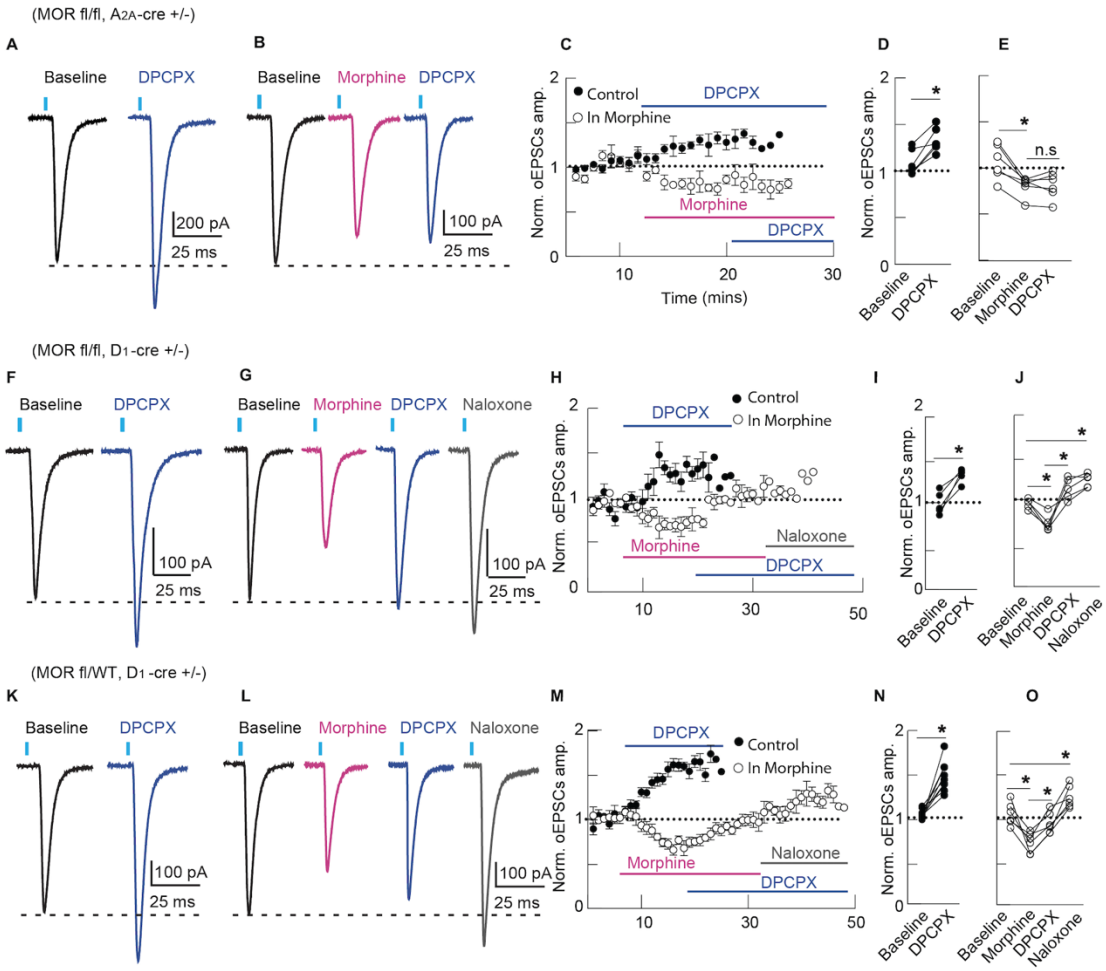


Figure 4.7. MORs in D_1R expressing MSNs, but not D_2R expressing MSNs, regulate tonic A_1R activation.

(A) Representative traces of oEPSCs evoked by 470 nm light (black label), facilitation of oEPSC amplitude by DPCPX (100 nM; blue label mice lacking MORs in D_2R expressing MSNs.

(B) Representative traces of oEPSCs evoked by 470 nm light (black label), inhibition of oEPSC amplitude by morphine (1 μM; pink label), and lack of facilitation by DPCPX (200 nM; blue label) in mice lacking MORs in D_2R expressing MSNs.

(C) Plot of the time course of normalized oEPSC amplitude for cells superperfused with DPCPX (dark circles; n = 6 cells, 4 mice), and for cells superperfused with morphine and then DPCPX (clear circle; n = 6 cells, 4 mice).

(D) Mean summary data of normalized oEPSC amplitude in control and after DPCPX (DPCPX: 1.3 ± 0.05 fraction of baseline, p = 0.0049, n = 6 cells, 4 mice, t(5) = 4.787, ratio paired T-test).

(E) Mean summary data of normalized oEPSC amplitude in control, after morphine superperfusion, and after DPCPX superperfusion. Morphine significantly inhibited oEPSC amplitude (morphine 0.76 ± 0.03 fraction of baseline, p = 0.0001), but there was no facilitation by DPCPX in the presence of morphine (DPCPX: 0.75 ± 0.02 fraction of baseline, and 0.99 ± 0.04 fraction of morphine, p = 0.9969, n = 6 cells, 4 mice, $F_{(2, 10)} = 30.38$, repeated measures one-way ANOVA, Tukey's multiple comparisons test).

(F) Representative traces of oEPSCs evoked by 470 nm light (black label) and facilitation of oEPSC amplitude by DPCPX (200 nM; blue label), in slices from mice lacking MORs from D_1R expressing MSNs.

(G) Representative traces of oEPSCs evoked by 470 nm light (black label), inhibition by morphine (1 μM; pink label), and facilitation of oEPSC amplitude by DPCPX (200 nM; blue label), in slices from mice lacking MORs from D_1R expressing MSNs.

(H) Plot of the time course of normalized oEPSC amplitude for cells superperfused with DPCPX (dark circles; n = 5 cells, 3 mice), and for cells superperfused with morphine and then DPCPX (clear circle; n = 5 cells, 3 mice).

(I) Mean summary data of normalized oEPSC amplitude in control and after DPCPX (DPCPX; 1.4 ± 0.09 fraction of baseline, p = 0.006, n = 5 cells, 3 mice, t(4) = 5.253, ratio paired T-test).

(J) Mean summary data of normalized oEPSC amplitude in control, after morphine superperfusion, and after DPCPX superperfusion. Morphine inhibited oEPSC amplitude (morphine 0.72 ± 0.04 fraction of baseline, p = 0.013;) and there was facilitation by DPCPX in the presence of morphine (1.14 ± 0.06 fraction of baseline, p = 0.06 and 1.51 ± 0.08 fraction of morphine, p = 0.0001, 5 cells, 3 mice, $F_{(3, 12)} = 25.36$, repeated measures one-way ANOVA, Tukey's multiple comparisons test). Naloxone caused an over-reversal of oEPSC amplitude (1.30 ± 0.03 fraction of baseline, p = 0.004). Line and error bars represent mean ± SEM; * denotes statistical significance; ns denotes not significant.

(K) Representative traces of oEPSCs evoked by 470 nm light (black label) and facilitation of oEPSC amplitude by DPCPX (200 nM; blue label), in slices from mice with a partial MOR knock-out from D_1R expressing MSNs.

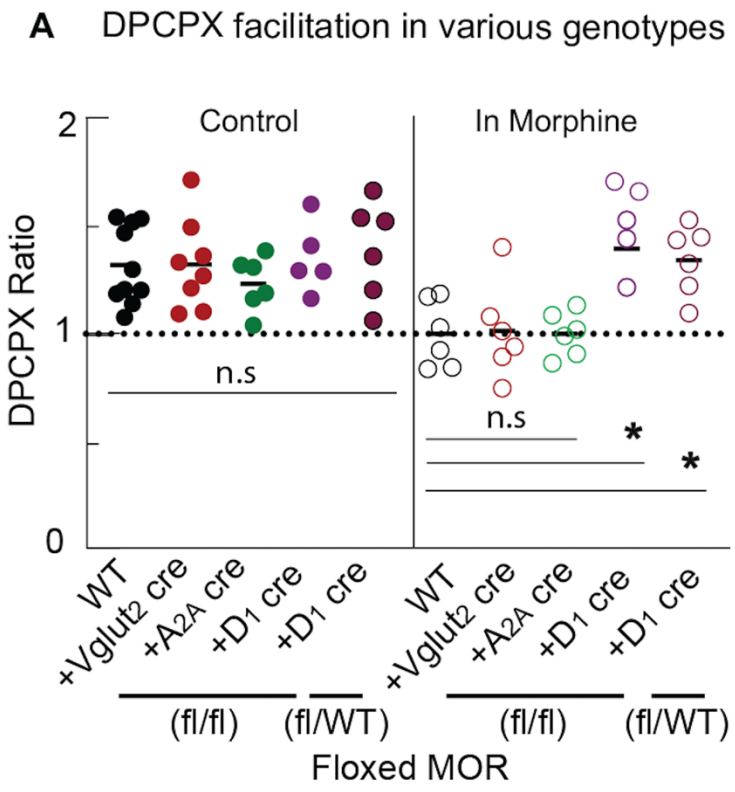
(L) Representative traces of oEPSCs evoked by 470 nm light (black label), inhibition by morphine (1 μM; pink label), and facilitation of oEPSC amplitude by DPCPX (200 nM; blue label), in slices from mice with a partial MOR knock-down from D_1R expressing MSNs.

(M) Plot of the time course of normalized oEPSC amplitude for cells superperfused with DPCPX (dark circles; n = 3 cells, 2 mice), and for cells superperfused with morphine and then DPCPX, followed by naloxone (clear circle; n = 3 cells, 2 mice).

(N) Mean summary data of normalized oEPSC amplitude in control and after DPCPX (1.38 ± .22 fraction of baseline, $p < .001$, n = 6 cells, 5 mice, $t(5) = 4.466$, ratio paired T-test).

(O) Mean summary data of normalized oEPSC amplitude in control, after morphine superperfusion, after DPCPX superperfusion, and after naloxone superperfusion. Morphine inhibited oEPSC amplitude (morphine .70 ± .05 fraction of baseline, p = .0003) and there was facilitation by DPCPX in the presence of morphine (DPCPX: .91 ± .06 fraction of baseline, p = .39, and 1.34 ± .06 fraction of morphine, p = .0086, 6 cells, 4 mice, $F_{(3, 15)} = 27.12$, repeated measures one-way ANOVA, Tukey's multiple comparisons test). Naloxone caused an over-reversal of oEPSC amplitude (1.2 ± .05 fraction of baseline, p = 0.0224). Line and error bars represent mean ± SEM; * denotes statistical significance; ns denotes not significant.

A summary of the effects of selective deletion of MOR from various neuronal populations demonstrates that while the effect of DPCPX was similar in the absence of morphine across all genotypes, only selective knockout of MOR in D_1R-positive cells resulted in a significant effect of DPCPX in the presence of morphine compared to WT mice (Fig 8, *Oprm1*$^{fl/fl}$, D_1-*cre*$^{+/-}$ p = 0.0004). Additionally, there was no statistical difference between *Oprm1*$^{fl/fl}$, D_1-*cre*$^{+/-}$ mice and global MOR KO mice in morphine condition (p = 0.3653, unpaired T-test, t(10) = 0.9485). Combined, these results demonstrate that morphine's regulation of adenosine signaling in this thalamo-striatal circuit critically requires MORs in D_1R positive MSNs and that, under these experimental conditions, these D_1R-positive neurons are the likely source of extracellular adenosine accumulation in dorsomedial striatum.



Discussion

This study explored how the opioid and adenosine signaling systems interact to inhibit glutamate release in a thalamo-striatal circuit. Consistent with previous findings, activation of MORs but not DORs inhibited glutamate release from thalamic terminals (Birdsong et al. 2019b). There also was no effect on glutamate release when KOR agonists were superperfused, suggesting the lack of KORs in the thalamo-striatal projections from medial thalamus to dorsomedial striatum. Additionally, activation of A_1Rs also inhibited glutamate release and antagonism of this receptor revealed endogenous adenosine tone that activated the A_1Rs. Opioids inhibited this tonic A_1R activation through MOR, but not DOR or KOR, via a cAMP-mediated mechanism. When MORs were selectively knocked-out from presynaptic terminals and D_2R positive postsynaptic medium spiny neurons (MSNs), morphine-mediated inhibition of tonic A_1R activation remained. In contrast, in mice lacking MORs in the D_1R positive MSNs, morphine no longer inhibited the tonic activation of A_1Rs. Thus, morphine-sensitive tonic endogenous adenosine in the thalamo-striatal circuit likely arises from D_1R positive MSNs.

Interaction between opioids, cAMP, and adenosine

There is evidence for increased basal endogenous adenosine after chronic morphine treatment and withdrawal (Bonci and Williams 1996a; Chieng and Williams 1998a; Matsui et al. 2014b), therefore, acute morphine application having an opposite effect of decreasing cAMP concentration, and subsequently adenosine release, is consistent with

the results of this study. However, it should be noted that there is also evidence for cell-type specificity in the striatum after acute and chronic treatment by morphine, with cAMP concentration increasing in the D_1R positive MSNs in acute morphine condition, and cAMP concentration increasing in the D_2R positive MSNs in chronic morphine condition (Muntean, Dao, and Martemyanov 2019a). Further, MOR activation is known to decrease AC activity and consequently, cAMP accumulation. The role of cAMP as a precursor for extracellular adenosine has been previously established in the hippocampus (Brundege et al. 1997; Brundege and Dunwiddie 1998; Dunwiddie, Diao, and Proctor 1997). Therefore, it is not surprising that fluctuation in cAMP concentration mediates tonic adenosine levels in dorsal medial striatum as well. Previous studies have shown that cAMP metabolism and transport alter adenosine concentration, and that the regulation of extracellular adenosine depends, in part, on the balance between mechanisms that increase and decrease cAMP concentration (Rosenberg and Dichter 1989; Krupinski et al. 1989).

Additionally, in the hippocampus, endogenous adenosine inhibits glutamate release and the basal concentration of endogenous adenosine is about 200 nM (Dunwiddie and Diao 1994). Similarly, there is evidence for basal endogenous adenosine affecting some striatal synapses. For example, there was a potentiation by DPCPX in glutamate release in nucleus accumbens core and GABA release in nucleus accumbens core and shell (Brundege and Williams 2002b). This study confirmed that DPCPX also potentiated glutamate release from thalamic terminals in the dorsal medial striatum and a similar cAMP-dependent mechanism mediated adenosine accumulation like in the hippocampus.

Opioid selectivity in mediating adenosine release in thalamo-striatal circuit

Consistent with previous findings, activation of MOR, but not DOR, led to inhibition of glutamate release in the thalamo-striatal circuit (Birdsong et al. 2019b). Furthermore, lack of presynaptic inhibition of glutamate release in FloxedMor-Vglut$_2$-*cre* mice corroborates previous finding that MORs in thalamic glutamate terminals regulate transmitter release (Reeves et al. 2020). Additionally, though opioids did not inhibit glutamate release in presynaptic MOR KO mice, morphine still inhibited adenosine tone. Though the thalamic terminals may not express detectable levels of functional DORs, both the D_2R positive MSNs, and cholinergic interneurons are enriched in DORs (Bertran-Gonzalez et al. 2013), with DORs in the patch region of the striatum inhibiting GABA release from MSN collaterals (Banghart et al. 2015a). Additionally, activation of KOR did not inhibit glutamate release, suggesting that effect of opioids on this thalamo-striatal circuit is agonist specific. The dynorphin system in the nucleus accumbens has been implicated in both aversive and rewarding behavior (Al-Hasani et al. 2015b), but the circuit and cell-type specificity driving these opposing behaviors is unknown and a potential avenue of future studies. Additionally, neither the activation of DOR nor KOR inhibited tonic adenosine release, suggesting that the MOR uniquely interacts with the adenosine system. The lack of effect of DOR agonists on tonic adenosine release also supports the observation that DORs appear to be enriched in D_2R expressing rather than D_1R expressing MSNs (Banghart et al. 2015a). Lastly, while the results here have been consistent and reproducible across slices and animals, all experiments were performed in the dorsomedial striatum. It is possible that regional heterogeneity exists in opioid-

regulation of adenosine tone such that differences may exist between medial and lateral dorsal striatum or nucleus accumbens.

Source of opioid-sensitive adenosine tone

Selectively knocking-out the MORs from the presynaptic terminals, D_1R positive MSNs, and D_2R positive MSNs revealed that morphine-induced inhibition of adenosine release was due to morphine's action on MORs in the D_1R positive MSNs, but not D_2R positive MSNs. This finding is consistent with previous work showing that D_1R and D_2R positive MSNs differentially modulate striatal activity (Lobo and Nestler 2011), and that the somatodendritic region of neurons can release adenosine and retrogradely bind presynaptic A_1Rs (Lovatt et al. 2012). Furthermore, MORs in D_1R positive MSNs and D_2R positive MSNs also differentially modulate opioid responses. MOR deletion from D_1R positive MSNs inhibits opioid-induced hyperlocomotion while deletion from D_2R positive MSNs increase opioid-induced hyperlocomotion (Severino et al. 2020). Additionally, MOR expression in the D_1R positive MSNs was shown to be necessary for opioid self-administration and reward (Cui et al. 2014) Thus, a novel role for MORs in regulating adenosine release in the striatum in a cell-type specific way can have profound implication for opioid dependence and addiction. It is also important to note that there is evidence for astrocytes mediating adenosine release in nucleus accumbens, though the mechanism behind adenosine release is through increases in Ca^{2+} activity, and not through an increase in cAMP concentration (Corkrum et al. 2020). The similarities and differences in ways adenosine is regulated to maintain homeostasis in striatal neuron signaling could be a potential new area of study.

The present results indicate that morphine inhibits tonic adenosine release by activating MORs, and subsequently inhibiting cAMP. This effect of opioid-induced inhibition of adenosine release was specific to MOR and not mediated by DOR or KOR. Selective KO of MORs from presynaptic terminals showed that though opioids presynaptically inhibit glutamate release, presynaptic MORs do not modulate extracellular adenosine accumulation and adenosine signaling in the thalamo-striatal circuit. Rather, tonic adenosine release was no longer inhibited by morphine when MORs were knocked-out from D_1R positive MSNs, but not D_2Rs positive MSNs or from glutamate terminals. Thus, the endogenous adenosine that tonically activates the A_1R comes only from D_1R positive MSNs in the medial thalamus-dorsomedial striatum circuit.

Chapter 5 Presynaptic Tolerance

Preface

This chapter describes work towards understanding adaptations to MORs in the presynaptic compartment after chronic morphine treatment. It begins with direct follow-up studies to investigate upregulation of adenyl cyclase after chronic morphine treatment. Then experiments are done to examine tolerance to MORs in the presynaptic compartment.

I conducted this study under the mentorship and assistance of Dr. William T. Birdsong, and Dr. John T. Williams. I designed and performed experiments, and analyzed and illustrated the data. Experiments in this chapter show four key preliminary findings: 1) Chronic morphine treatment does not result in upregulation of cyclase activity as measured by extracellular adenosine concentration. 2) Naïve MORs in the presynaptic compartment do not acutely desensitize in response to a saturating concentration of agonist. 3) Withdrawal from chronic morphine treatment increases the efficacy of MORs in the presynaptic compartment.

In the context of this dissertation, this chapter is a direct follow-up to results described in chapter 4, and a comparative study to look at similarities and differences between somatodendritic and presynaptic MORs. Chronically activating the MORs does not augment the level of adenosine in striatal synapses. Additionally, unlike their somatodendritic counterpart, after chronic morphine treatment and withdrawal, these receptors have increased efficacy.

Results

Naïve Animals

MORs in the presynaptic terminal region of neurons undergo vastly different regulation upon agonist binding. One key difference is that these receptors do not undergo acute desensitization (Blanchet and Lüscher 2002a; Fyfe et al. 2010a; Pennock, Dicken, and Hentges 2012b; Jullié et al. 2020a). In these studies, desensitization was measured by opioid agonist's ability to continually inhibit GABA release; thus, experiments were done to examine if inhibition of glutamate release from medial thalamus also did not desensitize. Channelrhodopsin was expressed in the medial thalamus and recordings were made from striatal MSNs as mentioned in Chapter 4. oEPSCs were induced by 470 nm light to cause glutamate release. Saturating concentration of DAMGO (10 uM) reduced oEPSC amplitude (Fig 5.1A, B DAMGO: 0.53 ± 0.07 fraction of baseline). oEPSC amplitude continued to remain inhibited throughout the course of DAMGO application (Fig 5.1 A, B), suggesting that MORs in thalamo-striatal glutamate terminals also do not acutely desensitize.

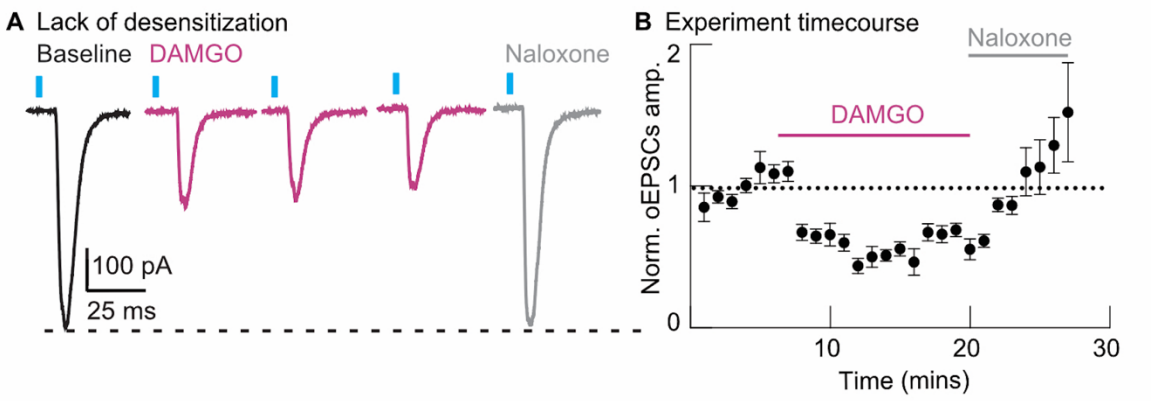


Figure 5.1. Presynaptic MORs do not undergo acute desensitization

(A) Representative oEPSCs evoked by 470 nm light (black label), inhibition of oEPSC amplitude by DAMGO (10 μM; pink label), and reversal by naloxone (1μM; gray label).

(B) Plot of the time course of normalized oEPSC amplitude for cells treated with DAMGO for 10 minutes, followed by naloxone.

Morphine treated animals

A hallmark of cellular tolerance is the upregulation of adenyl cyclase upon acute withdrawal (Sharma, Klee, and Nirenberg 1975b). This upregulation of adenyl cyclase leads to overproduction of cAMP which can have numerous downstream effects. Since cAMP can be metabolized into adenosine (chapter 4), it was hypothesized that higher level of adenosine tone could be observed after chronic morphine treatment. Higher adenosine tone has previously been observed after chronic morphine treatment in rats (Bonci and Williams 1996b; Matsui et al. 2014a), and when recording from striatal cholinergic interneurons (Chieng and Williams 1998b). Mice were treated with morphine as previously described (Chapter 2), and recordings were made from MSNs after two hours of acute withdrawal. DPCPX (A_1R antagonist) was used to measure tonic

adenosine inhibition of glutamate release. DPCPX increased oEPSC amplitude (Fig 5.2 DPCPX: 1.3 ± 0.04 fraction of baseline), but this increase was not higher compared to untreated animals (Chapter 4, DPCPX: 1.3 ± 0.08 fraction of baseline; $p > 0.05$; unpaired t-test), suggesting that chronic morphine treatment did not increase adenosine tone.

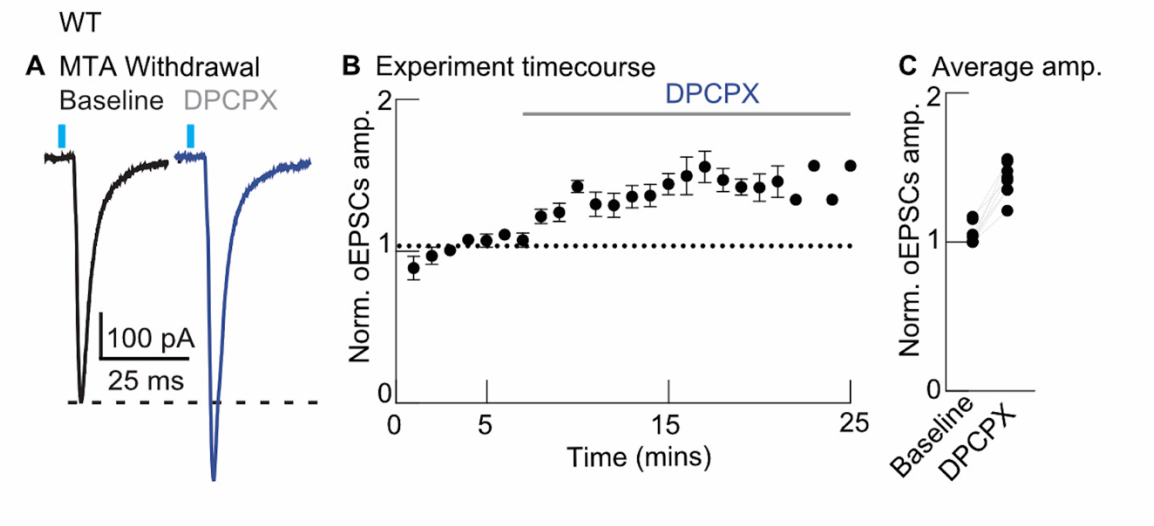


Figure 5.2. Adenosine tone does not increase after chronic morphine treatment

A) Representative traces of oEPSCs evoked by 470 nm light (black label) and facilitation of oEPSC amplitude by DPCPX (200 nM; blue label).

(B) Plot of the time course of normalized oEPSC amplitude for cells superperfused with DPCPX.

(C) Mean summary data of normalized oEPSC amplitude in control and after DPCPX.

In vivo desensitization of MORs after chronic treatment was next assessed. Slices from morphine treated animals were prepared and incubated in ACSF containing morphine (1 uM). Recordings were made in the presence of morphine and inhibition of glutamate release was revealed upon application of the opioid antagonist naloxone (1 uM) (Fig. 5.3 A, B C, naloxone: 1.33 ± 0.11 fraction of baseline). The morphine-mediated current was not statistically different than the current produced by the same concentration of morphnie (1 uM) in slices from untreated animals ($p > 0.05$, unpaired t-test). Next, in order to examine changes to receptor-effector coupling morphine inhibition of glutamate release (1 uM) was assessed. In naïve animals, morphine inhibits glutamate release by approximately 20 percent (morphine: 0.80 ± 0.05 fraction of baseline; chapter 4). In morphine treatment animals, morphine inhibited glutamate release by 34 percent (Fig 5.3D, E, F, morphine: 0.66 ± 0.04 fraction of baseline, $p < 0.05$, unpaired t-test), suggesting that chronic morphine treatment increases the efficacy of presynaptic MORs. A similar result has previously been reported in the periaqueductal gray where chronic morphine treatment enhances morphine's efficacy after chronic treatment by inducing a new effector that involves adenyl cyclase and protein kinase A (Ingram et al. 1998b). Examining the role of phosphorylation and kinases that mediate MOR regulation in

presynaptic terminal will provide key insights to mechanism mediating enhanced MOR efficacy after chronic treatment.

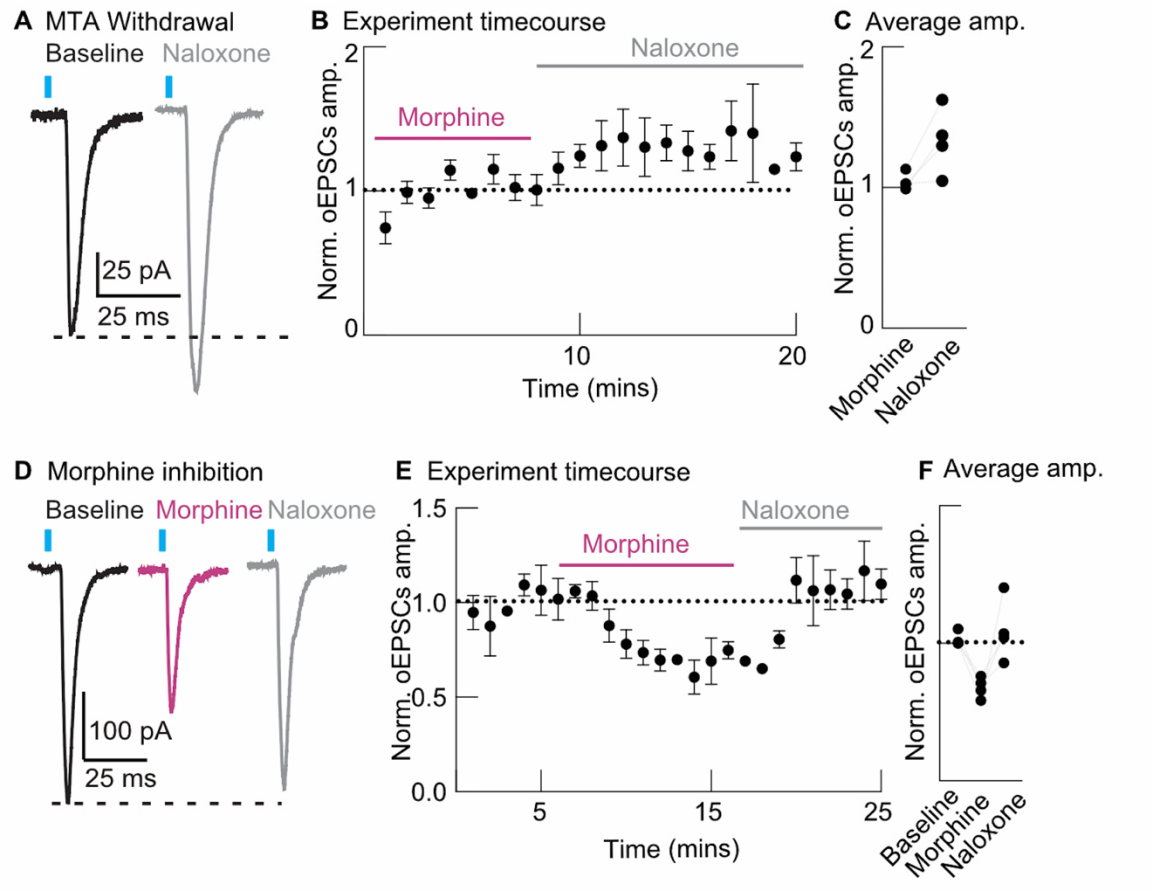


Figure 5.3. Chronic morphine treatment increases opioid efficacy in presynaptic terminals

(A) Representative traces of oEPSCs evoked by 470 nm light (black label) and facilitation of oEPSC amplitude by Naloxone (1 uM; gray label).

(B) Plot of the time course of normalized oEPSC amplitude for cells that were continuously perfused with morphine, and fascilitation of oEPSC amplitude after naloxone.

(C) Mean summary data of normalized oEPSC amplitude in morphine and upon reversal with naloxone.

(D) Representative oEPSCs evoked by 470 nm light (black label), inhibition of oEPSC amplitude by morphine (1 μM; pink label), and reversal by naloxone (1μM; gray label).

(E) Plot of the time course of normalized oEPSC amplitude for cells treated with morphine, followed by naloxone.

(F) Mean summary data of normalized oEPSC amplitude in baseline condition, after morphine perfusion, followed by naloxone.

Summary

The work outlined in this dissertation focus on the regulation of MORs in both the somatodendritic and presynaptic compartments of neurons. Chronically treating animals with opioid agonists of varying efficacies revelated how each agonist exerts its effect on MORs uniquely. Morphine and oxycodone are both low-efficacy partial agonists that share regulatory properties (low MOR desensitization and internalization). While chronic morphine treatment causes both desensitization *in vivo* and increases MOR desensitization in slice, chronic oxycodone treatment failed to do either. However, though oxycodone did not induce changes to receptor-effector coupling, similar to morphine, it did induce disruption of kinase regulation of the SST receptor. Alternatively, chronic treatment with fentanyl resulted in a dramatic uncoupling of MOR from its effectors, presumably due to down regulation of spare receptors in the LC. Consequently, there was no change in kinase regulation of SST receptor after fentanyl treatment. When animals with TPD MORs were treated with fentanyl, disruption of the kinase regulation of the SST receptor was induced, suggesting that sustained signaling by partial agonists promotes disruption in GRK2/3 activity.

MOR regulation in the presynaptic compartment was vastly different. Acutely, MORs in the presynaptic compartment did not desensitize after 10 minutes of agonist exposure, or after two hours of incubation in morphine. However, after chronic morphine treatment, instead of causing tolerance, there was a facilitation of MOR signaling. This facilitation

was not correlated with an enhanced cAMP levels or increased adenosine tone, although acutely morphine inhibited adenosine tone. Morphine's inhibition of adenosine tone was dependent upon MOR expression on D_1R positive MSNs. Therefore, a cell-type specific modulation of adenosine release after chronic treatment cannot be ruled out.

Discussion

The stark difference in MOR-effector uncoupling between fentanyl and oxycodone needs to be put into a larger a context of development of antinociceptive tolerance. In whole animals, tolerance to morphine and oxycodone develops much faster than to fentanyl or etorphine, while in the LC, fentanyl, but not oxycodone, causes a rapid downregulation of MOR. This difference suggests that the mechanism governing tolerance in the whole animal might not just be receptor downregulation but also other adaptive responses that counteract the persistent signaling by partial agonists. Though receptor downregulation might contribute to tolerance in the long-run, perhaps the upregulation of kinases and the heterologous disruption of SST receptor (and maybe others) contribute to the development of tolerance more rapidly. The significance of the heterologous effect on the SST receptor is also important to put into a larger context. First, is it SST receptor in particular or can other $G_{i/o}$ coupled GPCRs that are dependent on GRK phosphorylation also be disrupted? Second, chronic opioid use having unrealized consequences to other GPCRs raises the question of whether other neurons that contain MOR also can develop undergo heterologous disruption of kinase regulation. And if they do, then it's not only the downstream effectors influenced by MORs that undergo profound changes, but also

effectors that do not directly interact with the opioid system. This dysregulation of GRK2/3 can therefore can lead to systems-level tolerance that go beyond the receptor level and may contribute to *in vivo* antinociceptive tolerance.

Furthermore, the fact that the TPD MOR treated with fentanyl induced adaptation to the SST receptor suggests that continued signaling plays a crucial role in mediating cellular tolerance. The TPD MOR can be thought of as a G protein biased receptor as it fails to engage the arrestin pathway. This finding goes against the biased-signaling hypothesis because inhibition of the arrestin pathway should lead to reduced tolerance, but here, it induced another adaptive response that can mediate tolerance.

MOR signaling having effects on other GPCRs also applies to the adenosine system. Although MOR regulate glutamate release from the thalamic terminals, postsynaptic MORs seems to also indirectly influence other neuromodulatory systems and influence striatal dynamics. This modulation was through MORs effects on cAMP, therefore second messenger systems having unrealized effects on other GPCRs can also be consequential. This modulation is particularly important when one considers using design receptor exclusively activated by designer drugs (DREADDs), to silence neurons. Though DREADs can inhibit neuronal firing, they can also affect other GPCRs through inhibition of cAMP, suggesting their effects might not be cell type or circuit specific.

Lastly, chronic morphine treatment surprisingly did not increase adenosine tone in thalamo-striatal synapse. However, most studies that have reported increased adenosine release after chronic treatment has been done either in rats or guinea pigs. Therefore, perhaps there is a species dependent regulation of adenyl cyclase. Chronic morphine treatment also facilitated, instead of reducing, morphine's inhibition of glutamate release. This finding is particularly interesting because acutely presynaptic MORs do not desensitize due to lateral reserve of axonal receptors (Jullié et al. 2020a). Perhaps, when morphine is continuously bound to MORs, more receptors are recruited to the terminals, resulting in facilitation after morphine washout.

Future Directions

Many experiments and potential projects could expand upon the work presented here.

1. In relation to oxycodone, it would be interesting to chronically treat animals for more than one week to see if there is a greater uncoupling of MORs from its effectors and how this uncoupling relates to regulation of SST receptor desensitization. Specifically, if two weeks of treatment induces loss of receptor-effector coupling, would heterologous adaptation still be observed?

2. In relation to fentanyl, the profound downregulation of MORs is striking. Chronic morphine treatment induces desensitization of MORs that is regulated by protein-phosphatase-1 (Levitt and Williams 2012b). It would be interesting to know the time course of MOR reinsertion back into the plasma membrane and the role of phosphatases in mediating resensitization.

3. Direct visualization of endogenous MORs in tissue preparation is now possible (Arttamangkul, Plazek, et al. 2019). Though visualization of endocytosis of native receptors in the LC is difficult, the trafficking of virally expressed WT receptors can be done. It would be valuable to visualize the receptors after chronic morphine and fentanyl treatment to assess if cellular tolerance is due to receptor downregulation.

4. One day of morphine treatment did not induce disruption of kinase regulation, but the drug delivery mechanism was through intraperitoneal (IP) injection and not through an osmotic mini-pump. There is evidence for differences in MOR regulation after chronic treatment based on the method of drug delivery, therefore, the lack of effect after one day of treatment has this built in confound. IP injection of morphine for a week to study MOR and SST regulation, and the time course of development of tolerance, could give interesting results.

5. The homogenous distribution of MORs in the LC makes it an excellent brain region to study regulation of these receptors, however, the role of LC in mediating whole animal tolerance in controversial. Additionally, it is clear that regulation of GPCRs varies considerably depending on the cell-type and environment being used to study these mechanisms, therefore, expanding these results to brain regions that directly modulate antinociceptive pain would be valuable. Regions

such as a spinal cord prep with the dorsal root ganglion or the trigeminal ganglion could provide key insights.

6. It is clear that there are compartment specific regulation of MORs both acutely and after chronic treatment. Morphine seems to sensitize MORs after chronic treatment and it would be useful to know if other agonists of varying potencies like fentanyl cause similar adaptation. Additionally, the role of phosphorylation in mediating this sensitization is crucial to study as phosphorylation does a play a role in presynaptic MOR desensitization (Jullié et al. 2020a).

7. Additionally, examining compartment specific modulation of opioid receptors within the same brain region could provide unique results. Inducing norepinephrine release with electrical stimulation and measuring opioid inhibition of this release after chronic treatment could be useful.

8. Chronic treatment with morphine did not increase extracellular adenosine tone. Since adenosine release is mediated by MORs in D_1R positive MSNs, it would be interesting to see if adenosine release is change in these cells only. PKA activity seems to be differentially modulated in D_1 vs D_2 receptor positive MSNs *in vivo* during reward seeking (Lee et al. 2021), therefore using a PKA sensor to monitor activity after chronic morphine treatment will shed some light on role of adenosine post chronic exposure. Furthermore, there is evidence for cell-type

specific regulation of cAMP after chronic morphine treatment (Muntean, Dao, and Martemyanov 2019b).

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Appendix Recipes

Modified Krebs Buffer Solution

1. Add the following to 1 L nanopore H2O to obtain 2 L modified Krebs buffer
    a. 4.0 g D-glucose (11 mM)
    b. 3.6 g NaHCO3 (25 mM)
    c. 200 mL 10x stock solution (1 L 10x stock recipe below)
        i. 7.363 g NaCl (126 mM)
        ii. 0.186 g KCl (2.5 mM)
        iii. 0.244 g MgCl2 (1.2 mM)
        iv. 0.353 g CaCl2 (2.4 mM)
        v. 0.166 NaH2PO4 (1.2 mM)
        vi. 1 L ddH2O
    d. Constitute to a final volume of 2 L with nanopore H_2O
    e. Incubate in a 35° C water bath, while oxygenating with 95%/5% O2/CO2 gas

Internal Solutions

KMS-based BAPTA internal solutions

1. Add the following to 10 mL nanopure H_2O to obtain 50 mL of KMS 10 BAPTA intracellular solution
    a. 770 mg potassium methanesulfonate (115 mM)
    b. 0.5 mL 2M NaCl (20 mM)
    c. 75 μL 1M $MgCl_2$ (1.5 mM)
    d. 69 mg HEPES (K) (10 mM)
    e. 328 mg BAPTA (K4) (10 mM)
2. Constitute to a final volume of 50 mL
3. Filter with a 0.45 μm filter into 10 mL aliquots
4. Store aliquots at -20 C until needed

5. Before use, thaw a 10 mL aliquot and add the following:
    a. 1.6 mg Na-GTP (0.3 mM)
    b. 10 mg Mg-ATP (2 mM)
    c. 25.5 mg phosphocreatine (10 mM)
6. Add pH until 7.35
7. Adjust osmolarity until between 275-280 mOsm.
8. Aliquot into 1 mL fractions
9. Store unused aliquots at -20 C

K Gluconate-based internal solution

1. Add the following to 10 mL nanopure H_2O to obtain 50 mL of K-Gluconate 1 EGTA intracellular solution
    a. 1.29 g potassium gluconate (110 mM)
    b. 0.5 mL 1M KCl (10 mM)
    c. 0.375 mL 2M NaCl (15 mM)
    d. 75 μL 1M $MgCl_2$ (1.5 mM)
    e. 138 mg HEPES (K) (10 mM)
    f. 19 mg EGTA (1 mM)
2. Constitute to a final volume of 50 mL
3. Filter with a 0.45 μm filter into 10 mL aliquots
4. Store aliquots at -20 C until needed
5. Before use, thaw a 10 mL aliquot and add the following:
    a. 2 mg Na-GTP
    b. 10 mg Mg-ATP
    c. 20 mg phosphocreatine
6. Adjust pH until 7.35 – 7.45
7. Adjust osmolarity until between 275 mOsm.
8. Aliquot into 1 mL fractions
9. Store unused aliquots at -20 C