OPIOID AGONISTS DIFFERENTIALLY ENGAGE AND REGULATE MU OPIOID RECEPTOR DESENSITIZATION, TRAFFICKING AND RECOVERY FROM DESENSITIZATION

By

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ABSTRACT

Morphine has a potency and efficacy for producing analgesia and a pharmacokinetic profile that make it favorable for use in pain treatment. Continued use of morphine and other opioids results in some degree of tolerance, such that the dose needs to be escalated to achieve the same effect. This can become problematic in the treatment of chronic pain, as elevated doses of opioids can produce unwanted side effects such as respiratory depression and dependence. Methadone, a synthetic agonist, is being increasingly used in the treatment of pain. In cancer patients, less dose escalation is required for those who are receiving sustained release methadone versus morphine. Thus the development of tolerance may differ depending on the agonist being used and understanding the adaptations that produce tolerance will be useful for pain treatment.

The goal of this work is to understand the cellular mechanisms involved in desensitization and resensitization of mu opioid receptors in the locus coeruleus (LC) and how these processes are regulated by acute and long-term administration of various opioids. To do this electrophysiological recording was used to measure mu opioid receptor (MOR) stimulated hyperpolarizations and 2-photon imaging of epitope-tagged receptors was used to monitor MOR trafficking in neurons in live brain slices.

Desensitization and internalization induced by various agonists was examined in opiate naïve LC neurons. [Met^5]-enkephalin, methadone and etorphine resulted in both desensitization and internalization while oxycodone caused neither. Morphine

and oxymorphone were unique in that they caused desensitization without internalization of MOR, demonstrating that these processes are separately engaged.

Chronic treatment with morphine resulted in reduced recovery from ME-induced desensitization that was associated with less recycling of internalized receptors back to the plasma membrane. Reduced recovery from desensitization did not occur in slices from mice lacking functional G protein receptor kinase (GRK) or ß-arrestin2 (ß-arr2). Thus, chronic morphine treatment altered GRK2 and ß-arr2 function resulting in inhibited recovery from desensitization. Changes in the GRK2/ß-arr2 pathway were specifically engaged by morphine and reduced recovery from desensitization and receptor recycling were not observed after treatment with methadone.

The frequency of presynaptic excitatory inputs onto LC neurons was enhanced following chronic morphine treatment and this was dependent on PKA activity. However, no post-synaptic changes in efficacy or potency of ME were observed in mouse LC neurons after chronic morphine treatment. Thus, reports of increased excitability in LC neurons during withdrawal are likely due to a presynaptic mechanism that involves an upregulation in the cAMP/PKA pathway.

The results of this work highlight the complexity of signaling mechanisms involved in mu-opioid receptor regulation. Acute application of opioid agonists can specifically engage regulatory mechanisms to various degrees, and differences in acute regulation may contribute to the morphine specific adaptations. Interestingly, morphine, which does not induce internalization of opioid receptors, alters

trafficking induced by other agonists in such a way that MOR cannot recover from desensitization, and thus long-term signaling is dampened.

CHAPTER 1

INTRODUCTION

I. Acute actions of opioids

Opioid receptor ligands

Opioid receptors belong to the family of 7 transmembrane G protein coupled receptors (GPCR). There are 3 opioid receptor subtypes: mu, kappa and delta. Mu opioid receptor (MOR) activation is essential for producing the analgesic and rewarding properties of morphine, as demonstrated in MOR knock-out mice (Matthes *et al*., 1996; Sora *et al*., 1997; Kieffer, 1999). In trying to develop an opioid analgesic that lacks the unwanted outcome of tolerance and dependance there is now a rich pharmacology for opioid receptors that include endogenous and synthetic peptides and alkaloids. The endogenous peptide ligands for opioid receptors are cleaved from 3 prohormones. Proenkephalin is the precurson to met-enkephalin (ME) and leu-enkephalin. Proopiomelanocortin is cleaved to produce β-endorphin, β-lipotopin and ME. Prodynorphin yields dynorphin A and B, α and β neoendorphin and leu-enkephalin (*Neuropsychopharmacolgy*. Sinauer, 1997). Endomorphin 1 and 2 are also endogenous opioid peptides, however their precursors are not known (Zadina et al., 1999). In general, the peptide agonists have a preference for particular receptor subtype, but are considered to be fairly non-

selective. The enkephalin analog DAMGO is more selective for the mu receptor. The alkaloid agonists are derived from the opium poppy and include morphine and codeine. Thebaine also comes from the opium poppy and is used to synthesize oxycodone, oxymorphone, etorphine and buprenorphine, as well as the antagonists naloxone and naltrexone. Methadone and fentanyl are synthetic agonists. CTOP is a highly selective antagonist for the mu opioid receptor, and the irreversible antagonists ß-CNA, and ß-FNA are less so (Williams *et al*., 2001).

Opioid receptor coupling and effectors

Binding of an agonist to the opioid receptor results in activation of pertussis toxin sensitive heterotrimeric G proteins, Gi/o, causing decreases in neuronal excitability and transmitter release (Williams *et al*., 2001). This dampening of activity is achieved by the α and $\beta\gamma$ subunits of the activated G protein acting on multiple pathways. Adenylyl cyclase is inhibited by the α subunit of $G_{i/o}$, resulting in decreased cAMP and PKA activity. The $\beta\gamma$ subunits of the G protein go on to inhibit voltage-gated calcium channels and activate G protein linked inwardly rectifying potassium channels (GIRK). These are only a few of the many signaling pathways and ion channels that are acted on by opioid receptors.

Opioid receptor activation in response to various agonists has been studied *in vivo* and *in vitro*. When administered *in vivo* to rats, opioids are antinociceptive, as demonstrated by increased response times in the hot plate test and warm-water withdrawal assay. Opioids also produce euphoria, respiratory depression and

constipation. Agonists for MOR differ in their potency and efficacy to produce analgesia. Morphine, methadone and DAMGO, an enkephalin analog, all produce robust antinociception in rats. Interestingly, when opioid efficacy was examined *in vitro*, morphine is often found to be a partial agonist for stimulating GTPγS accumulation. Using this same assay, methadone and enkephalins are full agonists at the mu opioid receptor (Selley *et al*., 1998; Rodriguez-Martin 2008).

Acute activation of MOR in LC neurons

Differential activation of the mu opioid receptor by various opioid agonists has been investigated in neurons in the locus coeruleus (LC). The LC is located in the pons and is the primary noradrenergic nucleus in the brain. Neurons in the LC serve as a useful model system for cellular studies of opioid receptors, as they are a homogeneous population that expresses only the mu-receptor subtype. When activated mu-opioid receptors in the LC couple to activation of GIRK channels to produce membrane hyperpolarizations (Williams and North, 1984). Coupling to GIRK activation can be measured using intracellular recording of membrane potential or by whole cell measurement of outward currents. ME, morphine and methadone produce a maximal membrane hyperpolarization of about 30 mV in locus coeruleus neurons (Alvarez *et al*., 2002). However, when opioid activated currents are measured morphine and methadone appear to be partial agonists relative to ME, which is a full agonist (Virk and Williams, 2008; Matsui and Williams, 2010).

II. Acute desensitization and internalization

During prolonged agonist exposure desensitization occurs, a process in which agonist-bound receptors become uncoupled from their effectors. Electrophysiological measurement of GIRK activation provides the temporal resolution necessary to measure desensitization of MOR, which occurs within minutes of application of a saturating concentration of agonist. In LC neurons a decline in the peak amplitude of the membrane hyperpolarization produced by a saturating concentration of agonist can be observed over several minutes. This was demonstrated for concentrations of ME that exceeded 1 μM (Harris *et al*., 1991). Even brief exposures to saturating concentrations of ME result in a reduction in the amplitude of the hyperpolarization produced by an EC50 concentration of ME (Dang and Williams, 2004). Understanding the mechanisms that mediate opioid receptor desensitization is of interest, as these processes will impact the long-term actions of opioid agonists.

Classic model for desensitization of GPCRs: the ß-adrenergic receptor

The classic model for GPCR desensitization involves phosphorylation of the receptor by a G protein receptor kinase (GRK) and subsequent arrestin binding. GRK/ß-arrestin dependent desensitization was first described for the $ß_2$-adrenergic receptor but seems to be a conserved mechanism between many GPRC's. GRK's specifically phosphorylate ligand-bound receptors on their C-terminal tails and/or third intracellular loop of receptors. Phosphorylation by GRK increases the affinity of ß-arrestin for the receptor and thus promotes its binding (Lohse *et al*., 1990).

Phosphorylation and ß-arrestin binding to GPCRs results in uncoupling of the receptor from G protein activation and promotes internalization of the receptors into early endosomal compartments (Ferguson *et al*., 1998). Mutation of a putative phosphorylation site on the $ß_2$AR ($ß_2$AR-Y326A) resulted in decreased agonist induced phosphorylation and internalization. When GRK2 was overexpressed, agonist-induced phosphorylation and internalization of $ß_2$AR-Y326A were restored (Ferguson *et al*., 1995). Likewise, overexpression of ß-Arr resulted in enhanced internalization in $ß_2$AR-Y326A even though there was little phosphorylation of the receptor. When mutant ß-arrestins were expressed, internalization of wild-type $ß_2$AR was blocked, suggesting these molecules are required for agonist-induced internalization (Ferguson *et al*., 1996). ß-arrestins promote GPCR internalization by acting as an adaptor protein that recruits molecular machinery involved in endocytosis, such as AP-2 and clathrin, to the activated receptors. GRK/ß–arrestin dependent regulation of the $ß_2$AR has been demonstrated in native tissues and is particularly important in cardiac pathophysiology where GRK expression is upregulated, resulting in a tonic uncoupling of $ß_2$AR from G protein activation. This molecular scheme for agonist-induced desensitization likely represents a common regulatory mechanism for GPRCs.

Agonist-selective regulation of mu-opioid receptors

Mu-opioid receptor regulation by GRK and ß-arrestin has been well demonstrated in heterologous systems, however how these molecules impact regulation of MOR in

neurons is less clear. A particularly interesting aspect of GPCR regulation by GRK and ß-arrestin is the ability of different agonists to engage these pathways differentially. Agonist-selective regulation of MOR has been demonstrated in many cell types. In LC neurons, rapid and robust desensitization is observed with ME and methadone, while morphine causes much less (Alvarez *et al*., 2002; Dang and Williams, 2004). Immunohistochemical studies in myenteric neurons have shown that a single injection of etorphine causes internalization of receptors while morphine does not (Sternini *et al*., 1996). Similar results have been obtained in various cell lines examining agonist-induced internalization of epitope-tagged mu-opioid receptors, where etorphine, enkephalins and methadone caused receptor endocytosis (Keith *et al*., 1996; Alvarez *et al*., 2002; Borgland *et al*., 2003; Bailey *et al.,* 2003). Morphine- bound receptors are weakly phosphorylated and poor at recruiting ß-arrestin, and thus produce less desensitization and internalization. Limited GRK/ß-arrestin regulation of morphine-bound receptors can be overcome by over-expression of GRK2 or ß-arrestin 2 in HEK293 cells (Zhang *et al*., 1998; Whistler and von Zastrow, 1999). Interestingly, studies in cultured neurons have demonstrated that an acute application of morphine is capable of causing MOR internalization in the dendrites of nucleus accumbens neurons and throughout the cell body, dendrites and distal axons of striatal neurons (Haberstock-Debic *et al*., 2003; Haberstock-Debic *et al*., 2005). Internalization of MOR was dependent on ß-arrestin as neither DAMGO nor morphine induced receptor endocytosis in cultured striatal neurons that were transfected with a dominant negative form of ß-arrestin. These results suggest that differences in the local expression of regulatory proteins

may contribute to agonist-selective endocytosis and emphasizes the importance of studying this process in neurons. One of the goals of this work is to investigate further the role of GRK and ß-arrestin in regulating acute and chronic opioid actions in neurons, either by pharmacological inhibition or knock-down of expression.

It should be noted, that alternative mechanisms have been described for opioid receptor desensitization. Phosphorylation of the MOR by PKA and PKC has been described. In particular, desensitization of MOR in response to morphine has been reported to be dependent on phosphorylation by PKC rather than GRK2 (Johnson *et al*., 2006; Bailey *et al*., 2009). The pattern of phosphorylation induced by PKC is likely very different than that induced by GRK2 and could explain why morphine does not induce arrestin recruitment or internalization of the receptor. Inhibition of ERK has also been shown to alter ME-induced desensitization, suggesting multiple mechanisms contribute to this process (Dang *et al*., 2009). Regulators of G protein signaling, which accelerate deactivation of the G protein through hydrolysis of GTP to GDP may also play a role in opioid receptor desensitization. The work in this thesis will focus on testing the classical model for desensitization through GRK and arrestin dependent processes.

A link between efficacy, desensitization and internalization?

Multiple studies have examined the relationship between agonist efficacy at activating, desensitizing and internalizing MOR, with conflicting results, however experiments were often carried out under different conditions and in different cell

types. A positive correlation between efficacy at inhibiting adenylyl cyclase and producing MOR phosphorylation was observed in CHO cells (Yu *et al*., 1997) and between analgesic efficacy and in vitro ß-arr recruitment (Bohn *et al*., 2004). In contrast, coupling of MOR to GIRK activation in LC neurons does not appear to predict the extent of agonist-induced desensitization or internalization observed in HEK293 cells (Alvarez *et al*., 2002). Coupling of MOR to calcium channel inhibition in AtT20 cells was also not correlated with desensitization or internalization (Borgland *et al*., 2003).

There is, however, a positive correlation between an agonist's capacity for causing desensitization and internalization (Kovoor *et al*., 1998; Alvarez *et al*., 2002; Borgland *et al*., 2003; Walwyn *et al*., 2006). This led to the hypothesis that the two processes are closely linked and that receptor internalization may actually be required for acute desensitization of opioid responses. One difficulty in assessing this relationship in previous studies was that measurements of desensitization and internalization were often not carried out in the same cells under identical conditions. The development of a fluorescent-labeled agonist allowed for these two processes to be studied simultaneously in neurons. A dermorphin peptide agonist that was tagged with Alexa594 (Derm-A594) was applied to cultured LC neurons and membrane hyperpolarization due to activation of GIRK resulted (Arttamangkul *et al*., 2006). With a continued application, responses to Derm-A594 desensitized and intracellular fluorescence, indicative of receptor internalization, was observed.

Interestingly, when internalization of receptors was blocked with conconavalinA, intracellular fluorescence was no longer observed but desensitization occurred normally. Thus desensitization of MOR by DermA594 in LC neurons appears to be independent of internalization. At the molecular level, desensitization and internalization can be attributed to different domains of MOR (Celver et al., 2004). Elucidation of the relationship between desensitization and internalization for various agonists in neurons is necessary.

Resensitization

Internalization of receptors is theorized to be important for allowing them to recover from the desensitized state, termed resensitization (Koch *et al*., 2005). This process likely involves dissociation of arrestin, dephosphorylation of the receptor by a phosphatase and reinsertion of receptors to the plasma membrane. Recovery from desensitization occurs in LC neurons following a desensitizing application of ME (30μM for 10 minutes) and is nearly complete in approximately 30 minutes (Harris *et al*., 1991; Osborne and Williams, 1995). When internalization of MOR was blocked in LC neurons with concanavalinA, recovery from desensitization induced by Derm-A594 was unaffected, suggesting that resensitization can occur at the plasma membrane (Arttamangkul *et al*., 2006).

The rate and the extent of recovery from desensitization induced by agonists other than ME is unknown. Given that there is agonist-selective desensitization and trafficking of MOR, it is not unreasonable to think that recovery from desensitization induced by other opioid agonists would be different. An antagonist must be used to reverse the activation of MOR induced by many opioid agonists, which limits measurement of recovery from desensitization. When applied for a short duration, the irreversible antagonist ß-CNA can reverse the actions of opioids applied to LC slices while leaving some receptors available for signaling. Immediately after desensitizing with various agonists and reversal with ß-CNA, ME applied to LC slices results in a small outward current. When the same concentration of ME is tested again 45 later, the current is larger, indicating that some recovery from desensitization has occurred. The amount of recovery measured after applying saturating concentrations of several agonists was proportional to the amount of desensitization induced by the agonist. Thus there was more recovery following desensitization with ME and methadone, than was observed for morphine (Virk and Williams, 2008). Still recovery from morphine-induced desensitization did occur; providing further evidence that internalization of MOR is not necessary for resensitization.

While recovery from desensitization can occur at the plasma membrane, recycling of internalized receptors must still occur for those receptors to be available for signaling. In HEK293 cells that express MOR1, most receptors that were internalized in response to DAMGO returned to the plasma membrane within 45 minutes of removal of agonist (Tanowitz and von Zastrow, 2003; Tanowitz *et al*.,

2008). Less efficient recycling was observed for c-terminal splice variants of MOR1 and this was associated with a slower recovery resensitization (Tanowitz *et al.*, 2008). The time course and extent of recycling of MOR and the impact of loss of GRK and ß-arrestin function on resensitization in LC neurons is unknown and will be addressed in the present thesis.

III. Long-term actions of opioids

Analgesic tolerance

When administered to subjects in vivo, opioids produce antinociception. With repeated administration of the same dose, the amount of analgesia that results is decreased and a rightward shift in the dose-response curve is observed. Measurements of hotplate response latency and tail flick withdrawal in the rat show that analgesia produced by 20mg/kg of systemically administered morphine begins to decline after the second day and is reduced to 13.8% of the maximum effect after 8 days (Yaksh *et al*., 1977). An inverse relationship between agonist efficacy for producing analgesia and the amount of tolerance produced by chronic administration has been described (Stevens and Yaksh, 1989; Paronis and Holtzman, 1992; Duttaroy and Yoburn, 1995; Pawar et al., 2007). High efficacy agonists like etorphine and fentanyl produce less tolerance than lower efficacy agonists like morphine and meperidine. There has been extensive research aimed at understanding the adaptations that occur with chronic opioid treatment to produce tolerance and explain the difference in the amount of tolerance observed

with various agonists. Changes in molecular machinery, synaptic transmission and gene expression have been reported and likely contribute to the chronic actions of opioids. Some, particularly those relevant to opioid receptor signaling in the LC, are described below.

Cellular adaptations

Decreased G-protein coupling and effector activation following chronic opioid treatment has been reported in many brain areas. This decrease in coupling is both agonist- and region-specific, suggesting that multiple mechanisms contribute to tolerance. No changes in MOR mRNA expression or binding have been observed with chronic morphine treatment, however decreased binding is observed following chronic treatment with the highly potent agonist etorphine (Tao *et al*., 1987; Nishino *et al.*, 1990; Brodsky *et al*., 1995; Sehba *et al*., 1997; Shen et al., 2000). Despite the lack of downregulation with chronic morphine treatment, there was widespread decrease in G protein activation, as demonstrated by reduced GTPgS binding, throughout the CNS (Sim et al., 1996; Selley et al., 1997; Maher et al., 2000; Sim-Selley et al., 2007).

Spare opioid receptors have been demonstrated in several intact tissue preparations (Chavkin and Goldstein, 1984; Williams and North, 1984; Christie *et al*., 1987; Adams et al., 1990). That is to say there is an excess of functional receptors present, such that only a fraction of those receptors need be occupied to achieve a maximal effect. A decrease in receptor reserve was observed in the guinea pig ileum myenteric plexus, rat locus coeruleus and hippocampal CA1 neurons

following chronic morphine treatment (Chavkin and Goldstein, 1984, Christie *et al*., 1987; Wimpey *et al*., 1989). A higher fraction of receptors must be occupied to achieve a given effect, and therefore a higher concentration of drug is required.

This uncoupling of receptors from G protein activation is analogous to the acute desensitized state of the receptor. Interestingly, acute desensitization in the LC is altered by chronic morphine treatment in rats. The maximum morphine-induced desensitization is increased, and the time course of desensitization produced by ME and morphine is accelerated. Finally, the recovery from ME-desensitization is incomplete, or at least dramatically slowed compared to untreated animals (Dang and Williams, 2004). The mechanisms contributing to increased desensitization and decreased recovery during chronic-morphine treatment unknown and are addressed in this work.

cAMP/PKA up-regulation

Another adaptation caused by chronic morphine treatment is changes in the cAMP/PKA pathway. Following chronic treatment there is an up-regulation in adenylyl cyclase expression and activity to maintain normal levels of cAMP (Sharma *et al*., 1977; Duman *et al*., 1988). Acutely, opioids decrease cAMP levels and therefore reduce cAMP-dependent protein kinase (PKA) activity. In order to return to homeostatic levels, there is a compensatory increase in AC activity. Upon removal of opioid or application of antagonist, the increase in activity remains, resulting in higher than normal cAMP levels and PKA activity (Nestler *et al*., 1988).

These adaptations are thought to contribute, in part, to tolerance and have been linked to withdrawal symptoms. An increase in cAMP-dependent synaptic release is observed at several CNS synapses during withdrawal (Bonci and Williams, 1997; Chieng and Williams, 1998; Ingram et al., 1998; Williams *et al*., 2001; Hack *et al*., 2003). Upon systemic injection of the opioid receptor antagonist naltrexone into morphine-dependent rats, there is an increase in LC firing rate compared to control animals that is correlated in time with withdrawal behaviors (Aghajanian 1978; Valentino *et al*., 1989; Rasmussen *et al*., 1990). It has been proposed that counteradaptations in the cAMP/PKA pathway cause increased excitability of LC neurons and are responsible for some physical opiate withdrawal behaviors (Rasmussen *et al*., 1990; Nestler, 1994; Punch *et al*., 1997). While there have been a number of in vivo studies that support a role for the LC in initiating withdrawal symptoms it remains controversial whether adaptations within LC neurons are responsible for hyperexcitability (Esposito *et al*., 1987; Maldonado *et al*., 1992; Maldonado *et al*., 1993).

Ligand-specific adaptations

All opioids produce some degree of tolerance. The differences observed in the rate and extent of tolerance and in the cellular adaptations produced by different opioids are a topic of great interest. Can agonist-specific adaptations be explained solely by ligand efficacy or is there another property that contributes to its long-term actions? This question is particularly relevant for morphine, as it has a relatively lower

efficacy, but also differs from other ligands in that it is poor at recruiting the GRK/ß-arrestin for desensitization and internalization. There have been several studies aimed at addressing this issue. Morphine-induced analgesia and tolerance were examined in mice lacking ß-arrestin2 (ß-arr2 KO). Because morphine was unable to recruit ß-arr2 in heterologous systems, one might expect to see no differences in ß-arr2 KO mice. Surprisingly, morphine analagesia was enhanced and tolerance was delayed (Bohn *et al*., 1999, 2000). This effect was specific to morphine and was not observed for the internalizing agonists methadone, fentanyl or etorphine (Bohn *et al*., 2002).

Methadone is particularly interesting when compared to morphine because it has a similar efficacy at producing analgesia and activating cellular effectors, but unlike morphine it also induces robust receptor desensitization and internalization. Sustained release forms of morphine or methadone were administered to cancer patients and those receiving methadone required no dose escalation, while many of those receiving morphine did. When administered to mice at equi-analgesic doses, morphine (10mg/kg/day) tolerance is observed over 5 days while there is none with methadone (4 mg/kg/day) (Kim *et al*., 2008). Trafficking of MOR does appear to play a role in the development of analgesic tolerance. Expression of a mutant MOR that internalizes and recycles with morphine results in a lack of morphine induced analgesic tolerance and cAMP upregulation (Kim *et al*., 2008; Madhaven *et al.*, 2010). It remains unclear whether the cellular adaptations to MOR coupling and desensitization that occur in the LC following chronic morphine treatment are also

induced by treatment with an internalizing agonist like methadone, and this subject is addressed in the present work.

IV. Summary

Acute activation and desensitization of mu-opioid receptors has been well characterized, but the mechanisms involved in desensitization in neurons remains unclear. Receptors that are expressed in heterologous systems appear to be desensitized through a GRK2-dependent mechanism that involves arrestin binding and internalization of receptors. This thesis aims to understand the relationship between agonist-induced desensitization and internalization and to test the classic model for desensitization in LC neurons.

Tolerance and withdrawal to opioids develop in an agonist-specific manner. There have been many cellular adaptations described following chronic morphine treatment, however far less is known about the changes that occur in neurons following chronic treatment with other agonists. In vivo studies with methadone suggest that desensitization and internalization of activated receptors maintains normal signaling through MOR and prevents adaptations that are observed after chronic morphine treatment. In this thesis, morphine and methadone were used to treat animals to determine whether they caused similar changes in MOR signaling and desensitization and to elucidate the signaling pathways involved.

V. Goals

It remains unclear what properties of opioid agonists contribute to the development of tolerance. This thesis work aims to further our understanding the relationship between MOR desensitization and internalization and the contribution these processes make to cellular tolerance. Chapter 2 will focus on characterizing the desensitization and internalization profile for various opioid agonists in locus coeruleus neurons in brain slice. Chapter 3 will examine the adaptations produced by chronic treatment with two agonists with very different profiles for desensitization and internalization. Chapter 4 aims to identify the contribution of cAMP upregulation to the increased excitability observed in the LC following chronic morphine treatment.

CHAPTER 2

DIFFERENTIAL ACTIVATION AND TRAFFICKING OF MU-OPIOID RECEPTORS IN BRAIN SLICES

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Footnotes

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Activation and Trafficking of MOR

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Abbreviations: epitope-tagged (Flag) mu opioid receptor (FlagMOR), hemizygous transgenic mice (FlagMOR-Tg/+), homozygous mu-opioid receptor knockout mice MOR-KO crossed with the transgenic mice (FlagMOR-Tg/+, MOR-/-), homozygous ß-arrestin2-KO mice crossed with the transgenic mice (FlagMOR-Tg/+, Arr-/-), locus coeruleus (LC), [Met^5]enkephalin (ME), G protein-coupled receptor kinase (GRK), 5-bromo-6-[2-imidazolin-2-ylamino]quinoxaline (UK14304), ethylene glycol-bis(β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), tyrosine hydroxylase (TH), ß-chlornaltrexamine (ß-CNA),

I. *ABSTRACT*

The activation of G-protein coupled receptors results in a cascade of events that include acute signaling, desensitization and internalization and it is thought that not all agonists affect each process to the same extent. The early steps in opioid receptor signaling, including desensitization, have been characterized electrophysiologically using brain slice preparations, while most previous studies of opioid receptor trafficking have been conducted in heterologous cell models. This study used transgenic mice that express an epitope-tagged (Flag) mu opioid receptor (FlagMOR) targeted to catecholamine neurons by regulatory elements from the tyrosine hydroxylase gene. Brain slices from these mice were used to study of tagged MOR receptors in neurons of the locus coeruleus (LC). Activation of the FlagMOR with [Met^5]enkephalin (ME) produced a hyperpolarization that desensitized acutely to the same extent as native MOR in slices from wild type mice. A series of opioid agonists was then used to study desensitization and receptor trafficking in brain slices, which was monitored with a monoclonal antibody against the Flag epitope (M1) conjugated to Alexa594. Three patterns of receptor trafficking and desensitization were observed: 1. ME, etorphine and methadone resulted in both receptor desensitization and internalization; 2. Morphine and oxymorphone caused significant desensitization without evidence for internalization; 3. Oxycodone was ineffective in both processes. These results show that two distinct forms of signaling were differentially engaged depending on the agonist used to activate the receptor and support the hypothesis that ligand-specific

regulation of opioid receptors occurs in neurons maintained in brain slices from adult animals.

II. INTRODUCTION

The activation of G-protein coupled receptors results in a cascade of processes and different agonists acting on a single receptor can result in varying signaling patterns (Urban et al., 2007). The mu-opioid receptor (MOR) is a $G_{i/o}$-linked receptor that activates a potassium conductance, inhibits voltage dependent calcium conductance and inhibits adenylyl cyclase (Williams et al., 2001). In the continued presence of agonist, the MOR desensitizes by a mechanism that is thought to be similar to that originally described for the ß-adrenergic receptor (Krupnick and Benovic, 1998; Lefkowitz et al., 1998). The first step in receptor desensitization is thought to be a phosphorylation of the receptor by a G protein-coupled receptor kinase (GRK) followed by the binding to ß-arrestin with high affinity. This process also transforms the receptor into a state where it can no longer couple to G proteins and is therefore physiologically inactive. Acute desensitization can take place within a few minutes and requires a saturating concentration of agonist (Connor et al., 2004; Gainetdinov et al., 2004; vonZastrow et al., 2003).

Like the ß-adrenergic receptor, the MOR is trafficked into early endosomes followed by a multi-step process that recycles receptor back to the plasma membrane (Finn and Whistler, 2001; Tanowitz and vonZstrow, 2003). This trafficking pathway has been hypothesized to be a critical step in the recovery from desensitization where phosphatase activity is necessary for the reinsertion of receptors into the plasma membrane (vonZastrow et al., 2003; Ferguson et al., 1998; Luttrell and Lefkowitz 2002; Marie et al., 2006). The trafficking of MOR has been characterized in a variety of expression systems, although the ability to follow the

internalization of receptors in neurons under more physiological conditions in real time has been limited.

Receptor activation, desensitization and internalization have different concentration dependence or require distinct agonist/receptor dwell times. In order to understand the complexity of signaling from a single receptor it is necessary to study two or more processes simultaneously or at least under identical conditions with a variety of agonists. This study measured acute signal transduction (membrane hyperpolarization), acute desensitization and receptor internalization resulting from the activation of an epitope-tagged MOR in a brain slice preparation.

III. MATERIALS AND METHODS

FlagMOR-transgenic mice: The transgene was constructed by first ligating an 8.5 kb genomic fragment of the rat TH gene from plasmid pTH9000 (Min et al., 1994) containing 5' regulatory sequences, the basal promoter, and 26 bp from the 5' UTR in exon 1 of TH to a 0.7 kb cassette containing a heterologous intron 2 and splice donor/acceptor sites from the rabbit ß-globin gene. The 2.2 kb Flag epitope-tagged MOR cDNA with a bovine growth hormone polyA sequence was obtained from excision of plasmid pcDNA3 SSF-MOR (Kim and vonZastrow, 2003) and ligated 3' to the ß-globin intron in a pBluescript SK+ plasmid vector. The Flag epitope (DYKDDDA) is immediately preceded by a modified influenza hemaglutinin signal peptide sequence that facilitates translocation of the modified receptor into the endoplasmic reticulum and production of functional protein. Co-translational

cleavage of this sequence by signal peptidase is essential for subsequent binding of the M1 antibody to the free amino-terminal end of the Flag epitope. An 11.4 kb *Sal*I-*Not*I transgene fragment was purified from the final construct and used for pronuclear microinjection into zygotes of B6D2 F_2 mice by standard techniques. Two independent founders were identified by PCR genotyping of genomic DNA and backcrossed with C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) to obtain N_1 hemizygous progeny for initial characterization. The one strain with readily detectable levels of FlagMOR by immunofluoresence in brain slices was further maintained by continued backcrossing with C57BL/6J mice. All data were collected from these hemizygous FlagMOR-Tg/+ mice ranging from generations N_1 to N_5. FlagMOR-Tg/+ mice were also crossed with MOR (Schuller et al., 1999) and ß-arrestin2 knockout mice (Bohn et al., 2000) to generate mice that were hemizygous for the FlagMOR transgene and homozygous for either the MOR-KO (FlagMOR-Tg/+, MOR-/-) or the ß-arrestin2-KO (FlagMOR-Tg/+, Arr-/-). 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.

Fluorescent immunohistochemistry: Fixed brains from FlagMOR-Tg/+ mice perfused with 4% paraformaldehyde were prepared and sliced to 50 µm using a vibratome (Leica, Nussloch, Germany) as described previously (Ford et al., 2006). The primary antibody against the Flag epitope (M1, Sigma, St. Louis, MO) was chemically linked

to Alexa 555 (monoclonal labeling kit, Molecular Probes, Eugene, OR) and used at a 1:500 dilution. Immunostaining was done using a free-floating method and tissue slices were incubated at 4˚C for 18 hours. For tyrosine hydroxylase co-localization experiments, the tissues were permeabilized with 0.4% Triton X. The primary antibody against TH was a mouse-monoclonal antibody (1:5000, IncStar, Stillwater, MN) and the secondary antibody was Alexa 488-labeled goat-antimouse (1:1000, Molecular Probes, Eugene, OR). Images were collected from an Olympus microscope equipped with a confocal system, excitation/emission at 488 and 543 nm for Alexa 488 and Alexa 555, respectively.

Electrophysiolgy: Adult mice (4-10 weeks) were used for electrophysiology experiments. Animals were anesthesized with isoflurane, the brain was removed and sliced horizontally using a vibratome (Leica, Nussloch, Germany) in ice-cold artificial cerebro-spinal fluid (ACSF) containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl2, 1.2 NaH2PO4, 2.4 CaCl2, 21.4 NaHCO3 and 11 glucose. Slices (275 μm thickness) were incubated in warm (34˚C) oxygenated ACSF containing (+)MK-801 (10 μM, Sigma-Aldrich, St. Louis, MO) for at least 30 min before use in experiments. Sharp glass electrodes (50-60 MΩ) filled with KCl (2 M) were used for intracellular recordings of membrane potentials. Experiments were performed at 35˚C. Data was collected using Power Lab (Chart version 5.4, ADInstrument, Colorado Springs, CO) and acquired at 200Hz. Drugs were applied by perfusion at the rate of 1.5 ml/min.

Two-photon microscopy: Brain slices (200-220 μM) were prepared as those described for electrophysiological experiments. Slices were incubated in a solution containing M1 antibody (Sigma, St Louis MO) conjugated with Alexa 594 (Molecular Probes, Eugene, OR, 10 μg/ml, 45-60 min). In previous work the behavior of antibody-tagged receptors with unlabeled receptors was compared and there was no significant difference between the two conditions on the time scale of an hour (Tanowitz and vonZastrow, 2003; Whistler et al., 1999). The tissue was visualized with an upright microscope (Olympus, Center Valley, PA.) equipped with a custom-built two-photon apparatus. Data were acquired and collected using Scan Image Software (Pologruto et al., 2003). A z-series was collected at 1 μm intervals for 15 μm. Drugs were applied by perfusion. All experiments were done at 35˚C. Drugs used were ME, oxycodone (Sigma), oxymorphone (Mallinkrodt, Hazelwood, MO), methadone, morphine, and etorphine (NIDA).

Quantification of receptor internalization: Analyses were done off-line with Image J (NIH) software. Images were selected with the Stacks-Reducing Plugin algorithm for quantification. The stack was Z-projected using Sum-Slices method. Five random ROIs were selected and averaged for background fluorescence. The average background fluorescence was then subtracted from the total fluorescent intensity of the whole frame. In control, data were obtained from slices before drug application. This fluorescent intensity was considered as total fluorescent receptors (C). After

drug perfusion (15 min) followed by calcium free ACSF containing ethylene glycol-bis(β-aminoethylether) N,N,N',N'-tetraacetic acid (0.5 mM EGTA, 10 min), a stack of images was collected and analyzed. This fluorescent intensity was termed internalized receptors (I). Percentage of internalization was calculated by (I/C) x 100 and averaged fluorescence from the drug free controls were subtracted.

Radioligand binding assay: Slices containing the locus coeruleus nuclei were prepared (~500 μm) and the area of the LC was dissected. The tissue was homogenized using a Dounce Tissue Grinder in ice-cold Tris HCl (50 mM, pH 7.4). Tissue from 6-10 animals was prepared for a binding assay using [^{3}H]-diprenorphine (Amersham, Pittsburgh, PA) as described previously (Bunzow et al., 1995). Analyses were done using a one-site binding hyperbolic equation from Graph Pad Prism (San Diego, CA).

IV. RESULTS

FlagMOR-transgenic mice

A transgenic mouse strain was generated that expresses a fusion protein consisting of the MOR with an amino-terminal Flag epitope targeted to catecholamine neurons by an 8.5 kb segment of genomic DNA from the rat tyrosine hydroxylase (TH) gene (Figure 1A). Staining of FlagMOR on the plasma membrane of locus coeruleus (LC) neurons was demonstrated using the M1-antibody conjugated with Alexa 555 and was limited to the area of LC and co-localized with

TH immunoreactivity (Figure 1B). The FlagMOR was also detected in other catecholamine neurons in the olfactory bulb, arcuate nucleus, substantia nigra and ventral tegmental area (not shown). There was no obvious phenotypic difference between the transgenic mice and their wild type littermates. Hemizygous FlagMOR-Tg/+ mice grew and bred normally.

Opioid receptor expression and function

Receptor number determined by a binding assay using [^{3}H]diprenorphine indicated that LC neurons from the FlagMOR-Tg/+ mice expressed approximately 2-fold more MOR than wild type littermates (Bmax=732±220 fmol/mg protein, n=3 vs. 364±90 fmol/mg protein, n=4). The Kd from both groups of animals was the same (Kd=0.36±0.03 nM, n=3 for FlagMOR-Tg/+ and Kd=0.46±0.08 nM, n=4 for wild type, t-test $P>0.05$). This increased expression is similar to that reported recently for the delta opioid receptor (DOR) fused to EGFP (Scherrer et al., 2006). In homozygous DOR-EGFP knockin mice there was about a 2-fold increase in binding sites and a 3-fold increase in GTP-gamma-S binding (Scherrer et al., 2006).

Functional coupling of opioid receptors to a G protein-linked inwardly rectifying potassium channel (GIRK) was measured by intracellular recording of membrane potential in LC neurons (Figure 1C). The maximal hyperpolarization induced by [Met^5]enkephalin (ME, 30 μM) was 30.1±1.3 mV and 28.8±1.1 mV in FlagMOR-Tg/+ and wild type mice (n=14 and 19 neurons, respectively, t-test $P>0.05$, Figure 1D). The membrane hyperpolarization induced by the alpha$_2$-

adrenoceptor agonist, UK14304 (3 μM) was also the same in transgenic and wild type mice (FlagMOR-Tg/+=23.9±1.6 mV, n=14, wild type=25.8±0.9 mV, n=24, t=test P>0.05) suggesting that the higher receptor numbers in FlagMOR-Tg/+ neurons did not interfere with activity of GIRK. Increased receptor expression in FlagMOR-Tg/+ mice did, however result in an increase in the potency of ME. There was a 7-fold leftward-shift in the EC_{50} in recordings made from slices taken from FlagMOR-Tg/+ mice compared to wild type (FlagMOR-Tg/+ EC_{50}=94 nM; wild type EC_{50}=658 nM; Figure 1D).

Hemizygous FlagMOR-Tg/+ mice were crossed with MOR knockout (MOR-/-) mice for two purposes. First, if opioids caused a hyperpolarization in neurons in the compound mutant mice, this would demonstrate that FlagMOR was functional in the absence of all endogenous MOR. Second, this genetic cross would decrease the total number of receptors on LC cells. The maximum hyperpolarization in neurons from compound mutant FlagMOR-Tg/+, MOR-/- mice was similar (29.7±1.4 mV, n = 15) to that observed in wild type and FlagMOR-Tg/+ mice. The EC_{50} for ME in slices from the FlagMOR-Tg/+, MOR-/- mice was 151 nM, 50% greater than that in the FlagMOR-Tg/+ mice (94 nM) and only 4-fold less than the EC_{50} of wild type animals (658 nM, Figure 1D).

FlagMOR internalization in brain slices

A useful feature of the detection system combining a Flag-epitope tagged receptor and M1 antibody is that the binding affinity between the two is decreased

considerably upon the removal of calcium from the extracellular buffer. Slices were prepared identically to those used for electrophysiological experiments, incubated with the fluorescent-M1 antibody for 45 min and then placed in a tissue bath on the microscope for imaging. The fluorescent outlines of cell bodies were first visualized in each experiment (control, Figure 2A). When the superfusion solution was changed to one that was calcium free (EGTA 0.5 mM) for 10 min and the cells were imaged again, staining on the plasma membrane decreased to background levels (Figure 2A). This result confirmed that the fluorescence seen surrounding the cell body required calcium and that a 10 min wash with calcium free solution eliminated specific staining. In the next experiment an initial image was taken following staining and a second image was taken after treatment of the slice first with ME (30 μM, 15 min) followed by calcium free (EGTA) solution (10 min). The resulting image showed numerous fluorescent puncta remaining within the soma and processes (Figure 2C and D). To determine if the ME induced increase in intracellular puncta was receptor dependent, slices were treated with the irreversible opioid antagonist, ß-chlornaltrexamine (ß-CNA, 500 nM, 2 min) before the application of ME (30 μM, 15 min). The slice was then treated with calcium free (EGTA) solution and the result was a complete block of receptor internalization (Figure 2B, EGTA wash without ME treatment = 34.2±2.9 %, n=6; EGTA wash post ß-CNA+ME – 38.7±4.3%, n=3). The results indicate that the appearance of intracellular fluorescent puncta within the cell was dependent on the activation of FlagMOR.

The concentration dependence of internalization induced by ME was determined in slices from FlagMOR-Tg/+, MOR-/- mice (Figure 4). Application of ME (300 nM) did not result in an accumulation of fluorescence that was different from control. At higher concentrations (3 and 10 μM) more intracellular fluorescence was observed and the concentration that was required to cause a half-maximal amount of internalization was about 3 μM, well above the EC_{50} (151 nM) for the hyperpolarization. Thus the concentrations of ME required to induce desensitization and internalization were significantly greater than that required to induce hyperpolarization (Harris and Williams, 1991) indicating that less receptor occupancy is required to mediate the activation of the potassium current.

The time course of accumulation of intracellular fluorescence was examined by capturing images at 3 min intervals following the onset of superfusion with ME (30 μM, Figure 4, n=4). Within the first 3 min there was a detectable decrease in fluorescence at the plasma membrane and an increase in cytoplasmic fluorescent. Cytoplasmic puncta continued to increase over 12 min. This result demonstrated that detectable FlagMOR internalization was observed within 3 min, a period during which there is a significant amount of desensitization. With prolonged treatment, ME induced more cytoplasmic fluorescence in a perinuclear region.

Desensitization and recovery from desensitization

To determine if FlagMOR activity was regulated similarly to native receptors, desensitization was induced by a saturating concentration of ME (30 μM, 10 min).

Prior to the application of the desensitizing concentration of ME, a pre-pulse of ME (EC_{50}) was applied and the amplitude of the hyperpolarization was measured. Desensitization was measured in two ways. The first was to measure the decline in the peak hyperpolarization that occurred during the 10 min perfusion of ME (30 μM, Figure 5A). The amplitude of the hyperpolarization after 10 minutes decreased to 70%, 79% and 76% of the peak in wild type, FlagMOR-Tg/+, and FlagMOR-Tg/+, MOR-/- mice, respectively (Figure 5B, ANOVA $P>0.05$). Second, the amplitude of the hyperpolarization induced by an EC_{50} concentration of ME tested 5 min after washout of the desensitizing concentration of ME was decreased to 38±3% of the pre-pulse amplitude in wild type (ME 300 nM, n=9 neurons), 35±6% in FlagMOR-Tg/+ (ME 100 nM, n=5) and 40±7% in FlagMOR-Tg/+, MOR-/- (ME 100 nM, n=5, ANOVA $P>0.05$) mice.

The time-course of recovery from desensitization was measured by repeated applications of ME (100 or 300 nM) over a 30 min period (Figure 5A and C). All three genotypes showed a similar extent and time course of recovery from desensitization (ANOVA $P>0.05$). Thus, FlagMORs were functional and regulated similarly to endogenous receptors.

Recycling of receptors was observed in experiments where the preparation was treated with ME (30 μM, 5 min), followed by a wash with calcium free EGTA buffer (10 min) and a 45-min wash with ACSF. The results show that fluorescent antibody-receptor complexes reappeared along the plasma membrane (Figure 5D). Thus the FlagMOR/fluorescent-M1 bound complex was capable of recycling in a

time frame similar to the recovery from desensitization. Although only a fraction of the internalized receptors were able to traffic back to plasma membrane, the hyperpolarization induced by ME recovered almost completely. It is likely that the percentage of receptors that return to the plasma membrane is sufficient to result in a maximal hyperpolarization. The receptor-reserve reported in LC neurons suggests that only a small percentage of total receptors is necessary to elicit a maximal response (Christie et al., 1987).

Agonist-dependence of MOR desensitization and internalization

A series of opioid agonists was examined for the ability to induce desensitization and internalization in LC slices (Figure 6). As has been reported in other systems, ME, methadone and etorphine were all effective receptor-internalizing agents. The percent internalization induced by ME (30 μM) was 35±8% (n=6), etorphine (1 μM) 44±9% (n=6), and methadone (15 μM) 30±6% (n=6). The internalization induced by morphine (6±3%, n=6), oxymorphone (5±5%, n=6) and oxycodone (2±4%, n=6) was not different from background.

The ability of each opioid agonist to induce hyperpolarization and acute desensitization were also compared (Figure 6B and C). Each of the agonists produced a similar maximal hyperpolarization. Desensitization was measured as the decline in the amplitude of the hyperpolarization induced by each agonist during a 15 min application of a saturating concentration. The washout of all agonists except ME was very slow, so reversal of the hyperpolarization was obtained with

the perfusion of naloxone (1 μM). Following return of the membrane potential to control the alpha-2-adrenoceptor agonist, UK14304 (3 μM) was perfused and the amplitude of the resulting hyperpolarization was measured. During a 15-minute application of ME (30 μM), etorphine (1 μM), morphine (15 μM) and oxymorphone (15 μM) the hyperpolarization declined by 22±4, 23±3, 16±1 and 24±2%, respectively (Figure 6C). Methadone (15 μM) caused the greatest decline in hyperpolarization (34±8%), however this was probably the result of a blockade of the potassium channel because the hyperpolarization induced by the alpha-2-adrenoceptor agonist, UK14304 was also reduced (Rodriquez-Martin et al., 2008). The amplitude of the hyperpolarization induced by UK14304 (3 μM) was 29.1±1.0 (n=20) following desensitization with all opioid agonists except methadone (17.8±1.5, n=5, p=0.002). Thus with the exception of methadone, the opioid induced desensitization was homologous. Oxycodone (15 μM) resulted in a decline of only 9±2%. The rank order for acute homologous desensitization was ME=etorphine=oxymorphone≥morphine>>oxycodone. Thus, oxymorphone and morphine caused desensitization without inducing internalization.

Internalization in ß-Arrestin2-KO mice

The elimination of ß-arrestin might be expected to eliminate or at least decrease the amount of desensitization and internalization. Hemizygous FlagMOR-Tg/+ mice were crossed with ß-arrestin2-KO (Arr-/-) mice to generate compound mutant FlagMOR-Tg/+, Arr-/- animals to determine the role of ß-arrestin2 in

desensitization and internalization. Intracellular recording of neurons in slices from Arr-/- animals showed that ME (30 μM) induced a hyperpolarization to 26.7±1.0 mV that declined to 76±3% of its initial amplitude after 10 min application (Figure 7B); values not significantly different from those observed in wild type mice (t-test; P>0.5). Morphine-induced desensitization in slices from the Arr-/- animals and was not different from that in slices from wild type animals (Arr-/- decreased to 84.6±2.6% of the peak, wild type 84.0±1.0) despite the fact that the maximum hyperpolarization was reduced (13.5±0.9 mV). Internalization induced by ME was examined in slices from FlagMOR-Tg/+, Arr-/- animals. Slices from these mice were treated with ME (30 μM, 15 min) and the percent internalization calculated. Internalization of FlagMOR did not differ between littermates lacking or expressing ß-arrestin2 (FlagMOR-Tg/+, Arr-/-=28±3%, n=9 neurons; FlagMOR-Tg/+=26±5%, n=10, see Figure 7A and C). Thus elimination of ß-arrestin2 had no effect on desensitization or internalization.

V. DISCUSSION

In the present study, brain slices from transgenic mice were used to examine desensitization and trafficking of mu opioid receptors. Epitope-tagged (Flag) mu opioid receptors (FlagMOR) were targeted to locus coeruleus neurons using regulatory elements from the tyrosine hydroxylase gene. Using ME, the FlagMOR was activated and regulated in a similar way as the endogenous receptor. A series of opioid agonists was used to determine if different agonists activated distinct

pathways. All agonists activated a potassium conductance that resulted in the same maximum hyperpolarization. The demonstration of agonist-selective regulation of desensitization and internalization indicates that the two processes are independent and are not necessarily serial events. Originally, morphine was described as the agonist that caused neither desensitization nor internalization (Alvarez et al., 2002), however recent results indicate that it can cause both desensitization (Borgland et al., 2003; Dang and Williams, 2005) and internalization (Haberstock-Debic et al., 2005). In contrast, the present results based on brain slices containing LC neurons show that morphine causes desensitization but no internalization. Oxymorphone was similar to morphine in that it caused robust desensitization in LC neurons from rats (Virk and Williams, 2008) and mice but was ineffective at evoking internalization of FlagMOR. The processes of internalization and desensitization have also been separated in experiments using cultured neurons treated with concanavalin A, where desensitization induced by ME and a dermorphin analog (dermorphin-Alexa 594) was induced in the absence of internalization (Arttamangkul et al., 2006). Thus it appears that although desensitization may be a precursor to internalization, internalization is not necessary for the expression of desensitization.

The agonists used in this study produced three distinct classes of effects. One group caused both desensitization and internalization and included ME, etorphine and methadone. Morphine and oxymorphone were distinct in that neither caused

detectable internalization but both were capable of inducing desensitization. Oxycodone was the only agonist that neither desensitized nor internalized the receptor. In spite of the fact that oxycodone and oxymorphone differ only in a single methyl group, there was a distinct functional difference between the two compounds. Oxymorphone was more efficacious and much more effective at inducing desensitization than oxycodone (Virk and Williams, 2008). Given that oxymorphone is a natural metabolite of oxycodone, the receptor dependent processes of both oxycodone and oxymorphone must be considered in vivo.

The only way that desensitization could be compared using a series of agonists was by measuring the decline in the hyperpolarization during an extended application of a saturating concentration of each agonist. The slow washout of most agonists from brain slices prevented a more quantitative measurement of a decrease in sensitivity that was possible with ME (Figure 5A, 6B). The decline in peak hyperpolarization is not the most sensitive assay, as receptor reserve and the different efficacy of agonists result in a variable amount of desensitization. Given that near saturating concentrations of agonist are required for both desensitization and internalization, the decline in peak hyperpolarization would be blunted in experiments where highly efficacious agonists, such as ME or etorphine, were examined. Thus although a quantitative measure of desensitization is not possible, the rank order of the ability of various agonists to induce desensitization was reliably determined with this measure.

Morphine, oxymorphone and oxycodone are small opiate alkaloids having morphinan base structure while ME and methadone are linear and more flexible compounds. Considering the widely varying differences in chemical structure of opioid agonists, one may predict that different agonists could stabilize a range of receptor conformations and thus differentially affect the mechanisms leading to desensitization and internalization. Similar agonist dependent effects on receptor trafficking of ß2-adrenoceptors (Swaminath et al., 2005) and dopamine D1 receptors (Ryman-Rasmussen et al., 2007) have been observed. Likewise, distinctive signaling induced by opioid agonists can vary depending on, perhaps subtle, differences in experimental conditions. Morphine is a good example in that it does not cause mu opioid receptor internalization in many cell types but did in cultured striatal neurons (Haberstock-Debic et al., 2005). It is also known that morphine can cause internalization in a system where G-protein receptor kinase (GRK) is over expressed (Zhang et al, 1998). Although phospho-MOR preferentially interacts with and has higher affinity to ß-arrestin2 than ß-arrestin1 (Bohn et al., 2000; Bohn et al, 2004; Oakley et al., 2000) the results with the ß-arrestin2 KO mice suggest a possible interaction with ß-arrestin1. It is clear that mice lacking ß-arrestin2 have a dramatically altered response to some opioids. For example the tolerance to morphine is reduced, but treatment with etorphine resulted in tolerance that was the same as in wild-type animals (Bohn et al., 2004). These results are consistent with the idea that agonists can produce different patterns of receptor signaling *in vivo* (Stafford et al., 2001).

Although little work has been done at the cellular level with neurons from the ß-arrestin2 knockout mice, the results in the present study are consistent with other studies that have reported that acute desensitization induced by opioid peptide agonists was not changed in either cultured dorsal root ganglion cells (Walwyn et al., 2007) or in LC neurons recorded in brain slices (Dang and Christie, personal communication). That desensitization and internalization of FlagMOR were both observed in brain slices from ß-arrestin2 knockout mice suggests that mu opioid receptor signaling can occur by redundant pathways such that the control of receptor dependent signaling is ensured.

CONCLUSION

This study demonstrates the advantage of transgenic mice expressing an epitope-tagged receptor for the combined study of acute signaling through the activation of GIRK channels, desensitization and receptor trafficking. This approach allowed direct study of three acute receptor-dependent processes in a brain slice preparation. The role that these early signaling pathways have in the development of tolerance and dependence remains the subject of controversy and intense interest. Given that all opioid agonists result in tolerance under a variety of conditions, it is clear that no one process can completely account for the whole animal response to opioids. It is also clear that a single opioid agonist, such as morphine, has variable actions that are dependent on the cell under study and even

the part of the cell under study. With the understanding of the mechanisms that underlie the early events following receptor activation it may be possible to develop a better understanding of the response of the whole animal to chronic opioid treatment.

Acknowledgments:

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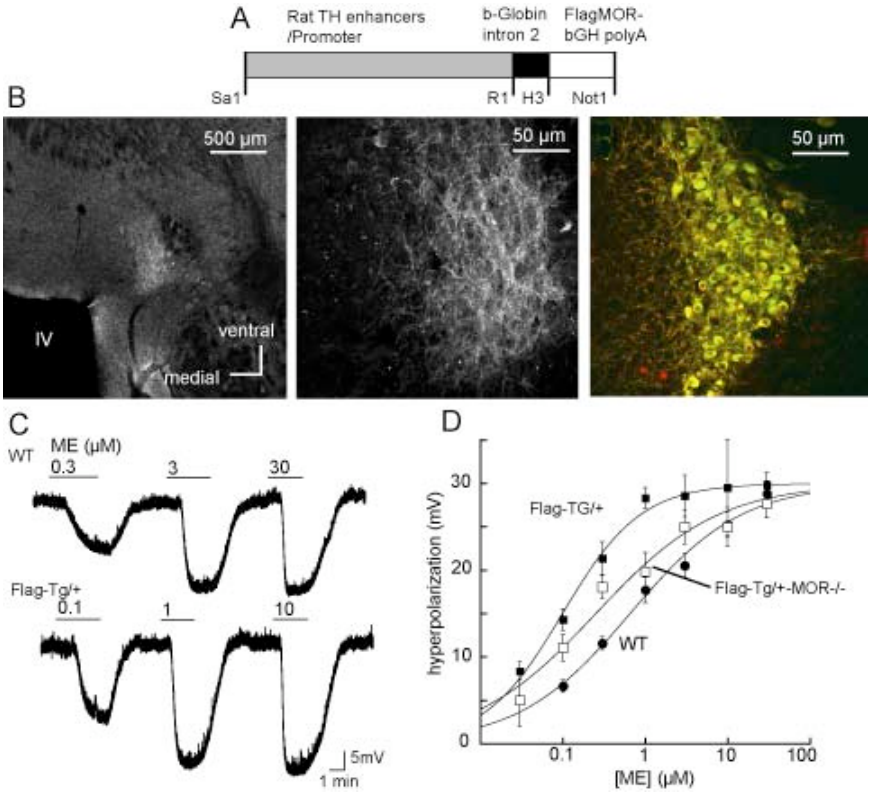

Figure 1. Expression and signaling of FlagMOR in locus coeruleus (LC) neurons. A) Restriction map of the tyrosine hydroxylase (TH)-FlagMOR-Tg construct. B) Left, low power micrograph of a slice containing the LC. The light area is the LC stained using the M1 antibody conjugated with Alexa 555. Middle, higher power image showing fluorescent staining of processes and cell bodies. Right, FlagMOR was stained in red and TH was stained with a secondary antibody labeled with Alexa488. C. [Met]^{5}enkephalin (ME) caused a concentration dependent hyperpolarization in slices from wild type and FlagMOR-Tg/+ mice. Cells from the FlagMOR-Tg/+ mice were more sensitive to ME. D. Concentration-response curves for ME in slices from wild type (solid circles, EC_{50}=658 nM), FlagMOR-Tg/+ (filled squares, EC_{50}=94 nM) and compound mutant FlagMOR-Tg/+, MOR-/- mice (open squares, EC_{50}=151 nM)

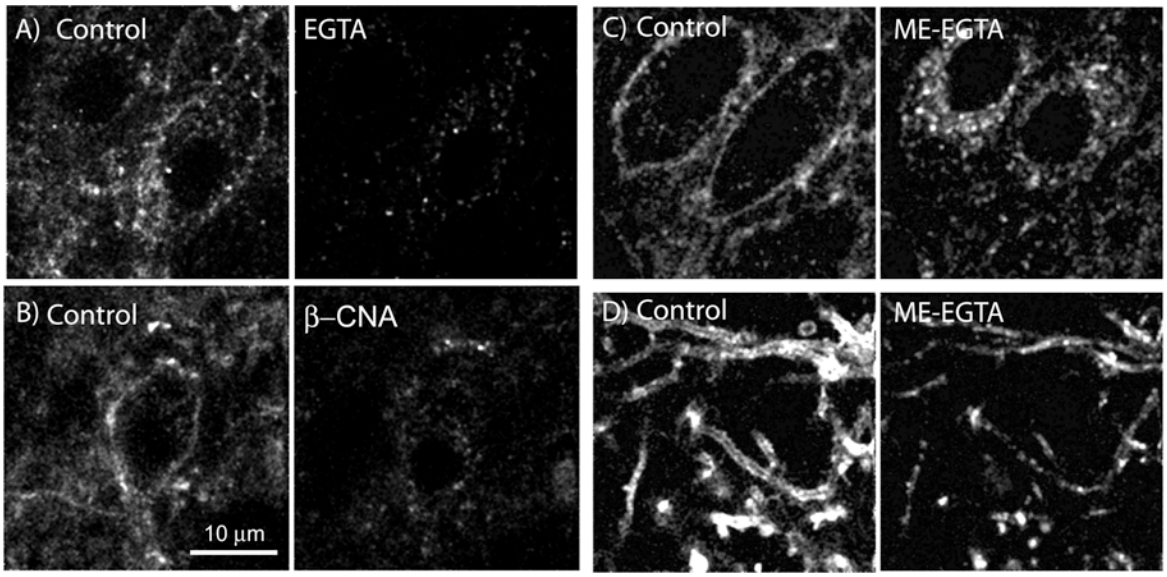

Figure 2. Internalization of FlagMORs induced by ME is receptor dependent.

A) Control (left) shows the initial staining pattern with an Alexa 594-conjugated anti-Flag antibody. Right, same slice after treatment with calcium-free (EGTA) solution. C&D) Internalization of FlagMOR induced by ME. Left, initial staining illustrating cell bodies (C) and dendrites (D). Right, staining following treatment of the slice with ME (30 µM, 15 min) followed by calcium-free (EGTA, 10 min) solution.

B) Pretreatment with ß-CNA (500 nM, 2 min) blocked internalization induced by ME. Left is control and the right side after ME (30 μM, 15 min).

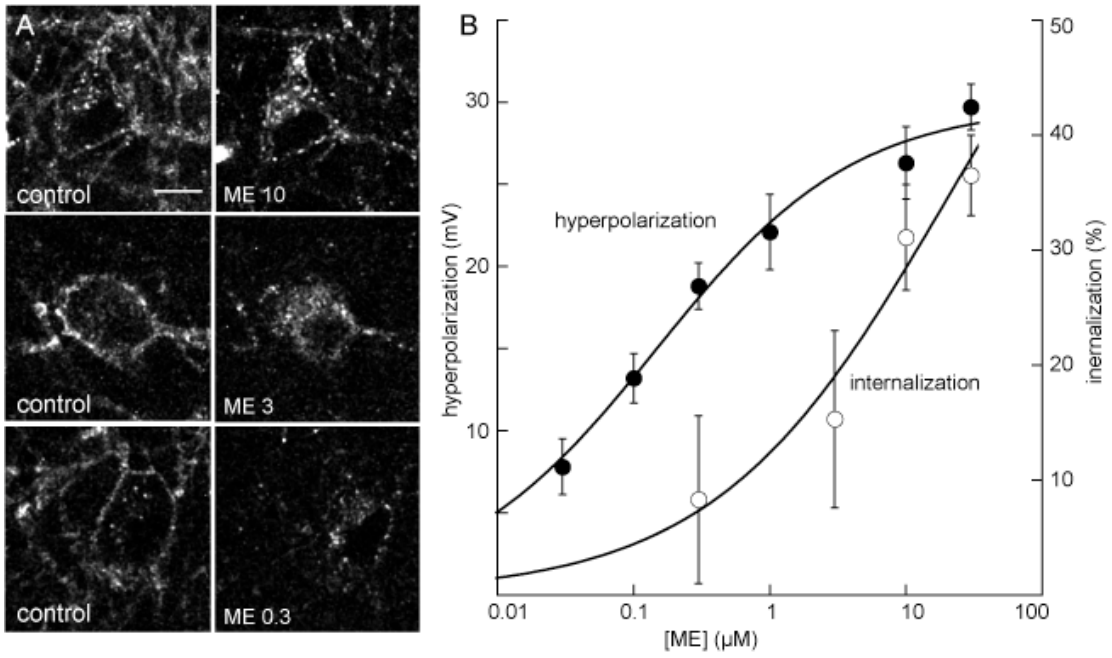

Figure 3. The concentration dependence of internalization induced by ME. A) Images were taken in control (left side) and after treatment of slices with different concentrations of ME for 15 min followed by a wash with calcium free solution (right side). B) Internalization required high concentrations of ME. The percent internalization caused by a 15-minute application of ME is plotted and compared with the concentration response to the hyperpolarization. All experiments were carried out using slices from FlagMOR-TG/+,MOR-/- animals.

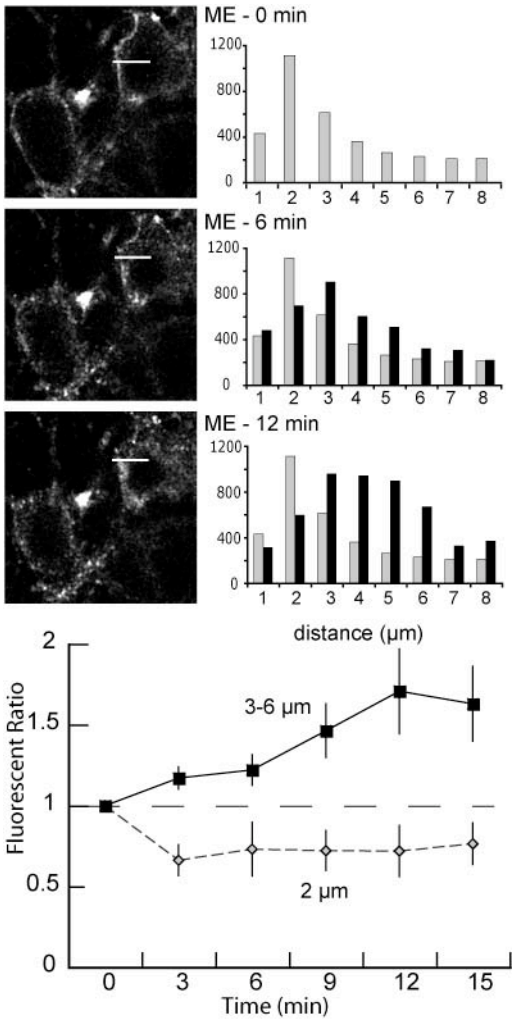

Figure 4. The time course of receptor internalization. A) An illustration of a single experiment showing the time course of internalization with images collected at 6 min intervals (top before ME, middle 6 min after ME, bottom 12 min after ME). Each image is a single Z-scan (1 μm). The white bar in each image represented the area used to determine fluorescent intensity from the plot profile (0.5 x 8 μm, ImageJ). The fluorescence measured over a distance of 1 μm was added and plotted

at the right for each image. Grey bars are the fluorescence measured before addition of ME. At 6 and 12 min the fluorescence moved away from the plasma membrane into the interior of the cell. B) Summary of the fluorescent ratio increasing in cytoplasm during 15 min perfusion of ME (30 μM, n=4). The fluorescence ratio was determined by dividing the counts measured after the addition of ME by the counts in the same area before addition of ME. Fluorescence decreased at the plasma membrane (2 μm, grey diamonds) and increased in the interior of the cell (3-6 μm, solid squares).

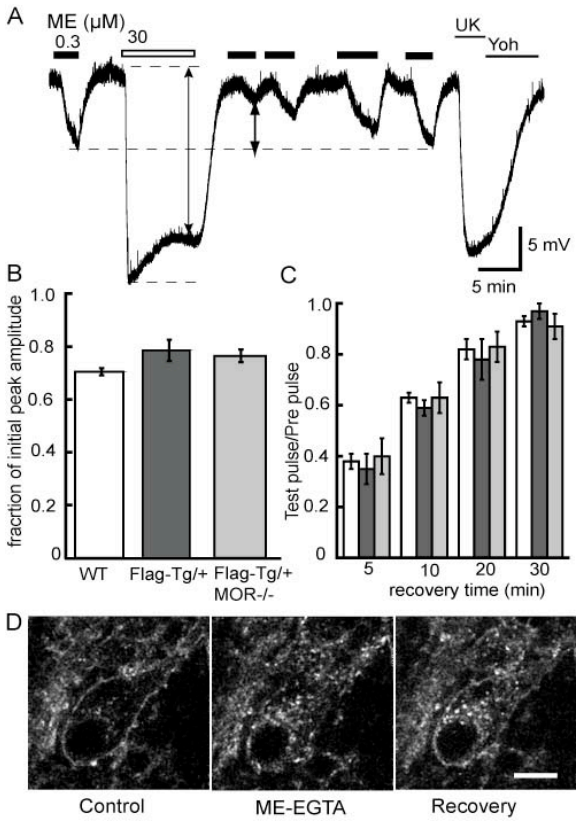

Figure 5. Recovery from desensitization and reinsertion of FlagMOR into plasma membrane. A) Voltage trace from a wild type mouse showing the hyperpolarization induced by ME (300 nM) before and after treatment of the slice with ME (30 μM) for 10 min. Following the washout of ME (30 μM) the hyperpolarization induced by ME (300 nM) was depressed and recovered completely after 30 min. At the end of the recording a saturating concentration of UK14304 (3 μM), an alpha-2-adrenoceptor agonist, and its blockade by the antagonist yohimbine (Yoh), was tested. B. Summary of the change in membrane potential during the 10-min treatment with ME (30 μM). The results indicate that

the decline in the hyperpolarization was the same in slices from all three genotypes. C. Summary of the time course and extent of recovery from desensitization. There was no difference between genotypes. Given that the FlagMOR-Tg/+ and FlagMOR-Tg/+ MOR-/- were more sensitive to ME, the test concentration used to measure the recovery from desensitization was reduced to 100 nM. D) Return of receptors to the plasma membrane of cell bodies. Left panels are the initial staining. Middle taken after application of ME (30μM, 5 min) and EGTA solution (10 min). Right taken after a 45 min wash.

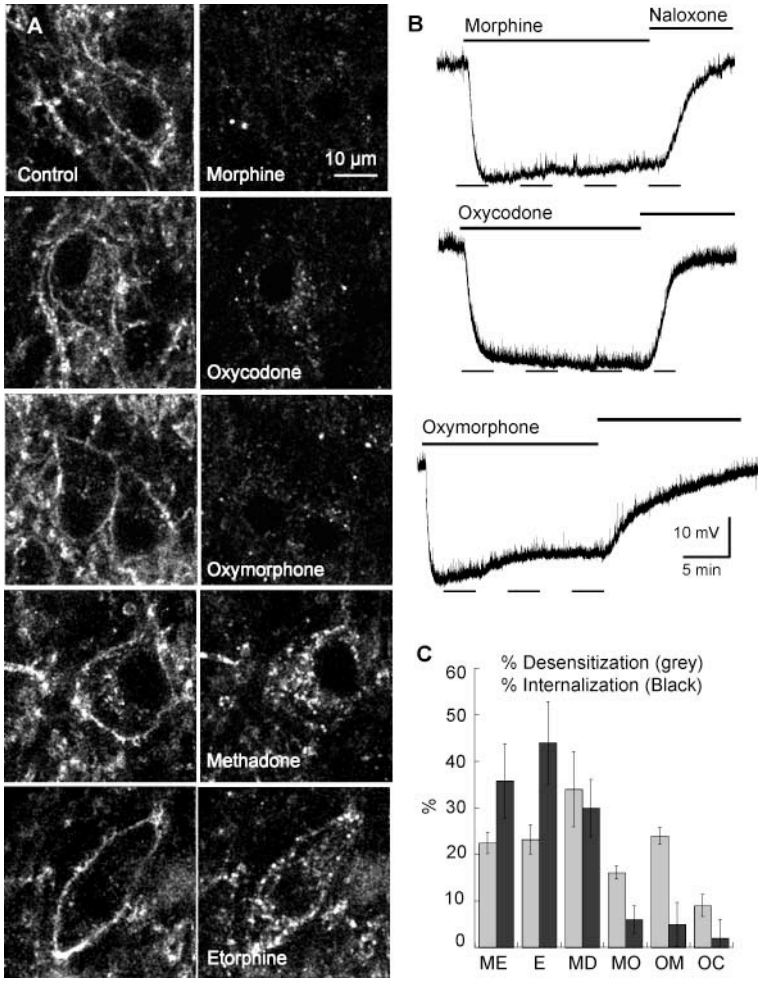

Figure 6. Agonist-selective internalization. A) Images of the internalization. Left, control; right, after agonist (15 min) followed by calcium-free (EGTA) wash. Etorphine (E) and methadone (MD) caused dramatic internalization, while morphine (MO), oxymorphone (OM) and oxycodone (OC) caused very little. B) Example traces of the hyperpolarization induced by morphine, oxycodone and oxymorphone (15 µM each) all agonists that caused little or no internalization. C) Summary plot showing the decline in hyperpolarization (% desensitization, gray bars) and internalization (% internalization, black bars). The results show that

different opioids produce different patterns of receptor regulation.

[Met]^{5}enkephalin, ME.

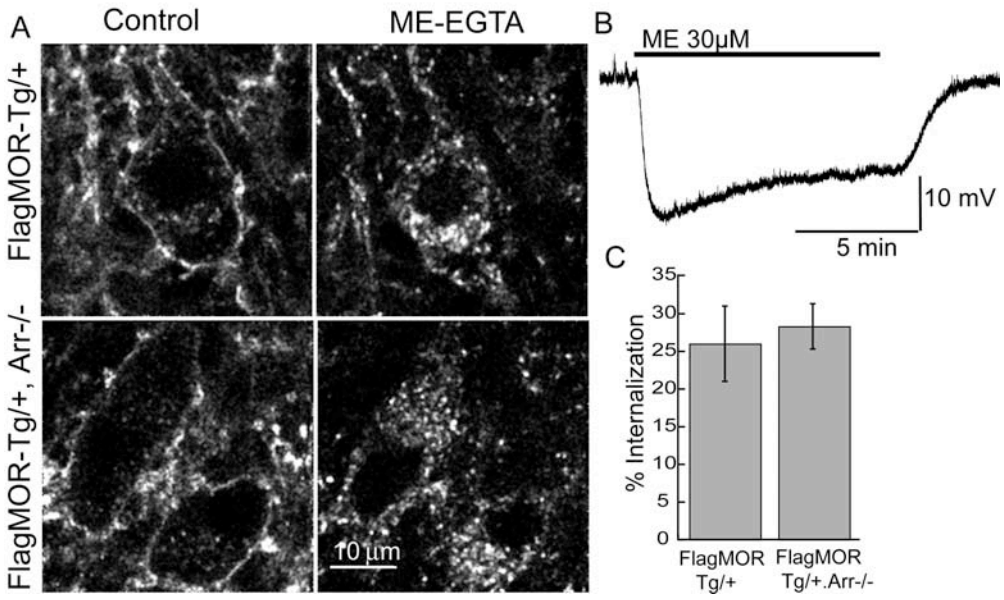

Figure 7. [Met]^{5}enkephalin (ME) induced MOR internalization occurs in absence of ß-arrestin2. A. Image from FlagMOR-Tg/+ (top) and FlagMOR-Tg/+, Arr-/- LC slices in control (left) and following application of ME (30µM, 15 min) and calcium-free (EGTA) solution (right). Intracellular puncta were observed in both genotypes. B. Intracellular recording of the hyperpolarization produced by application of ME (30 µM). The response declines to approximately 76% of the initial amplitude during the 10 min application. C. Summary of the percent internalization caused by ME in FlagMOR- Tg/+, Arr-/- and their FlagMOR-Tg/+ littermates.

CHAPTER 3

MU-OPIOID RECEPTOR DESENSITIZATION AND TRAFFICKING FOLLOWING CHRONIC TREATMENT WITH MORPHINE AND METHADONE

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Running title: Mu-opioid Receptor Desensitization and Trafficking

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I. ABSTRACT

Chronic treatment with morphine results in a decrease in mu-opioid receptor sensitivity, an increase in acute desensitization and a reduction in the recovery from acute desensitization in locus coeruleus neurons. Morphine is unlike many other opioid agonists in that it does not mediate robust acute desensitization or the induction of receptor trafficking. This study compares mu-opioid receptor desensitization and trafficking in brain slices taken from rats and mice treated chronically with morphine and methadone. In morphine treated animals, recovery from acute ME-induced desensitization and receptor recycling was diminished. In contrast, recovery and recycling from methadone treated animals were not different from experiments done in slices from untreated animals. In mice lacking ß-arrestin2, the recovery from desensitization and recycling were not affected by chronic-morphine treatment. Likewise, pharmacological inhibition of GRK2 resulted in complete recovery from desensitization following chronic morphine treatment. Thus, chronic treatment with morphine results in ß-arrestin2- and GRK2-dependent processes that prevent the recovery from desensitization and limit the reinsertion of receptors on the plasma membrane. The results indicate that chronic treatment with morphine or methadone results in unique adaptive changes in mu-opioid receptor regulation.

II. INTRODUCTION

Opioids exert analgesic and rewarding properties through activation of the mu opioid receptor (MOR). One of the major problems with continued opioid use during treatment of pain is the development of tolerance, such that more drug is required to produce the same level of analgesia. Results from both in vivo and heterologous systems suggest that tolerance to opiates develops in an agonist-specific manner (Grecksch et al., 2006; Koch et al., 2005; Stafford et al., 2001; Walker and Young, 2001; Whistler et al., 1999). Agonist-selective signaling of MOR results from the activation of unique acute regulatory events that could account for differential development of long term tolerance (Blake et al., 1997; Bohn et al., 2004; Bohn et al., 2000; Borgland et al., 2003; Finn and Whistler, 2001; Schulz et al., 2004; Yu et al., 1997). Two such signaling processes are acute, receptor-dependent desensitization and internalization. The classical model for GPCR desensitization is dependent on receptor phosphorylation by a G protein receptor kinase (GRK) (Gainetdenov et al., 2004) that facilitates binding of ß-arrestin, AP-2 and clathrin resulting in the targeting of this complex for internalization. Receptors are then recycled to the plasma membrane or degraded.

Receptor endocytosis appears to play a protective role in the development of tolerance, perhaps by facilitating recovery from desensitization (Koch et al., 2001) or by limiting MOR signaling and thus preventing cellular adaptations (Finn and Whistler, 2001; Martini and Whistler, 2007). Methadone is therefore thought to

limit downstream adaptations because it causes both desensitization and internalization. Continued receptor activation with morphine has been proposed to not cause internalization, resulting in cellular adaptations that underlie tolerance and withdrawal. Neurons in the locus coeruleus (LC) express MORs that desensitize and internalize in response to a saturating concentrations of some but not all opioid agonists (Harris and Williams, 1991; Alvarez et al. 2002, Arttamangkul et al., 2008). Following chronic treatment of rats with morphine, acute desensitization was increased and the recovery from desensitization was decreased (Dang and Williams, 2004). It is not known whether an agonist like methadone that can induce MOR internalization produces similar adaptations in neurons of the LC.

In the present study, receptor activation, desensitization, recovery and trafficking were compared after chronic treatment with morphine or methadone. In slices taken from rats treated with both morphine and methadone the concentration-response curve of [Met^5]-enkephalin (ME) was shifted to the right and acute desensitization was increased. In slices from morphine treated rats and mice the recovery from desensitization was reduced whereas the recovery from desensitization in slices from methadone treated animals was not different from controls. The reduced recovery from ME-induced desensitization was absent in morphine treated ß-arrestin-2 knockout animals. Likewise, following inhibition of GRK2 in slices from morphine treated animals, recovery from acute desensitization was complete. The results suggest that after chronic morphine treatment the

reduced recovery from desensitization results from an activation of the GRK2/ ß-arrestin-2 pathway that limits the reinsertion of active MORs on the plasma membrane.

III. MATERIALS AND METHODS

Tissue Preparation. For all experiments using rats, adult (150-250 g) male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were used. Details of the method of slice preparation and recording have been published previously (Williams et al., 1984). Briefly, animals were anesthetized with isoflurane and killed. The brain was dissected, blocked and mounted in a vibratome chamber in order to cut horizontal slices (200-260 μm thick) containing locus coeruleus (LC). Slices were stored at 35°C in an artificial cerebro-spinal fluid (aCSF) containing (in mM) 126 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, and 11 D-glucose while being continuously equilibrated with 95% O_2 / 5% CO_2. Slices were incubated for a minimum of 1 hour in order to wash out drugs used in chronic treatment protocols that may have remained in brain tissue.

Recordings. Slices (260 μm) were hemisected and transferred to the recording chamber (0.5 ml) where they were superfused with 35°C aCSF at a rate of 1.5 ml/min. Whole-cell recordings were made from rat LC neurons with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in the voltage-clamp mode (cells held at -55 mV). Pipettes (1.7-2.1 MΩ) were filled with an internal solution containing the following (in mM): 115 Methyl Potassium Sulfate, 20 NaCl, 1.5 $MgCl_2$,

10 HEPES, 10 BAPTA, 2 Mg-ATP, 0.5 Na-GTP, and 10 phosphocreatine, pH 7.3. Intracellular recording in mouse LC were made with an Axoclamp 2A using sharp electrodes (50-60 MΩ) filled with KCl (2 M). DC current (0-200 pA) was applied to inhibit spontaneous firing and hold the membrane potential at approximately -60 mV. Data was collected with PowerLab (Chart version 4.2.3). Analysis was performed with Prism and Kaleidagraph software. Values are presented as arithmetic mean±SEM. One-way ANOVA followed by Dunnett's or two-way ANOVA followed by bonferonni's multiple comparison test were performed and differences for which $p<0.05$ were considered significant.

Drug Treatment. Rats were implanted with osmotic minipumps (Alzet, 2ML1) in order to deliver morphine (NIDA–Neuroscience Center), methadone (NIDA–Neuroscience Center), or carrier (control). The minipumps have a 2 ml reservoir and deliver their contents for 7 days at the rate of 10 µl/hour. Pumps were filled with the required concentration of drug, dissolved in water, based on the weight of the rat and the desired dosing parameter (morphine: 60, 30, 15 mg/kg/day; methadone: 60, 30, 5 mg/kg/day). Mice received 45 mg/kg/day of either morphine or methadone via osmotic minipumps (Alzet, 2001) that have a 200 µl reservoir and release at a rate of 1 µl/hour for 7 days. Animals were anesthetized with isoflurane and an incision was made in the mid-scapular region to insert the pump subcutaneously. Rats receiving 60 mg/kg/day of either morphine or methadone were first given IP injections of 5 mg/kg at 9 am and 7 mg/kg at 6 pm on Day 1. On

Day 2, they received 7 mg/kg IP at 9 am and the osmotic minipump implanted at 6 pm. Animals were returned to their housing facility upon recovery. Experiments were performed on day 6 or 7 following minipump implantation. Control animals consisted of naïve animals and those implanted with vehicle-filled pumps.

Drug Concentration Analysis. All brain and plasma samples were analyzed at the University of Utah, Center for Human Toxicology under the supervision of Dr. Roger Foltz in conjunction with NIDA. Plasma and whole brain samples were obtained for drug (morphine or methadone) concentration analysis at the time of brain slice preparation. Following isoflurane anesthesia, 3 ml whole blood was obtained via cardiac puncture with a heparinized syringe. Blood was centrifuged and plasma was collected. Brain tissue removed after blocking the LC was collected and homogenized in water. Samples were frozen at -20°C and shipped to University of Utah, Center for Human Toxicology for analysis. Samples were analyzed by liquid chromatography/tandem mass spectrometry using electrospray ionization and selected reaction monitoring. Samples from morphine treated animals were analyzed for morphine and the metabolites morphine-3-glucuronide, and morphine-6-glucuronide. The quantification range for these compounds was between 1.0 and 1,000 ng/ml. Samples from methadone treated animals were analyzed for R- and S-methadone and their respective metabolites R- and S-2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP). The quantification range for these compounds was between 2.5 and 500 ng/ml.

FlagMOR transgenic mice. Mice were bred and genotyped as previously described (Arrtamangkul et al, 2008). Data were collected from mice that were hemizygous for the FlagMOR transgene (Flag-TG/+) and backcrossed with C57Blk6 for at least 5 generations. Mice were also bred with MOR-/- or Arr-/- mice to generate mice that were hemizygous for the transgene and homozygous knock-outs (Flag-TG/+MOR-/- and Flag-TG/+Arr-/-).

Receptor internalization and recycling. Brain slices (200 μm) were incubated in aCSF containing M1 antibody (Sigma) that was conjugated to 10 ug/ml Alexa-594 (InVitrogen) for 25-45 min. Slices were visualized using an upright microscope with a custom built 2-photon apparatus. A 15 μm z-series was collected in 1 μm sections using ScanImage software. [Met]^{5}enkephalin (ME) and calcium-free aCSF containing EGTA (0.5 mM, calcium-free) were applied by superfusion and all experiments were performed at 35˚. A control image (C) was collected prior to drug application and a second image was collected after perfusion of ME (30 μM, 10 min). This was followed by perfusion of the calcium free solution (10 min) either immediately after washout of ME (I) or after a 30-min wash-out period (R). Images were analyzed off-line using ImageJ software. Images were z-projected using the sum-slices method. Five regions of interest were selected and averaged for background fluorescence. The average background fluorescence was subtracted from total fluorescence to yield the fluorescence intensity. Internalization and was

calculated as the fraction of fluorescence intensity remaining after perfusion of ME and the calcium-free solution, compared to control (I/C). The total fluorescence remaining after a 30 min wash and calcium-free solution (R/C) was subtracted from the average internalization to determine the fraction of internalized receptors that recycled to the plasma membrane.

GRK2as5 transgenic mouse. To acutely regulate GRK2 activity in tissue, a pseudo-knockin" mouse that expresses a previously described analog-sensitive version of GRK2 (Kenski 2005) in place of the endogenous kinase was generated. This mutant GRK2 is selectively inhibited by the nucleotide analog, 11NaPP1 (1-(1,1-Dimethylethyl)-3-(1-naphthalenyl)-1H-pyrazolo[3, 4-d]pyrimidin-4-amine, gift of Kevan Shokat, UCSF). The fosmid M1-1960 containing the entire murine Adrbk1 gene and conserved 5' region was selected from the EpiFOS library (Bacpac, CHORI) for targeted mutagenesis. Mutations coding for C221V and L271G, which confer sensitivity to the small molecule inhibitor 11NaPP1 (Kenski 2005), were introduced into Adrbk1 by homologous recombination using the Counter-Selection BAC Modification method (Genebridges). The modified fosmid was linearized and microinjected into C57Bl/6 blastocysts (Gladstone Transgenic Core at UCSF). Founders, identified by Southern blot and the presence of a silent mutation introducing a BstEII site in the transgene detectable by PCR amplification and restriction digest, were crossed to C57Bl/6 WT animals to identify founders with germ-line transmission. Founders with endogenous levels of grk2as5 transgene

expression were selected for crosses to Adrbk1+/- heterozygous knockout mice in a C57Bl/6 background (Jaber 1996) (gift of Marc Caron, Duke University), and additional crosses were continued until mice homozygous for the transgene in a homozygous Adrbk1-/- background were obtained. Verification that starting breeding pairs were double homozygous mice was confirmed by mating each partner to WT mice, and all resulting offspring were double heterozygotes.

Drugs. Drugs were applied by bath superfusion. ME, bestatin and yohimbine were dissolved in water. NaPP1 and UK14304 were dissolved in DMSO. Final concentrations of DMSO did not exceed 0.01%. For experiments with NaPP1 slices were incubated prior to recording and were also superfused with the inhibitor. Thiorphan was dissolved in ethanol (applied less than 0.00001%). Morphine and methadone used for treatment were obtained from NIDA–Neuroscience Center. All other drugs were obtained from Sigma-Aldrich.

IV. RESULTS

Chronic treatment with morphine and methadone in rats

Chronic treatment of rats with morphine produces analgesic tolerance that results in a rightward shift of the morphine dose-response curve (Mucha et al., 1979). Concentration-response curves were constructed for [Met]^{5}enkephalin (ME) to determine whether treatment of rats with morphine or methadone resulted in

tolerance in LC neurons. Osmotic mini pumps containing either morphine (60 mg/kg/day) or methadone (60 mg/kg/day) were implanted for 6 days and the outward current induced by various concentrations of ME were measured (Fig 1A, B). The ME-stimulated current was normalized to the peak current induced by the alpha-2 adrenoceptor agonist, UK14304 (3 μM), which couples to the same population of g-protein linked inwardly rectifying potassium (GIRK) channels. In rats that were opiate naïve (control) the maximum ME-induced current was 145±8% of the peak UK14304-induced current. The maximum current was not different after chronic morphine or chronic methadone treatment (153±8% and 144±7% respectively). The concentration of ME that produced a half-maximal outward current (EC50) in slices from control was 281±47 nM. Slices from chronic morphine treated rats showed a 2 fold right-shift in the ME concentration-response curve (578±85 nM) compared to control, consistent with what has been reported previously following treatment of rats using morphine pellets (Christie et al, 1987). Chronic methadone treatment also resulted in a 2 fold right-shift (500±78 nM), suggesting that the doses of morphine and methadone used to treat rats produced a similar amount of cellular tolerance.

ME-induced receptor desensitization is increased following chronic morphine treatment of rats (Dang et al., 2004). To determine whether chronic methadone treatment produced a similar adaptation, ME-induced desensitization of outward currents was measured in slices from rats that were implanted with morphine or

methadone pumps. To induce desensitization a saturating concentration of ME (30 μM) was perfused and the peak and decline in the outward current was measured during a 10-min application. In control slices the outward current that remained after 10 minutes was 65±2% of the initial peak amplitude (Fig 2A). The outward current declined to 53±2% and 54±2% of the initial peak current after chronic morphine and chronic methadone treatment, respectively (Fig 2B,C). Therefore treatment with both morphine and methadone increased the amount of ME-induced acute desensitization and shifted the concentration-response curve (Fig 2D).

Recovery from ME-induced desensitization was examined in slices from morphine and methadone treated rats by comparing the current amplitude resulting from a pulse of ME (300 nM) applied before and after desensitization. The amplitude of the ME (300 nM) current 5 min after desensitization was 22±3% of the pre-pulse amplitude in control slices (Fig 3A). The test pulse of ME (300 nM) was applied again at 15, 25, 35 and 45 minutes. In slices from untreated rats the current recovered to 71±5% of the pre-pulse amplitude after 45 minutes. Following chronic morphine treatment the ME-induced current measured at 5 minutes following desensitization was less than the current in untreated rats (10±2% vs 22±3 of prepulse, $p<0.05$). After 45 minutes the recovery from desensitization was reduced in slices from morphine-treated rats compared to untreated (42±4% of pre-pulse; $p<0.05$, Fig 3B). While the extent of recovery was reduced after chronic morphine treatment the rate of recovery was not different from control, confirming results

obtained using morphine pellets (Dang and Williams, 2004). In slices taken from animals that were treated chronically with methadone, there was an increase in the amount of desensitization observed after 5 minutes (15±2% of pre-pulse; $p<0.05$)(Fig 3C). However, the current recovered to 67±2% of the pre-pulse amplitude after 45 minutes, which was not different from control ($p>0.05$, Fig 3D). Thus, chronic treatment with morphine resulted in a unique adaptive change that prevented recovery of MOR function.

To ensure that the observed differences between chronic morphine and methadone treatment was not dependent on the dose of drug, several doses of each agonist were used for treatment and recovery from desensitization was examined. Chronic morphine treatment resulted in reduced recovery from desensitization at all doses tested (Supplemental Figure?). Recovery from desensitization following methadone treatment were not different from control at all doses tested. Drug levels in brain and plasma for the drugs were measured for the various doses and are presented in Table 1. Morphine brain concentrations as low as 9±1 ng/ml were sufficient to cause changes in desensitization and recovery.

To determine whether acute morphine was sufficient to induce the same changes in MOR desensitization and recovery as chronic treatment, rats were given two 10 mg/kg intra-peritoneal injections of morphine, nine hours apart and experiments were performed 24 hours after the first injection. After ME-induced desensitization

the current induced by a test pulse of ME (300 nM) was 22±2% of the pre-pulse at 5 min and recovered to 76±4% at 45 min. These values were not different from control ($p>0.05$), indicating that one day of treatment was not sufficient to produce the changes observed by chronic treatment for 6 days.

Chronic treatment in mouse

Desensitization and recovery

Receptor trafficking is an important aspect of MOR regulation that may also be differentially regulated by chronic opioid treatment. To directly monitor trafficking of MOR, a transgenic mouse with the tyrosine hydroxylase (TH) promotor driving expression of a Flag-tagged MOR was used. Intracellular recording of membrane potential was used to measure coupling of Flag-MOR to GIRK activation. In slices from these transgenic mice ME (100 nM) produced a membrane hyperpolarization of 16.8±2.9 mV. The maximum hyperpolarization induced by ME (30 μM) was 32.3±1.3 mV and declined to 77±3% of the initial peak amplitude during the 10-minute application (Fig 4A). The test pulse of ME (100 nM) applied 5 min after desensitization was 27.9±5% of the initial pre-pulse amplitude. Test pulses were applied again after 10, 20 and 30 minutes. After 30 minutes the test-pulse amplitude had recovered to 92.5±3.3% of the pre-pulse amplitude.

Desensitization and recovery from desensitization were measured in transgenic mice (Flag-TG/+) and mice lacking the endogenous receptor (Flag-TG/+MOR-/-). In slices from animals treated with morphine (45 mg/kg/day, 6 or 7 days) the amplitude of the membrane hyperpolarization produced by ME (100 nM) was 16.5±2.4 mV and ME (30 μM) was 28.6±1.9 mV, not different from untreated mice (Fig 4B, $p>0.05$). During the application of ME (30 μM) the hyperpolarization declined to 74.3±3.7% of the maximum (Fig 4B). The amplitude of the ME (100 nM) test pulse 5 min after desensitization was not different from untreated (33.6±2.8% of pre-pulse, $p>0.05$) but recovered significantly less (70.9±5.5% of prepulse, $p<0.05$) after 30 minutes. Thus as was found in the rat, recovery from desensitization was incomplete following chronic morphine treatment compared to control.

Chronic treatment with methadone was carried out only in mice lacking the endogenous receptor (Flag-TG/+MOR-/-, 45 mg/kg/day, 6 or 7 days). This allowed the use of a high dose of methadone without special precautions to limit lethality. Results from methadone-treated mice were consistent with what was observed in the rat. The maximum hyperpolarization induced by ME was 27.4±1.6 mV and declined to 77.1±2% of the maximum during the 10-minute application (Fig 4C). The test pulse amplitude at 5 minutes was 33.6±5% of pre-pulse and after 30 minutes recovered to 88.7±4.2%, which were not different from untreated ($p>0.05$). In summary, chronic treatment with morphine and methadone had no effect on the

acute ME-induced desensitization as measured by the decline in the maximum hyperpolarization or by the more sensitive measure of comparing of amplitudes non-saturating ME-stimulated hyperpolarizations 5 minutes after desensitization. Chronic morphine treatment did however result in a reduced recovery from desensitization that was not observed after chronic methadone treatment (Fig 4D), confirming that only morphine induced adaptive changes to MOR regulation.

Internalization and recycling

Receptor trafficking after chronic opioid treatment was examined using LC slices from Flag-TG/+ mice prepared as for electrophysiology experiments. Extracellular N-terminally-tagged MORs were labeled with an M1 anti-Flag antibody conjugated to Alexa594 (M1-A594) and imaged using a 2-photon microscope. Initially M1-A594 labeling was localized primarily to the plasma membrane of LC neurons. Application of ME (30 μM) for 10 minutes resulted in an increase in puncta that remained after stripping receptors that remained on the plasma membrane with EGTA-buffered calcium free solution, suggesting that these receptors had undergone internalization (Fig 5A). ME-induced internalization was quantified as the percent of total fluorescence remaining after stripping the extracellular labeling, compared to the initial control image. In untreated mice, ME induced internalization of 52.3%±2.6 of labeled receptors. In slices taken from animals that were treated chronically with morphine or methadone ME-induced internalization was 58.2±5% and 56.2±3.5%, not different from untreated controls ($p>0.05$, Fig 5C).

Recycling of internalized receptors is thought to be important for recovery from desensitization. To examine recycling, ME was perfused to induce internalization, then normal ACSF was perfused for 30 minutes after washout of the ME solution. As described previously, this time point allowed near complete recovery from desensitization. Following the 30 min wash a redistribution of fluorescence labeling to the plasma membrane occurred and this was removed using the calcium-free solution (Fig 5B). In slices from untreated mice, the total fluorescence remaining after 30 minutes and Ca-free solution (R/C) was 34.1±1.1%, significantly less than the total fluorescence remaining when Ca-free solution was applied immediately after ME-induced internalization (Fig 5C, $p<0.05$). In slices from mice treated chronically with morphine there was no significant difference in the fluorescence remaining immediately after internalization and 30 minutes later (58.2±5% vs 49.7±3.5%), suggesting that few receptors recycled in 30 minutes ($p>0.05$). In slices from mice treated chronically with methadone 40±2.5% of the total fluorescence remained after 30 min, which was less than the fluorescence remaining immediately after internalization ($p<0.05$, Fig 5C). Therefore chronic morphine treatment decreased the trafficking of receptors back to the plasma membrane, which was not observed following chronic treatment with methadone.

Role of arrestin in morphine induced adaptations

ß-arrestin-2 has been identified as a molecule important for MOR regulation in cell lines and is implicated in contributing to morphine tolerance in vivo (Bohn et al, 2000). To explore a role for ß-arr2 in morphine-induced adaptations to receptor regulation FlagMOR expressing mice were crossed with ß-arrestin-2 knock-out mice and desensitization and the recovery from desensitization were examined. The membrane hyperpolarization produced by application of ME (100 nM) in untreated Flag-TG/+Arr-/- mice (13.7±1.1 mV) was not different from Flag-TG/+ mice, nor was the maximum hyperpolarization produced by a saturating concentration of ME (28.4±0.8 mV, Fig 6A, $p>0.05$). As reported previously, the decline in the membrane hyperpolarization during a 10-minute application of ME (30 μM) was not affected by loss of ß-arrestin-2 (79.1±2% of peak, Arttamangkul et al, 2008). ME (100 nM) tested 5 minutes after desensitization produced a hyperpolarization that was 32.8±2% of the initial pre-pulse amplitude and recovered to 94.4±7% after 30 minutes. Therefore desensitization and complete recovery were observed in the absence of ß-arrestin-2.

Chronic morphine treatment in Flag-TG/+Arr-/- mice did not alter ME-induced hyperpolarization (12.6±1.8 mV and 29.9±1.3 mV for 100 nM and 30 μM respectively) compared to untreated Flag-TG/+Arr-/-, nor did it affect the decline from the peak during a 10 min application (75.6±4% of peak, $p>0.05$). The ME (100 nM) test-pulse amplitude recovered to 90.8±6% of pre-pulse after 30 minutes, which was not different from untreated mice ($p>0.05$, Fig 6B,C). Therefore the

reduced recovery from desensitization observed in rats and Flag-TG/+ mice following treatment with morphine was absent in slices from morphine-treated Flag-TG/+Arr-/- mice.

Internalization and recycling were compared in untreated and morphine treated Flag-TG/+Arr-/- mice. Despite the role of arrestin in mediating receptor endocytosis, no change in internalization was observed in untreated Flag-TG/+Arr-/- mice (59.3±5.8%, $p>0.05$). Likewise, internalization was not affected in slices from mice that were treated chronically with morphine (54.9±4.5%, $p>0.05$, Fig 7A,B). In slices from untreated and treated Flag-TG/+ Arr-/- there was significantly less fluorescence remaining (39.8±4.6% and 37.4±3.3%, respectively) when Ca-free solution was perfused after 30 min compared to immediately after ME-induced internalization, suggesting receptor recycling occurred in both groups (Fig 7C, $p<0.05$). Thus, in Flag-TG/+Arr-/- mice that were treated chronically with morphine, the recovery from desensitization and receptor recycling were the same as in untreated animals. These results suggest that adaptations in the processes that underlie the reduced recycling of receptors to the plasma membrane induced by chronic morphine treatment are ß-arrestin-2 dependent.

GRK2 inhibition

Phosphorylation of GPCRs by GRK contributes to desensitization and facilitates recruitment of ß-Arrestin-2 (Gainetdinov et al., 2004). Desensitization and the recovery from desensitization were examined following inhibition of GRK2 to

determine if phosphorylation by GRK2 is required for MOR desensitization and the adaptations produced by chronic morphine treatment. A GRK2 knockout transgenic mouse that expressed a modified GRK2 with an amino acid substitution in the active site of the kinase (GRK2as5) that rendered the kinase sensitive to an ATP analog, NaPP1 was used for these experiments. In the absence of NaPP1 the kinase activity of GRK2as5 is maintained (ref). Concentration-response curves for ME were constructed using slices from untreated GRK2as5 mice in the absence and presence of NaPP1. In the absence of NaPP1, a ME produced a maximal membrane hyperpolarization of 28±1.7 mV with an EC50 of 1.1±0.51 μM (Fig 8A). The maximum hyperpolarization in slices incubated with NaPP1 was not different (27.2±1.1 mV) and the EC50 was 917±100 nM. This is similar to what has been reported in wild-type mice (Torrecilla et al., 2008)

To determine whether GRK2-dependent phosphorylation is required for desensitization and the recovery from desensitization, experiments were performed either in the absence or presence of the inhibitor, NaPP1. In experiments without NaPP1, ME (500 nM) produced a membrane hyperpolarization of 11.3±2.1mV. The maximum hyperpolarization produced by ME (30 μM, 28±1.7 mV) declined to 74.3±2.1% of the peak during the 10 min application. Following a 5 min wash, ME (500 nM) produced a hyperpolarization that was 29.6% of the pre-pulse amplitude and after 30 min the amplitude of the hyperpolarization was 82.6±4.5% of the initial value. In slices that were incubated in NaPP1 (10 μM, 30 min) to inhibit GRK2, the

hyperpolarization produced by ME (500 nM) was (10.7±1.1 mV) and the hyperpolarization produced by ME (30 μM) was 26.9±1.2 mV. In slices from untreated animals there was no effect of NaPP1 on ME-induced desensitization or the time course and extent of recovery from desensitization ($p>0.05$, Fig 8C). Thus desensitization and the recovery from desensitization were independent of GRK2 activity in untreated mice.

Following chronic morphine treatment, desensitization induced by ME was not affected by incubation with NaPP1 (74.3±2.1% without and 67.8±2.8% with NaPP1, 10 μM 30 min). The test pulse amplitude 5 min after washing out the high concentration of ME was 47±6.2% of the control, however in slices that were incubated in NaPP1 the recovery from desensitization was more rapid and complete than slices from morphine treated animals in the absence of NaPP1 (Fig 8B,D). Therefore the activity of GRK2 was required to decrease the rate and extent of recovery from desensitization after chronic morphine treatment.

V. DISCUSSION

Acute morphine and methadone differentially affect receptor activation, desensitization and internalization (Whistler et al., 1999; Alvarez et al, 2002; Arttamangkul et al, 2008). The results of this study confirm that chronic treatment with morphine decreased recovery from acute desensitization. We further

demonstrate an inhibition of receptor recycling and go on to show that these adaptive changes were not present in morphine treated ß-arrestin-2 knockout animals. Likewise inhibition of GRK2 in slices taken from morphine treated animals resulted in complete recovery from desensitization. Finally, chronic treatment with methadone had no effect on the recovery from desensitization or receptor trafficking. The results suggest that following chronic treatment with morphine, ß-arrestin-2 and the activity of GRK2 prevent or slow the normal recovery from acute receptor desensitization and this adaptive change is agonist dependent.

Trafficking

The trafficking of receptors has been proposed to be an important process in the development of tolerance among different opioid agonists (Koch et al., 2005; Martini and Whistler, 2007; Koch and Hollt, 2008). In the present study, the extent of ME-induced internalization was not different after chronic treatment with morphine or methadone. Reduced analgesia with no changes in DAMGO-induced internalization has also been reported in the spinal cord of morphine tolerant rats (Trafton et al., 2004). Although desensitization measured by the coupling to GIRK in opiate naïve cells was independent of receptor trafficking (Arttamangkul et al., 2006), the recycling of receptors appears to be correlated with recovery from desensitization following chronic morphine treatment. Reinsertion of internalized receptors is hypothesized to be protective or a mechanism that slows the development of tolerance (Martini and Whistler, 2007). The reduction in opioid receptor recycling

in slices from morphine-treated mice in the present study may contribute to the development of tolerance.

ß-Arrestin/GRK2 dependent mechanism

ß-arrestin has many roles in GPCR regulation and signaling and the expectation was that receptor internalization would be inhibited. However, both desensitization and internalization were unaffected by loss of ß-arrestin-2. Although the expression of ß-arrestin-1 in LC neurons may compensate for the loss of ß-arrestin-2 and allow for normal receptor internalization, there is increasing evidence that opioid receptor desensitization occurs independent of ß-arrestin-2 (Walwyn et al., 2007; Arttamangkul et al, 2008; Dang et al., 2009). It is clear however that following chronic morphine treatment, ME-stimulated receptors engage a ß-arrestin-2 dependent process capable of inhibiting recovery from desensitization or recycling.

The effect of chronic morphine on recovery and recycling was lost in mice lacking ß-arrestin-2. This supports the idea that recycling and recovery from desensitization are related and suggests that ß-arrestin-2 inhibits both processes after chronic morphine treatment. Given that morphine does not acutely induce arrestin translocation or receptor internalization, the role of ß-arrestin-2 in morphine-induced adaptations is somewhat surprising. Tolerance to morphine in ß-arrestin-2 knock-out mice was only observed after extended treatment. It remains unclear

whether the observed changes in recovery from desensitization and recycling contribute to the delayed analgesic tolerance observed in ß-arrestin-2 knock-out mice in vivo (Bohn et al., 2000 and 2002). The present results from Flag-TG/+ß-Arr2-/- mice that were treated chronically with morphine support a role for ß-arrestin-2 in mediating morphine-induced adaptations. The results also indicate that this is an agonist dependent process, since chronic treatment with methadone did not result in the same effect.

G-Protein Receptor Kinase

Inhibition of GRK2-as5 with NaPP1 in slices from mice treated chronically with morphine resulted in complete recovery from desensitization. In these experiments GRK was active throughout the morphine treatment period, suggesting that an active GRK mechanism is involved in the reduced recovery from acute desensitization after chronic morphine treatment. Inhibition with NaPP1 had no effect on receptor desensitization in slices from untreated or treated mice. The use of an inhibitory peptide to reduce GRK2 activity in HEK293 cells and LC neurons resulted in a decrease in DAMGO-induced desensitization and had no effect on morphine-induced desensitization (Johnson et al., 2006; Bailey et al., 2009). The acute inhibition of GRK2 with NaPP1 suggests there are other mechanisms that contribute to ME-induced desensitization in LC neurons. Indeed, blockers of multiple pathways were required to inhibit ME-induced desensitization (Dang et al., 2009).

Morphine vs methadone

The actions of morphine and methadone differ in several ways in spite of the observation that the ED50 for analgesia is only slightly greater for morphine (2.17±0.3 mg/kg) than for methadone (1.49±0.2 mg/kg) in the warm-water tail withdrawal assay (Peckham and Traynor, 2006). Mice treated with 4 mg/kg of methadone did not exhibit analgesic tolerance over a period of 5 days whereas an equally effective analgesic dose of morphine (10 mg/kg/day) did result in tolerance (Kim et al., 2008). Likewise, acute receptor desensitization and internalization induced by morphine and methadone were very different (Dang and Williams, 2005; Alvarez et al, 2002; Arttamangkul et al, 2008).

The reduction in recovery from desensitization and recycling was unique to chronic treatment with morphine and was not observed after chronic methadone treatment. Plasma and brain drug levels rule out failure or inaccurate dose delivery from osmotic pumps as a source for these differences. The difference between these two agonists was maintained over a wide range of doses, suggesting that it was not the result of discrepancies between effective plasma and brain concentration. To determine whether these differences account for the unique adaptations caused by morphine treatment a group of animals was treated with a low dose of methadone (5 mg/kg/day, 6-7 days, Table 1). However even by keeping the concentration of

methadone in brain below the threshold for internalization and desensitization, the recovery from desensitization did not mimic morphine treatment.

There were some similarities in the adaptations produced by chronic morphine and methadone treatment. First, the concentration-response curves for ME-induced outward current in slices from rats were shifted 2-fold to the right after treatment with both agonists, suggesting a similar amount of cellular tolerance by treatment with morphine and methadone. Treatment of rats with time-release morphine pellets instead of osmotic mini pumps were similar in that the peak current induced by morphine was not decreased relative to that measured in slices from untreated animals (Dang and Williams, 2004). However in mice treated chronically with morphine pellets, no shift in the concentration response was observed (Torrecilla et al., 2008). Chronic treatment of mice with morphine but not methadone resulted in a rightward shift in the dose response curve using a measure of analgesia (Kim et al., 2008). Drug treatment protocols, particularly the dose of methadone used and the method of administration (continuous vs intermittent) may explain the difference in the shift in concentration-response curves (Madia et al., 2009).

Chronic treatment with morphine and methadone resulted in enhanced desensitization in rat LC. There was a greater decline in the outward current induced by ME (30 μM) during a 10-min application compared to untreated animals. Second, there was a greater depression of the current induced by ME (300 nM)

measured at the first time-point (5 min) following desensitization. Enhanced desensitization following chronic treatment with morphine and methadone was not observed in mouse LC. This is likely due to the use of intracellular recording of membrane potential. The decline in the outward current induced by a maximum concentration of ME provides a more sensitive measure of desensitization because the peak amplitude is not limited by the approach to the potassium reversal potential. Dialysis of the intracellular milieu into the whole-cell pipette may also contribute to the differences observed in rat. Chronic morphine treatment of HEK293 cells resulted in a decrease in DAMGO-induced desensitization and internalization. However this was dependent on receptors being occupied by morphine and no difference was seen 1 hour after washout of morphine (Eisenger et al, 2002). The present results indicate that enhanced desensitization and reduced recovery persists for hours after washout of morphine.

Summary

As has been reported for many other aspects of MOR signaling, the adaptations that are induced by chronic treatment are agonist-dependent. The delayed recovery from desensitization after chronic morphine treatment was not observed after chronic treatment with methadone. While the lack of receptor internalization appears to contribute to morphine tolerance in vivo (Kim et al., 2008) it cannot be ruled out that the unique adaptations produced by chronic morphine are the result of other signaling pathways that are differentially engaged. These chronic actions of

morphine require the activity of GRK2 and ß-Arr2. The results may suggest a more stable interaction of GRK2 and/or ß-arrestin-2 with MOR, which could prevent dephosphorylation and recycling of the receptor (Oakley et al., 1999).

Morphine-Treated

Matrix	Dose	Morphine	M-3-G	M-6-G
Plasma	60	317 ± 21	< LOQ	90 ± 20
	15	65 ± 3	505 ± 85	28 ± 5
Brain	60	36 ± 2	14 ± 2	3 ± 1
	15	9 ± 1	3	< LOQ

Methadone-Treated

Matrix	Dose	R-Methadone	S-Methadone	R-EDDP	S-EDDP
Plasma	60	101 ± 6	140 ± 9	18 ± 1	20 ± 1
	5	9 ± 1	8 ± 1	< LOQ	< LOQ
Brain	60	150 ± 11	197 ± 11	4	4
	5	9	12 ± 1	< LOQ	< LOQ

Table 1. The concentration (ng/ml) of morphine, methadone and metabolites measured in plasma and brain samples taken from animals after a 6-7 day treatment period. Morphine doses of 60 and 15 mg/kg/day (n=10 and 8, respectively); methadone doses of 60 and 5 mg/kg/day (n=13 and 6, respectively) were delivered via osmotic minipump. Data are expressed as mean values ± S.E.M. Level of Quantification (LOQ) for morphine: 1.0 – 1000 ng/ml. ROQ for methadone: 2.5 – 500 ng/ml.

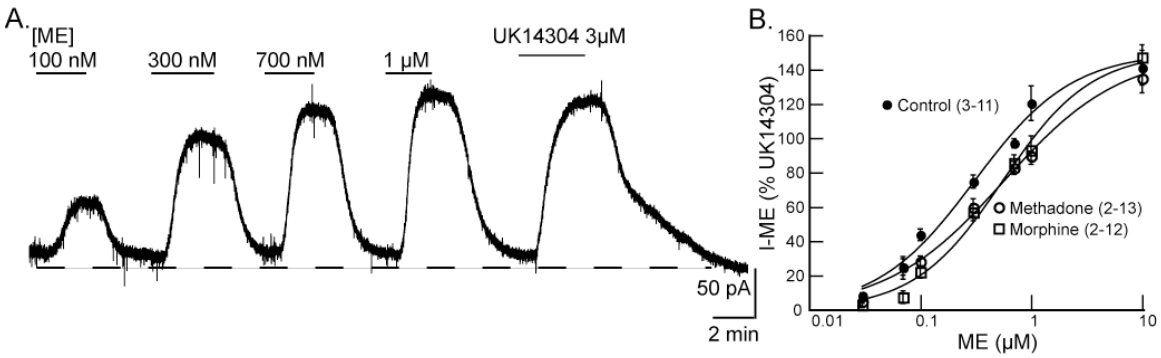

Figure 1. Chronic morphine and methadone treatment resulted in reduced potency of ME in rat LC neurons. A, Representative trace showing outward currents produced by perfusion of varying concentrations of ME. At the end of the experiment the outward current produced by UK14304 (3 µM) was measured and reversed by yohimbine (3 µM). B, Concentration-response curves for ME in slices from untreated, chronic morphine (60 mg/kg/day) and chronic methadone (60 mg/kg/day) treated rats. Outward currents induced by ME (30 nM – 10µM) were normalized to the peak current induced by UK14304. All points are mean ± standard error (n). The EC50 values from control (281±47 nM), chronic morphine (578±85 nM) and chronic methadone (500±78 nM).

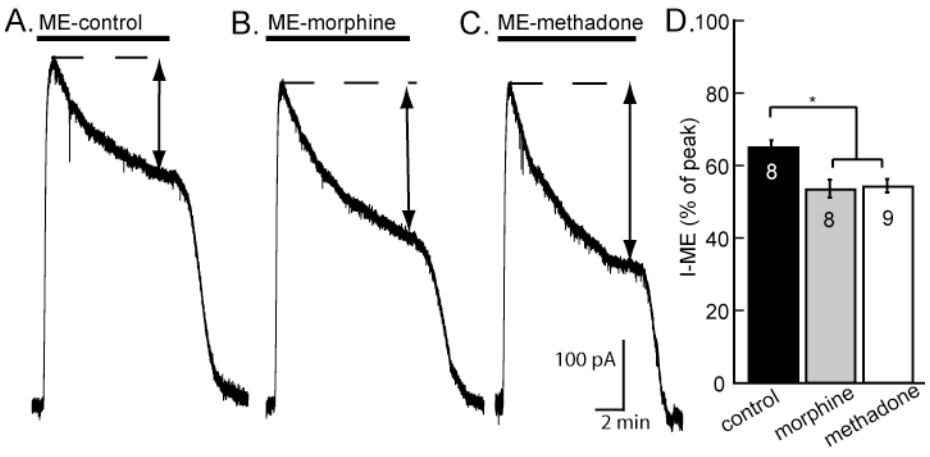

Figure 2. Enhanced desensitization observed after chronic treatment of rats with morphine or methadone. A-C, Representative traces showing the decline in the outward current during a 10-minute application of asaturating concentration of ME (30 μM). The peak current (dashed line) declined during the 10-minute application to a desensitized amplitude (double arrows). D, Bars represent the mean % of peak current ± standard error in control (black), morphine treated (gray), and methadone treated (white). n indicated in bars. One-way ANOVA followed by Dunnet's post-hoc test showed a significant difference between morphine-treated and methadone-treated vs. control (∗$p<0.05$).

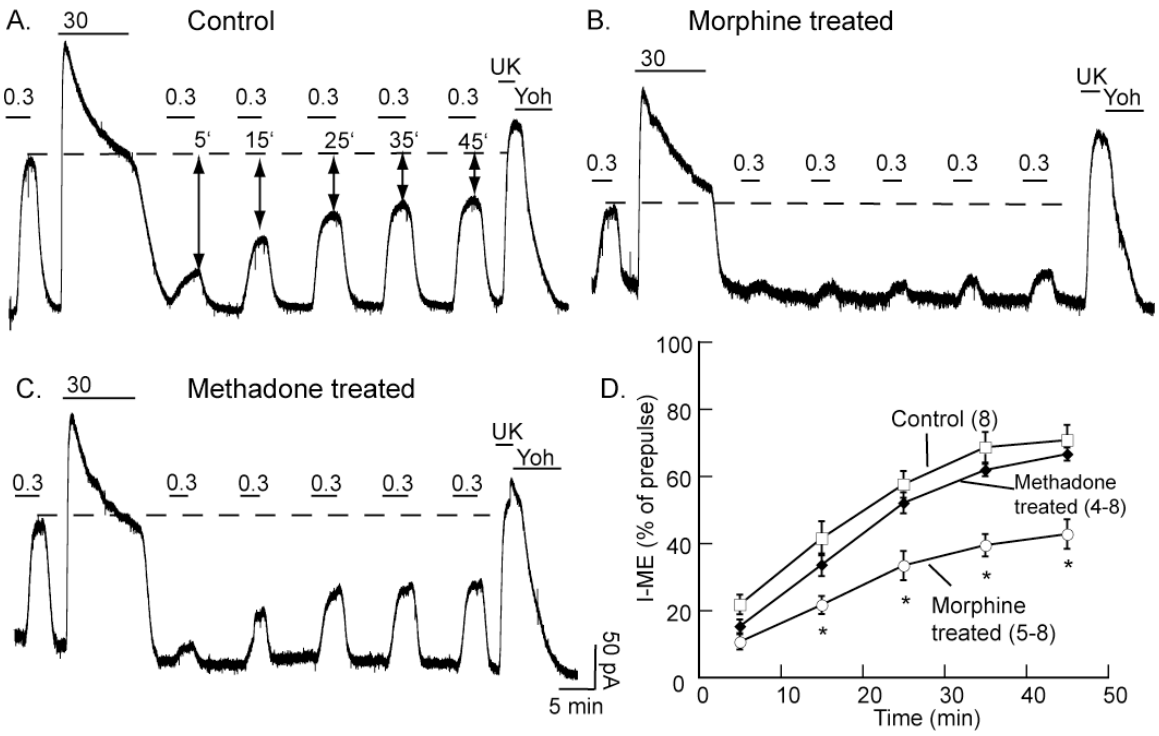

Figure 3. Recovery from desensitization is impaired after chronic morphine treatment but not chronic methadone. A, Representative traces of outward currents in slices from untreated mice. ME (300nM) was perfused prior to desensitizing with ME (30μM, 10 min). 5 minutes after washout of desensitizing concentration of ME (300nM) was tested again and amplitude was compared to the pre-pulse amplitude (dashed line). ME (300nM) was tested again at 15, 25, 35 and 45 minutes to measure recovery from desensitization (% of pre-pulse amplitude). At the end of the experiment the current induced by UK14304 (3μM) was determined and reversed with yohimbe (3μM). B, C, Representative traces from morphine treated and methadone treated rats. D, Summary of recovery from desensitization (% of pre-pulse amplitude). □ control, ○ morphine treated,

umethadone treated. Error bars represent SEM (n). Two-way ANOVA with bonferonni post-hoc comparison (∗ $p<0.05$ compared to control)

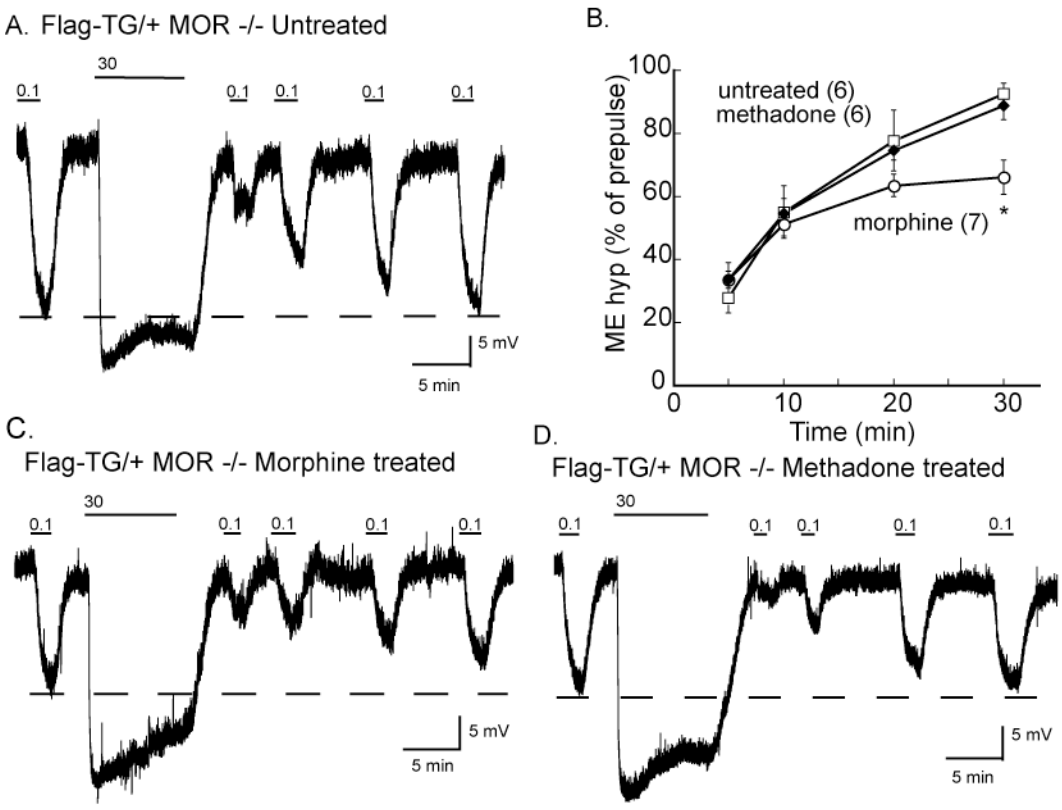

Figure 4. Recovery from desensitization impaired after chronic morphine treatment in Flag TG/+ mice. A, Representative trace of membrane potential in slices from an untreated Flag TG/+ mouse. ME (100nM) was perfused prior to and 5, 10, 20 and 30 minutes after desensitizing with a saturating concentration of ME (30μM). After 30 minutes recovery from desensitization was nearly complete (dashed line). B, Representative trace of desensitization and recovery in slices from morphine treated and C, methadone treated mice. D, Summary of recovery from desensitization (% of pre-pulse amplitude). □ control, ○ morphine treated, ◊ methadone treated. Error bars represent SEM (n). Two-way ANOVA with bonferroni post-hoc comparison (* $p<0.05$).

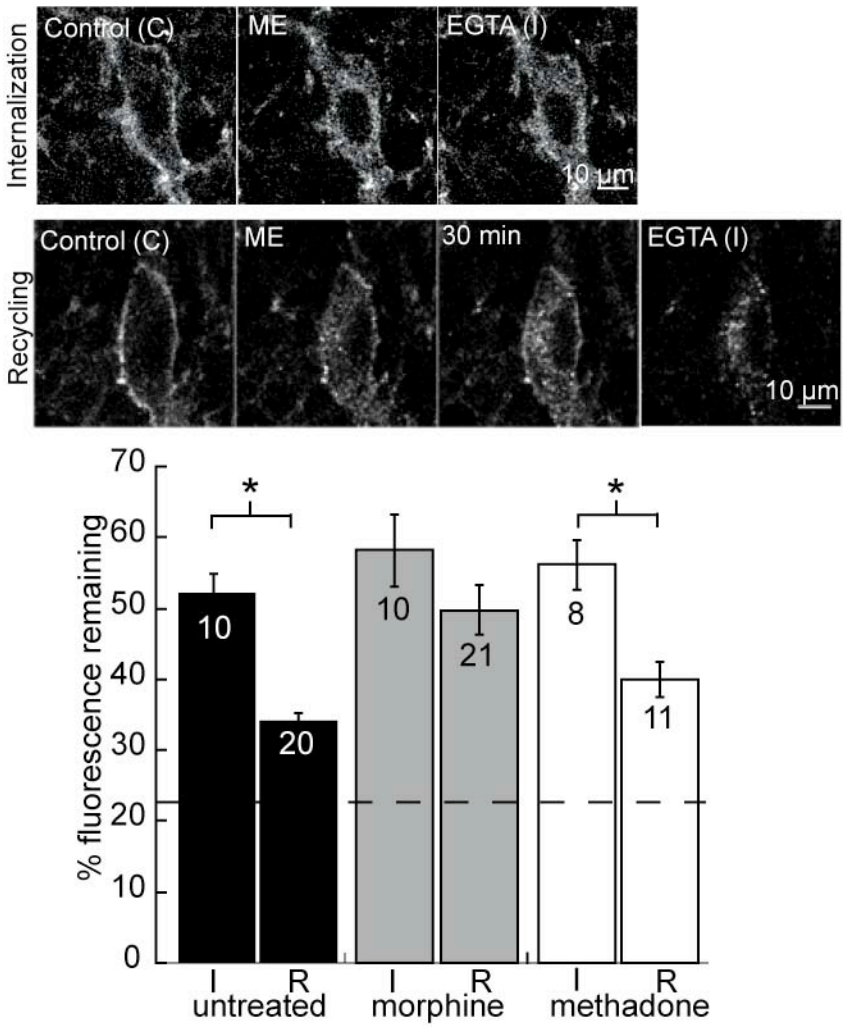

Figure 5. Internalization and recycling of FlagMOR in live brain slices. A. Representative images of ME-induced internalization. Control (C) is initial staining with an Alexa-594 conjugated anti-Flag M1 antibody prior to agonist treatment. Internalization was induced with a 10-min perfusion of ME (30µM). Immediately after washout of ME, calcium-free EGTA solution was perfused to remove M1-A594 from receptors on plasma membrane (I). B. Control (C) image of initial staining. Internalization was induced by ME and following a 30 min wash in normal ACSF

some receptors recycled to plasma membrane. Ca-free EGTA solution was then applied to strip antibody from the plasma membrane (R). C, Summarized data of total fluorescence remaining after perfusion of Ca-free EGTA solution immediately after internalization was induced (I/C) or after 30 minutes (R/C) in slices from untreated (black), morphine treated (gray) and methadone treated (white) mice. Dashed line indicates % fluorescence intensity remaining in drug-free experiment. Error bars represent SEM. I/C and R/C were compared within treatment groups using Student's t-test (* $p<0.05$)

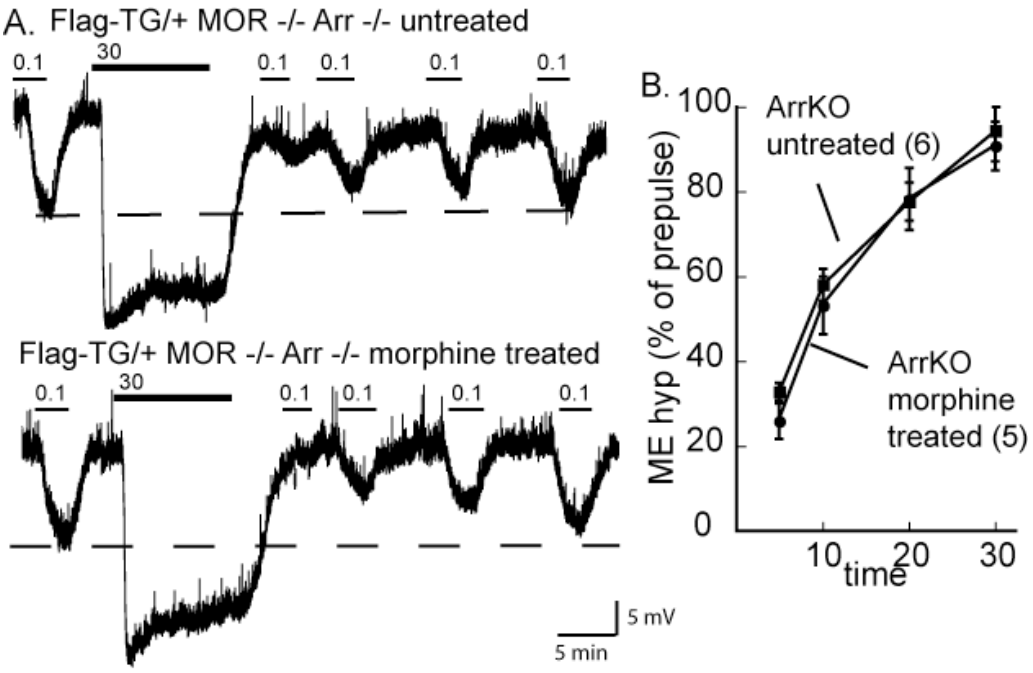

Figure 6. Recovery from desensitization is complete in slices from morphine-treated ßArr2 -/- mice. A, Representative traces showing desensitization and recovery in an untreated (top) and morphine-treated (bottom) mouse. Dashed lines represent pre-pulse amplitude of the hyperpolarization produced by ME (100nM). B, Summary of recovery (% of pre-pulse amplitude) for untreated (■) and morphine-treated (●) mice. Two-way ANOVA ($p>0.05$)

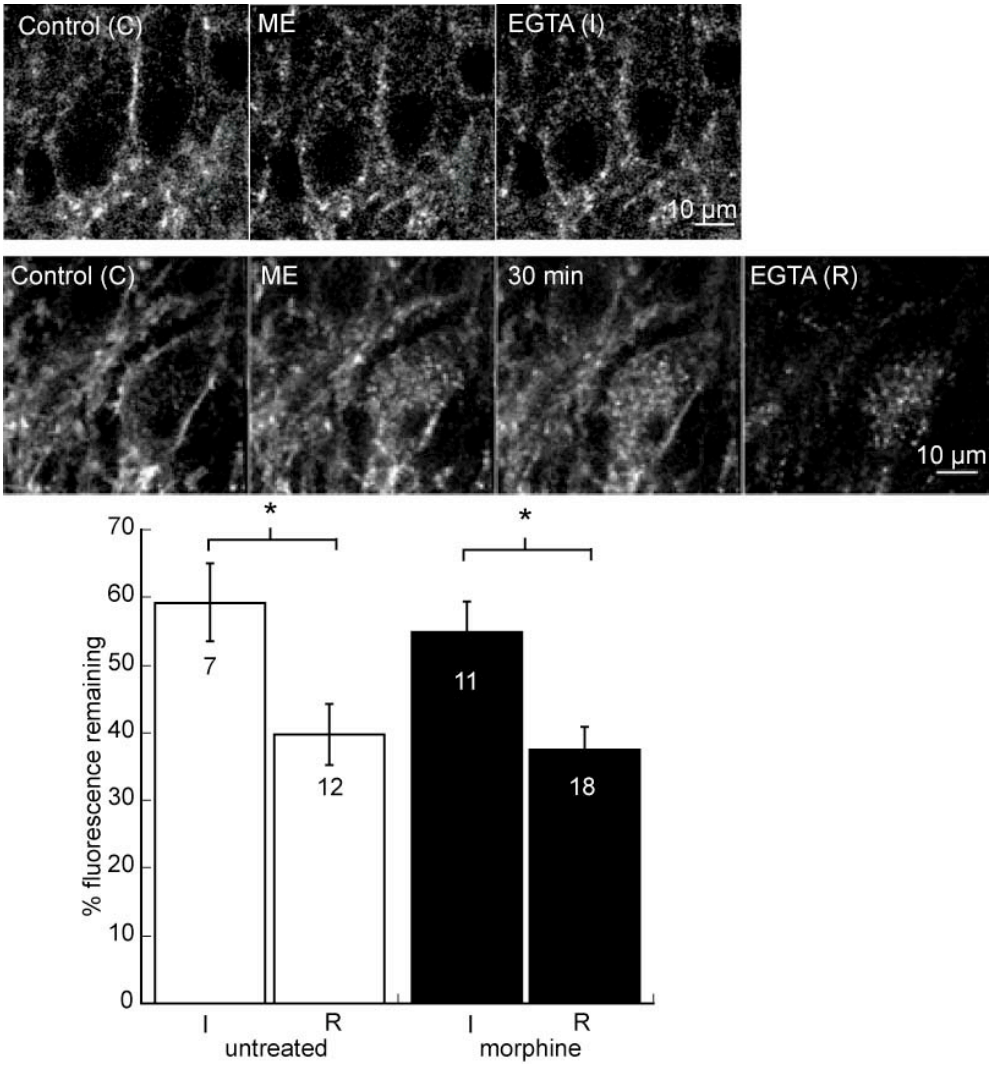

Figure 7. Internalization and recycling are not altered by chronic morphine treatment in ßArr2 -/- mice. A, Internalization induced by ME. Control (C) is initial staining with an Alexa-594 conjugated anti-Flag M1 antibody prior to agonist treatment. Internalization was induced with a 10 min perfusion of ME (30µM) and was followed immediately by Ca-free EGTA to remove M1-A594 from receptors on plasma membrane (I). B. Recycling of receptors following ME-induced internalization. Control (C) is initial staining and internalization was induced with

ME (30μM). After a 30 min wash in normal ACSF some receptors recycled to plasma membrane and Ca-free EGTA was applied to remove M1-594 from receptors on plasma membrane. C. Summarized data of total fluorescence remaining after Ca-free EGTA solution was applied immediately after ME-induced internalization (I/C) and following 30 min wash (R/C) in untreated (white) and morphine treated (black) Flag TG/+ Arr -/- slices. Dashed line represents fluorescence intensity remaining in drug-free experiment. Error bars represent SEM and Student's t-test was used to compare I/C with R/C within groups (* $p<0.05$).

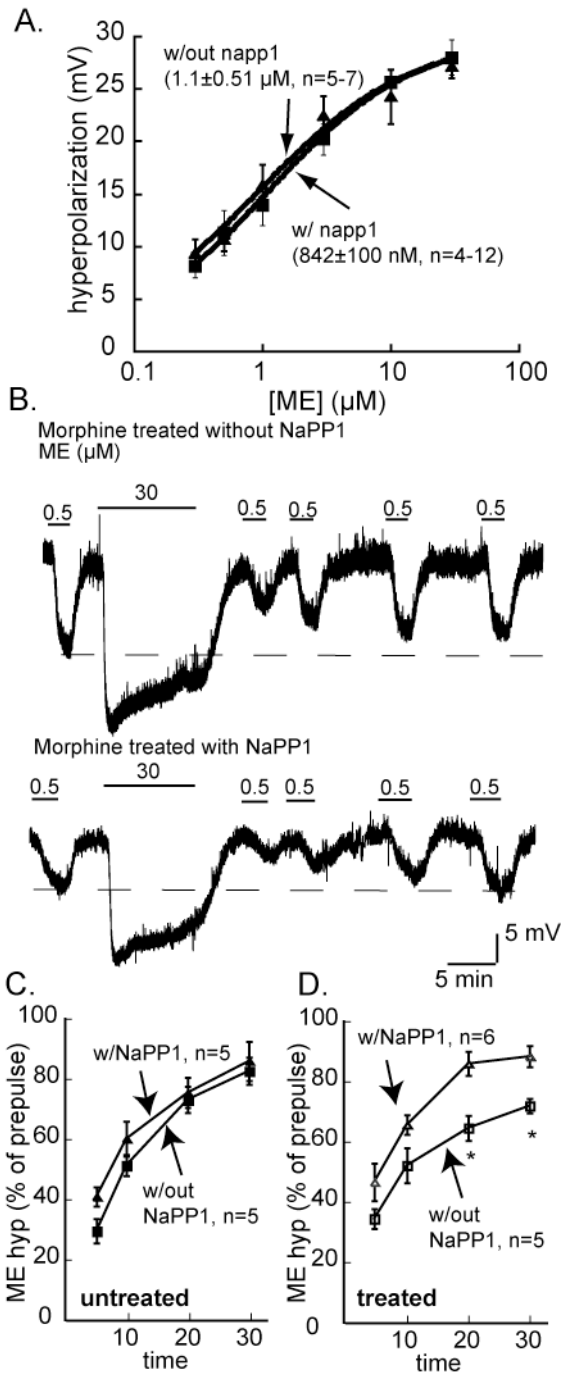

Figure 8. Inhibition of GRK2 with NaPP1 reverses the effects of chronic morphine treatment on recovery from desensitization A, Concentration-response curve for ME constructed from average membrane hyperpolarizations in

slices from GRK2-as5 mouse in the absence (■) or presence (▲) of napp1 (10 μM). B, Representative recordings in slices from morphine-treated GRK2-aa5 mice without NaPP1 (top) and slices incubated with NaPP1 (bottom, 10μM, 30 min) prior to desensitizing with ME. C, Summary of recovery (% of prepulse amplitude) in slices from untreated GRK-as5 without (■) or with (▲) NaPP1. C, Summary of recovery in slices from morphine treated GRK-as5 without (□) and with (△) NaPP1. Error bars represent SEM (∗ $p<0.05$).

CHAPTER 4

PRE- AND POSTSYNAPTIC REGULATION OF LOCUS COERULEUS NEURONS AFTER CHRONIC MORPHINE TREATMENT: A STUDY OF GIRK KNOCKOUT MICE

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I. ABSTRACT

While the acute inhibitory effect of opioids on locus coeruleus (LC) neurons is mediated primarily by the activation of G protein-gated inwardly-rectifying K^+ (GIRK) channels, the 3'-5'-cyclic adenosine monophosphate (cAMP)-system has been implicated in the effects of chronic morphine exposure. Presently, the impact of chronic morphine treatment on GIRK-dependent and GIRK-independent mechanisms underlying the opioid-induced inhibition of LC neurons is unclear. Here, opioid-induced postsynaptic inhibition was studied in LC neurons from wild-type and GIRK2/GIRK3$^{-/-}$ mice at baseline and following chronic morphine treatment. The postsynaptic inhibition of LC neurons caused by the opioid agonist [Met]^5 enkephalin (ME) was unaffected by chronic morphine treatment in mice of both genotypes. Furthermore, chronic morphine treatment had no effect on the forskolin augmentation of the ME-induced current in wild-type LC neurons, and only a minor effect on the ME-induced current in LC neurons from GIRK2/GIRK3$^{-/-}$ mice. Chronic morphine treatment did, however, lead to an increased frequency of spontaneous excitatory postsynaptic currents (EPSCs) in the LC. Interestingly, while forskolin augmented the EPSC frequency similarly in untreated and morphine-treated wild-type mice, as well as untreated GIRK2/GIRK3$^{-/-}$ mice, it failed to increase the frequency of EPSCs in morphine-treated GIRK2/GIRK3$^{-/-}$ mice. Altogether, the findings suggest that chronic morphine treatment exerts little impact on ion channels and signaling pathways that mediate the postsynaptic inhibitory effects of opioids, but does enhance excitatory neurotransmission in the mouse LC.

II. INTRODUCTION

Studies of the locus coeruleus (LC) have highlighted mechanisms underlying acute and chronic effects of opiates (Nestler & Aghajanian, 1997; Nestler, 2001; Williams *et al.*, 2001). Acutely, mu opioid receptor (MOR) agonists hyperpolarize LC neurons (Pepper & Henderson, 1980; Williams *et al.*, 1982). The hyperpolarization of LC neurons involves multiple channel types, the most dominant of which appears to be the G protein-gated inwardly-rectifying K^+ channel (GIRK/Kir3 channel) (Torrecilla *et al.*, 2002).

MOR agonists also inhibit adenylyl cyclase in LC neurons (Duman *et al.*, 1988). The activity of the cAMP system is enhanced during prolonged morphine exposure, an adaptation that parallels tolerance and may explain the recovery of LC neuron firing rates to pre-treatment levels (Duman *et al.*, 1988; Nestler & Tallman, 1988; Rasmussen *et al.*, 1990). In addition, application of MOR antagonists following chronic morphine exposure leads to hyper-stimulation of the cAMP system in LC neurons, a phenomenon implicated in the elevated firing rates of LC neurons seen in some studies during opiate withdrawal (Kogan *et al.*, 1992; Lane-Ladd *et al.*, 1997; Ivanov & Aston-Jones, 2001). Similar findings have been reported in other brain regions, supporting the contention that cAMP signaling contributes to the behavioral manifestations of opiate dependence, withdrawal, and reinforcement (Nestler, 2001; Williams *et al.*, 2001).

Though consensus has emerged that adaptations in the cAMP pathway occur during chronic morphine exposure, the impact on neuronal excitability is uncertain.

Some data argue that modifications intrinsic to LC neurons explain the enhanced activity noted during withdrawal, while other studies implicate enhanced afferent excitatory input. For example, antisense or dominant-negative suppression of the cAMP-response element binding (CREB) protein in LC neurons opposed the elevated firing rates and behavioral manifestations induced by opiate withdrawal (Lane-Ladd *et al.*, 1997; Han *et al.*, 2006). Nevertheless, the opiate withdrawal syndrome was observed following neurochemical lesion of the LC (Chieng & Christie, 1995; Caille *et al.*, 1999), and glutamate release was elevated in the LC during opiate withdrawal (Akaoka & Aston-Jones, 1991; Aghajanian *et al.*, 1994). Furthermore, glutamate receptor antagonism substantially blocked the withdrawal-induced enhancement of LC neuron firing rates (Akaoka & Aston-Jones, 1991). The latter observations are consistent with studies in other brain regions (Bonci & Williams, 1997; Chieng & Williams, 1998; Ingram *et al.*, 1998; Jolas *et al.*, 2000; Hack *et al.*, 2003; Bie *et al.*, 2005).

The relative contributions of intrinsic and extrinsic modifications to the LC resulting from chronic morphine administration remain unclear. Previously, we reported that the postsynaptic inhibitory effect of opioids (ME) was reduced in LC neurons from GIRK2/GIRK3$^{-/-}$ mice (Torrecilla *et al.*, 2002). Here, we examined the impact of chronic morphine administration on ME-induced current in LC neurons from wild-type and GIRK2/GIRK3$^{-/-}$ mice. We reasoned that adaptations to other opioid-modulated currents would be better resolved in the absence of the GIRK conductance. Our findings indicate that chronic morphine treatment does not alter

the opioid-induced postsynaptic inhibition of LC neurons, but rather leads to a significant increase in excitatory input to the LC.

III. MATERIALS AND METHODS

Experimental subjects: All experiments were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University and conducted according to guidelines established by the National Institutes of Health governing the use of animals in research. GIRK2/GIRK3$^{-/-}$ mice were generated as described previously (Torrecilla *et al.*, 2002). Wild type and GIRK2/GIRK3$^{-/-}$ male and female mice (3-8 weeks) were implanted with morphine pellets (25 mg morphine base, obtained from NIDA) under isoflurane anesthesia on Days 1 (1 pellet) and 3 (1-2 pellets). This protocol has been used extensively to induce tolerance and dependence in rats and mice (Christie *et al.*, 1986; Kolesnikov *et al.*, 1993; Shoji *et al.*, 1999; Georges *et al.*, 2006; Mouledous *et al.*, 2007). At the time of pellet implantation, each subject was given an intraperitoneal injection of lactated saline to prevent dehydration; we have observed that this approach leads to improved health of the animals during the study. All animals used in this study exhibited clear signs of opiate exposure following pellet implantation, including Straub tail and elevated motor activity. A second group of mice was implanted with placebo pellets (morphine-free pellets) following the same schedule. Data from the placebo group were indistinguishable from data from untreated mice. Data were pooled from

these control groups, and are referred to throughout as deriving from untreated or control mice.

Electrophysiology: Electrophysiological studies were performed on days 5-6 after pellet implantation. Mice were anesthetized with halothane and their brains were removed rapidly. Coronal and horizontal sections (240 μm) were used for whole-cell voltage-clamp experiments. Voltage-clamp experiments were performed at 34˚C using solutions and conditions described previously (Torrecilla *et al.*, 2002). Intracellular recordings were performed as described (Williams et al., 1982). In brief, slices (280 μm) were cut in the horizontal plane, and electrodes (50-60 MΩ) were filled with KCl (2 M). [Met]^{5}enkephalin (ME), UK-14304, forskolin, and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) were purchased from Sigma (St. Louis, MO, USA) were applied by superfusion. Forskolin was applied to slices at both 10 and 30 mM; no significant differences were seen with respect to electrophysiological outcomes with these two concentrations. Slices from both control and morphine-treated mice were incubated in morphine-free solution for at least 1 hr prior to recordings. Whole-cell currents, synaptic events, and membrane potentials were acquired with an Axopatch-1D amplifier (Axon Instruments, Inc.; Foster City, CA, USA). Data were low-pass filtered at 5 kHz, digitized with an ITC-16 Computer Interface (Instrutech Corporation; Long Island, NY, USA), and sampled at 10 kHz with Axograph 4.9 (Axon Instruments, Inc.). Whole-cell currents and spontaneous EPSCs were measured in voltage-clamp mode with the membrane potential (V_{hold}) held at -60 mV. Current-voltage relationships

were obtained by stepping the membrane potential from –50 to –130 mV in –10 mV increments (100 ms/step). EPSC frequencies were determined from 30 s of continuous recordings made in presence of picrotoxin (100 μM) to isolate excitatory activity. Frequency information was extracted using a template function (Axograph X; Berkeley, CA, USA), with deflections < 7 pA excluded from analysis.

Data analysis: Data analysis was performed with PRISM software (GraphPad Software, San Diego, CA). All results are presented as the mean ± SEM. EC_{50} values for agonist-induced hyperpolarization of LC neurons were determined by fitting concentration-response curves with a logistic function using KaleidaGraph (Synergy Software, Reading PA). Statistical significance was assessed using analysis of variance (two-way ANOVA) for multiple comparisons and Student's *t* test for pair-wise comparisons of the action of forskolin. Spontaneous EPSC frequency and amplitude under each condition were pooled and plotted as cumulative histograms, and analyzed with the Kolmogorov-Smirnov test. The level of significance was considered as $p<0.05$.

IV. RESULTS

Previously, we reported that the ME-induced current in LC neurons from GIRK2/GIRK3$^{-/-}$ mice was significantly smaller than that seen in wild-type controls (Torrecilla *et al.*, 2002). The small residual current in slices from GIRK2/GIRK3$^{-/-}$ mice was partially blocked by barium leaving a residual current of ~15 pA that was

insensitive to membrane potential. This residual current may be the result of the inhibition of a cAMP-dependent inward current reported previously in rat LC neurons (Alreja & Aghajanian, 1993). This possibility was tested by measuring whole-cell currents induced by ME in LC neurons from wild-type and GIRK2/GIRK3$^{-/-}$ mice prior to and 10 min after the application of forskolin, which stimulates adenylyl cyclase in a receptor-independent manner (Osborne & Williams, 1996).

ME induced an outward current in LC neurons from wild-type mice (61±7 pA at -60 mV, n=15; Fig. 1A,B). Subsequent application of forskolin produced a small inward current and an accompanying increase in excitatory synaptic input (described in detail below). In the presence of forskolin, the current induced by ME in LC neurons from wild-type mice was significantly enhanced (74±8 pA, n=15, $p<0.001$). The ME-induced outward current was smaller in LC neurons from GIRK2/GIRK3$^{-/-}$ mice (14±2 pA at -60 mV, n=21; $p<0.001$). Similar to experiments involving wild-type animals, forskolin increased the ME-induced current by 10-15 pA in LC neurons from GIRK2/GIRK3$^{-/-}$ mice (Fig. 1A,B). Thus, there was no evidence of a significant genotype-dependent difference in cAMP-dependent signaling in LC neurons.

We next asked whether chronic morphine treatment caused an adaptation in the opioid-induced current. Following treatment of wild-type mice, the amplitude of the ME-induced current (55±6 pA at –60 mV, n=11) and the augmentation of the ME-induced current by forskolin were indistinguishable from those recorded in slices from untreated wild-type mice (Fig. 1B). Similarly, in slices taken from morphine-

treated GIRK2/GIRK3$^{-/-}$ mice, the ME-induced current (18±2 pA at -60 mV, n=18) was not significantly different from that measured in LC neurons from untreated GIRK2/GIRK3$^{-/-}$ mice. The ME-induced current, however, was not significantly enhanced by forskolin in slices from morphine-treated GIRK2/GIRK3$^{-/-}$ mice (Fig. 1B).

The current/voltage relationships in the absence and presence of forskolin were measured to determine the effect of forskolin on the reversal potential of the ME-induced current and to determine the voltage dependence of the current induced by forskolin (Fig 2). In slices from untreated wild-type mice, forskolin produced an increase in the slope of the ME-induced current with no change in reversal potential (Fig. 2A). A similar result was obtained in slices taken from animals that were treated with morphine (Fig. 2B). The current induced by forskolin was inward at all potentials and was not different between slices from untreated and morphine-treated animals (Fig. 2A,B). In slices from untreated and morphine-treated GIRK2/GIRK3$^{-/-}$ mice, the amplitude of the ME-induced current was not sensitive to membrane potential (Fig. 2C,D). Forskolin evoked a voltage-independent increase in the outward ME current in LC neurons from untreated GIRK2/GIRK3$^{-/-}$ mice (Fig. 2C), but had no effect on the ME-induced current in slices from morphine-treated GIRK2/GIRK3$^{-/-}$ mice (Fig. 2D). The observation that forskolin had no apparent effect on the ME-induced current suggests that there was no dramatic up-regulation in the cAMP signaling pathway following chronic morphine treatment in GIRK2/GIRK3$^{-/-}$ mice.

Though pharmacologic manipulations suggested that recording conditions were suitable for measuring the influence of cAMP-dependent signaling on the ME-induced current in mouse LC neurons, the dialysis of the intracellular milieu during whole-cell recording experiments may adversely impact cAMP-dependent processes. Indeed, spontaneous LC neuron firing has been observed to run down under standard whole-cell recording conditions (Alreja & Aghajanian, 1995). To address this concern, we next used an intracellular recording technique to measure the hyperpolarization induced by ME in LC neurons from untreated and morphine-treated mice.

To measure the ME-induced hyperpolarization of LC neurons, holding current (0-200 pA) was applied to inhibit spontaneous firing and to hold the membrane potential near -60 mV. Under these conditions, ME induced a dose-dependent hyperpolarization of LC neurons in slices from untreated wild-type mice, with a maximal response measured at 30 mM (29±1 mV) and an EC_{50} of 0.64±0.09 μM (n=14) (Fig. 3). Consistent with the voltage-clamp data, the ME-induced hyperpolarization was significantly blunted in LC neurons from GIRK2/GIRK3$^{-/-}$ mice (13.3±1.2 mV at 30 mM ME, n=9; Fig. 3A,B). The EC_{50} for ME-induced hyperpolarization in slices from the GIRK2/GIRK3$^{-/-}$ mice (1.96±0.29 μM) was about 3-fold greater than that measured in slices from wild-type mice, arguing that the residual conductance mediating this hyperpolarization in LC neurons from GIRK2/GIRK3$^{-/-}$ mice is slightly less sensitive to opioid receptor stimulation than the GIRK channel. After chronic treatment of wild-type mice with morphine, there was no change in the maximal ME-induced hyperpolarization (26±1 mV, n=7) or the EC_{50}

(0.59±0.13 μM; Fig. 3C). In GIRK2/GIRK3$^{-/-}$ mice treated chronically with morphine, there was also no significant change in the maximal ME-induced hyperpolarization (14± 1 mV, n=9) or the EC_{50} (3.29±0.43 μM).

The a_2-adrenoceptor agonist UK-14304 (3 μM) was applied at the end of each experiment to probe for opioid receptor-specific or effector-specific changes induced by chronic morphine exposure. The hyperpolarization induced by UK-14304 (3 μM) was significantly smaller in LC neurons from untreated GIRK2/GIRK3$^{-/-}$ mice (12.3±1.4 mV, n=7; $p<0.001$) as compared to wild-type controls (25.4±1.0 mV, n=19; Fig. 3A,B). Furthermore, there was no change in the hyperpolarization induced by UK-14303 following chronic morphine treatment for either genotype (Fig. 3B).

Consistent with findings from the voltage-clamp study, forskolin increased the amplitude of the ME-induced hyperpolarization (data not shown). For these experiments, a sub-saturating concentration of ME (1 μM) was used in experiments involving slices from wild-type mice such that the amplitude of the hyperpolarization would not be limited by the approach of the membrane potential toward the potassium equilibrium potential. The ME test concentration was increased to (10 μM) in experiments involving LC neurons from GIRK2/GIRK3$^{-/-}$ mice since the hyperpolarization was not dependent on the change in voltage and because the amplitude of the hyperpolarization induced by ME (1 μM) was very small. In slices from untreated wild-type mice, the hyperpolarization induced by ME increased from 19.1±2.0 mV in control to 21.7±2.2 mV after treatment with forskolin (n=17, $p<0.01$). In slices from GIRK2/GIRK3$^{-/-}$ mice, the hyperpolarization

induced by ME was 10.0±1.4 mV in control, and this increased to 12.2±1.7 mV in the presence of forskolin (n=5, $p<0.05$). Following chronic morphine treatment, forskolin increased the ME-induced hyperpolarization in LC neurons from both wild-type (13.7±2.0 mV vs. 15.3±2.2 mV, n=6; $p<0.01$) and GIRK2/GIRK3$^{-/-}$ (9.7±1.4 mV vs. 12.1±1.8 mV, n=9; $p<0.01$) mice. The forskolin-induced augmentation of the ME-induced hyperpolarization seen in LC neurons from morphine-treated GIRK2/GIRK3$^{-/-}$ mice was somewhat unexpected given the lack of forskolin effect on residual ME-induced current in LC neurons from these mice. Though there is no clear-cut explanation for the difference in results, there are several possibilities given the technical differences in the experiments. Alternatively, changes in membrane potential may constitute a more sensitive measure of opioid actions in LC neurons.

Though the subject of some debate, enhanced excitatory transmission has been implicated in the elevated excitability of rat LC neurons seen during opiate withdrawal (Akaoka & Aston-Jones, 1991; Aghajanian *et al.*, 1994; Ivanov & Aston-Jones, 2001). To determine whether such modifications occur in the mouse, spontaneous EPSCs were measured in LC neurons from wild-type and GIRK2/GIRK3$^{-/-}$ mice at baseline and following chronic morphine treatment. Mouse LC neurons exhibited spontaneous EPSCs that were abolished by the glutamate receptor antagonist NBQX (5 μM, data not shown). The frequency, but not amplitude, of spontaneous EPSCs was elevated in slices from morphine-treated wild-type mice (Fig. 4). There was a similar elevation in the frequency of spontaneous EPSCs measured in slices from morphine-treated GIRK2/GIRK3$^{-/-}$ mice

(Fig. 5). Thus, an increase in excitatory input to LC neurons was evident in slices from both wild-type and GIRK2/GIRK3$^{-/-}$ mice during withdrawal from chronic morphine treatment.

The mean frequency and amplitude of spontaneous EPSCs measured in LC neurons were indistinguishable between untreated wild-type (10.6±1.8 Hz & 22.8±1.6 pA, n=12) and GIRK2/GIRK3$^{-/-}$ (12.3±1.5 Hz & 17.7±1.1 pA, n=20 unpaired t-test $p>0.05$) mice, suggesting that constitutive ablation of GIRK2 and GIRK3 did not elevate excitatory input to the LC (Fig. 6, Fig. S1). Forskolin (10 μM) increased the EPSC frequency in slices from both untreated wild-type (19.1±1.6 Hz, n=12; $p<0.01$) and GIRK2/GIRK3$^{-/-}$ (17.9±2.8 Hz, n=13; $p<0.01$) mice (Fig. 6B). Forskolin had no effect on the amplitude of spontaneous EPSCs in slices from morphine-treated animals of either genotype (Fig. S1). These observations support the contention that significant genotype-dependent differences in cAMP-dependent signaling contributing to excitatory neurotransmission were not evident at baseline. Following chronic morphine treatment, forskolin increased the frequency of spontaneous EPSCs in slices from wild-type animals, but had no significant effect in slices from GIRK2/GIRK3$^{-/-}$ mice (Fig 6B, Fig. S1).

V. DISCUSSION

While a consensus has emerged that adaptations in cAMP-signaling underlie the cellular and behavioral consequences of chronic morphine administration, many questions remain. This study began by asking whether chronic morphine treatment resulted in altered opioid-induced postsynaptic currents in the mouse LC. Among

the ion channels involved in the inhibitory effect of opioids on LC neurons, GIRK channels appear to mediate most of the postsynaptic inhibition (Torrecilla *et al.*, 2002). Nevertheless, opioids also inhibit voltage-gated calcium channels in LC neurons, and both barium-sensitive and insensitive components of the residual opioid-induced current are discernable in LC neurons from GIRK2/GIRK3$^{-/-}$ mice (Torrecilla *et al.*, 2002). The present work shows that exogenous activation of adenylyl cyclase can also impact the postsynaptic opioid-induced current in LC neurons. One key finding in this study is that neither the postsynaptic inhibitory effect of ME, nor the forskolin-induced augmentation of the ME-induced inhibitory current, was significantly impacted by chronic morphine treatment. Thus, alterations occurring in the LC as a consequence of prolonged morphine exposure do not appear to significantly influence the signaling pathways and channels mediating the postsynaptic inhibitory effect of opioids.

Parallel studies with mice lacking functional GIRK channels permitted the direct evaluation of modifications to less-pronounced components of the opioid-induced current that resulted from chronic morphine treatment. Though there was a slight discrepancy in the forskolin-induced augmentation of opioid inhibition measured in LC neurons from GIRK2/GIRK3$^{-/-}$ mice using voltage-clamp and intracellular recording techniques, both approaches indicated that any postsynaptic modification involving the cAMP-system following chronic morphine treatment is either minimal or uncoupled from the opioid-induced postsynaptic inhibition. Recent microarray studies have identified many genes exhibiting altered regulation in the rodent LC following chronic morphine treatment, including several directly involved in

synaptic transmission, such as the glutamate receptor subunit GluR1 (McClung *et al.*, 2005). Given the apparent lack of effect of chronic morphine treatment on postsynaptic opioid-dependent signaling, altered regulation of these targets is more likely to explain any change in the intrinsic excitability of LC neurons linked to chronic morphine exposure and precipitated withdrawal.

As the GIRK knockout mice used in this study were both global and constitutive in nature, it is important to consider that findings made with these mice may reflect a combination of developmental alterations or mechanisms designed to compensate for the loss of the gene(s) in question. Indeed, mice lacking GIRK2 exhibit blunted postsynaptic inhibition throughout the central nervous system (Luscher *et al.*, 1997; Slesinger *et al.*, 1997; Cruz *et al.*, 2004; Koyrakh *et al.*, 2005; Marker *et al.*, 2006; Labouebe *et al.*, 2007), and display a wide variety of behavioral phenotypes (Blednov *et al.*, 2001; Blednov *et al.*, 2002; Blednov *et al.*, 2003; Morgan *et al.*, 2003; Marker *et al.*, 2004; Costa *et al.*, 2005; Marker *et al.*, 2005). Nevertheless, the lesion in LC neurons from GIRK2/GIRK3$^{-/-}$ mice appears relatively selective for GIRK channels. For example, previous work showed that ME-induced suppression of voltage-gated calcium channels is preserved in LC neurons from GIRK2/GIRK3$^{-/-}$ mice (Torrecilla *et al.*, 2002). The forskolin-dependent augmentation of the opioid-induced current is normal in LC neurons from untreated GIRK2/GIRK3$^{-/-}$ mice. Furthermore, baseline excitatory input and the augmentation of this input by forskolin are normal in slices taken from GIRK2/GIRK3$^{-/-}$ mice. Thus, the most peculiar findings in this study were the lack of an action of forskolin on postsynaptic opioid-induced current in, and excitatory input to, LC neurons from morphine-

treated GIRK2/GIRK3$^{-/-}$ mice. While the different actions of forskolin found in untreated and morphine-treated GIRK2/GIRK3$^{-/-}$ mice suggest that an alteration occurred in the cAMP-system, one might have reasonably predicted that forskolin would have larger actions after chronic morphine treatment. It is possible, however, that the tone of the cAMP-system is elevated in LC neurons from morphine-treated GIRK2/GIRK3$^{-/-}$ mice. In this case, the stimulatory effect of forskolin endpoints would be muted.

There has been some debate as to whether the elevated firing rate of LC neurons noted during opiate withdrawal reflects intrinsic modifications or alterations in afferent input (*e.g.*, Ivanov, 2001 #2044}). Support for intrinsic modifications has come from multiple approaches, including the failure of various inhibitors of synaptic transmission to normalize the elevated LC neuron firing rates during withdrawal (Ivanov & Aston-Jones, 2001), and manipulation of CREB levels and activity in individual LC neurons. Indeed, LC neurons expressing a constitutively-active version of CREB show elevated firing rates, while LC neurons injected with antisense oligonucleotides targeting CREB or expressing dominant-negative forms of CREB show decreased firing rates (Lane-Ladd *et al.*, 1997; Han *et al.*, 2006). On the other side of the debate, elevated glutamate levels have been measured in the rat LC during withdrawal (Aghajanian *et al.*, 1994), and glutamate receptor antagonists have been shown to reduce LC firing rates to pre-treatment levels (Akaoka & Aston-Jones, 1991).

The present work supports the idea that elevated excitatory transmission facilitates activity of LC neurons during withdrawal. Indeed, the most notable

modification resulting from chronic morphine treatment seen in this study was the increase in excitatory afferent input to the LC. The frequency of spontaneous EPSCs measured in LC neurons was elevated significantly following chronic morphine treatment in both wild-type and GIRK2/GIRK3$^{-/-}$ mice. This enhanced excitatory input seen during withdrawal is predicted to contribute to elevated LC neuron firing rates, particularly *in vivo* where all afferent connections are intact. It is possible, however, that an increase in inhibitory input mitigates the elevated excitatory input to the LC following chronic morphine administration. Future studies will explore this possibility and examine the net impact of chronic morphine treatment on the *in vivo* firing rates of LC neurons from wild-type and GIRK2/GIRK3$^{-/-}$ mice.

In summary, chronic morphine treatment did not significantly influence the composite postsynaptic conductance or net inhibitory effect of opioids on LC neurons. Instead, enhanced excitatory transmission was the primary consequence of chronic morphine exposure. As such, these data support the contention that extrinsic adaptations induced by chronic morphine treatment play a significant role in the elevated excitability of LC neurons observed during opiate withdrawal.

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Abbreviations. cAMP, cyclic adenosine-5'-monophosphate; CREB, cAMP-response element binding protein; EPSC, excitatory postsynaptic current; GIRK, G-protein-gated inwardly rectifying K^+ channel; LC, locus coeruleus; ME, $[Met]^5$-enkephalin; MOR, mu opioid receptor; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione.

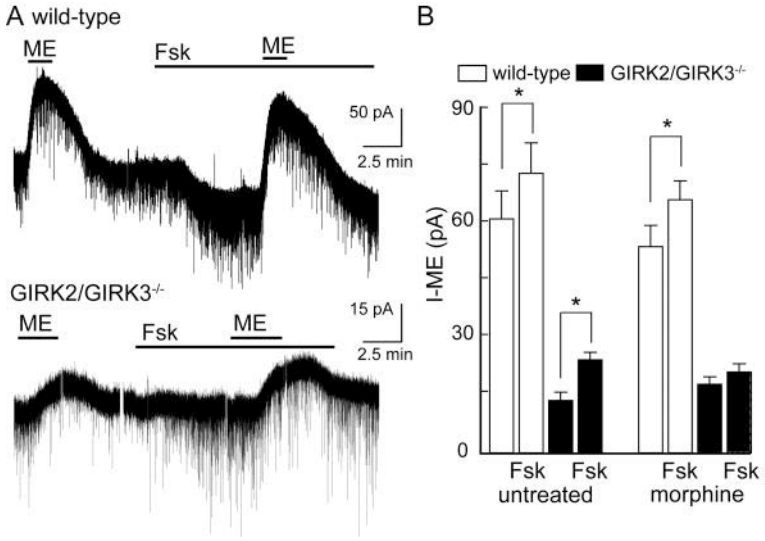


Figure 1. Opioid-induced outward currents in LC neurons from wild type and GIRK2/GIRK3$^{-/-}$ mice. A) Current traces showing the outward current induced by ME (30 μM) in the absence and presence of forskolin (Fsk, 30 μM) in slices from wild-type mouse (top trace) and a GIRK2/GIRK3$^{-/-}$ mouse (bottom trace). The holding potential (V_{hold}) was -60 mV. Forskolin (Fsk) induced a small inward current and increased spontaneous excitatory input. **B)** Summary showing the amplitude of the current induced by ME (30 μM) in slices from wild-type (white bars) and GIRK2/GIRK3$^{-/-}$ (black bars) mice, and the impact of chronic morphine treatment and Fsk on the ME-induced current (V_{hold} = -60 mV). In the interest of clarity, only within-genotype differences are noted on the plot; genotype-dependent differences were clearly evident and noted in the text. Symbols: * $p<0.05$ vs. Fsk, within genotype.

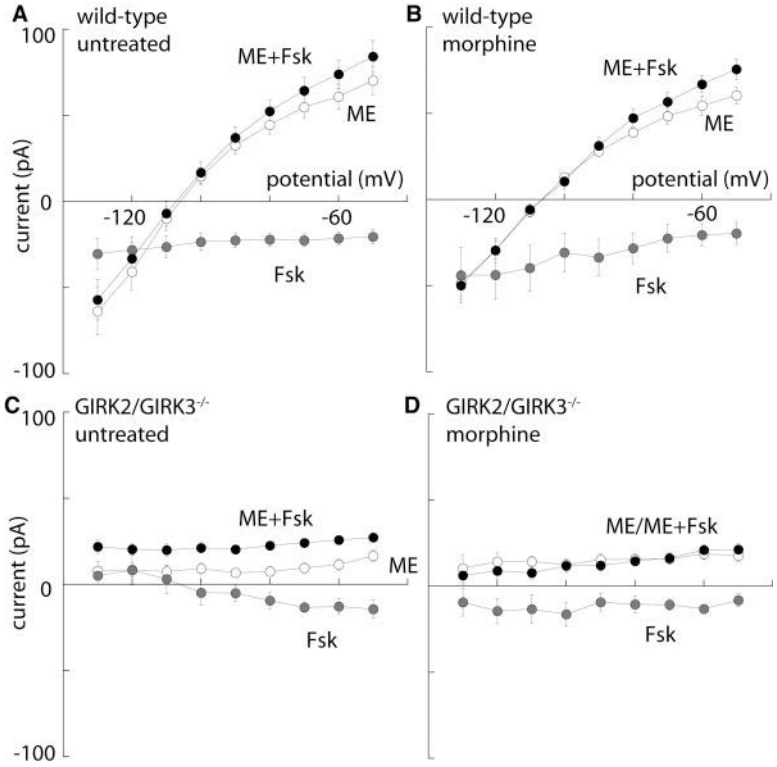


Figure 2. Current-voltage relationships in LC neurons from untreated and morphine-treated mice. The voltage dependence of the whole-cell currents measured following application of ME (30 mM; white circles), Fsk alone (gray circles), or ME in the presence of Fsk (ME+Fsk; black circles) are plotted for LC neurons from untreated and morphine-treated wild-type (**A,B**) and GIRK2/GIRK3$^{-/-}$ (**C,D**) mice. While the small Fsk-induced current measured at V_{hold} = -60 mV was similar across the four groups tested, some differences in the I-V profiles were evident (*e.g.*, compare C and D). Also note that the I-V plots for the ME-induced current measured in the absence or presence of Fsk were largely overlapping in panel D, reflecting the lack of effect of Fsk on ME-induced current in LC neurons from morphine-treated GIRK2/GIRK3$^{-/-}$ mice.

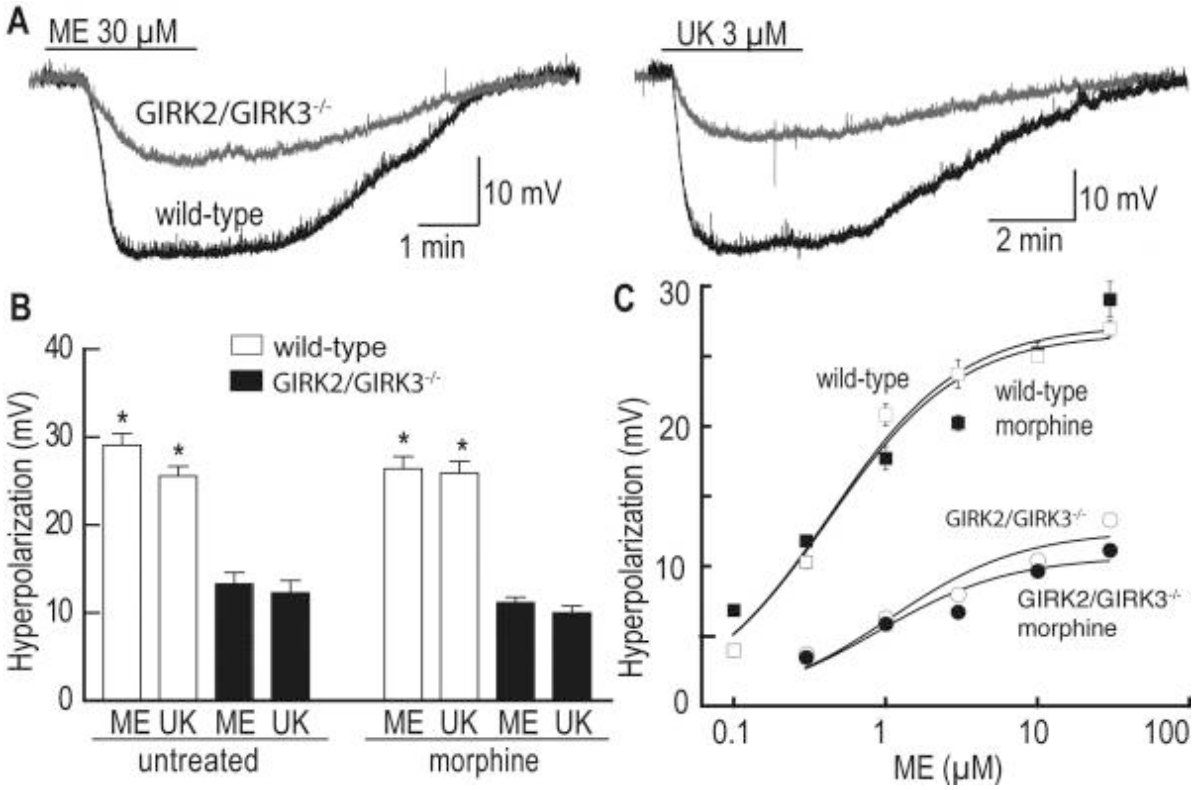


Figure 3. ME-induced hyperpolarization in LC neurons from untreated and morphine-treated mice. A) The superimposed traces on the left show the hyperpolarization induced by 30 µM ME in LC neurons from a wild-type (black trace) and GIRK2/GIRK3$^{-/-}$ mouse (grey trace). The superimposed traces on the right show the hyperpolarization induced by 3 µM UK-14304 in LC neurons from wild-type (black trace) and GIRK2/GIRK3$^{-/-}$ (grey trace) mice. **B)** Summary plot showing the hyperpolarization induced by ME (30 µM) and UK-14304 (UK, 3 µM) in LC neurons from untreated and morphine-treated wild-type (white bar) and GIRK2/GIRK3$^{-/-}$ (black bar) mice. **C)** Concentration-response curves describing the hyperpolarization induced by ME in LC neurons from untreated (white symbols) and morphine-treated (black symbols) wild-type and GIRK2/GIRK3$^{-/-}$ mice. Symbols: * $p<0.05$ vs. GIRK2/GIRK3$^{-/-}$ mice.

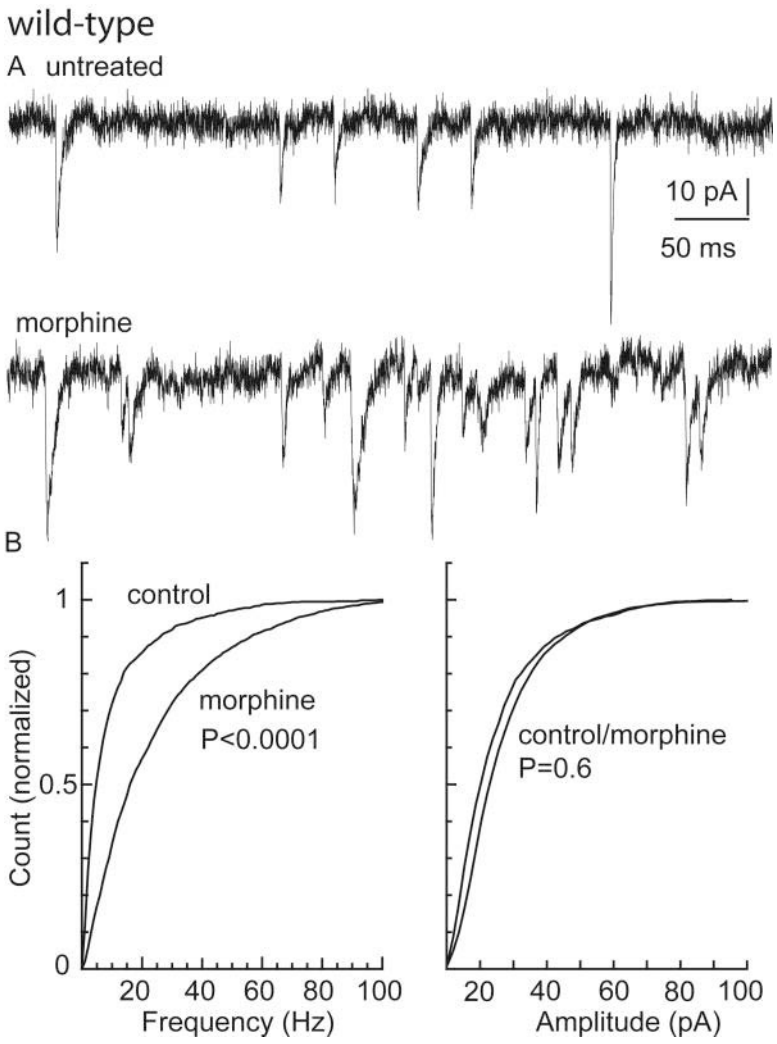


Figure 4. Excitatory input to LC neurons in untreated and morphine-treated wild-type mice. A) Representative current traces showing spontaneous EPSCs in slices from untreated (top) and morphine-treated (bottom) wild-type mice (V_{hold} = -60 mV). **B)** Cumulative histograms of frequency (left panel, >2000 EPSCs; 13 slices from 7 animals) and amplitude (right panel, >2000 EPSCs; 15 slices from 7 animals) showing that the frequency of spontaneous EPSCs is increased in slices from morphine-treated animals, while there was no significant change in the amplitude distribution.

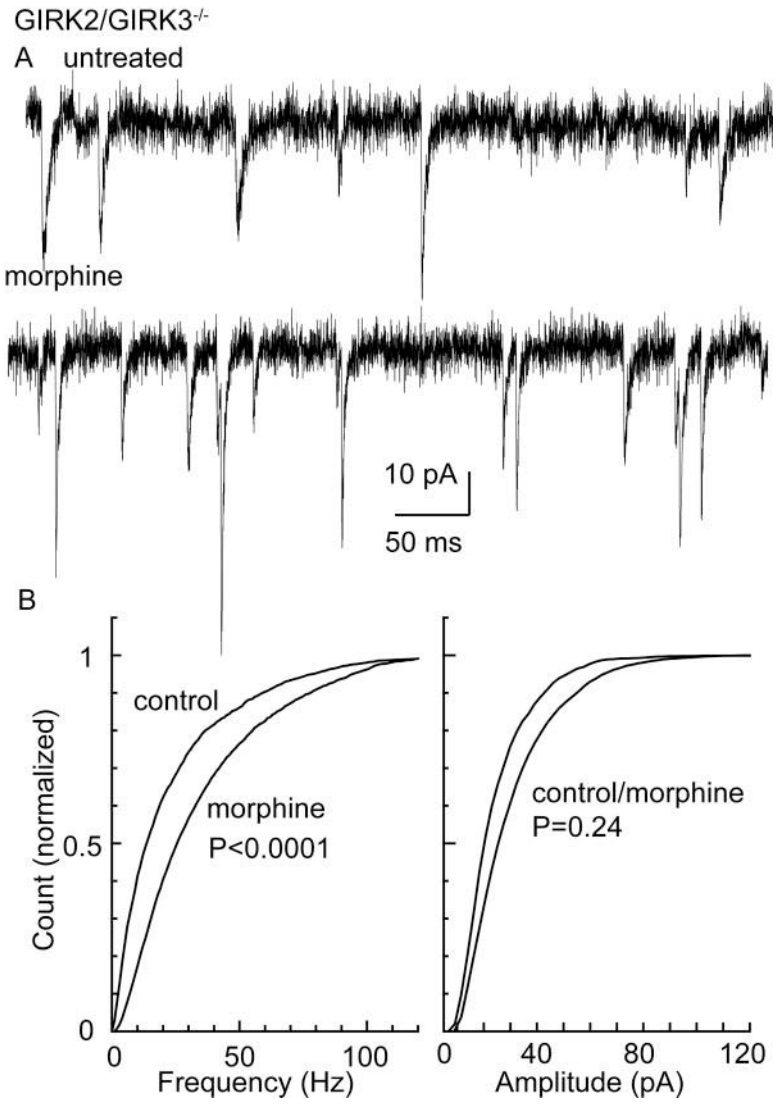


Figure 5. Excitatory input to LC neurons in untreated and morphine-treated GIRK2/GIRK3$^{-/-}$ mice. A) Representative current traces showing spontaneous EPSCs in slices from untreated (top) and morphine-treated (bottom) GIRK2/GIRK3$^{-/-}$ mice (V_{hold} = -60 mV). **B)** Cumulative histograms of frequency (left panel, >2000 EPSCs; 20 slices from 8 animals) and amplitude (right panel, >2000 EPSCs; 18 slices from 8 animals) showing that the frequency of spontaneous EPSCs is increased in

slices from morphine-treated animals, while there was no significant change in the amplitude distribution.

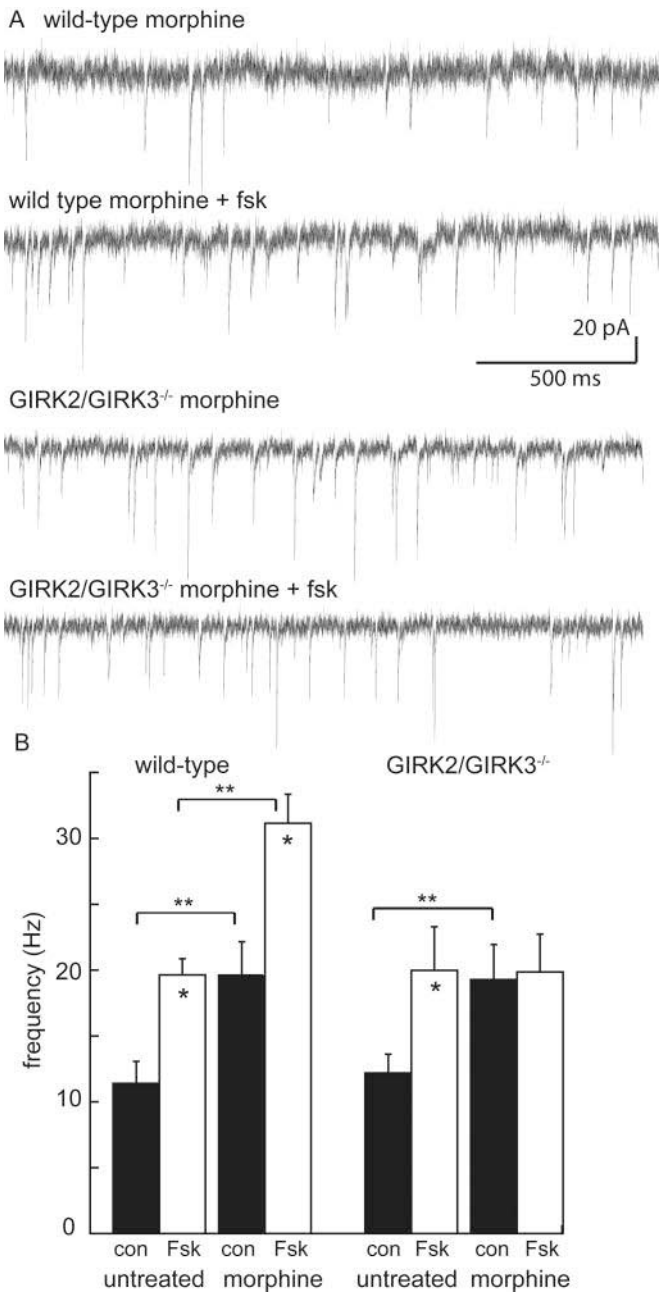
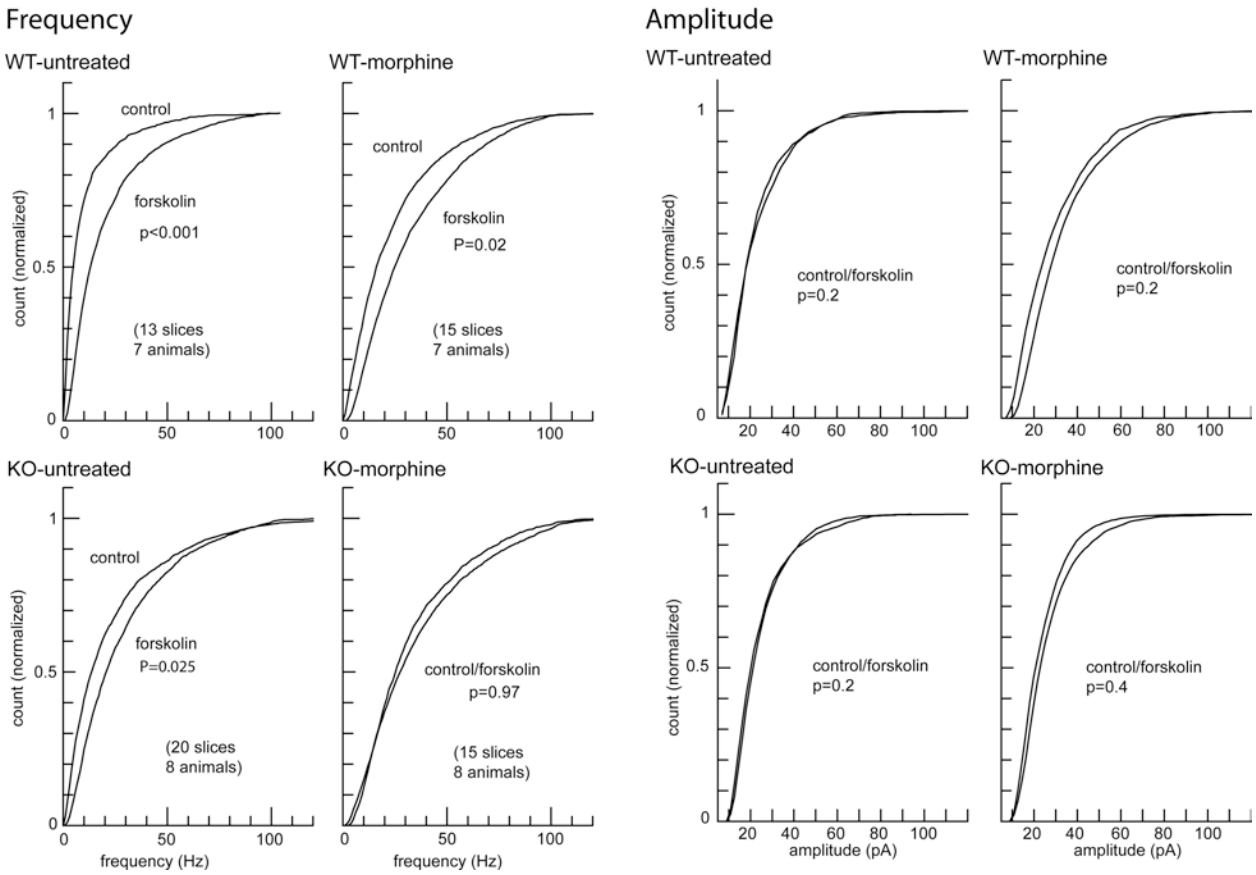


Figure 6. Forskolin increases the frequency of spontaneous EPSCs. A)

Representative traces from morphine-treated wild-type and GIRK2/GIRK3$^{-/-}$ mice

(V_{hold} = -60 mV). The traces show the increase in EPSC frequency induced by Fsk in the slice from a morphine-treated wild-type mouse (top two traces) and the lack of action of Fsk in a slice taken from a morphine-treated GIRK2/GIRK3$^{-/-}$ mouse (lower two traces). **B)** Summary plot of the frequencies (in Hz) of spontaneous EPSCs measured in LC neurons from untreated and morphine-treated wild-type (left panel) and GIRK2/GIRK3$^{-/-}$ (right panel) mice, in the absence (black rectangle, con) or presence (white rectangle) of Fsk (10 mM). Symbols: * $p<0.05$ vs. con group, within genotype and morphine-treatment groups; ** $p<0.01$ vs. untreated group, within Fsk treatment group.



Supplemental Figure (Fig. S1). Cumulative histograms illustrating the impact of forskolin on spontaneous EPSCs in slices from wild type and GIRK2/GIRK3$^{-/-}$ mice. Fsk increased the frequency of EPSCs in all groups except for morphine-treated GIRK2/GIRK3$^{-/-}$ mice. Forskolin had no significant effect on the amplitude of EPSCs for any group.

CHAPTER 5

CONCLUDING REMARKS

I. Summary of Results

The primary findings of this work support agonist-specific regulation of MOR following both acute and chronic agonist administration that results in varying degrees of desensitization, internalization and cellular tolerance. Based on their acute actions, opioid agonists were separated into 3 groups. Group I are those that caused robust desensitization and internalization and include ME, methadone and etorphine. Group II included morphine and oxymorphone, which caused some desensitization without internalization. Oxycodone, which caused no desensitization or internalization, was grouped separately. Morphine and methadone were selected for comparison in chronic treatment studies because of their frequent use clinically and unique profiles for causing acute desensitization and internalization of MOR. While some aspects of cellular tolerance in the LC were not different between the two drugs (reduced ME sensitivity and enhanced desensitization) treatment with morphine, but not methadone, resulted in reduced recovery from desensitization and recycling. This work also examined the role of GRK2 and ß-arr2 in acute and chronic MOR regulation. While acute desensitization was not changed in slices that did not have functional GRK2 or ß-arr2, morphine-induced inhibition of MOR recycling and recovery from desensitization were dependent on these molecules. Finally, morphine treatment also resulted in

enhanced pre-synaptic excitatory transmission onto LC neurons, which likely accounts for the increased excitability that is observed during withdrawal. An important aspect of MOR signaling is the cycle of activation, desensitization, internalization and subsequent recycling and recovery from desensitization. These processes should be thought of as separate effectors of MOR that can be engaged differentially by agonists and altered individually by chronic agonist treatment.

II. From rat to mouse

Opioid actions in the LC from rat have been extensively characterized. Here, mouse LC was also used to take advantage of transgenic and knock-out mice. ME-induced activation of MOR in wild-type mice was comparable to that observed in the rat, with a slightly lower potency but similar efficacy of ME-mediated membrane hyperpolarizations. The main difference between mouse and rat was the amplitude of the outward currents measured using whole-cell recording. Maximum currents produced by GIRK activation in the rat are between 300 and 600 pA while those in the mouse are rarely above 100 pA (Torecilla *et al.*, 2002). However, these relatively small currents resulted in membrane hyperpolarizations as large as those observed in the rat, approximately 30 mV. Therefore, intracellular recording of membrane potential was used to construct ME concentration-response curves and measure desensitization in the mouse.

Assessment of desensitization and internalization induced by various agonists in brain slices was possible using a novel transgenic mouse. The tyrosine hydroxylase

promoter drove expression of Flag-MOR, and resulted in MOR expression approximately two-fold higher than observed in wild-type mice. This mouse line was useful for cellular studies in the LC, particularly imaging experiments, because it allowed for immunohistochemical detection of Flag-MOR selectively expressed in LC neurons without expression in neighboring nuclei. However, since expression of Flag-MOR was limited to catecholamine neurons these mice cannot be used to examine trafficking in other brain regions or for *in vivo* studies of MOR regulation. To address these issues, future studies may employ a knock-in mouse model, which should result in expression in neurons that normally express MOR. This would also allow for examination of agonist-induced internalization in various regions of the brain, including those involved antinociception, such as periaquaductal gray and spinal cord. This approach has been used with a GFP-tagged delta-opioid receptor, allowing for correlation of trafficking and *in vivo* behaviors (Scherrer et al., 2006; Pradhan et al., 2009). It would be of interest to compare changes in MOR trafficking observed in the LC with other neuronal types or if there are cell-specific adaptations.

ß-arrestin2 knock-out mice and GRK2-as5 mice were used to assess the function of these molecules in acute desensitization and morphine-induced adaptations. The GRK2-as5 line was generated on a GRK2 knockout background so that only the modified GRK2 was expressed. Loss of GRK2 normally results in embryonic death and expression of GRK2-as5 rescues this phenotype. ß-arr2 KO mice were crossed

with Flag-MOR mice so that trafficking could be examined. This unfortunately could not be accomplished with GRK2-as5 mice, as the breeding scheme was quite complicated. It remains to be determined if GRK2 dependent phosphorylation alters MOR trafficking in the LC in response to ME or other opioid agonists.

III. Acute activation, desensitization and internalization in naïve animals

Agonist-specific regulation has been demonstrated for many GPCRs, including MOR (Kelley et al., 2008; Gilchrist, 2007). Here, we were able examine multiple aspects of MOR signaling under similar conditions in neurons. This work expands on trafficking studies in heterologous systems showing that agonists can activate the receptor without desensitizing or internalizing (Keith et al., 1996; Whistler and von Zastrow 1998; Alvarez et al, 2002). It remains unclear whether the differences in agonist-induced desensitization and internalization involve recruitment of different regulatory elements to the receptor. Results with morphine and oxymorphone demonstrate that desensitization mechanisms can be engaged without causing internalization of MOR. This confirms previous reports that desensitization and internalization are separable processes (Arttamangkul et al., 2006). It remains unclear still if internalization plays any role in the acute regulation of MOR signaling. It may only be important when there are longer exposures to agonist (hours-days) or for other cellular signaling, such as MAPK activation or gene regulation.

GRK2 and ß-arr2 function is required for desensitization and trafficking in heterologous systems, but this is not the case in LC neurons. This may be due to

molecular redundancy. GRK3 is expressed at the highest levels in the LC, followed by GRK2, 5 and 6 (Erdtmann-Vourliotis et al., 2001). The engineered GRK2 used in our studies is a useful tool because of the specificity it allows, but it would also be useful if an inhibitor was available that acted on all GRKs expressed in the LC. One potential way to inhibit phosphorylation of MOR is with a high concentration of staurosporine, resulting nonspecific inhibition of multiple kinases, including PKA, PKC and GRKs. Unpublished results from our lab show that desensitization occurs normally in slices incubated in staurosporin (10 μM), but trafficking of receptors is altered. Interestingly, recovery from desensitization is accelerated in the presence of staurosporine, suggesting phosphorylation stabilizes the desensitized state of the receptor and slows recovery.

Results from knockout mice suggest that GRK isoforms are differentially recruited to agonist-bound receptors. GRK3 knockout mice have reduced analgesic tolerance to acute application of fentanyl, however morphine tolerance was not affected (Kenski et al., 2005). There is no change in morphine antinociception or analgesic tolerance in GRK6 KO mice but there is enhanced locomotion, sensitization and reduced constipation (Raehal et al, 2009). Thus, there is agonist or cell-type specific recruitment of the different GRKs which may produce a unique pattern of phosphorylation that will engage specific pathways. Results using GRK2as5 mice suggest specificity of GRK2 in regulating ME-induced signaling following chronic morphine treatment. Further studies in GRK2-as5 mice may provide more

information about the specificity of different agonists for this GRK isoform. The profiles for desensitization and internalization observed in Flag-MOR mice may be attributable to specific GRK's and therefore a more robust effect of GRK2 inhibition may occur with an agonist other than ME.

Similar to GRK-as5 mice, there were no differences in desensitization or internalization in ß-arr2 knock-out mice. LC neurons express ß-arr1 which may compensate for the loss of the other isoform. Knock-out of both ß-arrestin isoforms is embryonic lethal, therefore it is difficult to test this hypothesis. Mouse embryonic fibroblasts lacking ß-arr1 and ß-arr2 have been used to clarify arrestin's role in the desensitization and trafficking of GPCRs (Kohout et al., 2001). DAMGO-induced desensitization was absent in this cell line while morphine-induced desensitization was unaffected (Chu et al., 2008). Thus there appears to be arrestin-dependent and arrestin-independent mechanisms for desensitization. Therefore, loss of GRK2 or ß-arr2 function may have no impact on MOR coupling because desensitization is independent of phosphorylation and arrestin binding. Experiments using staurosporine to inhibit kinase activity support the hypothesis that desensitization is independent of phosphorylation. Alternatively, agonist binding may induce a conformational state of the receptor that does not couple well to G proteins. Phosphorylation and arrestin binding may instead serve to stabilize the desensitized state and recruit other signaling pathways to the receptor.

IV. Acute activation, desensitization and internalization after chronic opioid treatment

Reduced recovery from desensitization after chronic morphine treatment was observed in rat and mouse. In contrast, complete recovery from desensitization was observed after chronic treatment with methadone. The main differences between these agonists are their profiles for desensitization and internalization. Thus it appears that reduced desensitization and lack of internalization during chronic morphine treatment results in more adaptations that prevent recovery from desensitization. This supports the hypothesis that agonist-induced internalization is protective in the development of tolerance. A more direct way to test this hypothesis in the LC would be to either block methadone-induced internalization during treatment, which should inhibit recycling and recovery from desensitization or to internalize receptors in response morphine, which should result in complete recovery from desensitization. Recently, a knock-in mouse that expresses a mutated MOR (RMOR) that internalizes and recycles in response to morphine was generated (Kim et al., 2008). Analgesic tolerance is not observed in these mice, nor is there an upregulation in cAMP/PKA pathways. Desensitization of RMOR in response to morphine was observed in GABAergic midbrain neurons (Madhavan et al., 2010). There have not yet been cellular studies of desensitization and recovery from desensitization after chronic treatment using these mice. If the changes in recovery from desensitization observed here are dependent on morphine-activated receptors remaining on the plasma membrane, one would expect complete recovery from desensitization in RMOR mice that are treated chronically with morphine.

Following chronic morphine treatment in the mouse, there was no change in ME potency or efficacy to induce membrane hyperpolarizations or in the amount of acute desensitization caused by ME. This is unlike what was observed in the rat, where the concentration-response for ME was shifted to the right and desensitization was enhanced following chronic morphine treatment. Differences in the amount of ME-induced desensitization are likely a result of recording configuration. When intracellular recording was used to measure desensitization in slices from rats, there was no difference in the amount of desensitization induced by a 10 minute application of ME between control and morphine-treated (Dang and Williams, 2004). It is unclear as to whether the lack of shift in the concentration-response is also due to the recording method or if it is a true species difference.

Relating the results of studies in the LC to analgesic tolerance is difficult because the role of LC in tolerance is unclear. If reduced recovery from desensitization is also observed in brain areas that are important for analgesia there may be more tolerance observed with morphine than with methadone because of this mechanism. This may be particularly evident in situations where a patient is receiving continuous morphine with a second opioid that causes desensitization, like fentanyl, for breakthrough pain. If recovery from desensitization is incomplete then morphine may be less efficacious after the second drug is administered. Based on the results presented here, I hypothesize that this would not occur with a patient receiving methadone to treat pain.

V. Recovery from desensitization and recycling inhibited by GRK2/ß-arr2 after chronic morphine treatment

Chronic morphine treatment did not prevent complete recovery from desensitization in slices lacking functional ß-arr2 and GRK2-as5. Thus, these molecules are critical in mediating the adaptations produced by chronic morphine treatment. However, no differences were observed in untreated mice suggesting that chronic morphine induces an up regulation in these pathways. Upregulation of ß-arr2 mRNA has been reported after chronic morphine treatment (Terwilliger et al., 1994) and GRK2 mRNA levels have been shown to decrease with chronic morphine treatment, but then increase during withdrawal (Fan et al., 2002). Because morphine washed out of brain slices prior to experiments, the slices are in a withdrawn state and may express more GRK2. It is also possible that chronic morphine treatment results in post-translational modifications of these proteins that alter their function.

Recycling of MOR is proposed to be a key step in the resensitization process. These studies suggest ß-arr2 inhibits the recycling, and thus the recovery from desensitization of MOR after chronic treatment. One potential mechanism is that chronic morphine treatment increases the stability of the interaction between MOR and ß-arr2, thus preventing dephosphorylation and recycling of the receptor. Alternatively, there are several endosomal-associating proteins that regulate GPCR recycling that may interact with ß-arrestin 2 to alter the intracellular trafficking of MOR to other sorting pathways.

VI. cAMP/PKA dependent increase in excitatory inputs to the LC

A GIRK conductance produces the majority of the hyperpolarization that is observed in response to MOR activation in the LC. The presence of a sodium channel that is inhibited by MOR activation through a cAMP-dependent mechanism has been reported. The rationale for using the GIRK 2/3 KO mice was that a relatively small conductance would be better resolved in the absence of GIRK channels. While there was a small hyperpolarization that remained in GIRK 2/3 KO mice, it did not appear to be modulated to a greater extent by forskolin than in WT mice. It cannot be ruled out that changes to the cAMP/PKA pathway do occur in LC neurons, but if so, these changes could not be detected using electrophysiology.

Excitatory transmission onto the LC was enhanced by chronic morphine treatment. This has been observed at multiple CNS synapses and is mediated by an upregulation in the cAMP/PKA pathway. It is unclear whether the adaptations that mediate changes in synaptic transmission occur with methadone treatment. Following chronic treatment with equianalgesic doses, upregulation of cAMP is not observed with methadone like it is with morphine (Kim et al., 2008). Therefore methadone treatment may not alter synaptic transmission.

Conclusions

The goal of this work was to elucidate the role of acute desensitization and trafficking of mu opioid receptors in mediating the decreased coupling to effector systems. Chronic morphine treatment produced more adaptations in the LC than methadone, perhaps because of differences in desensitization and internalization profiles. Morphine induced adaptations were mediated through GRK2 and ß-arr2, and demonstrate a novel role for these molecules in regulating MOR signaling. The reduction in recovery from desensitization and recycling likely represents just one of the many adaptations that occur in response to chronic morphine treatment that contribute to the development of tolerance to opioids.

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