AGONIST-SPECIFIC μ-OPIOID RECEPTOR REGULATION FOLLOWING ACUTE AND CHRONIC TREATMENT

by

Michael S. Virk

A DISSERTATION

Presented to the
Neuroscience Graduate Program
and the
Oregon Health & Science University School of Medicine
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

August 2007

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

MICHAEL S. VIRK

has been approved

[redacted]

Jonh T. Williams

[redacted]

Craig W. Jahr

[redacted]

James Maylie

[redacted]

David L. Farrens

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I am proud and fortunate to be able to write this page. The last four years were rich in opportunity and it was a privilege to be fully immersed in it all. Many people helped me get here, guided me along the way, and supported me throughout. I want to thank them.

John Williams, you are a mentor, advisor, teacher and friend. I could not have asked for a finer role model. You are in it for all the right reasons – the thrill of the chase, the imagination and creativity – and you possess enviable ability to laugh at failure. You have huge energy and it sets the tone in the lab and on the road. The common vision has been thoroughly enjoyed. Thanks.

John Adelman and Jim Maylie, you opened the door to discovery. John, the hard-nosed tactics were necessary. You have showed me the value of tenacity and the importance of keeping many irons in the fire. Jim, thanks for the words. I thank you (and hold you responsible) for pointing me in the right direction when I was transitioning to the next level. Thank you both for your time on the committee.

Craig Jahr and Dave Farrens, for your guidance and time on the committee, I am indebted. Craig, I hope some of your precision and even keeled demeanor have rubbed off. Dave, I have appreciated your ability to effortlessly weave science, philosophy and social commentary into conversation from my admission day interviews onward.

Chris Bond, thank you for helping develop my hands, appreciating experimental integrity, and helping me remember to stay balanced.

To past and present members of the lab, the successes were that much sweeter with the laughter. Thanks for all the help proof reading, result interpreting, experiment designing, and excellent excursions. If you ever need anything, just ask.

My family – mom, dad, bros, sister – you are the grounding and centering force. Thanks for casting the wide perimeter safety net and keeping the fire warm at home.

And to Lauren, for infinite patients, support and encouragement.

Thanks to you all. Without forgetting roots, I look forward to the next chapter.

ABSTRACT

Opioids are the most effective analgesics known, but they are also widely abused due to prescription diversion and the availability of illicit formulations, such as heroin. A fundamental feature of opiate use is tolerance and this property both compromises clinical utility and escalates the degree of dependence in opiate abusers. The effects of opioids are mediated through the μ-opioid receptor (MOR), and following agonist binding, MOR becomes the substrate for several regulatory events. These events – desensitization, internalization and re-sensitization – represent the cellular corollaries of tolerance. However, tolerance is a complex physiological adaptation and the specific cellular mechanisms contributing to its development remain elusive.

In addition to peptide agonists, there are several alkyloid opiates that signal differently through MOR and induce different degrees of regulation. Despite their prevalent use, few studies have examined their potential for inducing rapid desensitization and subsequent MOR recovery, particularly in neurons. In this study, whole-cell patch clamp recordings were made in slices containing locus coeruleus neurons and G protein coupled inward rectifying potassium (GIRK) currents were measured to monitor MOR signaling. The results of this study indicate that efficacy for inducing GIRK currents was correlated with the degree of desensitization induced for any given agonist. Furthermore, for each agonist, the degree of desensitization measured correlated with the amount of recovery observed. Oxycodone and buprenorphine demonstrated unique effects. Oxycodone was the only agonist that failed to induce desensitization or any subsequent recovery. Buprenorphine was capable of causing a small membrane hyperpolarization, but did not

induce any measurable current. While bound to MOR, however, it completely inhibited the otherwise robust desensitization characteristic of [Met]^{5}enkephalin (ME). Despite the attenuation of ME-induced desensitization, MOR signaling was facilitated in buprenorphine pre-treated cells exposed to repeat ME applications. These findings indicate that buprenorphine has a novel influence on MOR regulation.

Chronic treatment with five different opioid agonists revealed different persistent adaptations specific to acute MOR regulation. Drugs were delivered via osmotic minipumps for 6-7 days before brain slices containing LC neurons were prepared for whole-cell voltage clamp recordings. Morphine- and methadone-treatment induced the most discrepant findings for MOR recovery following ME-induced desensitization: morphine treatment inhibited the recovery of signaling, whereas recovery following methadone-treatment was the same as observed in sham-treated animals. Oxycodone and fentanyl treatment yielded intermediate results on MOR recovery. Finally, buprenorphine treatment had several effects that were different from the other 4 agonists used for chronic treatment: ME-induced current was inhibited, ME-induced desensitization was eliminated, and MOR signaling was preserved after long exposures to supersaturing ME concentrations.

Taken together, these results suggest that there is no single property of an opiate predictive of its potential to induce tolerance. An agonist's ability to induce acute MOR regulatory events and long-term, persistent cellular adaptations as well as

pharmacokinetics and active metabolites must be considered in order to effectively manage pain and successfully treat opiate addiction.

INTRODUCTION

Opiates are the most effective analgesic agents known to modern medicine. They are also recognized for their extreme addiction potential and are particularly dangerous because of their disproportionately high death rate compared to other drugs of abuse. All opioids, endogenous or opium derivatives, can produce euphoria and can be used as analgesics. These effects, as well as the propensity to induce dependence and reward, are mediated through the μ-opioid receptor (MOR), and the deletion of the gene that codes for MOR eliminates all three properties of opiates in mice. Continuous activation of MOR produces tolerance, a complicating feature of chronic opioid use. The onset of tolerance can occur within minutes, and this time course coincides with MOR regulatory events at the cellular level. Importantly, different opioid agonists exert different effects on MOR regulation. The focus of this dissertation is to examine MOR regulation – desensitization and recovery from desensitization – in two different contexts: 1) Acute regulation by 10 opioid agonists, several of them frequently prescribed for pain management; and 2) Acute regulation following chronic treatment with 5 commonly used opioids.

Initial studies demonstrated that cellular mechanisms involving MOR regulation underlie physiological tolerance. Tolerance is the precursor to dependence, and it is the most significant among adaptations that complicates pain management and potentiates abuse

liability. This introduction will begin with a brief update of current concerns and strategies in opioid use and the state of opioid dependence. The next section will review the initial events following MOR agonist binding and subsequent regulatory events. Finally, these events will be discussed in the context of animal physiology.

I. OPIOIDS AND HUMANS

The Sumerians cultivated poppies and isolated opium in lower Mesopotamia as early as the third millennium B.C. By the sixteenth century, manuscripts documenting opium tolerance and abuse emerged in Turkey, Egypt, Germany, and England. Morphine, the active ingredient in opium, was isolated in 1806 and, along with the invention of the hypodermic syringe, found a regular application as an adjunct to anesthetics in surgery and pain management (Brownstein, 1993). The problem of tolerance and abuse was not resolved with the invention of morphine. Great efforts were subsequently dedicated to synthesizing safer, more efficacious opiates that had lower abuse liability. Heroin was synthesized in 1898 and proclaimed to be more potent than morphine but free from addiction potential. This was the first in a series of opiate agonists pronounced to be the ideal compound. As a result of this research there now exists an array of opioid narcotics with significantly different profiles. Though the ideal compound has yet to be developed, prevalent use of opiates continues in the clinic, and their abuse is widespread.

Opiates remain the most effective analgesics known. They are widely used as adjuncts to anesthetics in surgery, in minor surgical procedures, and to control post-operative, acute

and chronic pain. Chronic pain – pain of at least 6 months duration – is a common cause of disability and affects 1 in 5 adult Americans (Chou et al., 2003). While there are several non-pharmacological and non-opioid pharmacological treatments available to relieve pain, opiates are the cornerstone of severe pain management. Between 1997 and 2002, the medical use of 3 frequently prescribed opiate analgesics, oxycodone, fentanyl and morphine increased by 403%, 227% and 73%, respectively (Gilson et al., 2004). Despite increased efforts to effectively manage pain, inadequate pain relief and under-prescription continues to be a problem (Kutner et al., 2001; Teno et al., 2001; Weiss et al., 2001; Marlowe and Chicella, 2002; Ballantyne, 2007; Mercadante, 2007). Concerns related to drug abuse are most frequently cited for the under-treatment of pain; however, these are unfounded when proper medical care is exercised (McQuay, 1999). Another problem commonly encountered in chronic pain management is that there are few data regarding the comparative efficacy and adverse event profiles associated with specific opioid compounds (Chou et al., 2003).

Opiate therapy directed at treating chronic pain is best studied in the context of cancer and palliative care. The introduction of multiple opiate agonists has changed the approach to analgesia by making rotation or alternation between agonists and/or routes of administration available. This is considered an effective strategy and is used to achieve the goals of high efficacy with minimal side effects, as well as safe and easy administration (Muller-Busch et al., 2005). While opiate rotation is improving pain management, there is no comprehensive regimen, but rather a formulation of guidelines that help with selection of particular agents and routes (Hanks et al., 2001). There is a

paucity of studies providing evidence for selection of baseline opiates or of indications for switching to alternative opiates (Hanks et al., 2001; Muller-Busch et al., 2005; Mercadante, 2007).

Opiates are highly addictive and both heroin as well as prescription opiates are abused by people across the globe. As a chronic, relapsing disorder, with broad social implications, opiate addiction has vast public health ramifications. The prevalence of heroin addiction is approaching 1 million users, however, as of 2003 only 281,000 people received treatment (2003 National Survey on Drug Use and Health: National Findings; Office of National Drug Control Policy, Heroin Fact Sheet, 2003). Between 1997 and 2002, abuse of oxycodone, fentanyl, and morphine increased by 347%, 642% and 113%, respectively (Gilson et al., 2004). When compared to all classes of abused drugs, these opioid analgesics showed the greatest increase (120%), though they were the second smallest combined category (10%), with alcohol-combinations (17%) and other illicit drugs (37%) representing larger categories. Finally, opiate analgesics are among the most rapidly growing class of abused drugs among high school students (Office of National Drug Control Policy, Prescription Drug Fact Sheet, 2003).

Opiate agonist maintenance treatment is the most effective treatment modality for opiate dependence and is first line therapy used by clinicians practicing in many countries (Collins et al., 2005; Haasen and van den Brink, 2006; Sung and Conry, 2006). The rationale behind this approach is to replace the opiate-occupied MOR in order to manage physiological and psychological components of withdrawal, craving, and

euphoria. Long-term maintenance with methadone, buprenorphine or levo-acethylmethadol (LAAM) have proven more effective than opiate detoxification with respect to decreased drug use, treatment retention, improved health outcomes, and improved social functioning (Joranson et al., 2000). While an ideal end-point is cessation or abstinence from opiate use, the successful treatment of opiate dependence can require indefinite maintenance, but there is little data examining long-term outcomes.

The population of people using opioids, whether in the context of pain management or addiction, is vast and warrants a thorough investigation of the physiological consequences that result from long-term use. One of the central phenomena encountered by all long-term opioid users is tolerance. With repetitive exposures, opiates induce adaptive physiological changes that prevent equivalent doses from producing the desired effect. Tolerance can be compensated for with dose escalation, however, both toxicity and dependence eventually results. Additionally, tolerance to some effects of opiates (analgesia, euphoria) can occur faster than to others (respiratory depression, constipation), which diminishes utility while increasing side-effect liability. Following cessation of opiate use, a spectrum of withdrawal symptoms can result. The severity of these symptoms varies but generally defines the degree of dependence. It is important to note that tolerance and dependence are the inevitable outcome of chronic opioid use, however, they do not necessarily result in addiction – the recurring compulsion to use a substance despite harmful consequences to personal health, mental state or social life. Thus, the mechanisms underlying the development of tolerance are a point of intersection between research and clinical application. Understanding these mechanisms is necessary

in order to increase the efficacy of analgesic therapy and to effectively manage opiate addiction.

II. OPIOID RECEPTORS

Structure

There are four cloned opioid receptors, the μ-opioid receptor (MOR), the κ-opioid receptor (KOR), the δ-opioid receptor (DOR), and the orphanin FQ/nociceptin receptor (OFQ/N-R). All four receptors belong to the class A (Rhodopsin) family of G_i/G_o protein-coupled receptors with 7 trans-membrane helical domains connected by three extracellular and three intracellular domains. The N-terminal domain is extracellular, while the C-terminal is intracellular. The opioid receptors are approximately 60% identical to each other with the greatest homology in the transmembrane helices and the greatest diversity in their N and C termini, and in their extracellular loops (Chen et al., 1993b; Chen et al., 1993a). Ligand-binding studies on the cloned OFQ/N-R initially revealed low affinity for all known opioid ligands, hence the names "orphanin FQ" or "ORL-1" (opioid receptor-like 1) receptor. The endogenous peptide, nociceptin, however, proved to be a hectadecapeptide closely related to the KOR-selective peptide dynorphin A (Meunier et al., 1995; Reinscheid et al., 1995). OFQ/N is considered here because it is expressed on locus coeruleus neurons and because one of the primary agonists of interest, buprenorphine acts as an agonist via this receptor.

Function

Transgenic mice have allowed for the selective deletion of coding regions specific to each receptor and have thus permitted specific functional roles to be assigned to each one. Essentially, MOR and DOR selective agonists are analgesic and rewarding, while KOR agonists are dysphoric. Activation of MOR elicits analgesia, hyperlocomotion, respiratory depression, constipation and immunosupression, all of which are eliminated in MOR-deficient mice (Mathes HWD, Maldonado R, Slowe S, 1996; Sora I, Uhl GR, 1997; Loh HH, Wei LN, 1998; Fuchs PN, Raja SN, 1999; Qiu C Sora I, Dubner R, 2000). Both OFQ/N-R and nociceptin knock-out animals have unaltered basal nociceptive responses and analgesic responses to morphine (Mogil JS, Pasternak GW, 2001).

MOR knock-out mice are unresponsive to all MOR-specific agonists and show no analgesia, dependence, or reward following their administration (Sora et al., 1997). Thus, the physiological effects of interest are all mediated by MOR activation, and the regulation of this opiate receptor is the focus of the studies presented here.

III. MOR SIGNALING AND REGULATION

Signaling

Opioid receptors are coupled to pertussis toxin-sensitive, heterotrimeric Gi/Go proteins. As such, activated receptors, via G-protien α and $\beta\gamma$ subunits, inhibit adenylyl cyclases and voltage-gated Ca^{2+} channels and stimulate G protein-coupled inwardly rectifying K^+ channels (GIRKS) and phospholipase Cβ (Loh and Smith, 1990; Connor et al., 1999;

Williams et al., 2001). The net effect of signaling through these effector systems is to reduce the excitability of neurons and to inhibit neurotransmitter release (Williams et al., 2001). In addition to signaling through downstream effectors, opioid-bound MORs become the substrate for regulatory machinery.

Desensitization

The acute regulation of MOR signaling by agonist-induced desensitization is a complex process that may occur through several mechanisms. These will be discussed here. There are four other concerns regarding desensitization that will be noted where relevant. First, MOR desensitization is a widely accepted phenomena, but the rate and extent reported depends on agonist identity, cellular environment, and experimental methodology. Second, homologous desensitization is a process that effects the activated receptor, whereas heterologous desensitization generalizes to other receptors or to changes in signaling cascades that reduces the response of other receptors. The focus of this work is homologous MOR desensitization, and efforts were taken to demonstrate this experimentally. Third, fast desensitization overlaps temporally with internalization and shares common mechanisms. The two events will be discussed separately whenever possible though the overlap in implicated functional molecular machinery does not allow for complete isolation of these two events by experimental design or result interpretation. This problem is particularly challenging in rat brain slices. Finally, tolerance is a complex physiological adaptation that involves, at the very least, receptor, cellular, synaptic, network, and behavioral changes. Events occurring at MOR are the most

upstream and, therefore, represent the first among many events that lead to the larger phenomenon.

Fast desensitization: a summary

The highly efficacious peptide agonist DAMGO (Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol enkephalin) will be used to summarize the basic events involved in desensitization. Following binding, heterotrimeric G_i/G_o proteins are activated and signal via downstream effectors. In a matter of seconds to minutes following continued agonist exposure, MORs are rapidly phosphorylated, and the addition of phosphates provides binding sites for arrestins. The binding of a kinase, phosphorylation of MOR, or arrestin binding to the phosphorylated receptor, could physically prevent ligand-bound receptors from interacting with G proteins as either as individual events or in combination. This rapid desensitization, or uncoupling of MORs from G proteins, is responsible for the initial loss of receptor sensitivity. However, desensitization also encompasses the events that immediately follow arrestin binding, which include the removal of receptors from the cell surface. MOR internalization will be discussed in subsequent section.

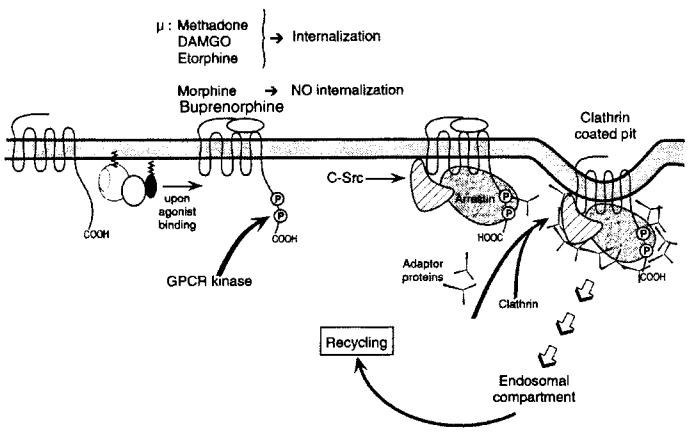


Figure 1. An illustration of the sequence of events leading to receptor desensitization and internalization. Not all agonists activate the internalization component of the pathway. (Adapted from Williams et al., 2001)

Phosphorylation: the initial event

Phosphorylation of MORs increases following ligand binding, though it does so to different extents depending on ligand identity (Arden et al., 1995; Zhang et al., 1996; Yu et al., 1997; Whistler et al., 1999; Koch et al., 2001). Morphine and buprenorphine have been shown to induce phosphorylation to a lesser extent than the peptide agonist, DAMGO, and the alkyloids, sufentanil and etorphine (Yu et al., 1997; Schulz et al., 2004). Phosphorylation is detectable within a few minutes and reaches a maximum after 10-15 minutes.

Multiple kinases implicated

The identity of the kinase responsible for phosphorylation following agonist binding to MOR is still a matter of debate, though the G protein receptor kinase 2 (GRK2) is a leading candidate. Protein kinase C (PKC), cyclic AMP dependent protein kinase A (PKA), calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase G (PKG), have all been implicated in desensitization, but it is unclear whether these kinases have a direct interaction with MOR or function in a second-mesenger-linked cascade (Wang and Wang, 2006). MORs have approximately 20 serine, threonine and tyrosine residues that could be accessible to protein kinases. The C-terminus of MOR contains putative GRK phosphorylation sites; mutation of these sites reduces phosphorylation following agonist binding (Deng et al., 2000; El Kouhen et al., 2001; Wang et al., 2002). By overexpressing GRK2 or GRK3, DAMGO- and morphine-induced phosphorylation is potentiated (Elliott et al., 1997; Zhang et al., 1998). In the absence of overexpression, morphine-bound MORs are a poor substrate for GRK phosphorylation. In neurons, the

identity of kinases involved in desensitization, whether involved in direct MOR phosphorylation or anywhere else, is severely lacking. A single study reports that in nucleus raphe magnus neurons, desensitization following DAMGO or morphine application could be reduced by intracellular application of a GRK2 inhibitory peptide (Li and Wang, 2001).

Arrestin binding: a link between desensitization and internalization

Arrestin binding to MORs uncouples them from G proteins and targets them for internalization. The affinity of arrestin for MORs is greatly increased by multiple phosphate groups added to the receptor and when the agonist remains receptor bound (Gurevich et al., 2004). Activated MORs recruit arrestin 3 (β-arrestin 2), but not arrestin 2 (β-arrestin 1), from the cytosol to the plasma membrane in HEK 293 cells (Zhang et al., 1998; Whistler and von Zastrow, 1999; Oakley et al., 2000; Bohn et al., 2004). Another important finding in this system was that morphine only promotes the translocation of arrestin 3 in the presence of overexpressed GRK2 (Zhang et al., 1998; Bohn et al., 2004). Agonist-specificity has been demonstrated in mouse embryonic fibroblasts lacking arrestin 2 and 3. Here, morphine was shown to promote translocation of GFP-tagged arrestin 3, while the high potency/efficacy agonist, etorphine (but not morphine), induced translocation of GFP-tagged arrestin 2.

Measuring desensitization

Functional measurements of MOR desensitization have demonstrated agonist-specific dependence. When GIRK currents are measured in LC neurons, sustained applications of

either met-enkephalin or DAMGO induce a decline from peak of approximately 50% after 10 minutes (Harris and Williams, 1991; Connor et al., 1996; Fiorillo and Williams, 1996; Alvarez et al., 2002; Bailey et al., 2003). While sustained applications of morphine showed no desensitization in initial studies (Alvarez et al., 2002; Bailey et al., 2003), improvements in experimental protocols revealed that morphine can cause a decline of approximately 10-35% following drug applications for 10 and15 minutes, respectively (Dang and Williams, 2005). Thus, morphine induced desensitization is slower and occurs to a lesser extent than to that induced by the peptides. Furthermore, methadone and etorphine, additional alkyloid agonists, have been shown to induce desensitization comaprable to that of DAMGO (Alvarez et al., 2002; Bailey et al., 2003). A study performed in AtT20 cells measuring Ca^{2+} demonstrated that DAMGO, methadone and morphine all produced maximal desensitization; however, desensitization occurred on the same time-course as GIRK currents in LC neurons (Borgland et al., 2003). Another study in the AtT20 cells measuring Kir3 currents showed that DAMGO pre-incubation, but not morphine, caused desensitization of a subsequent DAMGO-induce current. In all of the above studies, desensitization was primarily homologous. Interestingly, a recent study comparing agonist-specific desensitization in HEK 293 cells showed that DAMGO-induced desensitization is GRK2 dependent, whereas morphine-induced desensitization is PKC-dependent (Johnson et al., 2006). Thus, a possible mechanism has been suggested to explain the differences in MOR desensitization between different agonists. Taken together, these studies demonstrate that rapid MOR desensitization occurs in multiple systems, has an agonist-specific component, and may result from different mechanistic processes.

MOR internalization and trafficking

Fast desensitization begins with MOR phosphorylation and arrestin binding, however, within a few minutes of ligand-binding, MORs are directed to the highly conserved endocytotic pathway initially described for muscarinic and adrenergic receptors (Zhang et al., 1997; Tsao et al., 2001). Binding of the highly efficacious opioid peptides and certain alkyloids cause MORs to be concentrated in clathrin-coated pits that undergo dynamin-dependent internalization, fuse with early endosomes and cease signaling (Chu et al., 1997).

Distinct agonists also differ substantially in their ability to trigger MOR internalization, and this aspect of agonist-specific MOR regulation is best characterized particularly in heterologous systems. The peptide agonists met-enkephalin and DAMGO drive endocytosis of a massive population of receptors within minutes while morphine fails to cause detectable internalization after long incubations (Arden et al., 1995; Keith et al., 1996; Sternini et al., 1996; Keith et al., 1998; Abbadie and Pasternak, 2001). Internalization is time and concentration dependent with up to 50% of MORs that will be internalized endocytosed within 5 minutes and a dynamic steady state achieved in 30 min (Keith et al., 1998; Trapaidze et al., 2000; Borgland et al., 2003). Under certain conditions, however, morphine-induced MOR internalization has been observed. Overexpression of GRK2, or the over-expression of arrestin 2 or 3 without GRK overexpression, promotes morphine-bound MOR endocytosis (Whistler and von Zastrow, 1998; Zhang et al., 1998). Furthermore, morphine-induced internalization has been

observed in vivo in dendrites of nucleus accumbens neurons and in the cell bodies of striatal neurons in primary culture. This is further testament to the importance and variability of endogenous environments in the study of MOR regulation. The more comprehensive studies of agonist-induced internalization rank morphine, buprenorphine, hydromorphone and oxycodone as least capable; fentanyl and methadone as moderately capable; and DAMGO, Met-Enkephalin, etorphine and etonitazene as most capable (Keith et al., 1996; Zaki et al., 2000; Alvarez et al., 2002; Borgland et al., 2003; Koch et al., 2005). MOR antagonists, such as naloxone, have demonstrated receptor upregulation in vitro and in vivo; and the partial agonist buprenorphine has been shown to upregulate MOR expression in cell cultures (Zaki et al., 2000).

The function of MOR internalization and the molecular basis of endocytic sorting are unknown. Two generally accepted trafficking pathways following MOR endocytosis are receptor recycling back to the plasma membrane and proteolytic destruction (Koch et al., 1997; Law et al., 2000; Tsao et al., 2001). The recycling pathway is characterized by MOR dephosphorylation, re-insertion, and re-sensitization. The alternative route involves sorting to lysosomes, degradation, and down-regulation of MORs.

Resensitization

Recovery of signaling following MOR desensitization has largely been overlooked with the exception of re-sensitization after Met-enkephalin-induced desensitization in the LC, which is well characterized. ME washes rapidly from brain slice preparation, thus supersaturating concentrations can be applied in succession without residual agonist

remaining in the slice or bound to the receptor. In these studies, recovery following a 10 minute supersaturating application of ME occurred by 25-45 minutes (Harris and Williams, 1991; Osborne and Williams, 1995).

Most other studies assessed the contribution of MOR internalization to re-sensitization. Those performed in Neuro2A cells and HEK 293 cells demonstrated that any perturbation that interfered with MOR internalization increased desensitization and prolonged re-sensitization (Qiu et al., 2003; Koch et al., 2004). Furthermore, DAMGO activated phospholipase D2, proposed to facilitate MOR internalization, while morphine did not. Additionally, an MOR isoform that internalized more rapidly following DAMGO binding showed decreased desensitization and increased re-sensitization (Wolf et al., 1999). A single study compared MOR recovery following morphine or DAMGO desensitization and found that those following DAMGO desensitization recovered to a much greater extent (Schulz et al., 2004). In summary, results generated in these systems suggest that receptor internalization facilitates re-sensitization. In LC neurons, however, blocking MOR internalization had no effect on desensitization or recovery (Arttamangkul et al., 2006). This implies that the endogenous MORs expressed on neurons may be undergo significant modulation at the level of the plasma membrane.

IV. FUNCTIONAL MEASUREMENTS OF MOR DESENSITIZATION AND RECOVERY: EXPERIMENTAL CONCERNS

Functional measurements of MOR desensitization in a variety of systems have revealed that different systems are best suited to measure different components of MOR regulation

(Connor et al., 2004). The accurate interpretation of these studies, however, requires attention to three critical issues: temporal resolution, receptor expression levels, and receptor specificity. The component of MOR desensitization relevant in the present work is that occurring within 1-10 minutes of sustained agonist application. This requires continuous, real time monitoring of receptor activity and can be best accomplished with the temporal resolution afforded by electrophysiology. Receptor expression levels are of immense importance to desensitization because the stoichiometry of receptors and intracellular regulatory components influence function. Eliminating "spare receptors" increases the amount of acute desensitization measured and shifts dose-response curves such that probing re-sensitization with a fixed concentration of agonist can yield altered measurements. The slice preparation controls for this by preserving endogenous cellular environments and maintaining native expression levels. Finally, MOR desensitization must involve regulatory processes specific to MOR, not those that generalize to other receptors either directly or via common second-messenger cascades. Proper experimental design measures the degree of homologous and heterologous desensitization.

A sizeable number of studies examining MOR desensitization have been performed in various heterologous systems by measuring opioid induced inhibition of adenylate cyclase (Chakrabarti et al., 1995; Elliott et al., 1997; Koch et al., 1997; Chakrabarti et al., 1998; El Kouhen et al., 1999; Law et al., 2000; Koch et al., 2001; Koch et al., 2004; Koch et al., 2005). Desensitization happens slowly when using biochemical assays and often requires 1-24 hours of pre-incubation with agonists. Desensitization is then quantified by

measuring the ability of an agonist to inhibit adenylate cyclase following pre-incubation and compared with the ability of that same agonist in the absence of pre-incubation. Studies of this kind have examined the most diverse array of MOR agonists and have documented profound differences in their effects on MOR desensitization. However, because adaptations may have occurred during such extensive pre-incubations, the time-course is radically different, and the MOR-specificity is rarely included, these studies will not be included in a discussion of MOR desensitization.

V. TOLERANCE: CELLULAR MECHANISMS

Initial in vivo models: historical precedents

Cellular correlates of opiate tolerance have long been accepted as the locus of initial adaptive responses to chronic opioid treatment. MOR agonists reversibly inhibit AC during brief applications, however following their sustained presence a delayed, but stable, upregulation of AC activity occurs. This was initially demonstrated in neuroblastoma x glioma hybrid cells following morphine treatment and the state of AC upregulation was interpreted as a tolerant state because it counteracted the acute, inhibitory effect of morphine. Furthermore, these cells became dependent in 1-2 days because when morphine was removed, a profound overproduction of cAMP was observed, indicative of withdrawal (Sharma et al., 1975). This remains a favored model to study chronic opioid treatment in heterologous systems, though it is now known that this in vivo phenomenon is likely involves increased AC protein as opposed to increased activity alone (Williams et al., 2001).

A second study found that ileum taken from guinea pigs undergoing chronic morphine treatment displayed a reduction in μ-opioid receptor reserve, as measured by dose response curves to normorphine (Chavkin and Goldstein, 1984). This decrease in spare receptors – whether due to less binding sites or less receptor coupling – was proposed to be the basis of tolerance. Finally, following the discovery that enkephalin increases potassium conductance in LC neurons, it was demonstrated that chronic morphine treatment in the rat could also result in cellular tolerance (Williams et al., 1982; Christie et al., 1987). Thus, cellular correlates of tolerance were demonstrated in central neurons. Despite the fact that these systems are among the most important and significant, they are some of the most difficult to experimentally control. If a model is not confirmed in neurons and in vivo, it is of questionable relevance.

Chronic treatment: studies in neurons

Three current studies are of particular relevance to the work presented here as they looked at fast components of desensitization and recovery in neurons from chronically treated animals. In rats treated with morphine, MOR desensitization was facilitated while recovery from desensitization was inhibited when compared to untreated animals (Dang and Williams, 2004). Thus, a desensitizing stimulus applied to neurons following chronic morphine treatment causes subsequent MOR uncoupling to a much greater extent than those from untreated animals. Morphine-induced desensitization as measured by a decline in peak current during a sustained (10 min) application was also facilitated by chronic morphine treatment (Dang and Williams, 2005).

In a second study, I_{Ca2+} was studied in acutely isolated PAG neurons and GIRK was measured in PAG slices (Bagley et al., 2005). DAMGO was unable to inhibit I_{Ca2+} to the same extent in neurons from morphine-treated animals. Furthermore GIRK currents were smaller in morphine-treated animals than in control. Thus, two different measurements show a significant degree of tolerance induced by morphine-treatment.

These are two of the primary studies in neurons following chronic morphine treatment. They did not however look at the vast array of opiates used for analgesia and opiate maintenance.

Arrestin 3 and tolerance

The physiological consequences of endocytosis with respect to MOR regulation and opiate responsiveness are not yet understood. A series of studies in mice with the arrestin 3 gene deletion illustrate the complexity of these links. By measuring G protein activation in brain membranes, agonist-induced MOR desensitization was severely impaired (Bohn et al., 1999). The mice also demonstrated enhanced sensitivity to the analgesic effects of morphine and decreased development of anti-nociceptive tolerance to this opiate (Bohn et al., 1999; Bohn et al., 2000). Morphine tolerance was not completely blocked, however, and PKC was implicated in an alternative mechanism of tolerance (Bohn et al., 2002). The rewarding properties of morphine were also enhanced (Bohn et al., 2003). This mouse presents a paradoxical finding that antinociception was only enhanced for agonists that do not recruit arrestin 3 (morphine and heroin) in vitro, while there is no change in antinociception for agonists that recruit arrestin 3 more robustly

(etorphine, fentanyl and methadone). The subsequent explanation offered was that morphine-bound MORs do recruit arrestin 3 to a small extent and this becomes amplified when it is deleted, whereas the robust arrestin 3 recruiter can substitute arrestin 2 to fulfill the role in vivo (Bohn et al., 2004). Thus, the role of arrestin 3, as surmised by studies in mutants is difficult to ascertain because of the contradictions between in vitro and in vivo data. A double arretin 2/3 deletion would prove interesting, however, this mutant remains embryonic lethal.

VI. TOLERANCE: in vivo studies and current theories of cellular mechanisms

One of the central unanswered questions is what property of any given agonist determines how much tolerance will result from chronic administration? Though it is well documented that *in vivo* tolerance occurs at different rates and to different degrees depending on the agonist administered (Stafford et al., 2001; Walker and Young, 2001; Grecksch et al., 2006; Pawar et al., 2007), it remains a matter of great controversy as to whether this is a function of agonist efficacy or of another regulatory property triggered by an individual ligand (Kovoor et al., 1998; Walker and Young, 2001; Celver et al., 2004; Pawar et al., 2007). Morphine is the focal point of this controversy because its propensity to induce tolerance in vivo has been attributed to either, 1) its partial agonist properties in vitro or; 2) the observation that morphine-occupied MOR is a poor substrate for regulatory (desensitization/internalization) machinery.

The efficacy of any MOR agonist can be determined based on ability to bind GTPgS, to interact with one of several downstream effectors, or to provide analgesia in a variety of

behavioral tests. Most agonists are capable of providing a substantial degree of analgesia, particularly in opiate-naïve subjects. Two of the primary differences between agonists are potency and pharmacokinetic variables. The efficacy theory relies on the idea that analgesia is attained with high efficacy agonists (etorphine, fentanyl) by occupying fewer receptors than is required for low efficacy agonists (morphine, buprenorphine). This model, however, makes the assumption that occupied receptors become desensitized or permanently internalized. Some of the better evidence supporting this idea is that high dose etorphine downregulates MORs and upregulates the trafficking protein dynamin-2, while neither morphine, nor oxycodone have either effect (Patel et al., 2002; Pawar et al., 2007).

The second mechanistic explanation of tolerance is based on the observation that morphine bound MORs are a poor substrate for internalization. The classical theory postulates that morphine bound MORs are uncoupled by phosphorylation and that, because internalization is the only way for MORs to become dephosphorylated, agonists that induce phosphorylation in the absence of internalization remain desensitized (Koch et al., 1997; Zhang et al., 1998; Koch et al., 2001). A second theory suggests that morphine occupied MORs continue to signal at the plasma membrane because they are not internalized, thus counterregulatory cellular adaptations are promoted (Whistler and von Zastrow, 1998; Finn and Whistler, 2001). One of the major flaws in this idea is that if internalization serves to re-sensitize receptors, then agonists that induce rapid internalization will also continue to signal and produce the same adaptive changes. This

second idea, however, has had a significant impact on the field of opioid tolerance, and several labs have investigated its ramifications.

VII. LOCUS COERULEUS

The LC is the primary noradrenergic nucleus of the brain and, via a widespread efferent projection system, innervates more regions of the neuraxis than any other nucleus in the brain (Foote et al., 1983; Aston-Jones et al., 1996; Shipley et al., 1996). The nucleus proper contains LC cell bodies while dendritic arborization occurs primarily in the rostral-caudal plane, defining the pericoerulear region. The LC in rat has been characterized extensively and is comprised of a homogenous population of neurons all expressing MOR and not delta or kappa opioid receptors. LC neurons are eletrotonically coupled and fire tonically in awake behaving animals (Aston-Jones and Bloom, 1981; Ishimatsu and Williams, 1996; Aston-Jones et al., 1998). The LC and noradrenergic system initiate behavioral and neuronal activity states required to collect sensory information (Berridge and Waterhouse, 2003). In the waking state, LC neurons regulate states of attention, vigilance and sympathetic nervous system activity. This nucleus has been implicated in the actions of stress, antidepressants and opiates on the brain, particularly during the state of withdrawal. During opiate withdrawal, increased firing of LC neurons is responsible for many of the associated signs and symptoms (Aghajanian, 1978; Koob et al., 1992; Lane-Ladd et al., 1997).

VIII. COURSE OF DISSERTATION

The work presented here will use whole cell voltage clamp recordings on brain slices containing locus coeruleus neurons. Chapter 1 will focus on acute MOR regulation following the application of several different agonists. Chapter 2 will provide the results of chronic treatment with 5 of these agonists on acute MOR regulation. Chapter 3 will examine buprenorphine with respect pharmacology, acute signaling and adaptations that result from chronic treatment.

REFERENCES

Abbadie C, Pasternak GW (2001) Differential in vivo internalization of MOR-1 and MOR-1C by morphine. Neuroreport 12:3069-3072.

Aghajanian GK (1978) Tolerance of locus coeruleus neurones to morphine and suppression of withdrawal response by clonidine. Nature 276:186-188.

Alvarez VA, Arttamangkul S, Dang V, Salem A, Whistler JL, Von Zastrow M, Grandy DK, Williams JT (2002) mu-Opioid receptors: Ligand-dependent activation of potassium conductance, desensitization, and internalization. J Neurosci 22:5769-5776.

Arden JR, Segredo V, Wang Z, Lameh J, Sadee W (1995) Phosphorylation and agonist-specific intracellular trafficking of an epitope-tagged mu-opioid receptor expressed in HEK 293 cells. J Neurochem 65:1636-1645.

Arttamangkul S, Torrecilla M, Kobayashi K, Okano H, Williams JT (2006) Separation of mu-opioid receptor desensitization and internalization: endogenous receptors in primary neuronal cultures. J Neurosci 26:4118-4125.

Aston-Jones G, Bloom FE (1981) Nonrepinephrine-containing locus coeruleus neurons in behaving rats exhibit pronounced responses to non-noxious environmental stimuli. J Neurosci 1:887-900.

Aston-Jones G, Rajkowski J, Kubiak P, Valentino RJ, Shipley MT (1996) Role of the locus coeruleus in emotional activation. Prog Brain Res 107:379-402.

Aston-Jones G, Rajkowski J, Ivanova S, Usher M, Cohen J (1998) Neuromodulation and cognitive performance: recent studies of noradrenergic locus ceruleus neurons in behaving monkeys. Adv Pharmacol 42:755-759.

Bagley EE, Chieng BC, Christie MJ, Connor M (2005) Opioid tolerance in periaqueductal gray neurons isolated from mice chronically treated with morphine. Br J Pharmacol 146:68-76.

Bailey CP, Couch D, Johnson E, Griffiths K, Kelly E, Henderson G (2003) Mu-opioid receptor desensitization in mature rat neurons: lack of interaction between DAMGO and morphine. J Neurosci 23:10515-10520.

Ballantyne JC (2007) Opioid analgesia: perspectives on right use and utility. Pain Physician 10:479-491.

Berridge CW, Waterhouse BD (2003) The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. Brain Res Brain Res Rev 42:33-84.

Bohn LM, Lefkowitz RJ, Caron MG (2002) Differential mechanisms of morphine antinociceptive tolerance revealed in (beta)arrestin-2 knock-out mice. J Neurosci 22:10494-10500.

Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG (2000) Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. Nature 408:720-723.

Bohn LM, Dykstra LA, Lefkowitz RJ, Caron MG, Barak LS (2004) Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. Mol Pharmacol 66:106-112.

Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, Lin FT (1999) Enhanced morphine analgesia in mice lacking beta-arrestin 2. Science 286:2495-2498.

Bohn LM, Gainetdinov RR, Sotnikova TD, Medvedev IO, Lefkowitz RJ, Dykstra LA, Caron MG (2003) Enhanced rewarding properties of morphine, but not cocaine, in beta(arrestin)-2 knock-out mice. J Neurosci 23:10265-10273.

Borgland SL, Connor M, Osborne PB, Furness JB, Christie MJ (2003) Opioid agonists have different efficacy profiles for G protein activation, rapid desensitization, and endocytosis of mu-opioid receptors. J Biol Chem 278:18776-18784.

Brownstein MJ (1993) A brief history of opiates, opioid peptides, and opioid receptors. Proc Natl Acad Sci U S A 90:5391-5393.

Celver J, Xu M, Jin W, Lowe J, Chavkin C (2004) Distinct domains of the mu-opioid receptor control uncoupling and internalization. Mol Pharmacol 65:528-537.

Chakrabarti S, Law PY, Loh HH (1995) Neuroblastoma Neuro2A cells stably expressing a cloned mu-opioid receptor: a specific cellular model to study acute and chronic effects of morphine. Brain Res Mol Brain Res 30:269-278.

Chakrabarti S, Law PY, Loh HH (1998) Distinct differences between morphine- and [D-Ala2,N-MePhe4,Gly-ol5]-enkephalin-mu-opioid receptor complexes demonstrated by cyclic AMP-dependent protein kinase phosphorylation. J Neurochem 71:231-239.

Chavkin C, Goldstein A (1984) Opioid receptor reserve in normal and morphine-tolerant guinea pig ileum myenteric plexus. Proc Natl Acad Sci U S A 81:7253-7257.

Chen Y, Mestek A, Liu J, Yu L (1993a) Molecular cloning of a rat kappa opioid receptor reveals sequence similarities to the mu and delta opioid receptors. Biochem J 295 (Pt 3):625-628.

Chen Y, Mestek A, Liu J, Hurley JA, Yu L (1993b) Molecular cloning and functional expression of a mu-opioid receptor from rat brain. Mol Pharmacol 44:8-12.

Chou R, Clark E, Helfand M (2003) Comparative efficacy and safety of long-acting oral opioids for chronic non-cancer pain: a systematic review. J Pain Symptom Manage 26:1026-1048.

Christie MJ, Williams JT, North RA (1987) Cellular mechanisms of opioid tolerance: studies in single brain neurons. Mol Pharmacol 32:633-638.

Chu P, Murray S, Lissin D, von Zastrow M (1997) Delta and kappa opioid receptors are differentially regulated by dynamin-dependent endocytosis when activated by the same alkaloid agonist. J Biol Chem 272:27124-27130.

Collins ED, Kleber HD, Whittington RA, Heitler NE (2005) Anesthesia-assisted vs buprenorphine- or clonidine-assisted heroin detoxification and naltrexone induction: a randomized trial. Jama 294:903-913.

Connor M, Borgland SL, Christie MJ (1999) Continued morphine modulation of calcium channel currents in acutely isolated locus coeruleus neurons from morphine-dependent rats. Br J Pharmacol 128:1561-1569.

Connor M, Osborne PB, Christie MJ (2004) Mu-opioid receptor desensitization: is morphine different? Br J Pharmacol 143:685-696.

Connor M, Vaughan CW, Chieng B, Christie MJ (1996) Nociceptin receptor coupling to a potassium conductance in rat locus coeruleus neurones in vitro. Br J Pharmacol 119:1614-1618.

Dang VC, Williams JT (2004) Chronic morphine treatment reduces recovery from opioid desensitization. J Neurosci 24:7699-7706.

Dang VC, Williams JT (2005) Morphine-induced {micro}-opioid receptor desensitization. Mol Pharmacol.

Deng HB, Yu Y, Pak Y, O'Dowd BF, George SR, Surratt CK, Uhl GR, Wang JB (2000) Role for the C-terminus in agonist-induced mu opioid receptor phosphorylation and desensitization. Biochemistry 39:5492-5499.

El Kouhen R, Kouhen OM, Law PY, Loh HH (1999) The absence of a direct correlation between the loss of [D-Ala2, MePhe4,Gly5-ol]Enkephalin inhibition of adenylyl cyclase activity and agonist-induced mu-opioid receptor phosphorylation. J Biol Chem 274:9207-9215.

El Kouhen R, Burd AL, Erickson-Herbrandson LJ, Chang CY, Law PY, Loh HH (2001) Phosphorylation of Ser363, Thr370, and Ser375 residues within the carboxyl tail differentially regulates mu-opioid receptor internalization. J Biol Chem 276:12774-12780.

Elliott J, Guo L, Traynor JR (1997) Tolerance to mu-opioid agonists in human neuroblastoma SH-SY5Y cells as determined by changes in guanosine-5'-O-(3-[35S]-thio)triphosphate binding. Br J Pharmacol 121:1422-1428.

Finn AK, Whistler JL (2001) Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. Neuron 32:829-839.

Fiorillo CD, Williams JT (1996) Opioid desensitization: interactions with G-protein-coupled receptors in the locus coeruleus. J Neurosci 16:1479-1485.

Foote SL, Bloom FE, Aston-Jones G (1983) Nucleus locus ceruleus: new evidence of anatomical and physiological specificity. Physiol Rev 63:844-914.

Gilson AM, Ryan KM, Joranson DE, Dahl JL (2004) A reassessment of trends in the medical use and abuse of opioid analgesics and implications for diversion control: 1997-2002. J Pain Symptom Manage 28:176-188.

Grecksch G, Bartzsch K, Widera A, Becker A, Hollt V, Koch T (2006) Development of tolerance and sensitization to different opioid agonists in rats. Psychopharmacology (Berl) 186:177-184.

Gurevich EV, Benovic JL, Gurevich VV (2004) Arrestin2 expression selectively increases during neural differentiation. J Neurochem 91:1404-1416.

Haasen C, van den Brink W (2006) Innovations in agonist maintenance treatment of opioid-dependent patients. Curr Opin Psychiatry 19:631-636.

Hanks GW, Conno F, Cherny N, Hanna M, Kalso E, McQuay HJ, Mercadante S, Meynadier J, Poulain P, Ripamonti C, Radbruch L, Casas JR, Sawe J, Twycross RG, Ventafridda V (2001) Morphine and alternative opioids in cancer pain: the EAPC recommendations. Br J Cancer 84:587-593.

Harris GC, Williams JT (1991) Transient homologous mu-opioid receptor desensitization in rat locus coeruleus neurons. J Neurosci 11:2574-2581.

Ishimatsu M, Williams JT (1996) Synchronous activity in locus coeruleus results from dendritic interactions in pericoerulear regions. J Neurosci 16:5196-5204.

Johnson EA, Oldfield S, Braksator E, Gonzalez-Cuello A, Couch D, Hall KJ, Mundell SJ, Bailey CP, Kelly E, Henderson G (2006) Agonist-selective mechanisms of mu-opioid receptor desensitization in human embryonic kidney 293 cells. Mol Pharmacol 70:676-685.

Joranson DE, Ryan KM, Gilson AM, Dahl JL (2000) Trends in medical use and abuse of opioid analgesics. Jama 283:1710-1714.

Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ, von Zastrow M (1996) Morphine activates opioid receptors without causing their rapid internalization. J Biol Chem 271:19021-19024.

Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV, Monteillet-Agius G, Stewart PL, Evans CJ, von Zastrow M (1998) mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. Mol Pharmacol 53:377-384.

Koch T, Kroslak T, Mayer P, Raulf E, Hollt V (1997) Site mutation in the rat mu-opioid receptor demonstrates the involvement of calcium/calmodulin-dependent protein kinase II in agonist-mediated desensitization. J Neurochem 69:1767-1770.

Koch T, Schulz S, Pfeiffer M, Klutzny M, Schroder H, Kahl E, Hollt V (2001) C-terminal splice variants of the mouse mu-opioid receptor differ in morphine-induced internalization and receptor resensitization. J Biol Chem 276:31408-31414.

Koch T, Brandenburg LO, Liang Y, Schulz S, Beyer A, Schroder H, Hollt V (2004) Phospholipase D2 modulates agonist-induced mu-opioid receptor desensitization and resensitization. J Neurochem 88:680-688.

Koch T, Widera A, Bartzsch K, Schulz S, Brandenburg LO, Wundrack N, Beyer A, Grecksch G, Hollt V (2005) Receptor endocytosis counteracts the development of opioid tolerance. Mol Pharmacol 67:280-287.

Koob GF, Maldonado R, Stinus L (1992) Neural substrates of opiate withdrawal. Trends Neurosci 15:186-191.

Kovoor A, Celver JP, Wu A, Chavkin C (1998) Agonist induced homologous desensitization of mu-opioid receptors mediated by G protein-coupled receptor kinases is dependent on agonist efficacy. Mol Pharmacol 54:704-711.

Kutner JS, Kassner CT, Nowels DE (2001) Symptom burden at the end of life: hospice providers' perceptions. J Pain Symptom Manage 21:473-480.

Lane-Ladd SB, Pineda J, Boundy VA, Pfeuffer T, Krupinski J, Aghajanian GK, Nestler EJ (1997) CREB (cAMP response element-binding protein) in the locus coeruleus: biochemical, physiological, and behavioral evidence for a role in opiate dependence. J Neurosci 17:7890-7901.

Law PY, Erickson LJ, El-Kouhen R, Dicker L, Solberg J, Wang W, Miller E, Burd AL, Loh HH (2000) Receptor density and recycling affect the rate of agonist-induced desensitization of mu-opioid receptor. Mol Pharmacol 58:388-398.

Li AH, Wang HL (2001) G protein-coupled receptor kinase 2 mediates mu-opioid receptor desensitization in GABAergic neurons of the nucleus raphe magnus. J Neurochem 77:435-444.

Loh HH, Smith AP (1990) Molecular characterization of opioid receptors. Annu Rev Pharmacol Toxicol 30:123-147.

Marlowe KF, Chicella MF (2002) Treatment of sickle cell pain. Pharmacotherapy 22:484-491.

McQuay H (1999) Opioids in pain management. Lancet 353:2229-2232.

Mercadante S (2007) Opioid titration in cancer pain: A critical review. Eur J Pain.

Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, Butour JL, Guillemot JC, Ferrara P, Monsarrat B, et al. (1995) Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature 377:532-535.

Muller-Busch HC, Lindena G, Tietze K, Woskanjan S (2005) Opioid switch in palliative care, opioid choice by clinical need and opioid availability. Eur J Pain 9:571-579.

Oakley RH, Laporte SA, Holt JA, Caron MG, Barak LS (2000) Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. J Biol Chem 275:17201-17210.

Osborne PB, Williams JT (1995) Characterization of acute homologous desensitization of mu-opioid receptor-induced currents in locus coeruleus neurones. Br J Pharmacol 115:925-932.

Patel MB, Patel CN, Rajashekara V, Yoburn BC (2002) Opioid agonists differentially regulate mu-opioid receptors and trafficking proteins in vivo. Mol Pharmacol 62:1464-1470.

Pawar M, Kumar P, Sunkaraneni S, Sirohi S, Walker EA, Yoburn BC (2007) Opioid agonist efficacy predicts the magnitude of tolerance and the regulation of mu-opioid receptors and dynamin-2. Eur J Pharmacol 563:92-101.

Qiu Y, Law PY, Loh HH (2003) Mu-opioid receptor desensitization: role of receptor phosphorylation, internalization, and representation. J Biol Chem 278:36733-36739.

Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, Grandy DK, Langen H, Monsma FJ, Jr., Civelli O (1995) Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. Science 270:792-794.

Schulz S, Mayer D, Pfeiffer M, Stumm R, Koch T, Hollt V (2004) Morphine induces terminal micro-opioid receptor desensitization by sustained phosphorylation of serine-375. Embo J 23:3282-3289.

Sharma SK, Klee WA, Nirenberg M (1975) Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. Proc Natl Acad Sci U S A 72:3092-3096.

Shipley MT, Fu L, Ennis M, Liu WL, Aston-Jones G (1996) Dendrites of locus coeruleus neurons extend preferentially into two pericoerulear zones. J Comp Neurol 365:56-68.

Sora I, Takahashi N, Funada M, Ujike H, Revay RS, Donovan DM, Miner LL, Uhl GR (1997) Opiate receptor knockout mice define mu receptor roles in endogenous nociceptive responses and morphine-induced analgesia. Proc Natl Acad Sci U S A 94:1544-1549.

Stafford K, Gomes AB, Shen J, Yoburn BC (2001) mu-Opioid receptor downregulation contributes to opioid tolerance in vivo. Pharmacol Biochem Behav 69:233-237.

Sternini C, Spann M, Anton B, Keith DE, Jr., Bunnett NW, von Zastrow M, Evans C, Brecha NC (1996) Agonist-selective endocytosis of mu opioid receptor by neurons in vivo. Proc Natl Acad Sci U S A 93:9241-9246.

Sung S, Conry JM (2006) Role of buprenorphine in the management of heroin addiction. Ann Pharmacother 40:501-505.

Teno JM, Weitzen S, Wetle T, Mor V (2001) Persistent pain in nursing home residents. Jama 285:2081.

Trapaidze N, Gomes I, Cvejic S, Bansinath M, Devi LA (2000) Opioid receptor endocytosis and activation of MAP kinase pathway. Brain Res Mol Brain Res 76:220-228.

Tsao P, Cao T, von Zastrow M (2001) Role of endocytosis in mediating downregulation of G-protein-coupled receptors. Trends Pharmacol Sci 22:91-96.

Walker EA, Young AM (2001) Differential tolerance to antinociceptive effects of mu opioids during repeated treatment with etonitazene, morphine, or buprenorphine in rats. Psychopharmacology (Berl) 154:131-142.

Wang HL, Chang WT, Hsu CY, Huang PC, Chow YW, Li AH (2002) Identification of two C-terminal amino acids, Ser(355) and Thr(357), required for short-term homologous desensitization of mu-opioid receptors. Biochem Pharmacol 64:257-266.

Wang ZJ, Wang LX (2006) Phosphorylation: a molecular switch in opioid tolerance. Life Sci 79:1681-1691.

Weiss SC, Emanuel LL, Fairclough DL, Emanuel EJ (2001) Understanding the experience of pain in terminally ill patients. Lancet 357:1311-1315.

Whistler JL, von Zastrow M (1998) Morphine-activated opioid receptors elude desensitization by beta-arrestin. Proc Natl Acad Sci U S A 95:9914-9919.

Whistler JL, von Zastrow M (1999) Dissociation of functional roles of dynamin in receptor-mediated endocytosis and mitogenic signal transduction. J Biol Chem 274:24575-24578.

Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M (1999) Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. Neuron 23:737-746.

Williams JT, Egan TM, North RA (1982) Enkephalin opens potassium channels on mammalian central neurones. Nature 299:74-77.

Williams JT, Christie MJ, Manzoni O (2001) Cellular and synaptic adaptations mediating opioid dependence. Physiol Rev 81:299-343.

Wolf R, Koch T, Schulz S, Klutzny M, Schroder H, Raulf E, Buhling F, Hollt V (1999) Replacement of threonine 394 by alanine facilitates internalization and resensitization of the rat mu opioid receptor. Mol Pharmacol 55:263-268.

Yu Y, Zhang L, Yin X, Sun H, Uhl GR, Wang JB (1997) Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. J Biol Chem 272:28869-28874.

Zaki PA, Keith DE, Jr., Brine GA, Carroll FI, Evans CJ (2000) Ligand-induced changes in surface mu-opioid receptor number: relationship to G protein activation? J Pharmacol Exp Ther 292:1127-1134.

Zhang J, Ferguson SS, Barak LS, Aber MJ, Giros B, Lefkowitz RJ, Caron MG (1997) Molecular mechanisms of G protein-coupled receptor signaling: role of G protein-

coupled receptor kinases and arrestins in receptor desensitization and resensitization. Receptors Channels 5:193-199.

Zhang J, Ferguson SS, Barak LS, Bodduluri SR, Laporte SA, Law PY, Caron MG (1998) Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. Proc Natl Acad Sci U S A 95:7157-7162.

Zhang L, Yu Y, Mackin S, Weight FF, Uhl GR, Wang JB (1996) Differential mu opiate receptor phosphorylation and desensitization induced by agonists and phorbol esters. J Biol Chem 271:11449-11454.

CHAPTER 1

Agonist-Specific Regulation of μ-Opioid Receptor Desensitization and Recovery from Desensitization

Michael S. Virk[1] & John T. Williams[2]

Vollum Institute

Oregon Health and Science University

3181 SW Sam Jackson Park Road

Portland, OR 97239

Acknowledgements: This work was supported by the NIH (1F30-DA 021466)[1] and (DA 08163)[2] and NARSAD-Ritter Foundation[2]

Running title - Agonist selective MOR regulation

Corresponding author

John T Williams
Vollum Institute, L474
Oregon Health Sciences University
3181 SW Sam Jackson Park Rd
Portland OR 97239
503-494-5465
williamj@ohsu.edu

Text pages	30
Tables	0
Figures	6
References	36
Abstract	208
Introduction	588
Discussion	1475

Abbreviations: MOR - mu-opioid receptor, LC – locus coeruleus, ß-CNA – ß-chlornaltrexamine, ME – [Met]^{5}enkephalin, DAMGO - [D-Ala2,N-Me-Phe4-Gly5-ol]-enkephalin, M6G – morphine-6-glucuronide, GRK2 – G protein receptor kinase-2, PKC – protein kinase C, GIRK – G protein gated inwardly rectifying potassium, UK14304 - 5-bromo-6-[2-imidazolin-2-ylamino]quinoxaline, RAVE – relative activity versus endocytosis

ABSTRACT

Agonist-selective actions of opioids on the desensitization of μ-opioid receptors (MOR) have been well characterized, but few if any studies have examined agonist dependent recovery from desensitization. The outward potassium current induced by several opioids was studied using whole-cell voltage clamp recordings in locus coeruleus (LC) neurons. A brief application of the irreversible opioid antagonist, β-chlornaltrexamine (β-CNA), was applied immediately following treatment of slices with saturating concentrations of opioid agonists. This approach permitted the measurement of desensitization and recovery from desensitization using multiple opioid agonists including, [Met]^{5}enkephalin (ME), DAMGO, etorphine, fentanyl, methadone, morphine, morphine-6-glucuronide, oxycodone and oxymorphone. The results indicate that desensitization protects receptors from irreversible antagonism with β-CNA. The amount of desensitization was measured as the decline in current during a 10 min application of a saturating agonist concentration and was a good predictor of the extent of receptor protection from irreversible inactivation with β-CNA. Following desensitization with ME or DAMGO and treatment with β-CNA, there was an initial profound inhibition of MOR induced current that recovered significantly after 45 min. There was, however, no recovery of MOR-mediated current with time after treatment with agonists that did not cause desensitization, such as oxycodone. These results demonstrate that desensitization prevents irreversible inactivation of receptors by β-CNA.

Opiates are the most effective analgesics known. Activation of the μ-opioid receptor (MOR) belies their therapeutic efficacy as well as the euphoria and rewarding properties that lead to their abuse. Agonist-bound MORs activate G proteins and signal through downstream effectors. They also become substrates for the regulatory machinery responsible for agonist-induced MOR desensitization, endocytosis, and recovery from desensitization (reviewed in Bailey and Connor, 2005; Connor et al., 2004; von Zastrow, 2004; Williams et al., 2001). Different opioid agonists have widely varying signaling efficacies in each of these processes. For example, the highly efficacious endogenous peptide agonist, [Met]^{5}enkephalin (ME), causes both profound desensitization and internalization. However, morphine and some other alkaloid opiates are regarded as partial agonists that induce desensitization and endocytosis to a lesser degree (Alvarez et al., 2002; Borgland et al., 2003; Celver et al., 2004; Dang and Williams, 2005; Johnson et al., 2006; Keith et al., 1998; Koch et al., 2005; Schulz et al., 2004; Whistler and von Zastrow, 1998; Yu et al., 1997).

It is widely speculated that differences in acute MOR regulatory events underlie the profound agonist-selective differences observed in the development of tolerance *in vivo* (Grecksch et al., 2006; Patel et al., 2002; Pawar et al., 2007; Stafford et al., 2001; Walker and Young, 2001; Whistler et al., 1999). There is no consensus, however, on which elements of MOR regulation – signaling efficacy, desensitization, internalization or re-sensitization – are most directly correlated with tolerance (Bailey and Connor, 2005; Bohn et al., 2000; Connor et al., 2004; Koch et al., 2005; von Zastrow, 2004; Whistler et al., 1999; Williams et al., 2001). Understanding how MOR agonists, particularly those

employed in pain management, differ with respect to these fundamental aspects of MOR regulation, particularly in neurons, will contribute to the development of effective analgesic therapy.

Acute MOR regulation is characterized by the receptor-mediated components of desensitization and recovery from desensitization that occur within minutes of agonist exposure. Receptor-specific desensitization is thought to be dependent on agonist binding, phosphorylation and binding to β-arrestin followed by sequestration to clathrin coated pits, and dynamin dependent endocytosis (Connor et al., 2004; von Zastrow, 2004). These rapid receptor specific events are separate from the opioid-induced increase in activity of adenylyl cyclase following 1-2 hours of agonist treatment (Avidor-Reiss et al., 1997). One possible mechanism that may account for differences between DAMGO- and morphine-induced desensitization includes MOR phosphorylation by distinct kinases, GRK2 and PKC, respectively (Johnson et al., 2006). Whether differences in agonist-specific desensitization have an impact on the rate and extent of recovery of MOR signaling remains unclear.

In the present study, several different opioid agonists were used to measure potassium current (GIRK) amplitude, acute desensitization, and recovery from desensitization using whole cell recording from locus coeruleus neurons in brain slices. An experimental protocol that employed treatment of brain slices with the irreversible opioid antagonist β-CNA was used to measure recovery of functional receptors following desensitization. Application of β-CNA resulted in a dramatic inhibition of MOR-mediated current

following the pronounced desensitization induced by some agonists (ME, DAMGO, fentanyl, etorphine and methadone). This inhibition was transient and recovered substantially after 45 min. There was less recovery after treatment with agonists that caused an intermediate amount of desensitization (morphine and morphine-6-glucuronide). Oxycodone or a low concentration of ME (300 nM, EC_{50}) did not cause desensitization nor was there any recovery of signaling after treatment with β-CNA. This suggests that whether or not agonist-specific mechanistic differences govern desensitization, the degree of recovery is directly proportional to the amount of desensitization.

MATERIALS AND METHODS

Tissue Preparation and Recording. Adult (150-250 gm) male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were used for all experiments. Details of the method of slice preparation and recording have been published previously (Williams et al., 1984). Briefly, rats were anesthetized with halothane and killed. The brain was dissected, blocked and mounted in a vibratome chamber in order to cut horizontal slices (260 μm thick) containing locus coeruleus (LC). Slices were cut in cold (4°C) artificial cerebrospinal 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 subsequently incubated in a 25 ml glass tube at 35°C for a minimum of 30 minutes prior to experiments and constantly equilibrated with 95% O_2 / 5% CO_2. Slices were then 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 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. Data was collected with PowerLab (Chart version 4.2.3) and sampled at 100 Hz. Analysis was performed with Prism and Kaleidagraph software. Values are presented as arithmetic mean ± S.E.M. One-way ANOVA was performed and differences for which $p < 0.05$ were considered significant.

Drugs. Drugs were applied by bath superfusion. The following drugs were superfused: [Met^5]-enkephalin, DAMGO, oxycodone, UK14304, yohimbine, bestatin, thiorphan (Sigma), etorphine, methadone, fentanyl, oxymorphone, morphine, morphine-6-glucuronide (NIDA – Neuroscience Center), β-CNA (Tocris). Some compounds (UK14304, thiorphan, and β-CNA) were dissolved in dimethyl sulfoxide, ethanol or methanol. The final concentrations of these solvents did not exceed 0.01% DMSO, 0.00001% ethanol, 0.05% methanol, respectively. All other drugs were dissolved in water.

RESULTS

Protection from antagonist binding and recovery from desensitization

A saturating concentration of ME (30 μM) evoked an outward current that desensitized rapidly over the course of a 10-min application, as has been shown previously (Fig 1, (Fiorillo and Williams, 1996; Harris and Williams, 1991; Osborne and Williams, 1995). Peak currents measured 461±28 pA (n=17) and declined to 65±1% of the peak (295±18 pA) after 10 min. Following wash out of the desensitizing stimulus (ME, 30 μM, 10 min), short pulses of ME (30 μM) were applied after 5 and 45 minutes to assay recovery from desensitization (Fig 1A). The current activated by ME recovered from 65±1% at the end of the desensitization period to 75±2% at 5 min and 96±6% at 45 min (n=7). Thus, as reported previously, recovery from desensitization was complete by 45 minutes (Dang and Williams, 2004). A saturating concentration of the alpha-2-adrenergic agonist UK-14304 (3 μM) was superfused at the conclusion of the each experiment in order to activate the same potassium conductance by another G-protein coupled receptor. This allowed for the comparison between multiple opioid agonists that evoked different maximum currents and to detect heterologous desensitization.

To determine if recovery from desensitization involved re-activation of desensitized receptors, the irreversible opioid antagonist β-CNA was applied immediately after the desensitizing agonist application. In this experiment, ME (30 μM) was applied for 10 min followed by treatment with β-CNA (500 nM, 2 min, Fig 1B). As in the control experiment, two pulses of ME (30 μM) were applied 5 and 45 min after the end of the desensitizing agonist application. At 5 min, the current measured 10±2% (n=10) of the initial ME-induced current and recovered to 43±2% by 45 min. The two test-pulses

measured activation of receptors that were no longer desensitized and that were also protected from β-CNA binding.

Three additional experiments were performed to characterize the interaction between desensitization and antagonism by β-CNA (Fig 2). In all experiments test-pulses of ME (30 μM) were applied at 5 and 45 min to assay the state of MOR signaling. The current induced by the test pulses was then expressed as a percentage of the peak alpha-2-adrenergic-mediated current. In the first experiment (Fig 2A), β-CNA (500 nM) was tested in the absence of a prior agonist application. In this experiment, the current amplitude induced by ME (30 μM) after 5 min was 40±5% of the current induced by UK14304, indicating that the short treatment with β-CNA blocked a significant number of receptors. When the second ME (30 μM) test-pulse was applied after 45 min there was a further *decrease* in the current amplitude to 31±3% (total change, -9±2%; n=7). The decrease in current suggests that β-CNA remaining in the slice continued to react with MORs after the first test-pulse was delivered, such that more receptors were removed by the time of the second test-pulse. Therefore, the increase in test-pulse current amplitude that was observed following ME-induced desensitization (30 μM/10 min) and treatment with β-CNA was an underestimate of the total extent of desensitization-induced protection and, ultimately, of MOR recovery (Fig 1B).

In the second experiment ME (300 nM), an EC-50 concentration, was superfused for 10 minutes before application of β-CNA (500 nM, 2 min; Fig 2B). The peak current amplitude was 214±19 pA (n=8) and was 204±20 pA after 10 min, indicating that no

significant desensitization occurred. Test-pulses at 5 and 45 min following treatment with β-CNA were unchanged at 37±4% and 33±4% (total change, -3±3%). The results of this experiment illustrate two important measurements: first, the amplitude of the current induced by ME test-pulses (5 and 45 min) were the same as those observed in the experiment when β-CNA was applied without prior exposure to ME (Fig 2A), and second, there was no increase between the current induced by the first and second test pulse. Thus, without prior desensitization, there was no protection from β-CNA.

In the third experiment, a short application of high concentration ME (30 μM) was applied for 45 seconds before β-CNA (500 nM, 2 min, Fig 2C). This short treatment has been previously shown to induce a moderate amount of desensitization (Dang, Williams, 2004). The peak current amplitude induced by this brief application period was 400±62 pA. After β-CNA, the amplitude of the current induced by ME test-pulses was 18±5% of the UK14304 current at 5 min and increased to 34±5% after 45 min (total recovery 15±1%; n=6). The total recovery of 15±1% was considerably less than the 42±4% (12±3% to 53±4%) following the 10 min application of ME (30 μM).

The summarized results are presented in figure 2D, where the amplitude of the current induced by the two tests with ME (30 μM) are plotted as a percentage of the current induced by UK14304 (3 μM). The results show that without desensitization there was a decrease, or no change, in the relative amplitude of the current induced by ME measured at 5 and 45 min (Fig 2D, label A -9±2%; label B, -3±3%). Test-pulses applied at these two time points served as a measure of MOR re-sensitization. With increasing amount of

desensitization, the current induced by the ME test-pulse at 5 min was depressed and there was more recovery of the ME induced current after 45 min (Fig 2D, label ME+CNA 42±4%; label C, 15±1%). Taken together these results suggest that ME-induced desensitization protected MORs from irreversible antagonism by β-CNA and that as MOR desensitization increased, the amount of re-sensitization measured also increased.

Recovery from desensitization using other opioid agonists

Several opioid agonists were tested to compare agonist-induced desensitization and recovery specific to each compound (Fig 3). For each agonist, desensitization and recovery were measured as described in the previous section: a saturating concentration was applied for 10 min ("desensitizing stimulus"), followed by β-CNA (500 nM, 2 min), and the recovery from desensitization was assayed with test-pulses of ME (30 μM) at 5- and 45-min following the end of the desensitizing stimulus. Test-pulse amplitudes were expressed as a percentage of the peak UK-14304 current (Fig 3F). DAMGO (10 μM) evoked a peak current of 549±68 pA (n=5) that desensitized to 62±5% of peak after 10 min. Test-pulse amplitudes increased by 37±6% during the 45 min recovery period (from 9±2% at 5 min to 46±4% at 45 min; Fig 3A). Although an accurate measurement of desensitization induced by methadone (15 μM, 10 min) was not possible due to slow rise to peak, test pulse amplitudes following the desensitizing stimulus increased by 28±1% (from 12±2 to 40±3%; n=5; Fig 3B). This change was smaller than that observed with ME or DAMGO, but was larger than any other alkyloid agonists tested.

Morphine (15 μM/10 min) and M-6-G (15 μM/10 min) evoked peak currents of 263±24 pA (n=7) and 254±45 pA (n=6) that desensitized by 25±5% and 23±2%, respectively. Moreover, the two alkyloid agonists were similar with respect to the recovery from desensitization, as test-pulses following morphine desensitization increased by 17±2% (from 47±4% at 5 min to 64±4% at 45 min), while those following M-6-G increased by 13±5% (5min, 39±2% and 45 min, 53±6%). The peak current induced by oxycodone (15 μM, 278±29 pA; n=8) was the same as that induced by morphine and M-6-G, however it failed to desensitize after 10 min (1±2%). Likewise, there was no significant change between the test-pulse delivered at 5 min (45±4%) and 45 min (47±3%). Thus, despite evoking a large GIRK current, there was no evidence that oxycodone induced any MOR desensitization.

Desensitization using fentanyl, etorphine and oxymorphone

Fentanyl (10 μM/10 min), etorphine (1 μM/10 min), and oxymorphone (15 μM/10 min) were also used as desensitizing agonists. All three agonists evoked large outward currents with rapid onset and subsequent desensitization. Fentanyl (10 μM/10 min) evoked a peak current of 338±24 pA (n=16) that desensitized to 70±3% of the peak; etorphine (1 μM) evoked a peak current of 359±25 pA (n=9) that desensitized to 74±1% of the peak; and oxymorphone (15 μM/10 min) evoked a 348±54 pA (n=6) current that desensitized to 69±2% of the peak. Measuring recovery with these drugs in the brain slice preparation presented challenges that excluded them from the same analysis as the other ligands. In order to reverse the current evoked by these agonists, the concentration of β-CNA (1 μM, 2 min) was increased, while the duration was kept the same. This

treatment was sufficient to reverse the fentanyl-induced current (Fig 4A,C), however, reversal was incomplete for etorphine (Fig 4D). This concentration of β-CNA (1 μM, 2 min) had almost no effect on the current induced by oxymorphone (not shown).

In experiments with fentanyl and etorphine, following wash of β-CNA, an outward current developed in the absence of any applied agonist (Fig 4A,C,D). In control experiments where slices were not treated with β-CNA, a stable outward current was maintained for 45 min, indicating that the high affinity, lipophilicity and efficacy of these agonists sustained signaling (Fig 4B, supplemental Fig 1). Thus the increase in outward current that followed treatment with β-CNA resulted from re-activated receptors by agonist that remained present in the slice, suggesting that the desensitization induced by these agonists resulted in protection from β-CNA. As receptors recovered from desensitization, a sufficient concentration of each drug remained in the slice to activate those receptors. The recovery of the outward current is therefore similar to the recovery observed after desensitization induced by other agonists that was measured using the ME test-pulse protocol. While the amount of recovery is complicated by the fact that the agonist was present as the receptors recover from desensitization, it was possible to obtain a rough estimate of the rate of recovery. This rate was determined by fitting the increase in outward current to a single exponential to estimate a time constant. The time constant for recovery after desensitization with fentanyl was 7.1±0.6 min (n=5) and for etorphine was 4.6±1.1 min (n=4). This suggests that there is a fast phase of MOR recovery, analogous to the fast receptor desensitization that results from saturating concentrations of agonist applied for 10 minutes.

Summary

The results show that each agonist caused a different maximal activation of outward GIRK current and capacity to induce desensitization (Fig 5A). ME and DAMGO induced the largest outward currents and caused the greatest amount of desensitization, whereas morphine and M-6-G are less effective at both. Oxycodone stands alone in that it evoked a current as large as that induced by morphine and M-6-G, but induced no detectable desensitization (Fig 5A). In fact, the results with oxycodone were comparable to the experiments using the EC_{50} concentration of ME (300 nM/10min) in that there was no desensitization (Fig 5A). The concentration of oxycodone (15 μM) was saturating since the outward current induced by concentrations ranging from 10-30 μM were the same (% of the maximum UK14304 current; 10 μM 68±3.4%, n=6, 15 μM 72±6.4% n=6, 30 μM 69±3.2 n=6). When agonists were compared based on the degree of desensitization and recovery measured by the two ME test-pulses delivered at 5 minutes (ME pulse 1) and 45 minutes (ME pulse 2, Fig 5B), the two measurements correlated well.

DISCUSSION

The development of a protocol using the irreversible MOR antagonist, β-CNA, made the acquisition of the present results possible. Saturating concentrations of alkyloid agonists normally require an extended duration to wash from brain slices such that measuring the recovery from desensitization was not previously possible. The present results suggest

that recovery from desensitization may be near complete within 15 min following the end of the desensitizing treatment. The use of β-CNA reduced the number of active receptors. Following a 10-minute application of ME or DAMGO, the reduction in active receptors by β-CNA resulted in a dramatic inhibition of ME current induced at the 5 min test point. The only way that the test-pulse amplitude could increase after 45 min following β-CNA treatment is if un-occupied receptors became available throughout this period. Desensitization therefore resulted in a state where β-CNA was not able to bind to MORs, perhaps due to receptor endocytosis or because desensitized receptors have an increased affinity for receptor bound agonist. Finally, β-CNA likely remained in the slice and continued to inactivate MORs beyond the initial 2-minute application because in β-CNA control experiments (no prior desensitizing stimulus) the current induced by ME at the 45 min test was smaller than that measured after 5 min (Fig 2A). Thus, the measurement of MOR recovery from desensitization was likely an underestimate.

The results suggest that treatment with β-CNA resulted in a distribution of receptors into three possible configurations: free/unbound, irreversibly inactivated by β-CNA and desensitized/internalized (Fig 6). The treatment with β-CNA was short enough that not all receptors were inactivated. This was demonstrated in experiments where β-CNA was applied without a prior desensitizing stimulus and subsequent ME test-pulses resulted in a reduced but measurable current (Fig 2A). The depression of the maximum current indicated that a substantial pool of receptors was inactivated by β-CNA while others remained unbound (Christie et al., 1986). The third pool of receptors were desensitized and protected from binding to β-CNA and were thus capable of recovery and subsequent

activation. This pool of receptors may have been agonist bound and desensitized on the plasma membrane and therefore neither capable of signaling nor available for binding to β-CNA. It is also possible that desensitized receptors were internalized and thus physically inaccessible to β-CNA. Without high resolution imaging of the receptors it is not possible to distinguish the two possibilities. In cultures of mouse LC neurons, however, desensitization or the recovery from desensitization was not changed after blockade of internalization of a fluorescent opioid agonist with concanavalin A (Arttamankgul et al., 2006). This experiment indicated that internalization was not required for desensitization and suggests that the results using β-CNA could result from a process where receptors remain on the plasma membrane.

The degree of MOR recovery was directly related to the amount of initial desensitization prior to treatment of the tissue with β-CNA. Furthermore, the rank order of this series of agonists in the recovery process correlates with their ability to induce receptor internalization (Keith et al., 1998; Koch et al., 2005). In either case, within 45 min the pool of receptors recovered to a state that permitted agonist activation.

The results of this study indicate that the peak amplitude of the current evoked by a saturating concentration of several agonists generally correlated with the amount of acute desensitization (Fig 5). DAMGO and ME evoked the largest current and induced the greatest amount of desensitization, whereas the smaller maximum current induced by morphine and M-6-G caused significantly less desensitization. These results are in agreement with those reported previously in heterologous systems (Yu et al., 1997).

There were, however, two notable exceptions illustrated in experiments using either a saturating concentration of oxycodone (15 μM) or a low concentration of ME (300 nM). Oxycodone and ME (300 nM) evoked currents similar in amplitude to morphine and M-6-G, but induced no desensitization. Thus, the amount of desensitization induced by many, but not all, agonists can be predicted by the efficacy in activation of the GIRK conductance.

These results demonstrate that the amount of acute desensitization induced by a saturating concentration of any given agonist can be used to predict the amount of recovery from desensitization. When there is more desensitization, more recovery was obtained. While the degree of agonist-specific desensitization may be governed by different mechanisms, the present results indicate that MOR re-sensitization is directly related to the degree of desensitization. Moreover, the temporal component of these experiments suggests that there is a rapid phase of receptor re-sensitization that is analogous to rapid MOR desensitization. Desensitization was induced by a short agonist exposure (10 min) and the rate of recovery in the fentanyl and etorphine experiments was greatest in the first 15 min. These observations likely reflect acute MOR regulatory processes.

This is in contrast to the results from other studies showing that morphine and DAMGO induced the same degree of MOR desensitization, but that morphine-exposed receptors failed to recover after 60 min while DAMGO-treated receptors recovered completely after 40 minutes (Koch et al., 2004; Schulz et al., 2004). While our results agree

qualitatively in that MORs recover to a greater extent following DAMGO exposure than following morphine exposure, important differences prohibit direct comparison of our results. One significant difference between the present results and those obtained in HEK 293 cells expressing MORs was the duration of agonist exposure. The desensitization induced by a 4-hour exposure used in experiments with the HEK293 cells may have resulted in downstream adaptations that decreased signaling rather than direct receptor dependent desensitization observed in the present study. It is possible that these experiments address separate phenomena.

Oxycodone is different

Oxycodone is a frequently prescribed opiate analgesic used to control moderate to severe pain. It has approximately the same lipophilicity as morphine (partition coefficients of 0.91 and 1.07, respectively), but lower MOR affinity (K_i,1.7±0.5 and 43.9±7 nM) (Peckham and Traynor, 2006). Oxycodone has about the same efficacy as morphine as determined with by a GTPγS stimulation assay in rat thalamic brain slices (36.6±4.9 and 42.8±5.3% of the DAMGO induced activation, respectively)(Peckham and Traynor, 2006). Its analgesic efficacy is likely the consequence of high bio-availability, as well as the potency, affinity, and efficacy of its primary active metabolite, oxymorphone (Lemberg et al., 2006). Results presented here, show that oxymorphone evoked a large outward current that desensitized extensively while oxycodone-evoked currents are similar to morphine, but, in contrast to all other agonists tested, did not desensitize at all. Thus, the properties of both oxycodone and oxymorphone must be taken into account for

experiments involving chronic treatment via systemic administration because the properties of each are so different.

With respect to efficacy, desensitization and recovery, oxycodone is most similar to low concentration ME (300 nM), not morphine or M6G. The significance of this deviation is that oxycodone-bound MORs may elicit robust GIRK signaling, but somehow manage to elude desensitization machinery. It is the only agonist tested here capable of discriminating between these elements of acute agonist-specific MOR signaling and receptor regulation. Furthermore, it has been reported that oxycodone and morphine trigger the same amount of internalization in HEK 293 cells (Koch et al., 2005), though it remains unknown how the two drugs compare with respect to internalization in neurons. If oxycodone triggered less endocytosis than morphine in neurons, in the same way that it induced significantly less desensitization, it may prove to be an important tool for experiments testing the Relative Activity Versus Endocytosis (RAVE) hypothesis (Whistler et al., 1999).

Receptor number and ME concentration affect desensitization

The three experiments done with ME indicate that a saturating concentration is required in order to induce desensitization (Figs 2,6). A saturating concentration of ME (30 μM) induced desensitization to approximately 65% of peak current amplitude after 10 minutes. When an EC_{50} concentration of ME (300 nM) was used, the amplitude of the peak current was about 50% of that induced by a saturating ME (30 μM) concentration (215 pA and 460 pA, respectively), and this caused no desensitization (95% current

remaining after 10 min). These results demonstrate that desensitization is dependent on both receptor occupancy and receptor number, as has been shown for MOR and other G-protein coupled receptors (Law et al., 2000; Zhang et al., 1997).

Conclusions

Several strategies are currently used in the clinic to effectively manage pain and to treat opiate addiction. Regardless of the indication or treatment desired, tolerance is the central complication in opiate therapy, because compensation by dose escalation often results in toxic consequences. It is now appreciated that agonist-specific differences in the development of tolerance *in vivo* are profound and that there are cellular correlates of agonist-specific MOR regulation. Effective therapy can be maximized only by creating a thorough pharmacological profile of each opiate agonist, knowing how each differentially regulates MOR, and understanding how differential MOR regulation influences the development of tolerance.

FOOTNOTES

This work was supported by the NIH, 1F30-DA 021466 (MSV) and DA 08163 (JTW) and NARSAD-Ritter Foundation (JTW)

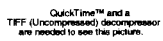


Figure 1. Treatment with β-CNA following ME-induced desensitization decreased the absolute MOR mediated current but did not inhibit recovery from desensitization. A, Current trace from control experiment where a saturating concentration of ME (30 μM, 10 min) resulted in an outward current that peaked and declined during the application. Following the wash ME (30 μM) was applied at 5 and 45 min. The resulting current partially recovered after 5 min and recovered completely after 45 min. At the end of the experiment the alpha-2-adrenoceptor agonist, UK14304 (3 μM) was applied to control for changes in the recording after the prolonged washout. B, β-CNA (500 nM, 2 min) was applied immediately after ME (30 μM, 10 min) induced desensitization and recovery was again measured with ME test-pulses at 5 and 45 min. In this experiment the current induced by ME (30 μM) was almost eliminated after 5 min and recovered significantly after 45 min.

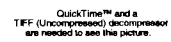


Figure 2. The amount of desensitization determines the degree of MOR protection from β-CNA blockade. A, Current trace of a control experiment where β-CNA (500 nM, 2 min) was applied without prior treatment of the preparation with ME. Application of ME (30 μM) at 5 and 45 min following the wash out resulted in current that decreased after 45 min. At the end of the experiment UK14304 (3 μM) was applied such that the currents induced by ME could be normalized. B, An experiment using an EC_{50} concentration of ME (300 nM, 10 min) applied prior to treatment with β-CNA. In this case the amplitude of the current induced by ME (30 μM) after 5 and 45 min did not change. C, An experiment where a saturating concentration of ME (30 μM) was applied for only 45 sec, followed by treatment with β-CNA (500 nM, 2 min). In this case the amplitude of the ME (30 μM) current increased between the 5 and 45 min test points, but not to the same extent as was observed with a longer (10 min) ME desensitizing application. D, Summarized results after treatment of slices with β-CNA (500 nM, 2 min) showing the change in the size of the current induced by the first test-pulse (5 min, gray bar) and the second test-pulse (45 min, black bar) of ME (30 μM) in different experiments. The current amplitudes were normalized to the current induced by UK14304 that was measured at the end of each experiment. The bars labeled ME+CNA are taken from the experiments illustrated in Figure 1B; CNA control illustrated in trace A; ME300nM/10min illustrated in trace B; ME30μM/45s illustrated in trace C. The * over the bars indicates a significant difference between the amplitude of the current induced by ME (30 μM) on first (5 min) and second (45 min) test-pulse ($p<0.05$). The only experiment where there was no difference between the two pulses was low concentration ME (EC_{50}, 300nM/10min) (B). The only experiment where there was a *decrease* between the two pulses was without prior ME treatment (A).

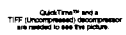


Figure 3. Agonist-selective protection from blockade by β-CNA. The protocol used for these experiments was the same of each agonist and is identical to that illustrated in Figure 1B. The indicated agonist (A-E) was applied at a saturating concentration for 10 min, followed by treatment with β-CNA (500 nM, 2 min) and then test-pulses of ME (30 μM, 2 min) were applied after 5 and 45 min. At the end of each experiment UK14304 (3 μM) was applied and used to normalize the opioid currents. F, A summary of the results obtained for each agonist. The * over the bars indicates a significant difference between the amplitude of the current induced by ME (30 μM) on first (5 min) and second (45 min) test-pulse ($p<0.05$). The only agonist where there was no difference between the two pulses was oxycodone.

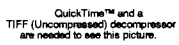


Figure 4. Desensitization with fentanyl and etorphine protects receptors from blockade by β-CNA. A, Fentanyl (FEN, 10 μM) was applied for 10 min followed by treatment with β-CNA (1μM, 2 min). The current induced by fentanyl declined during the 10 min application and was almost completely reversed after treatment with β-CNA. Following the washout of β-CNA an outward current was observed that was blocked by naloxone (NLX, 1 μM). B, A control experiment showing that the outward current induced by fentanyl did not decline upon washout. C, Summarized results of experiments using fentanyl with and without β-CNA (1 μM/2 min). D, Summarized results from experiments using etorphine (1 μM/10 min) in the same experimental protocol as with fentanyl.

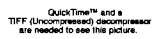


Figure 5. The amplitude of the current induced by agonists does not always correlate with the ability to induce desensitization, however, the extent of desensitization and the recovery from desensitization do correlate. A, Summary of results plotting the amount of desensitization as a function of the mean current induced by a series of agonist; 1 – M6G, 2 – morphine, 3 – fentanyl, 4 – oxymorphone, 5 – etorphine, 6 – ME, 7 – DAMGO, Oxyc – oxycodone, ME-L – ME (EC_{50}, 300 nM). B, The pulse2/pulse1 ratio of the current induced by test-pulses of ME (30 μM) plotted as a function of the amount of desensitization induced by a series of agonists.

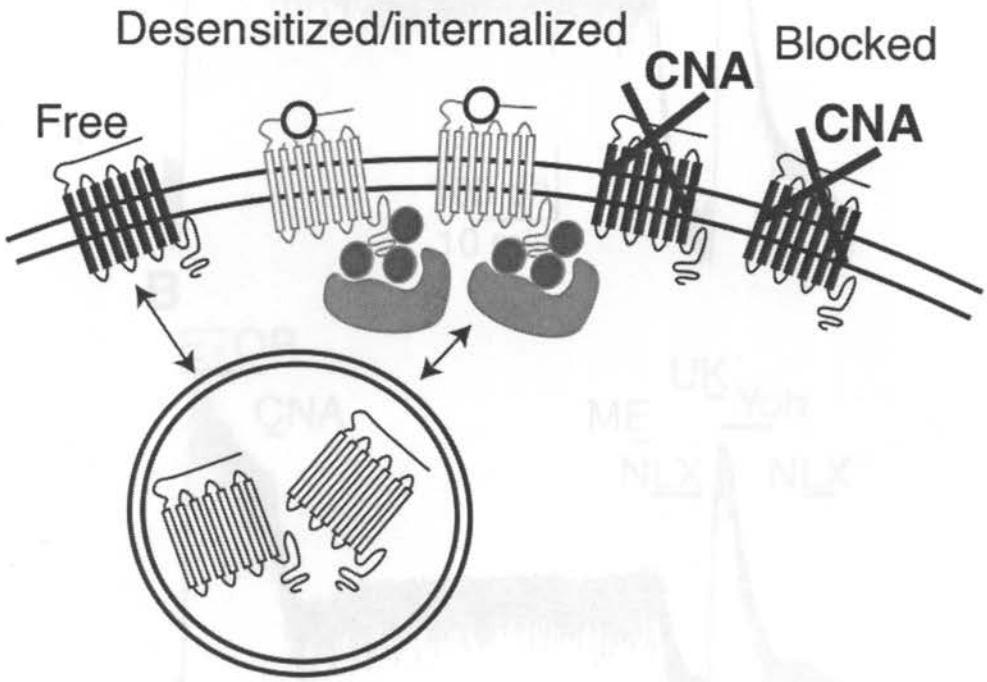
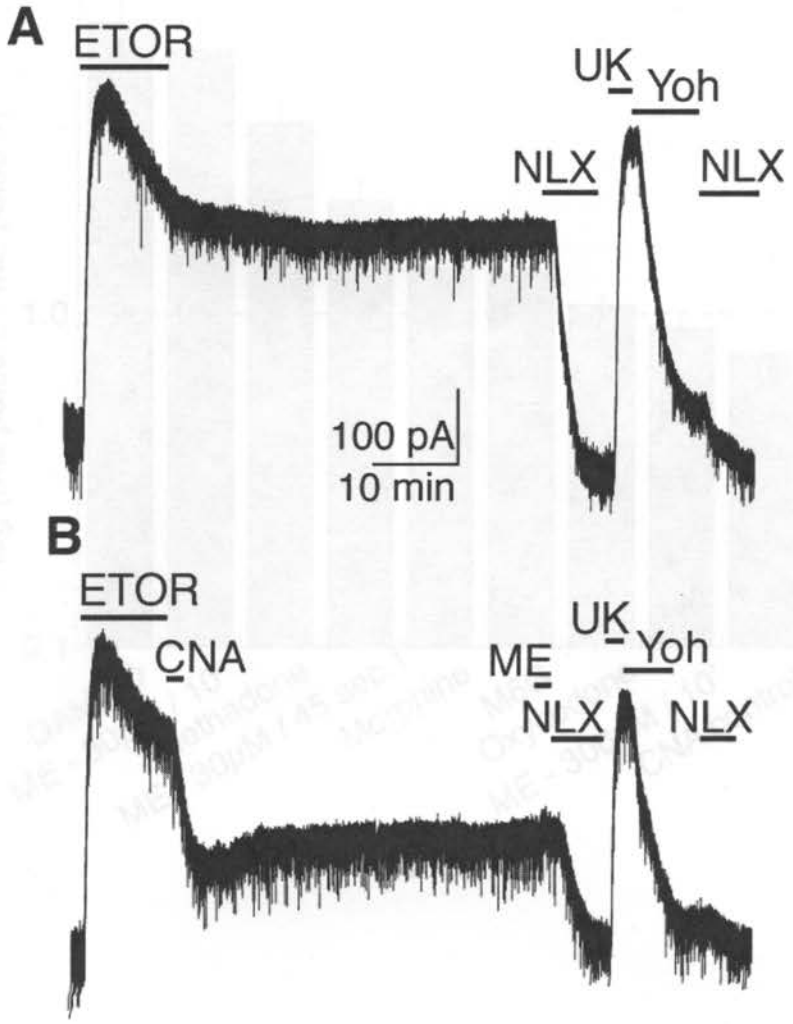
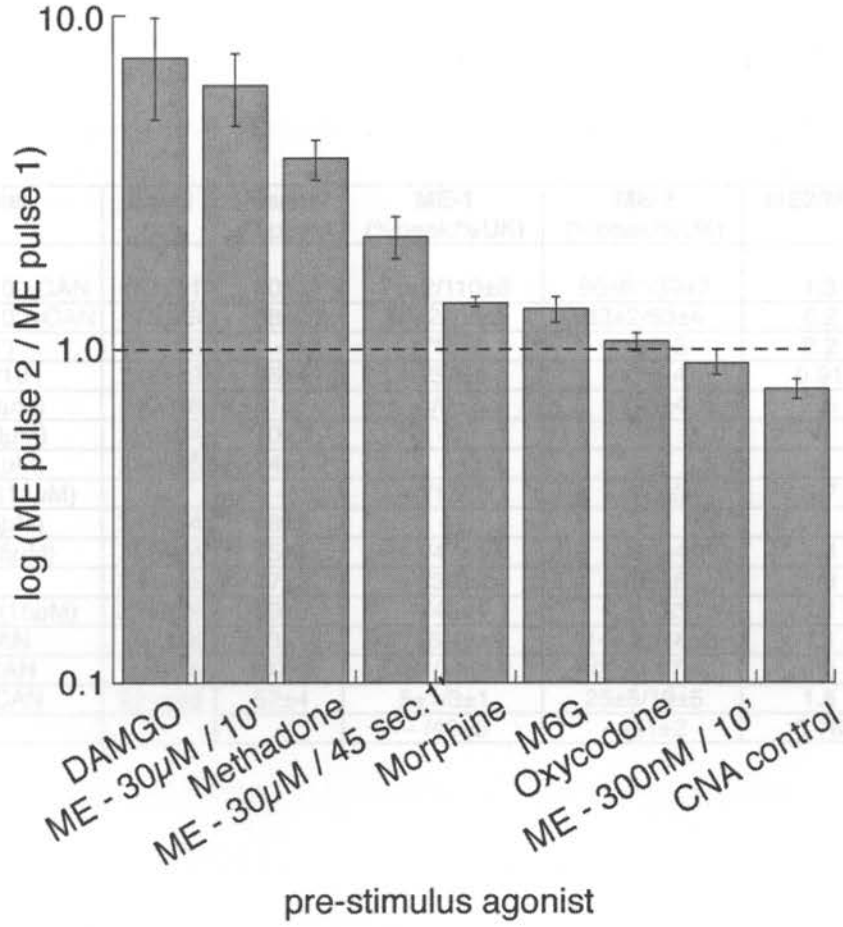


Figure 6. A schematic illustrating the three pools of MORs resulting from desensitization, treatment with β-CNA, and recovery from desensitization. A significant number of receptors are irreversibly bound to β-CNA. The pools of active/free receptors and those that are desensitized or internalized vary based on the agonist used for desensitization and the time (5 or 45 min) after the induction of desensitization.



Supplemental Figure 1. Rcovery from desensitization induced by etorphine. A, control experiment where etorphine (1 μM) was applied for 10 min. Following the washout there was no decline in current until the application of naloxone (1 μM). B, application of ß-CNA (1 μM, 2 min) resulted in a decrease in the outward current that paritally recovered.



Supplemental Figure 2. A direct comparison of agonist selective protection from blockade by ß-CNA. Summarized results showing the ratio of the current induced by ME (30 μM) 45 min (pulse 2) divided by the current induced 5 min (pulse 1) after the end of the 10 min agonist application. The ratio is plotted on a log scale and is greatest for DAMGO, ME and methadone, is greater than 1 for morphine and morphine 6-glucuronide (M6G) and is 1 for oxycodone. Ideally a ratio of 1 (no change in current) indicates that there was no protection from binding with ß-CNA. Given that the ratio is less than one in the experiment where no agonist was applied, a ratio one (found with oxycodone treatment) may suggest a small amount of protection.

Agonist	Ipeak (pA)	Desens. (%peak)	ME-1 (%peak/%UK)	ME-2 (%peak/%UK)	ME2/ME1
ME (30μM/10')-CAN	453±41	60±2	75±2/110±6	96±6/139±7	1.3
ME (30μM/10')+CAN	475±49	68±2	10±2/12±3	43±2/53±4	6.2
ME (30μM/1')	401±62	-	- /18±5	- /34±5	2.2
ME (300nM/10')	215±19	96±4	- /37±4	- /33±4	0.91
DAMGO (10μM)	549±68	61±5	- /9±2	- /46±4	7.5
Fentanyl (10μM)	339±24	70±3	-	-	-
Etorphine (1μM)	359±25	74±1	-	-	-
Methadone (15μM)	-	-	- /12±2	- /40±3	3.7
Oxymor. (15μM)	348±54	69±2	-	-	-
Morphine (15μM)	263±24	75±5	- /47±4	- /64±4	1.4
M-6-G	254±45	77±2	- /39±2	- /53±6	1.3
Oxycodone (15μM)	278±29	99±3	- /45±4	- /47±3	1.1
Bup-ME/ -CAN	182±18	103±10	97±6/43±2	114±13/48±2	1.1
Bup-ME/ +CAN	166±18	107±6	52±10/20±2	102±9/47±2	2.5
CAN-ME/ +CAN	221±48	52±4	5±1/3±1	25±5/19±5	1.5
control	-	-	- /40±5	- /31±2	0.76

Table 1. Summary data for agonist tested. Agonist and/or experimental condition is listed in Agonist column. Ipeak lists peak GIRK current induced by agonist (pA). Desensitization induced is expressed as a percentage of the peak current remaining after 10', unless otherwise stated. ME-1 and ME-2 aer test-pulse current amplitudes expressed as a percentage of the peak desensitizing stimuls or as a percentage of IUK. The ratio of ME-2/ME-1 is listed in the last column.

REFERENCES

Alvarez VA, Arttamangkul S, Dang V, Salem A, Whistler JL, Von Zastrow M, Grandy DK and Williams JT (2002) mu-Opioid receptors: Ligand-dependent activation of potassium conductance, desensitization, and internalization. *J Neurosci* **22**(13):5769-5776.

Avidor-Reiss T, Nevo I, Saya D, Bayewitch M and Vogel Z (1997) Opiate-induced adenylyl cyclase superactivation is isozyme-specific. *J Biol Chem* **272**(8):5040-5047.

Bailey CP and Connor M (2005) Opioids: cellular mechanisms of tolerance and physical dependence. *Curr Opin Pharmacol* **5**(1):60-68.

Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ and Caron MG (2000) Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* **408**(6813):720-723.

Borgland SL, Connor M, Osborne PB, Furness JB and Christie MJ (2003) Opioid agonists have different efficacy profiles for G protein activation, rapid desensitization, and endocytosis of mu-opioid receptors. *J Biol Chem* **278**(21):18776-18784.

Celver J, Xu M, Jin W, Lowe J and Chavkin C (2004) Distinct domains of the mu-opioid receptor control uncoupling and internalization. *Mol Pharmacol* **65**(3):528-537.

Christie MJ, Williams JT and North RA (1986) Tolerance to opioids in single locus coeruleus neurons of the rat. *NIDA Res Monogr* **75**:591-594.

Connor M, Osborne PB and Christie MJ (2004) Mu-opioid receptor desensitization: is morphine different? *Br J Pharmacol* **143**(6):685-696.

Dang VC and Williams JT (2004) Chronic morphine treatment reduces recovery from opioid desensitization. *J Neurosci* **24**(35):7699-7706.

Dang VC and Williams JT (2005) Morphine-induced {micro}-opioid receptor desensitization. *Mol Pharmacol.*

Fiorillo CD and Williams JT (1996) Opioid desensitization: interactions with G-protein-coupled receptors in the locus coeruleus. *J Neurosci* **16**(4):1479-1485.

Grecksch G, Bartzsch K, Widera A, Becker A, Hollt V and Koch T (2006) Development of tolerance and sensitization to different opioid agonists in rats. *Psychopharmacology (Berl)* **186**(2):177-184.

Hanks GW, Conno F, Cherny N, Hanna M, Kalso E, McQuay HJ, Mercadante S, Meynadier J, Poulain P, Ripamonti C, Radbruch L, Casas JR, Sawe J, Twycross RG and Ventafridda V (2001) Morphine and alternative opioids in cancer pain: the EAPC recommendations. *Br J Cancer* **84**(5):587-593.

Harris GC and Williams JT (1991) Transient homologous mu-opioid receptor desensitization in rat locus coeruleus neurons. *J Neurosci* **11**(8):2574-2581.

Johnson EA, Oldfield S, Braksator E, Gonzalez-Cuello A, Couch D, Hall KJ, Mundell SJ, Bailey CP, Kelly E and Henderson G (2006) Agonist-selective mechanisms of mu-opioid receptor desensitization in human embryonic kidney 293 cells. *Mol Pharmacol* **70**(2):676-685.

Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV, Monteillet-Agius G, Stewart PL, Evans CJ and von Zastrow M (1998) mu-Opioid receptor

internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. *Mol Pharmacol* **53**(3):377-384.

Koch T, Brandenburg LO, Liang Y, Schulz S, Beyer A, Schroder H and Hollt V (2004) Phospholipase D2 modulates agonist-induced mu-opioid receptor desensitization and resensitization. *J Neurochem* **88**(3):680-688.

Koch T, Widera A, Bartzsch K, Schulz S, Brandenburg LO, Wundrack N, Beyer A, Grecksch G and Hollt V (2005) Receptor endocytosis counteracts the development of opioid tolerance. *Mol Pharmacol* **67**(1):280-287.

Law PY, Erickson LJ, El-Kouhen R, Dicker L, Solberg J, Wang W, Miller E, Burd AL and Loh HH (2000) Receptor density and recycling affect the rate of agonist-induced desensitization of mu-opioid receptor. *Mol Pharmacol* **58**(2):388-398.

Lemberg KK, Kontinen VK, Siiskonen AO, Viljakka KM, Yli-Kauhaluoma JT, Korpi ER and Kalso EA (2006) Antinociception by spinal and systemic oxycodone: why does the route make a difference? In vitro and in vivo studies in rats. *Anesthesiology* **105**(4):801-812.

Mercadante S (2007) Opioid titration in cancer pain: A critical review. *Eur J Pain.*

Muller-Busch HC, Lindena G, Tietze K and Woskanjan S (2005) Opioid switch in palliative care, opioid choice by clinical need and opioid availability. *Eur J Pain* **9**(5):571-579.

Osborne PB and Williams JT (1995) Characterization of acute homologous desensitization of mu-opioid receptor-induced currents in locus coeruleus neurones. *Br J Pharmacol* **115**(6):925-932.

Patel MB, Patel CN, Rajashekara V and Yoburn BC (2002) Opioid agonists differentially regulate mu-opioid receptors and trafficking proteins in vivo. *Mol Pharmacol* **62**(6):1464-1470.

Pawar M, Kumar P, Sunkaraneni S, Sirohi S, Walker EA and Yoburn BC (2007) Opioid agonist efficacy predicts the magnitude of tolerance and the regulation of mu-opioid receptors and dynamin-2. *Eur J Pharmacol* **563**(1-3):92-101.

Peckham EM and Traynor JR (2006) Comparison of the antinociceptive response to morphine and morphine-like compounds in male and female Sprague-Dawley rats. *J Pharmacol Exp Ther* **316**(3):1195-1201.

Schulz S, Mayer D, Pfeiffer M, Stumm R, Koch T and Hollt V (2004) Morphine induces terminal micro-opioid receptor desensitization by sustained phosphorylation of serine-375. *Embo J* **23**(16):3282-3289.

Stafford K, Gomes AB, Shen J and Yoburn BC (2001) mu-Opioid receptor downregulation contributes to opioid tolerance in vivo. *Pharmacol Biochem Behav* **69**(1-2):233-237.

von Zastrow M (2004) A cell biologist's perspective on physiological adaptation to opiate drugs. *Neuropharmacology* **47 Suppl 1**:286-292.

Walker EA and Young AM (2001) Differential tolerance to antinociceptive effects of mu opioids during repeated treatment with etonitazene, morphine, or buprenorphine in rats. *Psychopharmacology (Berl)* **154**(2):131-142.

Whistler JL, Chuang HH, Chu P, Jan LY and von Zastrow M (1999) Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* **23**(4):737-746.

Whistler JL and von Zastrow M (1998) Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proc Natl Acad Sci U S A* **95**(17):9914-9919.

Williams JT, Christie MJ and Manzoni O (2001) Cellular and synaptic adaptations mediating opioid dependence. *Physiol Rev* **81**(1):299-343.

Williams JT, North RA, Shefner SA, Nishi S and Egan TM (1984) Membrane properties of rat locus coeruleus neurones. *Neuroscience* **13**(1):137-156.

Yu Y, Zhang L, Yin X, Sun H, Uhl GR and Wang JB (1997) Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. *J Biol Chem* **272**(46):28869-28874.

Zhang J, Ferguson SS, Barak LS, Aber MJ, Giros B, Lefkowitz RJ and Caron MG (1997) Molecular mechanisms of G protein-coupled receptor signaling: role of G protein-coupled receptor kinases and arrestins in receptor desensitization and resensitization. *Receptors Channels* **5**(3-4):193-199.

CHAPTER 2

Differential μ-Opioid Receptor Regulation Following Chronic Treatment with Different Opiate Agonists

Michael S. Virk[1] & John T. Williams[2]

Vollum Institute

Oregon Health and Science University

3181 SW Sam Jackson Park Road

Portland, OR 97239

Acknowledgements: This work was supported by the NIH (1F30-DA 021466)[1] and (DA 08163)[2] and NARSAD-Ritter Foundation[2]

Running title – μ-Opioid Receptor Desensitization after Chronic Treatment

Corresponding author:

John T Williams
Vollum Institute, L474
Oregon Health and Science University
3181 SW Sam Jackson Park Rd.
Portland OR 97239
503-494-5465
williamj@ohsu.edu

Text pages	21
Tables	1
Figures	7
References	28
Abstract	184
Introduction	556
Discussion	1337

Abbreviations: MOR - mu-opioid receptor, LC – locus coeruleus, ME – [Met]^{5}enkephalin, DAMGO - [D-Ala2,N-Me-Phe4-Gly5-ol]-enkephalin, M6G – morphine-6-glucuronide, GRK2 – G protein receptor kinase-2, PKC – protein kinase C, GIRK – G protein gated inwardly rectifying potassium, GPCR – G protein coupled receptor, LOQ – level of quantification, UK14304 - 5-bromo-6-[2-imidazolin-2-ylamino]quinoxaline, NOP – nociceptin/ophanin FQ peptide like receptor, RAVE – relative activity versus endocytosis

ABSTRACT

Several opiate agonists are used for the treatment of pain and addiction. The choice of agonist is dependent on the goal of treatment with primary consideration given to efficacy, pharmacokinetics and profile of side effects. Opiates also differ considerably in their capacity to induce tolerance. This study examines acute μ-opioid receptor signaling in neurons taken from animals that have been treated for one week with one of four commonly employed agonists: morphine, methadone, oxycodone and fentanyl. Acute desensitization of mu-opioid receptors induced by [Met]^{5}enkephalin was studied in locus coeruleus neurons maintained in brain slices. The results show that recovery from acute opioid desensitization varied considerably depending on the agonist. At the extremes were morphine, where recovery from desensitization was dramatically decreased, and methadone, where recovery was the same as in sham treated animals. Treatment with oxycodone and fentanyl had intermediate effects on the recovery from desensitization. Although the underlying mechanisms remain the subject of controversy, the results indicate that chronic treatment of animals with agonists having varying efficacy and propensity to cause desensitization result in different adaptive processes measured at the cellular level.

INTRODUCTION

Opioid-mediated activation of the μ-opioid receptor (MOR) is required for effective analgesic therapy, the treatment of opiate dependence, and is the central event leading to opiate abuse. Chronic opioid use, however, results in tolerance to both the analgesic and rewarding properties of these drugs. To contend with this phenomenon, dose escalation is necessary. This requirement both complicates clinical utility and increases the likelihood of experiencing toxic consequences. Another method used to achieve high analgesic efficacy while minimizing side effects during chronic opiate treatment is to rotate between agonists. Though this approach is improving pain management, there are few studies providing evidence for baseline opiate selection or which alternatives should be considered (Hanks et al., 2001; Mercadante, 2007; Muller-Busch et al., 2005). Strategic analgesic and opiate maintenance therapy would benefit from identifying agonist-specific MOR regulatory properties that are directly related to the development of opioid tolerance.

Morphine, methadone, oxycodone and fentanyl are used clinically as analgesics to treat acute and chronic pain whereas methadone is used in opiate maintenance therapy. Results from both in vivo and heterologous studies 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). The agonist-specific development of tolerance could result from the unique acute regulatory events that follow binding of different opioids to MOR (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). Morphine and methadone produce approximately the same maximum potassium current in locus coeruleus neurons and are therefore thought to have similar efficacy (Alvarez et al., 2002). Morphine, however, causes little desensitization as measured by the decline in peak response during a 10 min application of supersaturating concentration. Methadone and [Met] ^{5}enkephalin (ME), on the other hand, cause a 50% decline in current amplitude. Agonist-selective mechanisms for desensitization could explain such discrepant results. For example, in HEK 293 cells G protein-coupled receptor kinase 2 activity results in desensitization of the DAMGO-occupied MOR, whereas morphine-mediated desensitization is caused by protein kinase C activity (Johnson et al., 2006). MOR agonists also differ in their abilities to induce analgesic tolerance as measured by both hot plate and electrical stimulation tests (Grecksch et al., 2006) and can differentially upregulate MOR trafficking proteins following chronic treatment (Patel et al., 2002).

Given that different opioid agonists cause varying amount of acute desensitization, chronic treatment with these agonists would be expected to result in different persistent adaptations measured at the cellular level. This study examines acute desensitization and recovery from desensitization in locus coeruleus neurons taken from animals that have been treated chronically with morphine, oxycodone, methadone and fentanyl. The results show that chronic morphine treatment facilitated acute desensitization and decreased the recovery from acute desensitization. Following treatment with methadone, however, the recovery from acute desensitization was not different from saline controls. Treatment with either oxycodone or fentanyl had intermediate actions on recovery. The underlying

mechanism(s) responsible for the acute desensitization and recovery from desensitization is the subject of intense investigation. That agonist efficacy or the ability to induce desensitization alone does predict what adaptive processes are engaged following long-term treatment is a step toward an understanding of the processes involved in the development of opioid tolerance.

MATERIALS AND METHODS

Tissue Preparation and Recording. Adult (150-250 gm) male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were used for all experiments. Details of the method of slice preparation and recording have been published previously (Williams et al., 1984). Briefly, rats were anesthetized with halothane and killed. The brain was dissected, blocked and mounted in a vibratome chamber in order to cut horizontal slices (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. Slices were then 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 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. Data was collected with PowerLab (Chart version 4.2.3) and sampled at 100 Hz. Analysis was performed with Prism and Kaleidagraph software. Values are presented as arithmetic mean ± S.E.M. One-way ANOVA followed by Dunnett's or Tukey'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; oxycodone: 80 mg/kg/day; fentanyl: 2.6 mg/kg/day). Rats 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 implant at 6 pm. Rats receiving oxycodone and fentanyl were also given 3 priming IP injections on the same schedule as high-dose morphine and methadone. The doses for oxycodone and fentanyl delivered at all 3 time points were 3 mg/kg and 0.3 mg/kg, respectively. Rats subsequently received a constant infusion of drug subcutaneously. Rats 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 halothane 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. Samples from oxycodone treated animals were analyzed for oxycodone, oxymorphone and noroxycodone with a quantification range of 0.4 – 500 ng/ml (plasma) and 0.8 – 1000 ng/ml (brain). Samples

from fentanyl treated animals were analyzed for fentanyl and norfentanyl with a quantification range of 0.5 – 1000 ng/ml.

Drugs. Drugs were applied by bath superfusion. The following drugs were superfused: [Met^5]-enkephalin (Sigma), bestatin (Sigma), thiorphan (Sigma), morphine (NIDA – Neuroscience Center), methadone (NIDA – Neuroscience Center), UK14304 (Sigma), and yohimbine (Sigma). Applied concentrations of UK14304 and thiorphan did not exceed 0.01% dimethyl sulfoxide and .00001% ethanol, respectively. All other drugs were dissolved in water only.

Protocols. Two protocols were used to measure acute desensitization and recovery from desensitization in LC neurons after the induction of desensitization after short and more prolonged treatment. Both protocols used a saturating concentration of ME (30 μM) and allowed the continuous measurement of potassium current amplitude. The first protocol used a ME (30 μM for 10 min) to induce desensitization (Fig 1). The current induced by ME (300 nM, 2 min) was determined just before (pre-pulse) and 5 min following the wash out of the desensitizing application (ME 30 μM, 10 min). The current induced by ME (300 nM) was measured repeatedly every 10 min for 45 min. The extent of desensitization, was determined by the decrease in the current induced by ME (300 nM) during the first test-pulse (5'). An additional measurement of acute desensitization was a decline in the maximum GIRK current evoked during the continuous 10 min supramaximal ME (30 μM) application (Fig 4). The second protocol used the decline in the current induce by ME (300 nM) that followed a brief, supramaximal pulse of ME (30

μM, 1-2 min, Fig 3). The experiment started with a treatment with ME (300 nM), followed immediately with ME (30 μM for 1-2 min) after which the solution was returned to ME (300 nM) for 10 min. Two more test-pulses (ME, 300 nM) were delivered at 20 and 30 min following the desensitizing pulse. Desensitization was defined as the greatest depression of the ME (300 nM) current following high ME (30 μM) superfusion. Recovery from desensitization was plotted as the increase in ME (300 nM) current amplitude following desensitization. The current induced by ME (300 nM) alone did not cause any desensitization (Osborne and Williams, 1995).

RESULTS

Tolerance induced by chronic treatment with morphine and methadone

The initial experiments were aimed at determining whether treatment of animals using osmotic mini pumps containing either morphine or methadone for 6 days would render locus coeruleus neurons opiate tolerant. Concentration response curves to ME were constructed in slices from control and morphine (60 mg/kg/day) and methadone (60 mg/kg/day, Fig. 1A) treated animals. The ME induced current amplitude was normalized to the current induced by a saturating concentration of the alpha-2 adrenoceptor agonist, UK14304 (% peak I-UK14304). Peak UK14304 current amplitude was not affected by chronic drug treatment (control, 319±30 pA; methadone-treated 313±31 pA; morphine-treated, 306±20 pA). Neither treatment with morphine nor methadone reduced the maximum outward current produced by ME (10 μM, control 145±8%; morphine-treated 153±8%; methadone-treated 144±7%). However, the concentration-response curve was

shifted 2-fold to the right following treatment with either drug. The half-maximal effect (EC_{50}) of ME in control, morphine- and methadone-treated animals was 281±47 nM, 578±85 nM, and 500±78 nM, respectively. The results obtained in morphine-treated animals are in agreement with previous work where subcutaneous morphine pellets were used to treat animals chronically (Christie et al., 1987). Cellular tolerance has not been studied previously in methadone-treated animals. The shift in the concentration response curve to ME that was observed in slices from methadone treated animals suggests that methadone treatment produced about the same degree of tolerance as treatment with morphine.

To further characterize the effect of chronic treatment with methadone, concentration-response curves to methadone were constructed in slices from control and methadone-treated (60 mg/kg/day) animals (Fig 1B). Current amplitudes were normalized to the current induced by the alpha-2 adrenoceptor agonist, UK14304. Peak values, induced by methadone (10 μM) were not different in neurons from control and treated animals (control 90±2%; methadone-treated 91±6%). There was, however, a 2-fold right-shift in the concentration-response curve to methadone in sliced from methadone treated animals (EC_{50}: control 261±24 nM; methadone-treated 508±109 nM).

Desensitization is greater after methadone- and morphine-treatment

To examine the extent of acute desensitization the decline in current was measured during a continuous application of ME (30 μM, Fig 2). The amount of current remaining at the end of the 10 min application was significantly smaller in slices from morphine-

and methadone-treated animals than in slices from control animals. In control slices (Fig 2A), 65±2% of the maximum current remained, whereas in slices from morphine- (60 mg/kg/day) and methadone-treated animals (60 mg/kg/day) 53±2% and 54±2% of the maximum current persisted, respectively (Fig 2B, C and D). These results show that chronic treatment with both drugs had similar effects on acute MOR desensitization.

Recovery from desensitization

Morphine. The recovery from desensitization was first examined in morphine treated animals using two protocols. The first experiment examined the recovery after the induction of maximum desensitization (long protocol). A test pulse of ME (300 nM) was applied before and repeatedly after inducing desensitization using ME (30 μM, 10 min). Maximum desensitization was first measured 5 min after washing the saturating concentration of ME (30 μM). In slices from control animals, maximum desensitization (5') was 22±3% of the pre-pulse (Fig 3A). Forty-five minutes after washing ME (30 μM), the current induced by the final ME (300 nM) test-pulse had recovered to 71±5% of control. In morphine-treated (60 mg/kg/day) animals, however, maximum desensitization was 10±2% of the pre-pulse (5') and recovered to only 42±4% after 45 min (Fig 3B,D).

The inhibition of recovery from desensitization after chronic morphine treatment was also observed after a short application of a saturating concentration of ME (30 μM; Figure 4). This experiment measured the current induced by a pre-pulse of ME (300 nM, EC_{50}) just prior to a short application of a saturating concentration of ME (30 μM, 1-2

min). During this period the outward current peaked but did not decline (Figure 4). The perfusion solution was changed back to ME (300 nM) in order to measure the maximum desensitization at 5 min (5'). The amplitude of the ME (300 nM) test-pulse current at 10, 20 and 30 min was used to monitor the recovery from desensitization. In control animals, maximum desensitization was 41±2% of the pre-pulse (Fig 4A). Recovery, at 10, 20, and 30 min was 53±2%, 76±2%, and 81±2% of the pre-pulse, respectively. In morphine-treated animals (60 mg/kg/day), desensitization was facilitated and recovery was inhibited (Fig 4B). The ME current was 16±4% of the pre-pulse at the maximum desensitization point. During recovery the current was 31±5%, 48±5%, and 51±7% of the pre-pulse at 10, 20 and 30 min, respectively. The results from these two experiments show that the recovery from desensitization is depressed in morphine treated animals. Further, with the use of the short and long desensitization protocols, the blunted recovery from desensitization is independent of the degree of desensitization that is induced.

Although the extent of recovery was decreased in morphine treated animals, the time course over which there is recovery was not different from control (Fig 3D and 4D). The time required for 40% recovery ranged from 8.2 to 11.3 min and for 60% recovery, 15.5 to 18.4 min were required. These results extend similar results where both the treatment protocol and the method of recording were different (Dang and Williams, 2004).

Methadone. In experiments using animals that were treated with methadone (60 mg/kg/day), there was no significant effect on the extent of desensitization or recovery compared to control animals (Fig 3C and 4C). After desensitization induced by ME (30

μM, 10 min) the first test-pulse (5') was 15±2% that of the pre-pulse and recovered to 67±2% in 45 min. The recovery from desensitization was not significantly different from control (Fig 3D). Similarly results were obtained using a short desensitizing application of ME (30 μM, 1-2 min, Fig 4C and D). The current induced by the test-pulse concentration of ME (300 nM) measured 5 min after ME (30 μM), was 30±4% of pre-pulse (Fig 4D). Thus, short protocol experiments revealed more desensitization than recorded in slices from control animals. Recovery was similar, however, to control animals at each subsequent time point. Test-pulses, at 10, 20, and 30 min, were 48±4%, 70±3%, and 85±5% of the pre-pulse and all were significantly greater than those measured in morphine-treated animals (Fig 4D). These results indicate that following chronic methadone treatment ME-induced desensitization was increased. Unlike after chronic morphine treatment, however, there was no effect on the recovery from desensitization with either the short or long desensitization protocol. Thus, chronic treatment with morphine and methadone result in unique adaptive change in the regulation of MOR function.

Chronic morphine and methadone treatment actions are independent of dose

In order to determine whether or not dose dependent effects on desensitization and recovery exist, various doses of each drug were administered. Methadone was delivered at doses of 60, 30 and 5 mg/kg/day and recordings were made after 6-7 days of chronic treatment. Results obtained after desensitization with ME (30 μM, 10 min) showed no dose-dependent effect in slices from animals treated with methadone (Fig 5A). There

was no significant difference in desensitization or recovery between control rats and those treated with any dose of methadone.

The drug levels in brain and plasma (Table 1) are similar to those reported in previous work in rats treated with methadone (10 mg/kg/day) where the average plasma concentration was 123 ng/ml (Zhou et al., 1996). Both heroin- and cocaine-seeking were attenuated in rats maintained on methadone 30 mg/kg/day, (Leri et al., 2004). Humans that receive methadone for analgesia or maintenance therapy can achieve an average plasma concentration of 240 ng/ml (Kreek, 2000).

Morphine was administered to rats at doses of 60, 30, and 15 mg/kg/day for 6-7 days. Plasma and brain concentrations for morphine and the metabolites, morphine-3-glucuronide and morphine-6-glucuronide are presented in Table 1. All doses resulted in significantly greater ME-induced desensitization and inhibited recovery from desensitization as compared to either control or methadone-treated animals (Fig 5B). The lowest dose selected for morphine was 15 mg/kg/day, versus 5 mg/kg/day for methadone, because a smaller fraction of parent drug and metabolites traverse the blood-brain barrier. These results demonstrate that morphine brain concentrations as low as 9±1 ng/ml were sufficient to cause an increase in desensitization and reduced recovery.

One day morphine treatment did not affect desensitization or recovery

In order to determine whether an acute dose of morphine was sufficient to induce the same changes in MOR desensitization and recovery resulting from chronic treatment, rats

were given two 10 mg/kg intraperotoneal injections of morphine, one at 9 am and one at 6 pm. Experiments were performed 24 hours after the first injection. After ME-induced (30 μM/10 min) desensitization, the current induced by a test-pulse of ME (300 nM) was 22±2% that of the pre-pulse at 5 min and recovered to 76±4% at 45 min, (n=4, not shown). These values were not different from control, indicating that one-day of treatment was not sufficient to produce the changes observed by constant drug infusion for 6-7 days.

Desensitization in morphine- and methadone-treated animals is homologous

To determine whether morphine- and methadone-treatment resulted in an effect at the opioid receptor or a global cellular change that generalized to other GPCRs, experiments were performed with the alpha-2 adrenergic agonist UK14304 and the nociceptin/orphanin FQ peptide like receptor agonist nociceptin. Both of these GPCRs are expressed on LC neurons and couple to GIRK channels. As illustrated in Figures 2 and 3, a supersaturating concentration of UK14304 (3 μM) was applied at the conclusion of all desensitization and recovery experiments to provide a measurement of heterologous desensitization. If a heterologous desensitization component existed, peak UK14304 currents would be reduced following ME desensitization in morphine treated animals but not in controls. Peak UK14304 current amplitudes were thus normalized to peak ME amplitudes as measured in the long protocol. As predicted by the concentration-response results, peak ME (30 μM) induced current amplitudes were similar for all three groups (control, 423±41 pA; methadone-treated, 401±17 pA; morphine-treated, 410±24 pA). Furthermore, there was no difference in peak UK14304 current amplitudes following a

supersaturating ME (30 μM/10 minutes) application for any of the three treatment groups (control, 319±30 pA; methadone-treated 313±31 pA; morphine-treated, 306±20 pA). These results suggest that chronic morphine- or methadone-treatment was not capable of inducing sustained heterologous desensitization at alpha-2 adrenergic receptors.

The experiments with alpha-2 adrenoceptors may be limited because even prolonged exposure to UK14304 fails to induce desensitization. To more rigorously pursue the question of treatment-induced heterologous desensitization, experiments were performed with the nociceptin/orphanin FQ peptide like receptor (NOP) because NOP exhibits robust desensitization. Experiments were only performed on morphine-treated animals because prolonged homologous MOR desensitization was only found in this treatment group.

Two experiments were performed in slices from control and morphine-treated rats. First, nociceptin (3 μM/10 min) was used to desensitize NOP (Fig 6A). In that experiment the current induced by ME (300 nM) was measured before and after desensitization of the NOP. The current induced by ME (300 nM) was reduced to about 75% of control but that inhibition was the same in untreated and morphine treated animals (Fig 6B). The recovery of the current induced by nociceptin was slightly greater in slices from control animals. The second experiment measured the current induced by nociceptin before and after desensitization with ME (30 μM/10 min, Fig 6C). Following desensitization in this experiment there was a long lasting depression of the current induced by ME (300 nM) in slices from morphine treated animals (as expected), but the current induced by nociceptin

(50 nM) was not affected (fig 6D). The results of these experiments indicate that morphine treatment has an effect specific to MOR regulation. This effect was homolgous and failed to generalize to either alpha-2 adrenergic receptors or nociceptin/orphanin FQ peptide like receptors.

Oxycodone and fentanyl

The effects of chronic treatment with oxycodone and fentanyl were examined next (Fig 7). These two opioid agonists were chosen, first, because of their prolific clinical use and, second, based on the relative ability to induce desensitization and internalization. Oxycodone failed to induce MOR desensitization (Virk and Williams, submitted) leading to the expectation that chronic treatment with oxycodone would have a similar outcome as chronic morphine. Fentanyl induced robust acute desensitization and therefore chronic treatment with fentanyl was predicted to result in actions similar to those obtained with methadone. Rats were treated with high doses of oxycodone (80 mg/kg/day) or fentanyl (2.8 mg/kg/day) and resulting plasma and brain concentrations are presented in Table 1. Acute desensitization and recovery from desensitization were measured using the same protocol as described previously. While treatment with oxycodone resulted in an increase in MOR desensitization measured at the 5 min time point following desensitization, recovery was no different from control ($p>0.05$ - one-way ANOVA followed by a Dunnett's test). The first test-pulse with ME (5') was 13±2% that of the pre-pulse and recovered to 62±4% after 45 min (n=6). Thus, chronic treatment with oxycodone had an action more like methadone than morphine. Similar results were found in slices from fentanyl treated animals, desensitization and recovery from

desensitization were not significantly different from control. Fentanyl treatment resulted in an initial test-pulse (5') measurement of 17±2% that recovered to 53±7% at 45 min (n=9, Fig 7).

DISCUSSION

The results of this study indicate that chronic treatment with different μ-opioid agonists had significantly different effects on acute desensitization and recovery from desensitization. Chronic treatment of animals with fentanyl, oxycodone, methadone and morphine compromised the recovery from desensitization to different degrees. At the extremes, morphine treatment inhibited recovery while methadone had no effect. Although the recovery from desensitization was depressed only in slices from morphine treated animals, there were also several common effects of chronic treatment with either morphine or methadone. First, during a 10 min application of ME (30 μM), the peak outward current declined to the same extent in slices from morphine- and methadone-treated animals, and this was significantly greater than for untreated animals. Second, at all concentrations of morphine and methadone used, there was a greater depression of the current induced by ME (300 nM) measured at the first time-point (5') following desensitization. Finally, concentration-response curves constructed for both ME and methadone were right shifted 2-fold in neurons from methadone-treated animals, indicating the same degree of cellular tolerance to both morphine and methadone. A previous study using morphine pellets instead of osmotic mini pumps for chronic treatment obtained similar results in that the peak current induced by morphine was not

decreased relative to that measured in slices from untreated animals (Dang and Williams, 2005). Plasma and brain drug concentration measurements of all four compounds confirmed the presence of each drug used for chronic treatment. Thus, failure or inaccurate dose delivery from osmotic mini pumps was ruled out.

Homologous desensitization

A small component of heterologous desensitization between MORs and other GPCRs has been previously demonstrated (Fiorillo and Williams, 1996). However, in slices from morphine treated animals, there was no additional inhibition of the ME test-pulses following desensitization with nociceptin. Likewise there was no inhibition of the nociceptin test-pulse following desensitization with ME in slices from morphine treated animals. While nociceptin-induced desensitization compromised the current evoked by a subsequent nociceptin test-pulse in morphine-treated animals, the magnitude of that inhibition was small. Furthermore, ME induced MOR desensitization had no effect on subsequent alpha-2 adrenergic receptor current in slices from morphine treated or control animals. Thus, the mechanism by which morphine-treatment changes MOR regulation in LC neurons appears to be homologous and does not result in global changes that affect the desensitization of other GPCRs.

Signaling and efficacy

The most agonist-specific effect of chronic treatment is best illustrated by comparing the recovery from desensitization in slices from morphine and methadone treated animals. Although recovery from acute desensitization was not changed from control after

treatment of animals with methadone, there was a significant decrease in the recovery from desensitization in slices taken from morphine treated animals. 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 concentrations or efficacy. The ED_{50} 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) and the two drugs have the same efficacy for increasing a potassium conductance in the LC (morphine: 278±18 pA; methadone: 259±47 pA; see also Alvarez et al., 2002).

Differences in opioid agonists have been modeled with the RAVE (Relative Activity Versus Endocytosis) theory (Whistler et al., 1999) and tested for their significance with respect to tolerance (Finn and Whistler, 2001; Koch et al., 2005). Methadone has a value closer to the opioid peptides than morphine does because it induces endocytosis to a greater extent. The idea that arrestin-dependent endocytosis serves as a protective role in the development of tolerance and dependence is still a matter of debate (Bohn et al., 2000). This theory was tested in the present study in two ways. First, different concentrations of methadone were used and, second, agonists that induce internalization to varying extents were compared. A group of animals was treated with a low dose of chronic methadone (5 mg/kg/day) in order to activate MOR but to result in very low brain concentrations (Table 1). This dose of methadone was not expected to induce either desensitization or internalization. The hypothesis was that methadone brain concentrations maintained below the threshold for internalization and desensitization

would result in MOR recovery similar to that following morphine treatment. This hypothesis, however, was not supported.

Agonist selectivity after chronic administration was also examined with the use of oxycodone and fentanyl because they are similar in some respects to morphine and methadone, respectively. However, the results of chronic treatment with these agonists on desensitization and recovery were not predicted. Morphine and oxycodone are similar acutely in that the maximum outward current induced by saturating concentrations of each is the same and neither caused dramatic desensitization (Virk and Williams submitted). Additionally, neither morphine nor oxycodone has been reported to trigger MOR internalization in HEK 293 cells (Koch et al., 2005). Based on these observations, chronic treatment with oxycodone was predicted to increase MOR desensitization and inhibit recovery to the same extent as that with morphine. However, oxycodone treatment resulted in recovery that did not differ from control and that was more similar to methadone than morphine treatment.

One possible explanation for the intermediate values of recovery following desensitization in oxycodone treated animals is that oxymorphone, a highly potent metabolite, was found in significant concentrations in the brain (Table 1). Oxymorphone is more potent than oxycodone in hot-plate and paw-pressure tests and is considerably more potent than both oxycodone and morphine in GTPgS assays (Lemberg, et al., 2006). When applied acutely to LC neurons in brain slices, oxymorphone (15 μM) evoked an outward current that was similar in amplitude to fentanyl and etorphine, and significantly

larger than that evoked by oxycodone, morphine or the active metabolite of morphine, morphine N6-glucuronide. Additionally, the current induced by oxymorphone desensitized significantly during a 10 min application. It is not known to what extent oxymorphone induced MOR internalization, however agonists such as fentanyl, etorphine and methadone all induce receptor endocytosis (Keith et al., 1998; Whistler et al., 1999; Finn and Whistler, 2001; Koch et al., 2005). The presence of oxymorphone in the brain may therefore result in adaptations that are similar to methadone (Table 1). The net effect on the recovery from desensitization after chronic treatment with oxycodone may therefore be intermediate between methadone and morphine.

The results of chronic fentanyl treatment on desensitization and recovery were also not expected. Fentanyl has approximately the same intrinsic activity, as methadone as measured in GTPgS assays. It evoked a large outward current in LC neurons similar to that induced by ME or DAMGO and the current desensitized extensively during a 10 min application (Virk and Williams, submitted). Fentanyl also triggers significant internalization (Zaki, et al., 1999; Borgland, et al., 2003; Koch, et al., 2005). Finally, methadone and fentanyl have nearly identical RAVE values. Because of its similarities to methadone, chronic treatment with fentanyl was predicted to have no effect on MOR recovery from desensitization. One remarkable observation made while doing experiments with fentanyl treated animals was the degree of variability between animals. In experiments from some animals the recovery from desensitization was complete as in methadone treated animals. In other animals, however, there was no recovery from desensitization as found in morphine treated animals. There was no correlation between

these results and the amount of fentanyl found in the plasma or brain, and when more than one experiment was done using different slices from a given animal, the results from each slice was the same. The source of this variability is not known.

In conclusion, agonist-specific properties of MOR regulation have been demonstrated in native neurons. Chronic treatment with morphine and methadone resulted in different adaptations measured with the acute MOR desensitization and recovery from desensitization in LC neurons. Oxycodone and fentanyl-treatment resulted in intermediate effects on the recovery from desensitization, potentially due to active metabolites. These findings may have implications for strategic analgesic therapy aimed at minimizing the development of tolerance, and for understanding mechanisms underlying opiate addiction.

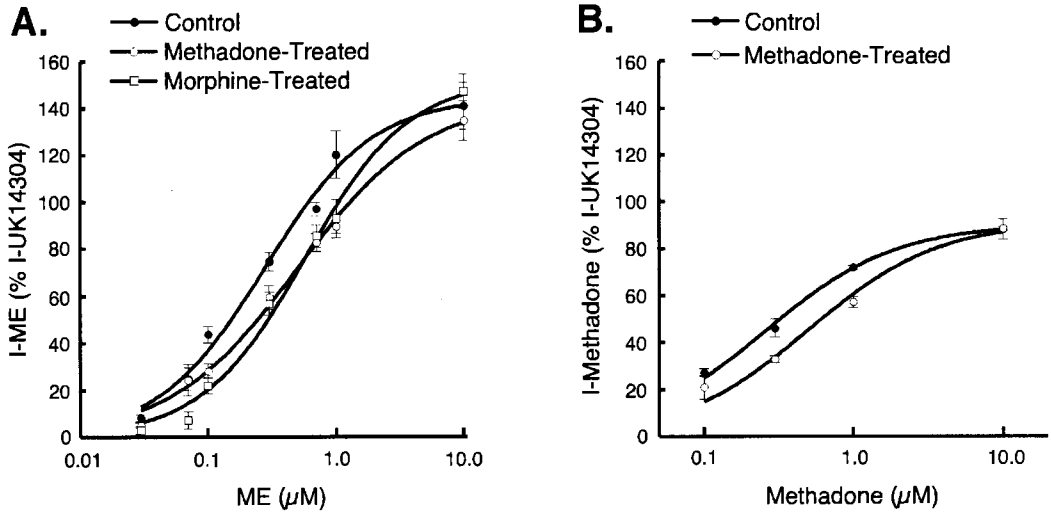


Figure 1. Concentration-response curves for ME and methadone show decreased sensitivity following drug treatement. *A,* ME (30 nM – 10 μM) applied to LC neurons from control, methadone-treated (60 mg/kg/day), and morphine-treated rats (60 mg/kg/day). ME currents (I-ME) are normalized to UK14304 currents (I-UK14304). All points are mean values with vertical bars representing standard error. The number of values at each ME concentration for control, methadone-treated and morphine-treated animals were n=4-11, n=4-13, and n=4-12, respectively. *B,* Methadone (100 nM – 10 μM) applied to LC neurons from control and methadone-treated (60 mg/kg/day). I-Methadone was normalized to I-UK14304. All points are mean values with vertical bars representing standard error. The number of values for each methadone concentration for control and methadone-treated were n=4-8 and n=4-6, respectively.

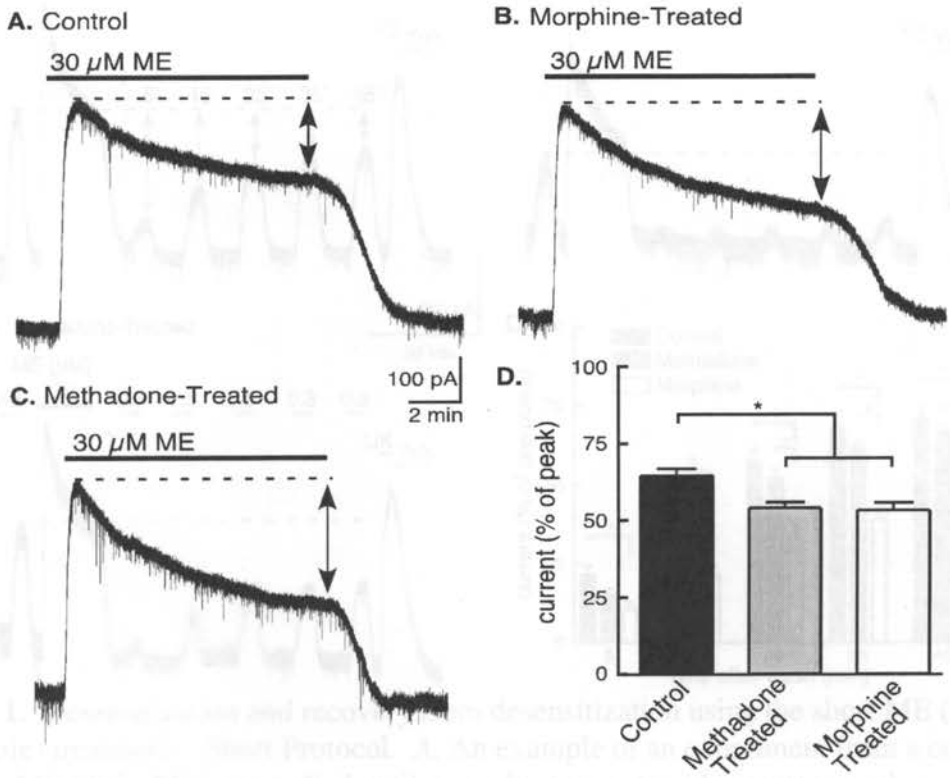


Figure 2. Morphine and methadone treatment increase the rate of acute ME-induced (30 μM/10 min) MOR desensitization as measured by the decline in peak. *A,* A saturating concentration of ME (30 μM) was superfused for 10 min on to an LC neuron from control animals. *B,* ME (30 μM) superfused for 10 min on to an LC neuron from methadone-treated rats (60 mg/kg/day). *C,* ME (30 μM) superfused for 10 min on to an LC neuron from morphine-treated rats (60 mg/kg/day). *D,* Summary of results for MOR desensitization. The amount of current remaining after 10 min is expressed as a percentage of the peak current (control, n=10; methadone-treated, n=9; morphine-treated, n=8). Scale bar represents traces A – C. Data are presented as mean values and standard error, and compared at each time point with one-way ANOVA and Dunnett's Multiple Comparison Test. * Significantly different ($P < 0.05$)

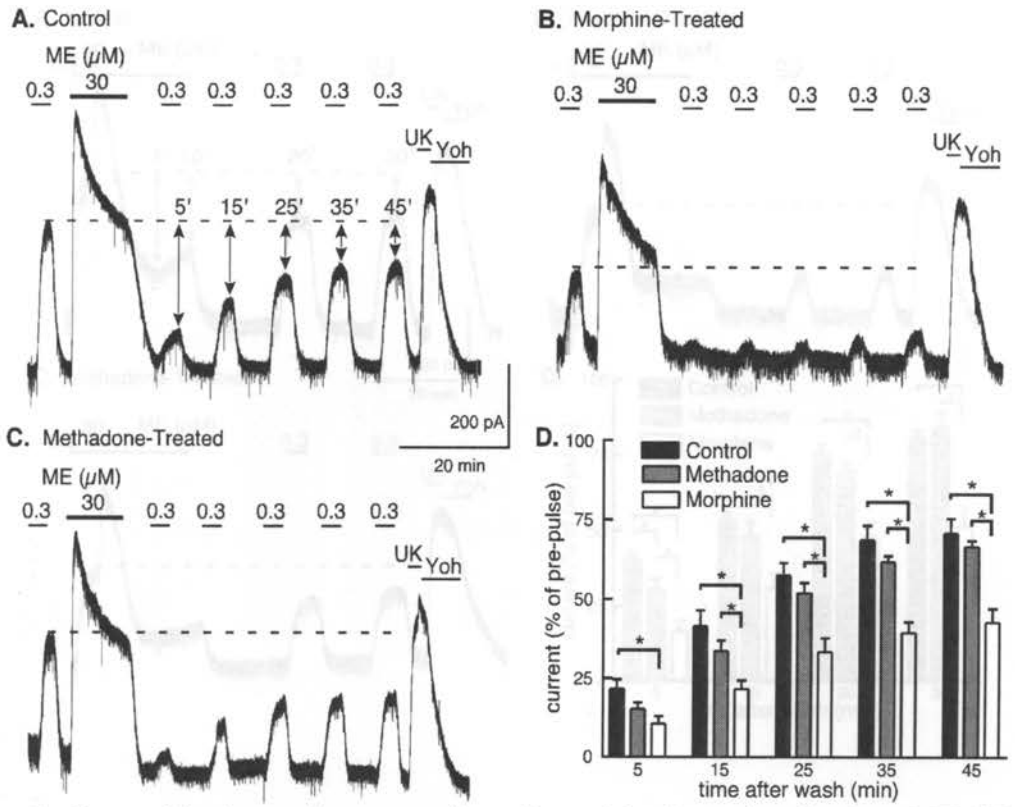


Figure 1. Desensitization and recovery from desensitization using the short ME (30 μM/1 min) treatment – Short Protocol. *A,* An example of an experiment from a control animal. ME (300 nM) was applied until a steady state outward current was observed (pre-pulse). The superfusion solution was then changed to ME (30 μM) to cause a maximal outward current. The superfusion solution was changed back to ME (300 nM) immediately after the current induced by the high concentration of ME reached steady state. The current induced by ME (300 nM) declined below the original value within about 5 min (5'), indicating maximum desensitization. ME (300 nM) was washed after 10 min (10'). Short applications of ME (300 nM, 1-2 min) were used to monitor the MOR recovery at 20 and 30 min. At the end of the experiment, UK14304 (UK, 3 μM) was applied and the resulting current was reversed with yohimbine (Yoh, 10 μM). *B,* An example of an experiment from a morphine-treated animal. Morphine treatment caused greater acute MOR desensitization and inhibited recovery. At 5 min (5') the GIRK current was smaller relative to the pre-pulse than those in both control and methadone-treated rats. MOR recovery remained compromised at each time-point throughout the 30-min period. *C,* An example of an experiment from a methadone-treated animal. Methadone treatment caused greater acute MOR desensitization. At 5 min (5') the ME (300 nM) current was significantly smaller than that of controls, there was, however, no effect on the extent of recovery. The current induced by the test-pulse at 30 min (30') was the same as in slices from control animals. *D,* Summary of results for acute MOR desensitization and recovery. Test-pulse amplitudes were normalized to pre-pulse values (control, n=13; morphine-treated, n=7; methadone-treated, n=6). Scale bar represents traces A – C. Data are presented as mean values and standard error (vertical bars), and compared at each time point with one-way ANOVA and Tukey's Multiple Comparison Test. * Significantly different ($P < 0.05$)

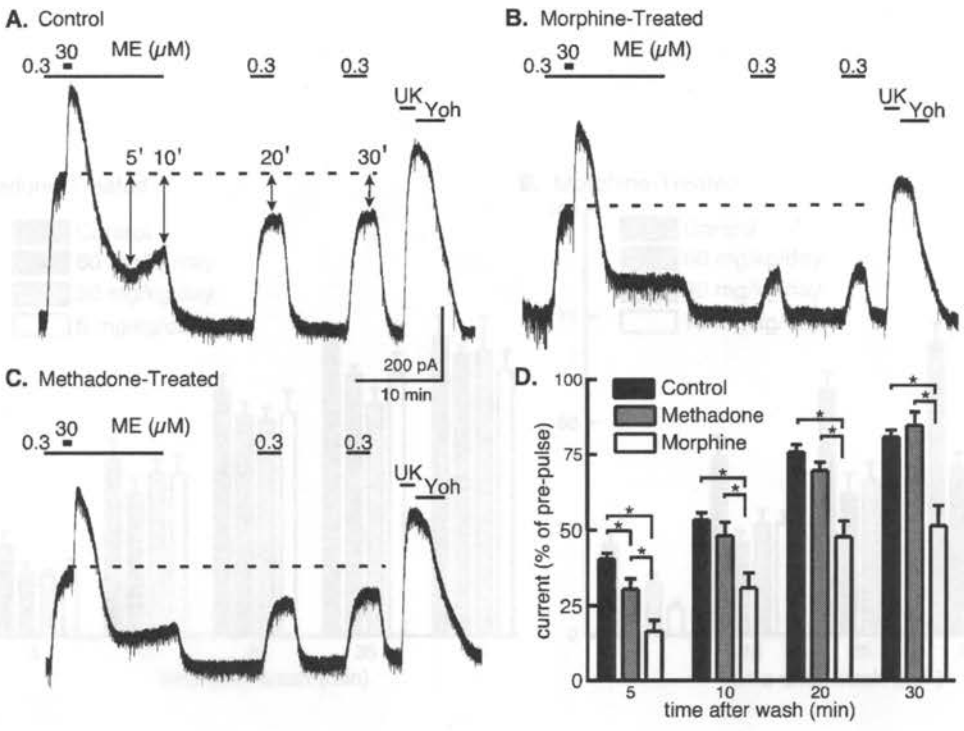


Figure 3. Desensitization and recovery following a long ME (30 μM/10 min) desensitization treatment – Long Protocol. *A,* An example from a control animal. ME (300 nM) was applied until steady state outward current was reached (pre-pulse) and allowed to wash. A saturating concentration of ME (30 μM) was superfused for 10 minutes. Test-pulses were delivered at 5 min (5') following wash out and every 10 min thereafter for 45 min (45') to monitor recovery. At the conclusion of the experiment, the current induced by UK 14304 (UK, 3 μM) was determined and reversed by yohimbine (Yoh, 10 μM). *B,* An example from a morphine-treated animal. Morphine treatment caused greater acute MOR desensitization and inhibited recovery. The test-pulse at 5 min (5') was diminished as compared with control values. At every subsequent time point, test-pulses evoked smaller currents relative to the pre-pulse than those from both control and methadone-treated animals. *C,* An example from a methadone-treated animal. Methadone treatment had no effect on acute MOR desensitization or on the extent of recovery. *D,* Summary of results for acute MOR desensitization and recovery. Test-pulse amplitudes were normalized to pre-pulse values (control, n=8; morphine-treated, n=8; methadone-treated, n=8). Scale bar represents traces A – C. Data are presented as mean values and standard error (vertical bars), and compared at each time point with one-way ANOVA and Tukey's Multiple Comparison Test. * Significantly different ($P < 0.05$)

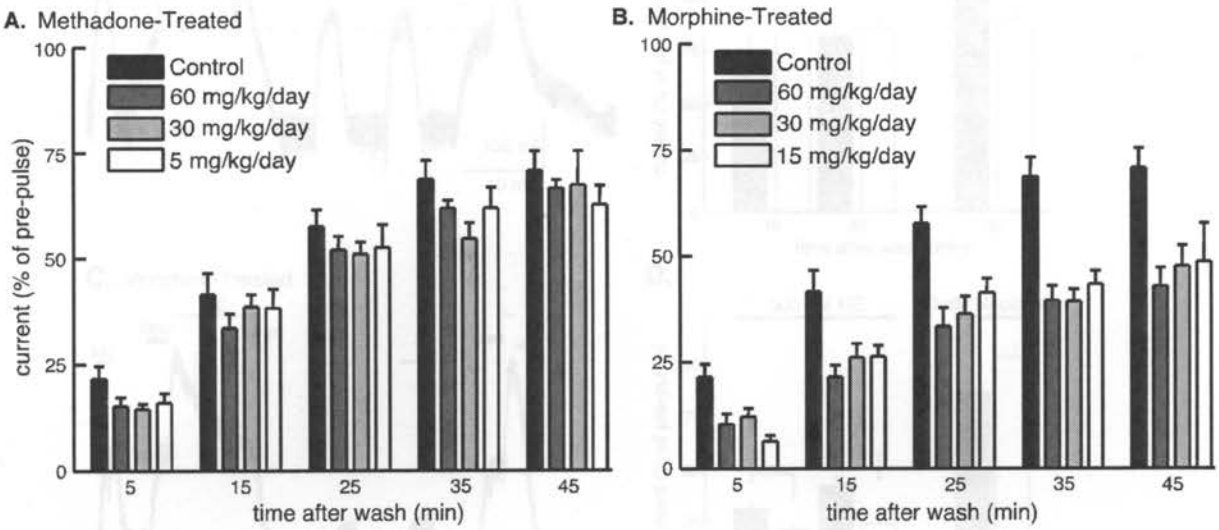


Figure 5. Methadone and morphine treatment show no dose-dependent effects on MOR recovery. *A,* Three different doses of methadone were delivered to rats via osmotic minipump: 60, 30, and 5 mg/kg/day (n=8, 10, 9, respectively). The extent of MOR recovery, as measured with the long protocol, did not differ from control regardless of the dose administered. *B,* Three different doses of morphine were delivered to rats via osmotic minimpump: 60, 30, and 15 mg/kg/day (n=8, 6, 6, respectively). The extent of MOR recovery was significantly inhibited as compared to control and methadone-treated animals in all three groups at each time point. MOR recovery did not differ between groups receiving different doses of morphine. The rate of recovery was the same as control for both drug treatment groups at all doses. Data are presented as mean values and standard error (vertical bars), and compared at each time point with one-way ANOVA and Dunnett's Multiple Comparison Test.

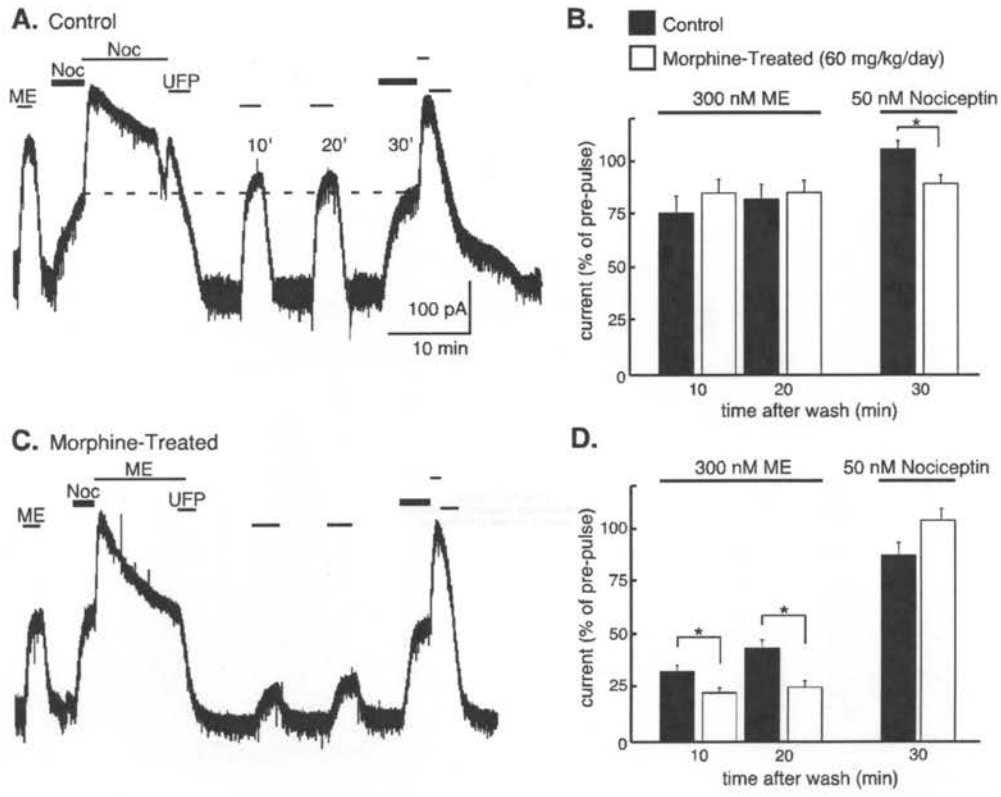
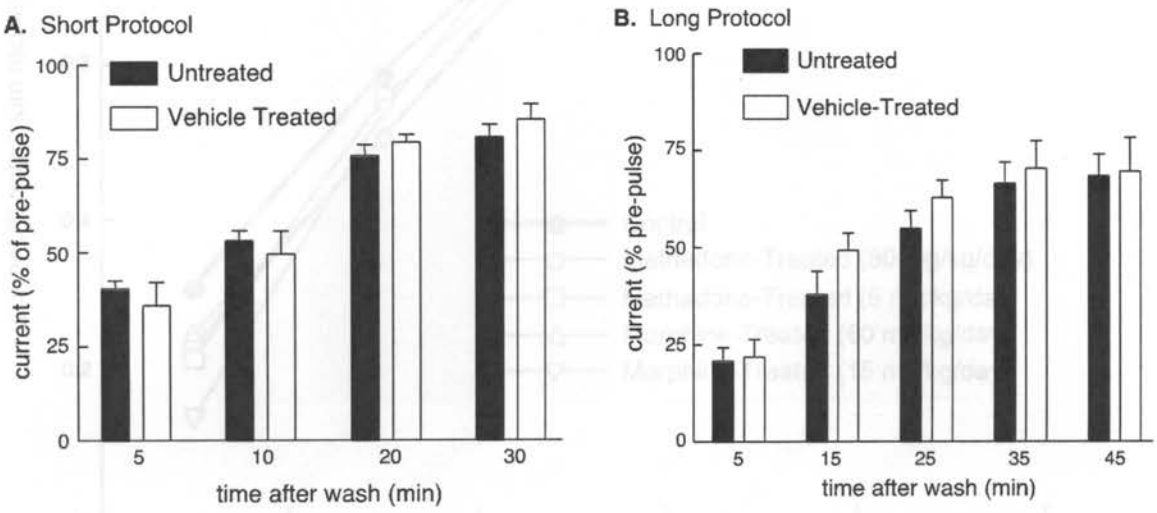
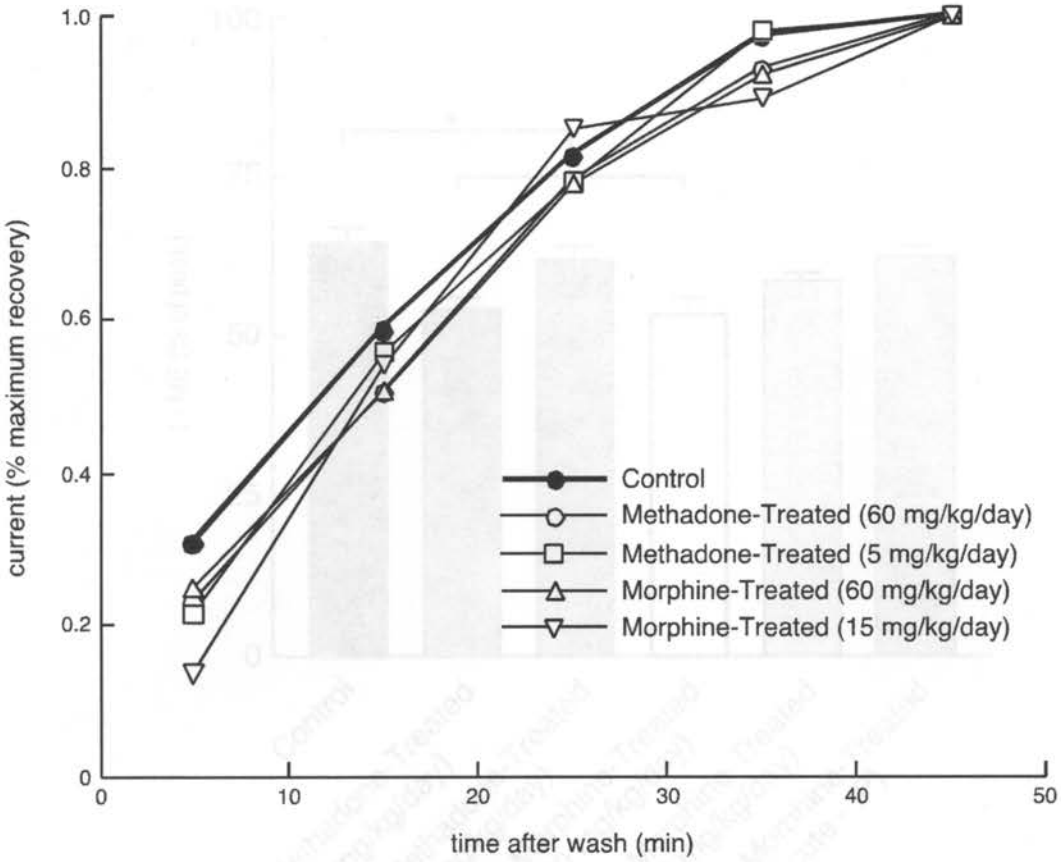


Figure 6. ME-induced MOR desensitization and inhibited recovery in morphine-treated animals is homologous. A, An example of a recording from a control animal. Pre-pulses of ME (300 nM) and nociceptin (Noc, 50 nM) were applied until a steady-state amplitude was reached. Noc (3 μM) was then applied for 10 min followed by treatment with UFP-101 (UFP, 2 μM). Test-pulses of ME (300 nM) were delivered at 10 and 20 min following treatment with UFP and at 30 min, Noc (50 nM and 3 μM) were applied. UFP was superfused to reverse Noc-induced GIRK currents. B, Summary of results in control and morphine-treated animals following Noc (3 μM) desensitization (control, n=7-9; morphine-treated, n=4). Test-pulse current amplitudes were normalized to pre-pulse values for their respective agonist. Data are presented as mean values and standard error (vertical bars), and compared at each time point with the Student's t-test. * Significantly different ($P < 0.05$). C, An example of a recording from a control animal. ME (300 nM) and Noc (50 nM) were applied until a steady-state current was reached. A saturating concentration of ME (30 μM) was applied for 10 min and washed with UFP (2 μM) to ensure that all residual Noc-induced current was reversed. Test-pulses of ME (300 nM) were delivered at 10 and 20 min and at 30 min, Noc (50 nM) was applied followed by ME (30 μM). UFP was superfused to reverse Noc-induced GIRK currents. D, Summary of results for homologous and heterologous desensitization in control and morphine-treated animals following ME (30 μM) desensitization (control, n=4; morphine-treated, n=6-7). Test-pulse current amplitudes were normalized to pre-pulse values for their respective agonist. Data are presented as mean values and standard error (vertical bars), and compared at each time point with one-way ANOVA. * Significantly different ($P < 0.05$).

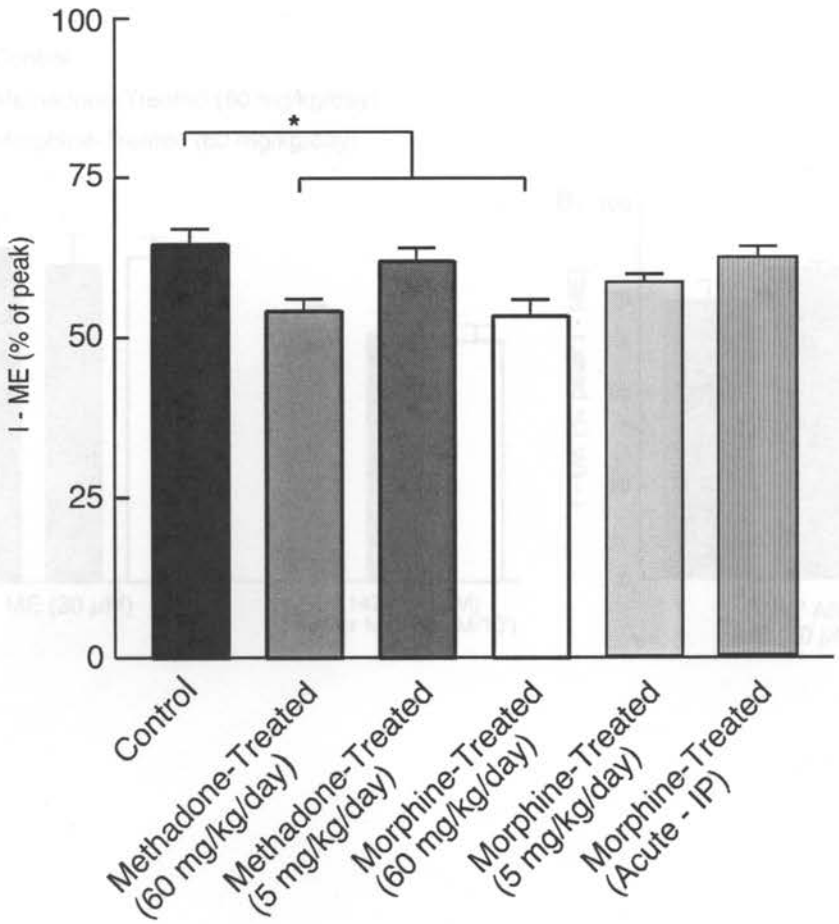
Figure 7. The extent of recovery from desensitization is dependent on the agonist used during chronic treatment. In slices from control animals (black bars) the current induced by ME (300 nM) recovers to about 70% of the initial value after about 35 min. The recovery from desensitization is the same as in control in animals treated chronically with methadone (dark gray bars), whereas the extent of recovery in morphine treated animals (white bars) is significantly less. Chronic treatment with fentanyl and oxycodone (lighter gray bars) lie between that of morphine and methadone.



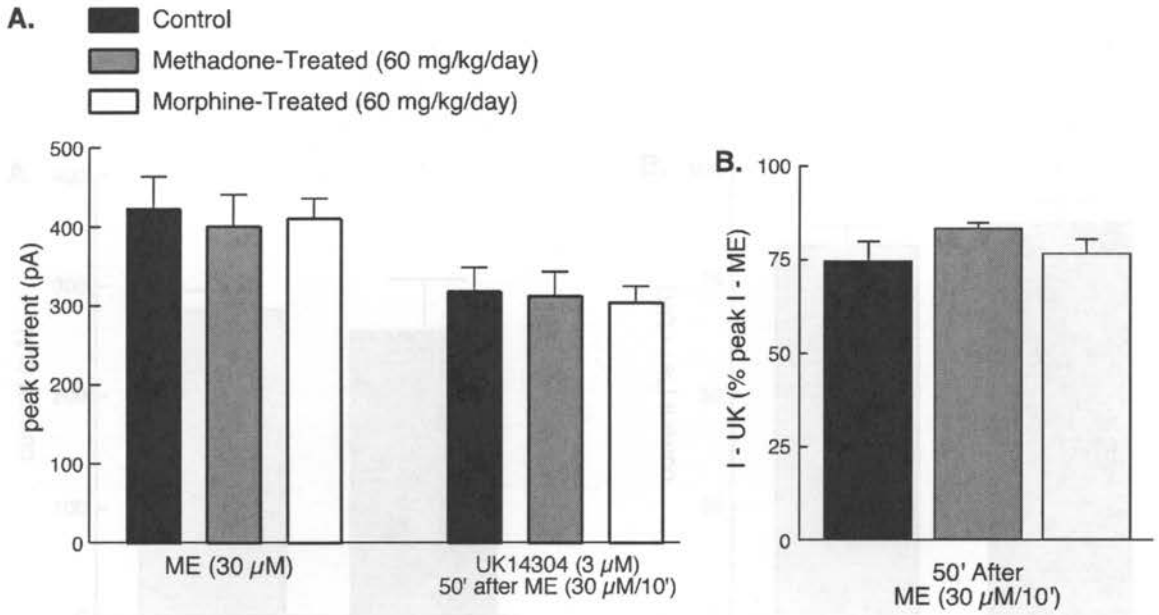
Supplementary Figure 1. The extent of MOR recovery as measured by the short and long protocols in control animals does not differ between control rats and those undergoing sham surgery. *A*, Summary of results showing the extent of acute MOR desensitization and recovery following the short protocol (Fig 1) between untreated rats and rats undergoing surgical implantation of vehicle-filled minipumps. Test-pulse amplitudes were normalized to pre-pulse values (untreated, n=10; vehicle-treated, n=3). Data are presented as mean values and standard error (vertical bars), and compared at each time point with the Students t-test. No significant differences were found ($P < 0.05$). *B*, Summary of results showing the extent of acute MOR desensitization and recovery following the long protocol (Fig 2) between untreated rats and rats undergoing surgical implantation of vehicle-filled minipumps. Test-pulse amplitudes were normalized to pre-pulse values (untreated, n=5; vehicle-treated, n=3). Data are presented as mean values and standard error (vertical bars), and compared at each time point with one-way ANOVA. No significant differences were found ($P < 0.05$).



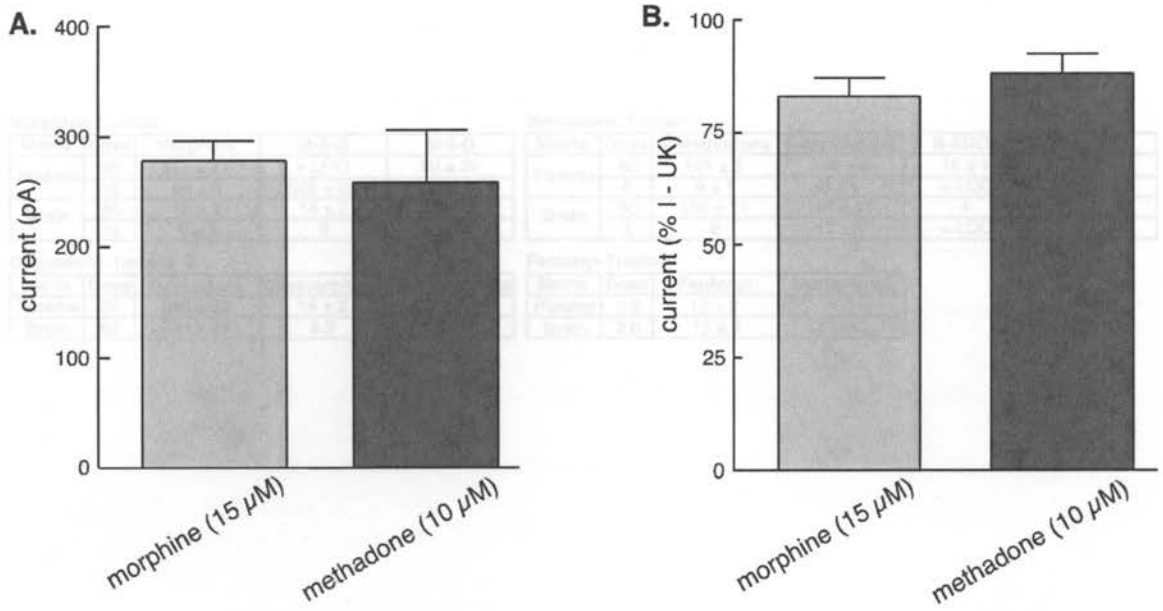
Supplementary Figure 2. The rate of MOR recovery following ME-induced (30 μM/10 min) desensitization is not affected by drug-treatment despite differences in the extent of recovery. MOR recovery measured in long protocol experiments is plotted as time versus current as a percentage of maximum recovery (Figs 2 and 3). Test-pulse values at all time points were normalized to the maximum recovery value in dose-dependence experiments.



Supplementary Figure 3. The rate of acute MOR desensitization is only affected by the highest methadone- (60 mg/kg/day) and morphine- (60 mg/kg/day) treatment doses. Summary of results for acute ME-induced (30 μM/10 minutes) MOR desensitization. The amount of current remaining after 10 min is expressed as a percentage of the peak current for each group (control, n=10; high methadone-treated, n=9; low methadone-treated, n=9; high morphine-treated, n=8; low morphine-treated, n=6, IP morphine-treated, n=4). Rats in the IP morphine-treated group received two IP morphine injections of 10 mg/kg separated by 9 hours. Experiments were performed 24 hours after the first injection. Data are presented as mean values and standard error, and compared at each time point with one-way ANOVA and Dunnett's Multiple Comparison Test. * Significantly different ($P < 0.05$).



Supplementary Figure 4. UK14304 (3 μM) currents are the same size following ME-induced (30 μM/10 minutes) desensitization regardless of morphine- or methadone-treatment. *A,* Peak ME (30 μM) and UK14304 (3 μM) current amplitudes measured in long protocol experiments (Fig 2). At the conclusion of all long protocol experiments (approximately 50 minutes post-wash), the alpha-2 adrenoceptor agonist UK14304 (3 μM) was applied (Fig 2). *B,* Peak UK14304 current amplitudes were normalized to peak ME-induced amplitudes. Data are presented as mean values and standard error, and compared at each time point with one-way ANOVA. No significant differences were noted ($P < 0.05$).



Supplementary Figure 5. Saturating concentrations of morphine and methadone evoke the same amplitude GIRK currents in LC neurons. *A*, Peak current amplitudes (pA) induced by morphine (15 μM) and methadone (10 μM) in control animals (morphine, n=6; methadone, n=5). *B*, Morphine- and Methadone-induced peak current amplitudes normalized to peak UK14304 current amplitudes in the same LC neuron. Data are presented as mean values and standard error (vertical bars), and compared at each time point with one-way ANOVA. No significant differences were found ($P < 0.05$).

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

Oxycodone-Treated

Matrix	Dose	Oxycodone	Oxymorphone	Noroxycodone
Plasma	80	265 ± 33	14 ± 2	122
Brain	80	341 ± 64	8.2	51

Fentanyl-Treated

Matrix	Dose	Fentanyl	Norfentanyl
Plasma	2.8	12 ± 2	1.4
Brain	2.8	13 ± 2	< LOQ

Table 1. The concentration (ng/ml) of morphine, methadone, oxycodone, fentanyl 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); oxycodone dose of 80 mg/kg/day (n=3); fentanyl dose of 2.8 mg/kg/day (n=4) 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; for methadone: 2.5 – 500 ng/ml; for oxycodone: plasma, 0.4 – 500 ng/ml, brain, 0.8 – 1000 ng/ml ; for fentanyl: 0.5 – 1000 ng/ml.

REFERENCES

Alvarez VA, Arttamangkul S, Dang V, Salem A, Whistler JL, Von Zastrow M, Grandy DK and Williams JT (2002) mu-Opioid receptors: Ligand-dependent activation of potassium conductance, desensitization, and internalization. *J Neurosci* **22**(13):5769-5776.

Blake AD, Bot G, Freeman JC and Reisine T (1997) Differential opioid agonist regulation of the mouse mu opioid receptor. *J Biol Chem* **272**(2):782-790.

Bohn LM, Dykstra LA, Lefkowitz RJ, Caron MG and Barak LS (2004) Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. *Mol Pharmacol* **66**(1):106-112.

Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ and Caron MG (2000) Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* **408**(6813):720-723.

Borgland SL, Connor M, Osborne PB, Furness JB and Christie MJ (2003) Opioid agonists have different efficacy profiles for G protein activation, rapid desensitization, and endocytosis of mu-opioid receptors. *J Biol Chem* **278**(21):18776-18784.

Christie MJ, Williams JT and North RA (1987) Cellular mechanisms of opioid tolerance: studies in single brain neurons. *Mol Pharmacol* **32**(5):633-638.

Dang VC and Williams JT (2004) Chronic morphine treatment reduces recovery from opioid desensitization. *J Neurosci* **24**(35):7699-7706.

Dang VC and Williams JT (2005) Morphine-induced {micro}-opioid receptor desensitization. *Mol Pharmacol.*

Finn AK and Whistler JL (2001) Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. *Neuron* **32**(5):829-839.

Fiorillo CD and Williams JT (1996) Opioid desensitization: interactions with G-protein-coupled receptors in the locus coeruleus. *J Neurosci* **16**(4):1479-1485.

Grecksch G, Bartzsch K, Widera A, Becker A, Hollt V and Koch T (2006) Development of tolerance and sensitization to different opioid agonists in rats. *Psychopharmacology (Berl)* **186**(2):177-184.

Hanks GW, Conno F, Cherny N, Hanna M, Kalso E, McQuay HJ, Mercadante S, Meynadier J, Poulain P, Ripamonti C, Radbruch L, Casas JR, Sawe J, Twycross RG and Ventafridda V (2001) Morphine and alternative opioids in cancer pain: the EAPC recommendations. *Br J Cancer* **84**(5):587-593.

Johnson EA, Oldfield S, Braksator E, Gonzalez-Cuello A, Couch D, Hall KJ, Mundell SJ, Bailey CP, Kelly E and Henderson G (2006) Agonist-selective mechanisms of mu-opioid receptor desensitization in human embryonic kidney 293 cells. *Mol Pharmacol* **70**(2):676-685.

Koch T, Widera A, Bartzsch K, Schulz S, Brandenburg LO, Wundrack N, Beyer A, Grecksch G and Hollt V (2005) Receptor endocytosis counteracts the development of opioid tolerance. *Mol Pharmacol* **67**(1):280-287.

Kreek MJ (2000) Methadone-related opioid agonist pharmacotherapy for heroin addiction. History, recent molecular and neurochemical research and future in mainstream medicine. *Ann N Y Acad Sci* **909**:186-216.

Leri F, Tremblay A, Sorge RE and Stewart J (2004) Methadone maintenance reduces heroin- and cocaine-induced relapse without affecting stress-induced relapse in a rodent model of poly-drug use. *Neuropsychopharmacology* **29**(7):1312-1320.

Mercadante S (2007) Opioid titration in cancer pain: A critical review. *Eur J Pain.*

Muller-Busch HC, Lindena G, Tietze K and Woskanjan S (2005) Opioid switch in palliative care, opioid choice by clinical need and opioid availability. *Eur J Pain* **9**(5):571-579.

Osborne PB and Williams JT (1995) Characterization of acute homologous desensitization of mu-opioid receptor-induced currents in locus coeruleus neurones. *Br J Pharmacol* **115**(6):925-932.

Patel MB, Patel CN, Rajashekara V and Yoburn BC (2002) Opioid agonists differentially regulate mu-opioid receptors and trafficking proteins in vivo. *Mol Pharmacol* **62**(6):1464-1470.

Peckham EM and Traynor JR (2006) Comparison of the antinociceptive response to morphine and morphine-like compounds in male and female Sprague-Dawley rats. *J Pharmacol Exp Ther* **316**(3):1195-1201.

Schulz S, Mayer D, Pfeiffer M, Stumm R, Koch T and Hollt V (2004) Morphine induces terminal micro-opioid receptor desensitization by sustained phosphorylation of serine-375. *Embo J* **23**(16):3282-3289.

Stafford K, Gomes AB, Shen J and Yoburn BC (2001) mu-Opioid receptor downregulation contributes to opioid tolerance in vivo. *Pharmacol Biochem Behav* **69**(1-2):233-237.

Walker EA and Young AM (2001) Differential tolerance to antinociceptive effects of mu opioids during repeated treatment with etonitazene, morphine, or buprenorphine in rats. *Psychopharmacology (Berl)* **154**(2):131-142.

Whistler JL, Chuang HH, Chu P, Jan LY and von Zastrow M (1999) Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* **23**(4):737-746.

Williams JT, North RA, Shefner SA, Nishi S and Egan TM (1984) Membrane properties of rat locus coeruleus neurones. *Neuroscience* **13**(1):137-156.

Yu Y, Zhang L, Yin X, Sun H, Uhl GR and Wang JB (1997) Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. *J Biol Chem* **272**(46):28869-28874.

Zhou Y, Spangler R, Maggos CE, LaForge KS, Ho A and Kreek MJ (1996) Steady-state methadone in rats does not change mRNA levels of corticotropin-releasing factor, its pituitary receptor or proopiomelanocortin. *Eur J Pharmacol* **315**(1):31-35.

FOOTNOTES

The analysis of the brain and plasma samples was performed at the Center for Human Toxicology at the University of Utah under the supervision of Dr. Roger Foltz and was supported by NIDA contract N01 DA-4-7737.

This work was supported by NIH: 1F30-DA 021466 (MSV), DA-08163 (JTW) and NARSAD-Ritter Foundation (JTW).

CHAPTER 3

Buprenorphine inhibits desensitization in locus coeruleus neurons

(Preliminary data and interpretation)

Michael S. Virk[1] & John T. Williams[1 & 2]

1 – Vollum Institute, L474
Oregon Health and Sciences University
3181 SW Sam Jackson Park Road
Portland, OR 97239
2 – Corresponding author (williamj@ohsu.edu)

# of figures:
# of tables:
# of pages:

Keywords: desensitization, recovery, MOR, locus coeruleus, opioids

Acknowledgements: This work was supported by the NIH (1F30-DA 021466)[1] and (DA 08163)[2] and NARSAD-Ritter Foundation[2]

INTRODUCTION

Buprenorphine is a semisynthetic thebaine derivative used in the treatment of opiate addiction and as an analgesic. The compound has demonstrated a broad analgesic profile for multiple types of pain in studies addressing both acute and chronic applications (Christoph et al., 2005; Meert and Vermeirsch, 2005). It is also an effective alternative to methadone in the treatment of heroin dependence (Vigezzi et al., 2006). Buprenorphine has been shown to have a bell shaped analgesic dose-response curve (Lutfy et al., 2003; Yamamoto et al., 2006) and a ceiling effect for respiratory depression (Dahan et al., 2005; Dahan et al., 2006). These two properties are unique among opioids, but attractive for clinical use. With repeated administration in animals, tolerance has been shown to develop with a time course similar to that found with morphine (Grecksch et al., 2006).

Buprenorphine is notable for slow receptor association/dissociation kinetics and a half-life of 2-5 hours, a property that contributes to low abuse liability and few withdrawal symptoms upon withdrawal (reviewed in (Tzschentke, 2002). Its high MOR affinity has been viewed as a double-edged sword. It may diminish the ability to experience opiate euphoria in patients on maintenance therapy, however, it be a liability in the same population if opiate analgesia is required for pain management. Additionally, because of the slow dissociation kinetics, naloxone does not displace buprenorphine from receptors thus raising concern over its safety. Though the in vivo properties of buprenorphine have been well-characterized, the underlying MOR-mediated pharmacology and signaling, particularly in neurons, remains poorly understood.

Buprenorphine is a partial agonist at MORs (Selley et al., 1997; Bloms-Funke et al., 2000; Lutfy et al., 2003) and ORL-1 receptors though some reports indicate that it is a full agonist activity at ORL-1 (Wnendt et al., 1999; Lutfy et al., 2003). It has also demonstrated mixed agonist/antagonist activity at KOR and antagonist activity at DOR. The bell shaped dose-response curve has been attributed to activation of MORs at low doses and activation of ORL-1 at higher concentrations. Buprenorphine has considerably greater affinity at MOR than most opiate agonists, though its efficacy in the systems tested thus far has been poor. It has shown weak activation of G protein inward rectifying potassium channels via MOR in oocytes and has not shown any desensitization. This might be expected given that concentrations required to phosphorylated MOR were 100,000 times greater than etorphine (Yu et al., 1997). MOR desensitization and MOR signaling following chronic treatment have not been examined.

LC neurons express both ORL-1 and MOR, but not KOR or DOR and brain slices containing these neurons are well suited to study the pharmacology, acute signaling, and adaptations following chronic treatment with buprenorphine. The results presented here show that buprenorphine is a partial MOR agonist that cannot be reversed with naloxone and that has no measurable activity at ORL-1. Exposing brain slices to very low concentrations of buprenorphine (5 nM) for a short interval (1 hr) is sufficient to eliminate the robust desensitization characteristic of the highly efficacious agonists [Met]^{5}enkephalin (ME) and etorphine. Moreover, in slices pretreated with buprenorphine, following washout of a normally desensitizing concentration of ME the

ME induced current increases with time. Chronic treatment with buprenorphine diminishes peak currents induced by ME, eliminates desensitization in a dose dependent manner. Buprenorphine stands alone among opiates because it is not efficacious in activating potassium currents, but completely inhibits desensitization by other opiate agonists.

METHODS

See Chapters 1 and 2.

RESULTS

Buprenorphine is a partial agonist at MOR and does not bind to ORL-1

Intracellular recordings of membrane potential were made from LC neurons. The application of opioid receptor agonists and nociceptin resulted in a hyperpolarization of the membrane potential (Fig 1). Buprenorphine (1 μM, 15 min) caused a slowly activating hyperpolarization (14.5±1.7 mV; n=8) that blocked the hyperpolarization induced by ME applied by pressure ejection (arrows, Fig 1). The inhibition of the ME hyperpolarization was not the result of occlusion because application of the ORL-1 agonist, orphanin FQ/nociceptin resulted in a further hyperpolarization (Fig1).

Supramaximal concentrations of ME (30 μM) hyperpolarized LC neurons by 34.5 ± 1.5 mV (n=5, Fig 2). However, when buprenorphine (1 μM, 15 min) preceded ME bath application, no additional response was observed (data not shown, n=3). This indicates that buprenorphine-occupied MORs blocked a further effect of ME. As a result of the

slow dissociation kinetics of buprenorphine, superfusion with naloxone (1 μM, 20 min) did not reverse the hyperpolarization. In order to determine if buprenorphine acted on MORs, slices that were pretreated with the irreversible antagonist, ß-CNA (1 μM, 10 min). Subsequent application of buprenorphine (1 μM, 15 min) had no effect on the membrane potential (0.8 mV, n=4). Taken together, the results suggest that the buprenorphine-induced hyperpolarization is mediated by the activation of MORs.

The ORL-1 agonist, OFQ/N (2 μM, 2 min), caused a 36.8 ± 0.6 mV hyperpolarization (n=6), similar to that induced by ME (30 μM). After treatment with buprenorphine, the OFQ/N-induced hyperpolarization was reduced to 22.6±1.5 mV, which when added to the 14.5 mV hyperpolarization induced by buprenorphine resulted in an additive effect. Thus buprenorphine activates MORs, but not ORL-1 receptors on locus coeruleus neurons.

To further characterize the buprenorphine-induced hyperpolarization, slices containing LC neurons were pre-incubated in low concentration buprenorphine (5 nM) for 1 hour before recordings were made. Pre-incubation resulted in a sustained resting membrane potential that was hyperpolarized by approximately 10 mV (-66 ±1.9 mV, n=5) as compared with untreated slices. Superfusion with a ME (30 μM) induced an additional hyperpolarization of 14±1.1 mV (n=7, Figure 2). Thus, after treatment with a low concentration of buprenorphine, the MOR mediated hyperpolarization was reduced and did not display additivity. In the same slices application of OFQ/N (2 μM) caused a

30±1.2 mV hyperpolarization (n=5), suggesting again that buprenorphine did not interact with ORL-1 receptors.

Buprenorphine remains bound to MORs

Voltage clamp recordings using whole cell electrodes were made to further investigate the interaction between buprenorphine and other opioid agonists. With this mode of recording, buprenorphine (1 μM, 40 min) alone had no effect on current that was required to hold the cell at -60 mV. As was observed with intracellular recordings, buprenorphine decreased the outward current induced by the application of ME. Buprenorphine (100 nM) applied for a period of 20-30 min resulted in complete inhibition of current induced by pulses of low concentration ME (300 nM, 2 min, n=3), and a dramatic reduction (>70%) in the current induced by ME (30 μM). All subsequent experiments were carried out after pretreatment of slices with buprenorphine (5 nM, 1 hr).

If buprenorphine dissociated from MOR, during a prolonged wash in drug-free aCSF, ME should cause a larger amplitude current. There was no change in the amplitude of the current induced by ME (30 μM) over a 4-hour period. The current induced by ME (30 μM) immediately following incubation with buprenorphine was 43±5% of the current induced by the alpha-2-adrenoceptor agonist, UK14304 (3 μM, n=6). In slices that were washed for 4 hours after incubation with buprenorphine the current induced by ME was 48±3% of the current induced by UK14304 (3 μM, n=6). Thus buprenorphine remained bound to MOR.

Buprenorphine eliminates MOR desensitization

ME (30 μM) caused a peak current of is 461±28 pA that desensitized over 10 min to 65±1% if the peak. Likewise etorphine caused an outward current of 359±25 pA that declined to 74±1% of the peak after 10 min (Virk and Williams, 2008). Acute desensitization induced by ME (30 μM, 10 min) and etorphine (1 μM, 10 min) were tested after pre-incubating slices in buprenorphine (5 nM, 1 hr). Following pre-incubation with buprenorphine, the peak ME (30 μM) current was reduced to 175±13 pA (n=18, Fig 3A). Unlike what was observed in control, there was no decline in the ME current during the 10 min continuous application (174± 9 pA, 105±6%). The current induced by etorphine (1 μM, 10 min) was similarly affected. The peak etorphine current was 67±9% of the current induced by UK14304 (3 μM) and was 64±5% after 10 min (n=5). The results indicate that the desensitization induced by both ME- and etorphine was completely blocked by pre-treatment with buprenorphine.

Agonist pre-treatment does not eliminate MOR desensitization

It was possible that the affinity and kinetics of buprenorphine binding to MOR could account for the blockade of desensitization. To test for this possibility slices were pre-incubated with low concentration etorphine (2 nM, 1hr) prior to testing for desensitization induced by ME. After treatment with etrophine slices were placed in the recording chamber, whole cell recordings were made and ME (30 μM, 10 min) was superfused. The current induced by ME (30 μM) measured 149±14 pA (n=7) and declined to 79±14 pA after 10 min (desensitized to 51±9%). After ME washed from the slice, naloxone (1 μM) was applied to determine if etorphine remained in the slice.

Naloxone caused an inward current of 151±42 pA (n-7), thus as has been shown previously, etorphine evoked a prolonged outward current long after removal of etorphine from the extracellular solution (Virk and Williams, 2008). These results indicate that pre-incubation with etorphine did not eliminate ME-induced desensitization and is therefore distinct from buprenorphine.

Antagonist pre-treatment increases MOR desensitization

It is possible that buprenorphine blocked desensitization because of the fact that it is such a weak partial agonist. To test this possibility slices were pre-incubated with the irreversible MOR antagonist β-chlornaltrexamine (β-CNA, 5–40 nM) for 1 hour. The reduction in the current induced by ME (30 μM) following treatment with β-CNA varied widely (Fig 3D). This variation was unlike that observed following pre-incubating with buprenorphine (5 nM). Multiple concentrations of β-CNA were tested in order to obtain a range of currents induced by ME that could be compared with the results obtained with buprenorphine. Regardless of the concentration of ß-CNA that was used, the peak current induced by ME (30μM, 10 min) desensitized (Fig 3 B). Moreover, when the peak current induced by ME was 200 pA or less, the amount of desensitization was *greater* (Fig 3D, triangles) than in untreated controls. When ME (30 μM) was applied without pre-incubation with ß-CNA, the range of peak current amplitude was 276–698 pA and after 10 min declined by 35±1% (Fig 3 C and D, squares). The center line of the shaded rectangle in Fig 3D represents the mean desensitization measured when ME was applied to cells incubated in aCSF alone and the borders delimit the 95% confidence interval (23-47%). Cells pre-incubated in buprenorphine (5 nM) for 1 hour showed that as peak

current declined, the current amplitude *increased* during a 10 min application of ME (30 μM, Fig 6D circles). This is the opposite result obtained using β-CNA to eliminate receptors. Thus, removal of receptors with the irreversible antagonist, β-CNA, increased the magnitude of ME-induced desensitization, whereas incubation with buprenorphine either eliminated desensitization or resulted in an increase in peak ME current.

Repeated applications of ME - after acute buprenorphine treatment

Experiments were done where ME (30 μM) was applied repeatedly in slices that were pre-incubated with buprenorphine (Fig 4A). In all recordings, ME (30 μM) was first applied for 10 min followed by short applications 5 and 45 min after the 10 min application. The current induced by short applications after 5 and 45 min were expressed as a percentage of the initial peak ME current (Fig 4D). In control (no drug pre-incubation, Fig 4D, "aCSF-CNA"), the current induced by ME (30 μM, 10 min) desensitized to 65±1% of the initial peak and following washout recovered to 75±2% in 5 min and was complete (96±6%) after 45 min (21±5% increase). After pre-incubation with buprenorphine (5 nM, 1 hr), the current induced by ME (30 μM) did not decline over the 10 min application and test-pulse amplitudes were not statistically different from the initial ME peak current amplitude (97±6% and 114±13%; Fig 4A). Given that there was no desensitization induced by ME (30 μM, 10 min), it was expected that the test-pulse amplitudes would be the same as the initial peak ME current amplitude.

In previous work the irreversible antagonist, β-CNA, was used to determine the extent to which desensitized receptors were protected from irreversible inactivation. The results of

that experimental approach indicated that agonists that induced greater desensitization resulted in greater protection from inactivation by ß-CNA (Fig 4C, Virk and Williams, 2008). After pre-treatment with buprenorphine (5 nM, 1 hr), there was no decline in the current induced by ME (30 μM) applied for 10 min (Figure 3A, Fig 4A), with subsequent applications of ME (30 μM) there was however a trend toward an increased current (Fig 4A). The ß-CNA protocol was used to investigate whether prior application of ME (30 μM, 10 min) induced a form of protection from ß-CNA that could be detected even in the absence of any change in the current during the 10 min application. As expected immediately (5 min) after treatment with ß-CNA (500 nM, 2 min) the current induced by ME (30 μM) was reduced (52±9% of the initial ME current). Surprisingly after 45 min ME (30 μM) induced a current that was 102±9% of the initial value (30 μM, Fig 4B,D). The total change between test-pulse amplitudes was 58±7%. When this same experiment was done in slices that were not pre-treated with buprenorphine, the recovery between the two test-pulses was 33±3% and the maximum recovery, measured after 45 min was 43±2% of the peak ME current (Fig 4C, Virk and Williams, 2008). The results indicate that β-CNA (500 nM, 2 min) removed a fraction of receptors and other receptors became available during the 40 min recovery period (Sup Fig 1).

As a control, recovery from desensitization was examined in neurons that were pre-incubated in the irreversible antagonist β-CNA (5-40 nM/1 hour). After treatment with β-CNA, ME (30 μM/10 min) caused substantial desensitization, and the test-pulses measured 5±1% and 25±5% at 5 and 45-min, respectively (Fig 4D). The total recovery between test-pulses was 21±4% and was similar to that observed in controls. Thus,

treatment with β-CNA (5-40 nM) did not affect recovery from desensitization. The increase in ME current observed in buprenorphine pre-treated slices is therefore unique.

Chronic Buprenorphine Treatment

Animals were treated with 3 different doses of buprenorphine (1, 5, and 10 mg/kg/day) for 6-7 days before slices were prepared. All experiments used a saturating concentration of ME (30 μM, 10 min) to induce desensitization followed by the application of a lower concentration to measure the extent of recovery from desensitization. The results of these experiments show that there were three effects of buprenorphine that were dose dependent: the peak current induced by ME (30 μM) was reduced, ME (30 μM/10 min) induced desensitization was inhibited, and the recovery from desensitization was facilitated (Fig 5). These effects are the opposite of those observed following treatment with any other MOR agonist (Virk and Williams, unpublished).

Buprenorphine remains in slices prepared from rats receiving chronic treatment

The amplitude of the ME induced current was reduced as the dose of buprenorphine increased (Fig 5 A, B, C). ME (30 μM) currents expressed as a percentage of UK-14304 currents measured 136±5% (n=20) in control animals. Peak ME (30 μM) induced currents decreased to 96±4% (n=6), 74±7% (n=6), and 47±4% (n=8) as buprenorphine treatment increased from 1, 5, and 10 mg/kg/day (Fig 5C). This dose-dependent decrease in current amplitude suggests that the percentage of buprenorphine occupied MORs increased as the treatment dose increased.

Buprenorphine prevents ME-induced MOR desensitization

Acute MOR desensitization was measured as a decline in maximum GIRK current during a 10 min application of ME (30 μM). The current remaining at the end of the 10 min period differed between treatment groups in a dose-dependent manner (Fig 5 A, B, D). In untreated animals, the current that remained after 10 min in ME (30 μM) was 65±2% of the peak. The current after 10 min was 55±3%, 76±5%, and 90±6% of the peak in the 1, 5, and 10 mg/kg/day buprenorphine treatment groups. Thus, desensitization was inhibited in animals treated with buprenorphine in a dose dependent manner.

Buprenorphine treatment facilitates recovery from desensitization

The reduction in ME-induced current following buprenorphine treatment required the use of a higher concentration of ME to monitor extent and recovery from desensitization. Normally the test-pulse of ME (300 nM) was repeatedly applied before and after the induction of desensitization with ME (30 μM, 10 min). In untreated animals the initial value of the ME (300 nM) current was 51±4% of the current induced by ME (30 μM). The current induced by ME (300 nM) was reduced to 37±3% of the maximum in animals treated with buprenorphine (1 mg/kg/day). In animals treated with 5 mg/kg/day the test pulse of ME was increased to 700 nM and the resulting current was 27±3% of the maximum current. In animals treated with 10 mg/kg/day the test-pulse ME concentration was increased to 1 μM and this evoked a current that was 32±3% of the maximum.

The recovery from desensitization was measured for 45 minutes following acute application of ME (30 μM/10 min), by applying a test-pulse every 10 minutes following

desensitization. The results of these experiments show that the current induced by the test-pulse of ME increased with time in spite of diminishing desensitization during the application of saturating concentrations of ME (30 μM, 10 min). The test pulse at 5 min measured 22±3% for controls and 19±2% for the 1 mg/kg/day treated group (n=6), while at 45 minutes the test-pulses measured 71±5% and 75±9%, respectively (Fig 5E). However, following treatment with 5 mg/kg/day (n=4), the test-pulse at 5 minutes measured 59±8% and completely recovered (97±8%) by 25 minutes. When animals were treated with 10 mg/kg/day (n=4-8), the first test-pulse measured 82±4% and had completely recovered to stable values by 15 min (103±11%). Thus, desensitization decreased and recovery occurred faster as the dose of buprenorphine used for treatment increased.

The results suggest that buprenorphine bound receptors are maintained in slices prepared after chronic treatment with buprenorphine for the duration of the entire experiment. Despite incubating the slices in aCSF for 1 hour prior to experiments, the slow dissociation rate of buprenorphine likely resulted in the continued occupation of MORs. Although there was no effect of chronic treatment with the lowest dose of buprenorphine (1 mg/kg/day), the amplitude of ME–induced current at both 300 nM and 30 μM was reduced. Thus buprenorphine remains in the slice but is only detectable as a shift in sensitivity to ME.

DISCUSSION

The results of the experiments presented here indicate that buprenorphine binding has unique functional consequences on MOR regulation. Neurons contained in LC brain slices permitted the isolation of MOR and ORL-1 specific effects because they lack KOR and DOR. Because there was no evidence of signaling through ORL-1 receptors, the effects of buprenorphine reported here were exclusively MOR mediated. At very low concentrations, buprenorphine eliminates the robust desensitization characteristic of ME and etorphine. Pre-incubation with this compound protects MOR from CNA binding, but has the additional effect of stimulating MOR availability over time. Finally, chronic treatment of animals with buprenorphine decreases MOR availability and inhibits MOR desensitization in a dose dependent manner.

Buprenorphine is a partial agonist at MOR. It was possible to measure an increase in GIRK conductance in response to high concentration of buprenorphine only when measuring membrane potential with sharp electrodes, given the greater sensitivity of this configuration. Nonetheless, high concentration buprenorphine (1 μM) failed to evoke an ORL-1 mediated response or to block the effect OFQ/N at this receptor. This compound binds to MOR with an affinity exceeding that of etorphine and fentanyl, as the signaling induced by the latter two compounds could be reversed with naloxone (Virk and Williams, 2008, in press), while that of buprenorphine could not. In vivo studies examining the ability of naloxone to reverse buprenorphine-induced analgesia, however, have found that this is possible, particularly with high or repetitive doses of naloxone (Kogel et al., 2005). Furthermore, it was not possible to displace buprenorphine with

highly localized pressure-ejected ME, despite significant increases in concentration and duration. Though slow to bind to MOR, once bound, buprenorphine did not dissociate during the time course of the recordings.

A brief incubation of slices with low concentration buprenorphine was sufficient to eliminate ME and etorphine induced desensitization. This effect was specific to buprenorphine because neither pre-incubation with etorphine nor β-CNA could produce this result. Etorphine was selected as a control because it is a structurally similar thebaine molecule that shares high potency, high lipid solubility and high MOR affinity with buprenorphine. β-CNA was selected because it is an irreversible opioid antagonist that permanently reduced the number of available MORs. Moreover two separate phenomena became apparent. The reduction of MOR reserve with β-CNA, indicated by reduced ME currents, was correlated with increased MOR desensitization. Peak currents that measured less than 200 pA decreased to a greater extent with time that those measuring greater than 200 pA. In contrast, peak ME currents that were reduced to less than 200 pA following buprenorphine pre-incubation, increased in amplitude over the course of a 10 min superfusion. Previous experiments in HEK 293 cells have demonstrated that reducing MOR reserve with an irreversible antagonist, β-FNA, increased etorphine-induced desensitization (Law et al., 2000), however, there are no reports of pre-treatment with any agonist or antagonist that serve to increase MOR sensitivity during continuous exposure.

Another set of experiments further addressed the observation that buprenorphine pre-incubation increased MOR-mediated signaling following exposure to high concentrations of ME. In control slices, ME (30μM) applied for 10 min desensitized a larger percentage of MORs and desensitization protected a subset of these MORs from β-CNA binding (Virk and Williams, 2008, in press). The test-pulse at 5 min was significantly diminished (10±2% of peak), but recovered substantially by 45 min (43±2% of peak). When the same experiment was done with neurons pre-incubated in buprenorphine, no desensitization occurred during the ME (30μM/10min) application. After β-CNA superfusion, the 5 min test-pulse (52±10% of peak) indicated that the MOR reserve was decreased compared to when β-CNA is not perfused (97±6% of peak). However, over the course of 40 min, the current increased to initial peak current values (102±9%). When this experiment was performed with oxycodone (15μM/10min), no desensitization was measured and the test-pulse currents at 5- and 45-min measured the same amplitude (Virk and Williams, 2008, in press). In the case of oxycodone, therefore, when there was no desensitization, the test-pulses remained equal. Buprenorphine pre-incubation alters MOR signaling such that after exposure to ME (30μM/10min), more receptors become available over time.

Chronic Treatment

Three different doses of buprenorphine were used to evaluate the effects of chronic treatment with this compound on MOR signaling. The drug remained bound to MOR after slices were prepared from rats undergoing treatment. Both peak ME current and desensitization were decreased in a dose-dependent fashion. Although 1 mg/kg/day

decreased peak currents, this dose had no effect on desensitization or recovery as compared with control animals. MOR recovery from desensitization in the control and 1 mg/kg/day groups plateaued at 45 mins and reached a maximum of and 71±5% and 75±9%. At 5 mg/kg/day, however, MOR recovery was complete and occurred rapidly (97±8% at 25 min). In the highest dosage group, desensitization was inhibited and recovery was complete by 15 min.

The results from chronic treatment studies indicate that more efficacious opioids can bind and signal via MOR despite high dose treatment. Moreover, in the presence of buprenorphine these opiates do not desensitize MOR. Interestingly, the 10 mg/kg/day treatment group yielded similar results to pre-incubation with buprenorphine (5nM/1hr). The doses used in this study (1, 5, 10 mg/kg/day) are in the range of those used to achieve maximal analgesia. In a study measuring analgesia to tail withdrawal, single subcutaneous doses of 2.5, 10 and 40 mg/kg induced maximal analgesia within 60 min (Meert and Vermeirsch, 2005). Beginning at 40 mg/kg and profoundly at 80 mg/kg the inverted U-shaped curve was observed. Finally, to confirm that buprenorphine doses were being faithfully delivered to the animals, plasma and brain samples were analyzed for drug concentration. The concentrations for 1, 5, and 10 mg/kg/day were as follows: Plasma – 5.2, 27.7, and 58.1 ng/ml; Brain – 12.2, 29.1, and 64.5 ng/ml. These numbers must be interpreted with caution because the limits of quantitation are 0.2 – 20 ng/ml.

Conclusion

Buprenorphine is high potency, partial agonist that binds to MOR at low concentrations and exerts novel functional effects that have not been described for any other opioid. In rat LC, there was no evidence of binding to ORL-1. This is in spite of the fact that several studies have attributed the downward arm of the inverted-U shape curve to ORL-1 mediated effects (Lutfy et al., 2003; Yamamoto et al., 2006). The mechanism underlying the elimination of ME-induced desensitization remains unclear, but could have significant implications for tolerance development in subjects maintained on buprenorphine. Finally, it is not known how MORs become available for binding after buprenorphine pre-treatment and high concentration ME applications. Regarding receptor trafficking, buprenorphine has been shown to down regulate MOR expression after chronic treatment in vivo (Belcheva et al., 1993), increase surface receptor expression following prolonged exposures in vitro (Yu et al., 1997), and fail to induce MOR internalization acutely (Koch et al., 2005). There is little know about how long term treatment affects analgesic tolerance in humans, whether or not the inverted U-shape curve described for animals exists in humans, or how long and at what cost long term opioid maintenance therapy affects patients. The compound is gaining increasing notoriety as a useful analgesic and narcotic abuse treatment, however, a mechanistic understanding of how these affects are achieved will require considerably more attention.

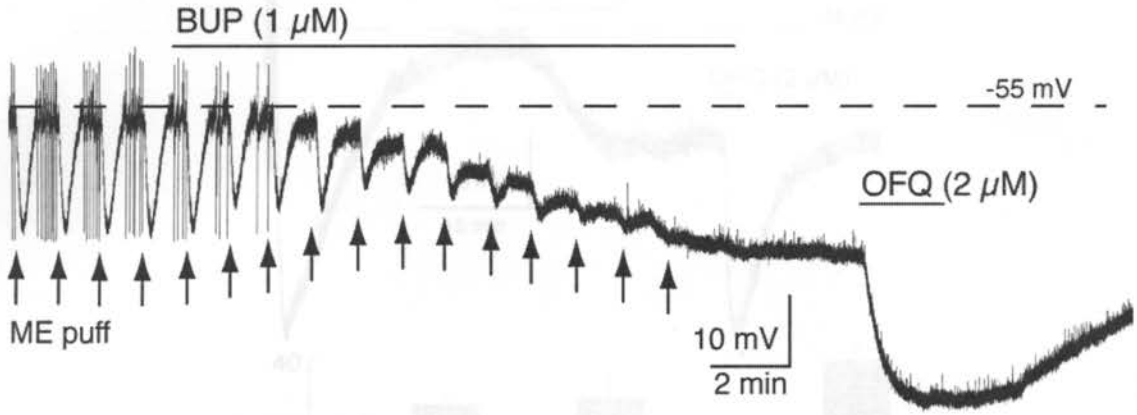


Fig 1. Buprenorphine causes a slow activating membrane hyperpolarization that blocks hyperpolarizations induced by ME, but not orphanin FQ/nociceptin. A representative trace of an experiment using sharp electrodes to record membrane potential in rat LC. ME (1 mM) was pressure ejected (arrows) before and during buprenorphine (1 μM/15 min) superfusion. The ME response is completely blocked as the maximum buprenorphine induced hyperpolarization is reached. The ORL-1 agonist, orphanin FQ/nociceptin, induces further membrane hyperpolarization despite buprenorphine treatment.

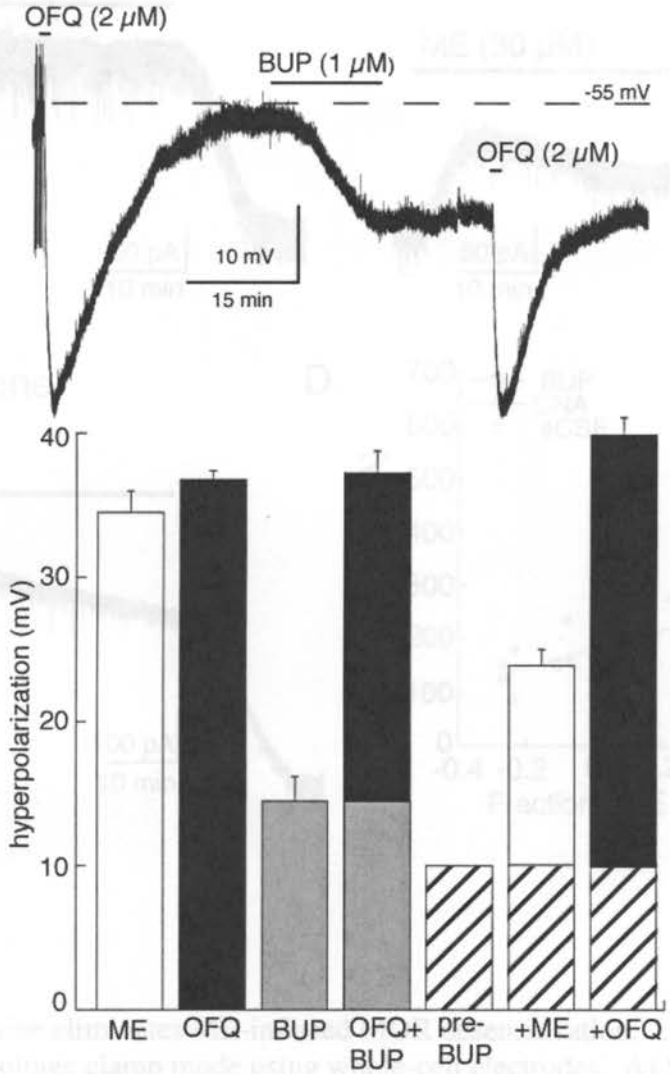


Fig 2. Buprenorphine blocks ME-induced hyperpolarization, but displays additivity with orphanin FQ/nociceptin at ORL-1. An example of a representative trace of membrane potential in LC neurons. Orphanin FQ/nociceptin (2 μM/2 min) caused a hyperpolarization similar in magnitude to ME (30 μM/2 min). Buprenorphine (1 μM/15 min) induced a slow hyperpolarization that did not block subsequent orphinanin FQ/nociceptin, but was completely additive. A similar finding was observed when brain slices were pre-incubated in low concentration buprenorphine (5 nM/1 hour). In these slices, LC neurons were hyperpolarized by approximately 10 mV. When ME (30 μM) was applied the hyperpolarization was inhibited by not blocked. Applying orphanin FQ/nociceptin still resulted in a maximal hyperpolarization.

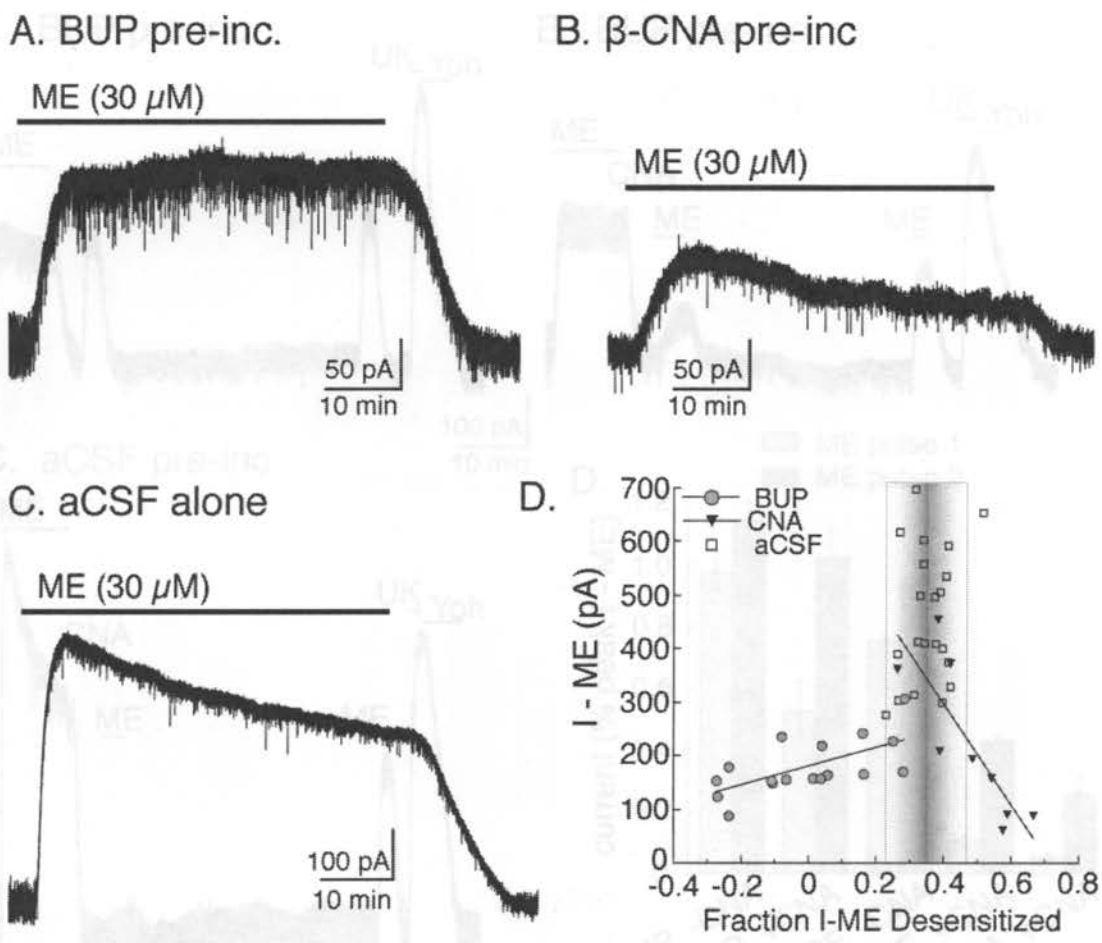


Fig 3. Buprenorphine eliminates ME-induced MOR desensitization. LC neurons were recorded from in voltage clamp mode using whole-cell electrodes. A) Slices were pre-incubated in buprenorphine (5 nM/1 hr). The peak amplitude current induced by ME (30 μM/10 min) was reduced and desensitization was eliminated. B) Slices were pre-incubated in the irreversible MOR antagonist, B-CNA (5-40 nM/1 hr). ME (30 μM/10 min) induced currents amplitudes were reduced while desensitization was equal to control cells or greater. C) When LC neurons were incubated in aCSF alone, ME (30 μM/10 min) induced maximal currents that desensitized by approximately 35%. D) A plot of desensitization vs peak current induced by ME following 3 different pre-incubation conditions. The vertical line marks the average MOR desensitization observed in response to ME (30 μM/10 min) while the shaded box outlines the 95% confidence interval. By eliminating MOR receptor reserve with an antagonist, B-CNA, desensitization increases, particularly when peak currents are less than 200 pA. Buprenorphine occupied MORs, however, display the opposite trend with desensitization decreaseing as peak currents decrease below 200 pA.

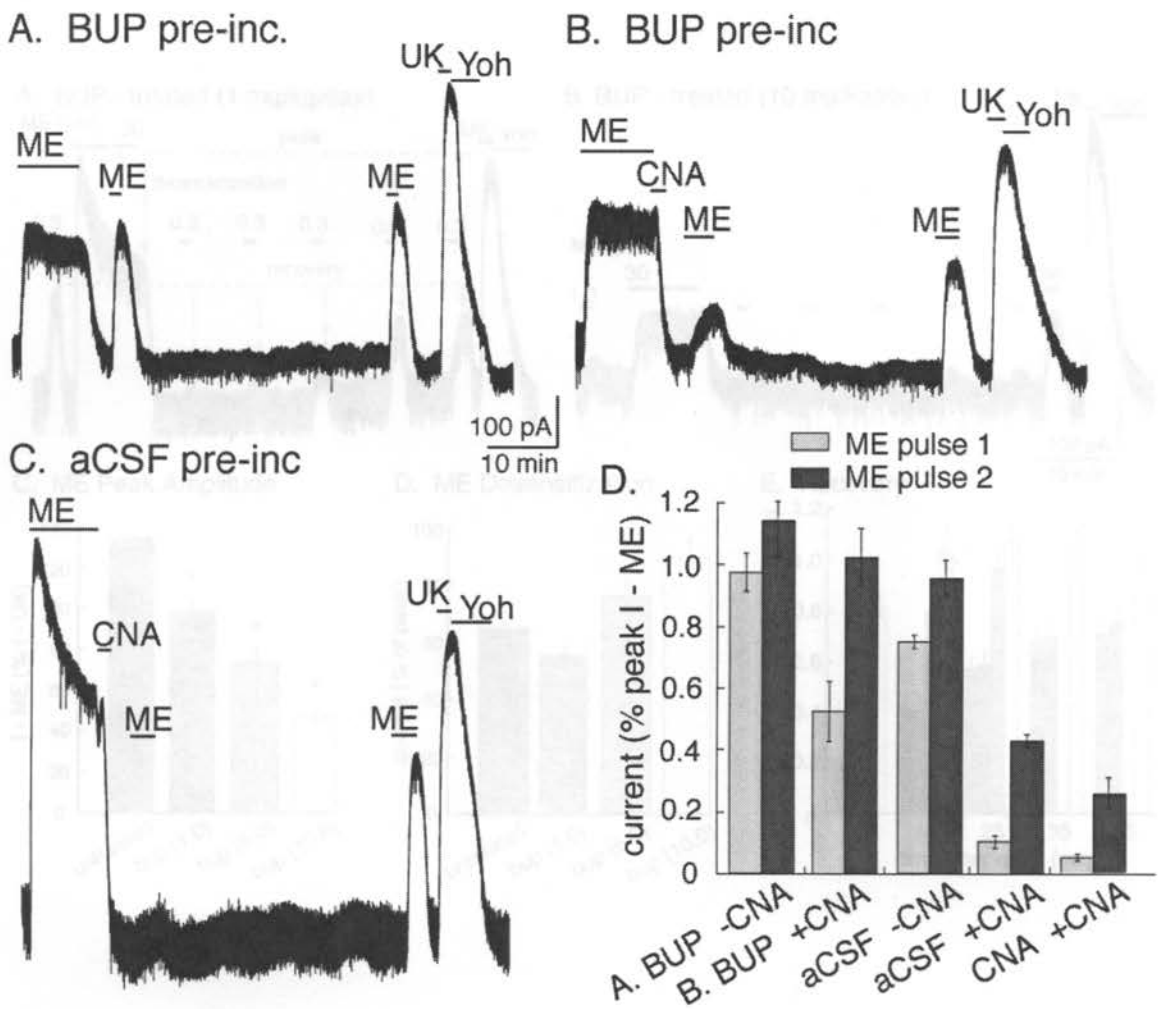


Fig 4. ME exposure increases subsequent ME induced currents following buprenorphine pre-incubation. In all recordings, ME (30 μM) was first applied for 10 min followed by short applications 5 and 45 min after the 10 min application. A) ME (30 μM/10 min) causes no desensitization and test pulses at 5 and 45 min are the same amplitude. B) ME (30 μM/10 min) causes no desensitization. B-CNA (500 nM/ 2min) reduces the ME test pulse at 5 min by 50%. By 45 min the ME test pulse has returned to its original amplitude. C) Control experiment where slices were pre-incubated in aCSF only and B-CNA (500nM / 2 min) was applied after the initial ME superfusion. ME (30 μM/10 min) desensitizes MOR and at 5 min the ME test pulse is 10% of the original value. After 45 min, the ME current has recovered to 40%. D) The current induced by short applications after 5 and 45 min were expressed as a percentage of the initial peak ME current. In control experiments with no B-CAN, (aCSF –CNA), the ME current returns to the original value by 45 min. When slices were pre-incubated in B-CNA and B-CAN was applied after the first ME application (CNA+CNA), absolute ME currents were reduced but the percent of recovery was the same as in conditions without buprenorphine pre-incubation.

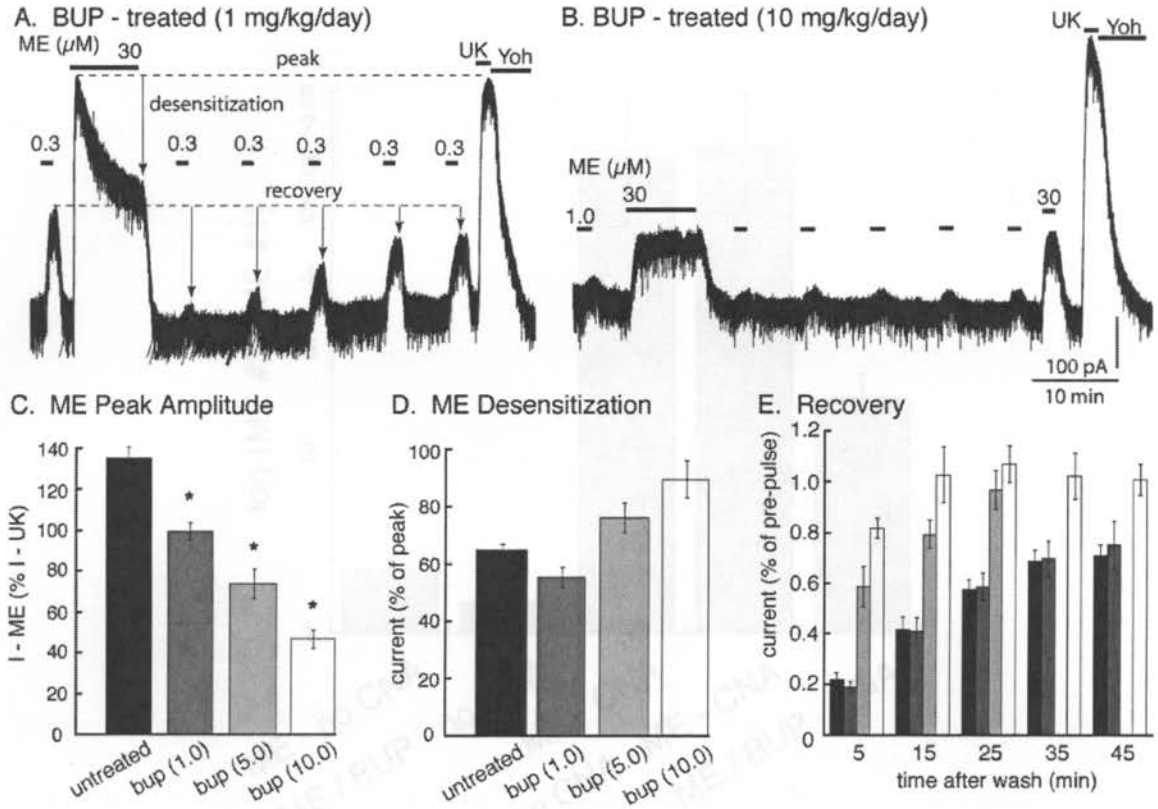


Fig 5. Chronic buprenorphine treatment (6-7 days) had three dose-dependent effects on MOR signaling: peak ME (30μM) currents were reduced, ME induced desensitization was inhibited and recovery following desensitization was facilitated. A) Sample trace of LC neuron taken from animal receiving low dose buprenorphine treatment (1 mg/kg/day). Peak ME current, desensitization and recovery measurements are labeled with dotted lines and arrows. B) Sample trace of LC neuron taken from animal receiving high dose buprenorphine (10 mg/kg/day). C) Summary of peak ME (30 μM) current expressed as a percentage of peak UK14013 current for control animals and all three treatment groups. D) Summary of MOR desensitization induced by ME (30 μM/10 min) where current at 10 min is expressed as a percentage of peak current. E) Summary of MOR recovery following desensitization where test-pulse amplitudes at each time point indicated are expressed as a percentage of the pre-pulse.

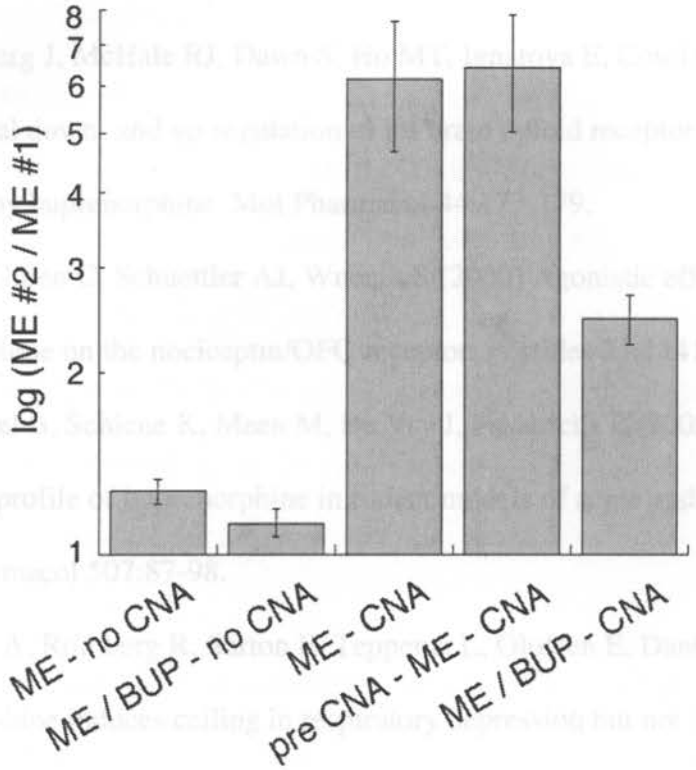


Figure 8. A summary of results showing the degree of protection from desensitization that is mediated by pre-incubation with buprenorphine. The ratio of the current induced by ME (30 μM) applied 5 and 45 min following a 10 min treatment with ME (30 μM) are plotted under different conditions. Pre-incubation with buprenophine does not change the pulse2/pulse1 ratio very much under conditions where ß-CNA was not used (compare no CNA with BUP – no CNA). The pulse2/pulse1 ratio was increased dramatically with the application of ß-CNA (500 nM, 2 min) immediately after the 10 min desensitization treatment with ME (30 μM) in control (CNA) and after the eliminating receptors with a pre-incubation with ß-CNA (preCNA/CNA). After pre-incubation with buprenorphine, although there was no desensitization induced by the 10 min treatment with ME (30 μM) the ratio of pulse2/pulse1 was observed, suggesting that receptors were protected from blockade by ß-CNA.

REFERENCES

Belcheva MM, Barg J, McHale RJ, Dawn S, Ho MT, Ignatova E, Coscia CJ (1993) Differential down- and up-regulation of rat brain opioid receptor types and subtypes by buprenorphine. Mol Pharmacol 44:173-179.

Bloms-Funke P, Gillen C, Schuettler AJ, Wnendt S (2000) Agonistic effects of the opioid buprenorphine on the nociceptin/OFQ receptor. Peptides 21:1141-1146.

Christoph T, Kogel B, Schiene K, Meen M, De Vry J, Friderichs E (2005) Broad analgesic profile of buprenorphine in rodent models of acute and chronic pain. Eur J Pharmacol 507:87-98.

Dahan A, Yassen A, Romberg R, Sarton E, Teppema L, Olofsen E, Danhof M (2006) Buprenorphine induces ceiling in respiratory depression but not in analgesia. Br J Anaesth 96:627-632.

Dahan A, Yassen A, Bijl H, Romberg R, Sarton E, Teppema L, Olofsen E, Danhof M (2005) Comparison of the respiratory effects of intravenous buprenorphine and fentanyl in humans and rats. Br J Anaesth 94:825-834.

Grecksch G, Bartzsch K, Widera A, Becker A, Hollt V, Koch T (2006) Development of tolerance and sensitization to different opioid agonists in rats. Psychopharmacology (Berl) 186:177-184.

Koch T, Widera A, Bartzsch K, Schulz S, Brandenburg LO, Wundrack N, Beyer A, Grecksch G, Hollt V (2005) Receptor endocytosis counteracts the development of opioid tolerance. Mol Pharmacol 67:280-287.

Kogel B, Christoph T, Strassburger W, Friderichs E (2005) Interaction of mu-opioid receptor agonists and antagonists with the analgesic effect of buprenorphine in mice. Eur J Pain 9:599-611.

Law PY, Erickson LJ, El-Kouhen R, Dicker L, Solberg J, Wang W, Miller E, Burd AL, Loh HH (2000) Receptor density and recycling affect the rate of agonist-induced desensitization of mu-opioid receptor. Mol Pharmacol 58:388-398.

Lutfy K, Eitan S, Bryant CD, Yang YC, Saliminejad N, Walwyn W, Kieffer BL, Takeshima H, Carroll FI, Maidment NT, Evans CJ (2003) Buprenorphine-induced antinociception is mediated by mu-opioid receptors and compromised by concomitant activation of opioid receptor-like receptors. J Neurosci 23:10331-10337.

Meert TF, Vermeirsch HA (2005) A preclinical comparison between different opioids: antinociceptive versus adverse effects. Pharmacol Biochem Behav 80:309-326.

Selley DE, Sim LJ, Xiao R, Liu Q, Childers SR (1997) mu-Opioid receptor-stimulated guanosine-5'-O-(gamma-thio)-triphosphate binding in rat thalamus and cultured cell lines: signal transduction mechanisms underlying agonist efficacy. Mol Pharmacol 51:87-96.

Tzschentke TM (2002) Behavioral pharmacology of buprenorphine, with a focus on preclinical models of reward and addiction. Psychopharmacology (Berl) 161:1-16.

Vigezzi P, Guglielmino L, Marzorati P, Silenzio R, De Chiara M, Corrado F, Cocchi L, Cozzolino E (2006) Multimodal drug addiction treatment: a field comparison of methadone and buprenorphine among heroin- and cocaine-dependent patients. J Subst Abuse Treat 31:3-7.

Wnendt S, Kruger T, Janocha E, Hildebrandt D, Englberger W (1999) Agonistic effect of buprenorphine in a nociceptin/OFQ receptor-triggered reporter gene assay. Mol Pharmacol 56:334-338.

Yamamoto T, Shono K, Tanabe S (2006) Buprenorphine activates mu and opioid receptor like-1 receptors simultaneously, but the analgesic effect is mainly mediated by mu receptor activation in the rat formalin test. J Pharmacol Exp Ther 318:206-213.

Yu Y, Zhang L, Yin X, Sun H, Uhl GR, Wang JB (1997) Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. J Biol Chem 272:28869-28874.

CONCLUSIONS

The work presented here examined 1) agonist-specific MOR regulation following the acute application of 10 different opioid agonists in opioid naïve preparations and 2) acute MOR regulation following chronic treatment with 5 different opioid agonists. In order to functionally monitor MOR signaling, whole-cell patch clamp recordings in the voltage clamp configuration were used to measure GIRK currents in brain slices containing locus coeruleus neurons. The results of these experiments indicate that opioid agonists commonly prescribed in clinics for acute and chronic treatment of pain have different profiles with respect to efficacy, desensitization, and re-sensitization. Additional properties such as receptor binding kinetics and lipophilicity must be considered in proper experimental design. It is also apparent that chronic treatment with different agonists resulted in different adaptations governing MOR regulation. These experiments further highlight the importance of opiate properties, particularly drug metabolism and the presence of active metabolites. Taken together, the results provide functional data supporting acute agonist specific MOR regulation and demonstrate cellular adaptations resulting from chronic treatment that may contribute to different levels of tolerance observed in animal models and experienced by patients in the clinic.

Acute agonist-selective MOR regulation: efficacy, desensitization and re-sensitization

The results of this study have demonstrated that 8 clinically relevant opioids and 2 opioid peptides have distinctly different profiles. The agonists tested here all demonstrated a

correlation between efficacy and desensitization, with some notable exceptions. Moreover, a closer examination reveals three distinct groups with respect to these two parameters. The endogenous peptide agonist, ME, and the synthetic peptide, DAMGO, comprise the first group with respect to inducing the largest amplitude GIRK currents, the most profound desensitization and the greatest recovery from desensitization. Etorphine, oxymorphone and fentanyl make up the second group. Methadone is likely a member of this group, but due to experimental limitations inherent in brain slice physiology, binding kinetics, and the possibility that methadone blocks GIRK channels, an accurate measure of peak current and desensitization was not possible. The second group is comprised of a thebaine (etorphine), a semi-synthetic (oxymorphone) and two fully synthetic (fentanyl and methadone) opioids. Thus, there is no clear relationship between method of derivation and binding-induced MOR regulation. The third group includes morphine, its metabolite, morphine-6-glucuronide, and the semi-synthetic opioid, oxycodone, induced the smallest GIRK currents. These were all approximately 50% of the amplitude induced by the peptide agonists, however differed with respect to both desensitization and recovery.

Oxycodone, rather than morphine, is firmly anchored at one pole of the ligand spectrum with respect to desensitization and recovery. Morphine has been at the center of tolerance controversies because it has high intrinsic efficacy, induces little desensitization, and causes no internalization when examined in heterologous systems. Yet, tolerance develops to morphine more rapidly than almost all other opioids. Oxycodone, however, may be a better agonist to use as an experimental prototype

because it is as efficacious as morphine, but induces *no* desensitization or subsequent re-sensitization. Results from one study that tested several agonists for their ability to induce internalization showed that oxycodone and morphine induced the same amount of MOR internalization and this was considerably less than DAMGO (Koch et al., 2005). In striatal neurons, however, DAMGO and morphine induced the same amount of internalization, again illustrating the significance of endogenous intracellular regulatory components (Haberstock-Debic et al., 2005). If oxycodone induced less MOR internalization in striatal neurons, it would be as efficacious as morphine with respect to evoking GIRK currents, but lacking in the ability to drive any endocytosis or desensitization. This would make it an important tool for investigating the leading hypotheses underlying tolerance. The primary limiting factor would be delivery route, due to the bioconversion of oxycodone to oxymorphone.

Buprenorphine is an important compound in managing opioid dependence. Currently, methadone is first line treatment for opioid abuse because it has been successfully used for over 40 years. However, methadone is a highly regulated Schedule II drug and can only be administered at specialized methadone clinics. Furthermore, estimates indicate that only 15-20% of US heroin addicts can be accommodated by established methadone clinics (Meehan WJ, webMD, 2006). Buprenorphine was approved for opiate addiction in 2002 and, as a Schedule III drug, can be administered from office-based practices as long as physicians take a one-day course and accept a regulated number of patients. Its ceiling effect with respect to respiratory depression and low abuse liability further

increase its appeal. However, studies investigating the cellular mechanisms underlying acute and chronic treatment with buprenorphine are few.

The data presented here show that buprenorphine is a unique compound among opioids. The ability to eliminate ME-induced desensitization following a low concentration, 1hour incubation was unexpected and is a novel interaction among opioids. Additionally, the interaction between buprenorphine pre-incubation, β-CNA application, and ME exposure reveals another novel pharmacological or signaling phenomena. The increase in MOR signaling following exposure to high concentration ME does not correlate at all with a failure to cause desensitization. β-CNA may not act as an irreversible antagonist at MOR following buprenorphine pre-incubation. Alternatively, MORs may undergo dimerization or unique endocytosis and trafficking pathways with this combination of compounds. In any case, experiments targeting mechanism will undoubtedly prove interesting for this compound.

Acute MOR regulation following chronic treatment with different opiate agonists

The results of this study indicate that chronic treatment with different opiate agonists causes different cellular adaptations that in turn modulate acute MOR regulation. Morphine, methadone, and buprenorphine were most rigorously investigated because they produced the most discrepant results.

Morphine and methadone produced two similar results: they both increased acute MOR desensitization and produced a 2-fold right shift in the dose response curve. This

indicates that desensitization machinery was more effective following chronic treatment. The shift in dose response curve indicates that treatment with either drug uncoupled the same number of MORs and, thus decreased the number of spare receptors to the same extent.

Treatment with morphine, however, inhibited subsequent MOR recovery, while methadone had no effect. This was an interesting result given that treatment with both drugs increased acute MOR desensitization. Thus, it was hypothesized that chronic treatment resulted in differential expression of MOR regulatory proteins, specifically GRK2. To test this hypothesis, rats were treated with the highest dose of morphine or methadone (60 mg/kg/day) by the protocols previously described. LC punches were taken on days 6 and 7, and western blots were done to monitor changes in GRK2 and tyrosine hydroxylase (TH) expression (as a control for protein loading). The results from our experiments showed NO consistent change in expression for either protein (Fig1 – western blot done by Joy Yu and Mark von Zastrow, UCSF). Work from other groups has focused on regulatory changes in mRNA abundance and expression of these proteins following chronic morphine treatment (Boundy et al., 1998; Fan et al., 2002; Guitart et al., 1990; McClung et al., 2005; Terwilliger et al., 1994). The

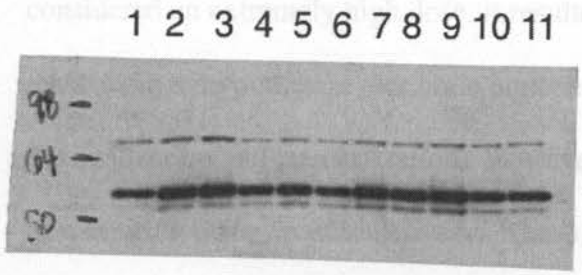


Fig 1. Western Blot of GRK2 and TH expression in LC punches taken from untreated, morphine- and methadone-treated rats. Lanes 1-3, control; lanes 4,5,8,9, morphine-treated (60 mg/kg/day); lanes 6,7,10,11, methadone-treated.

results have shown both up- and down-regulation GRK2, but primarily up-regulation for TH. Taken together, these observations further outline the differential modulation of mRNA and protein expression following drug treatment.

The experiments performed on slices from animals chronically treated with oxycodone and fentanyl highlighted two key issues with chronic treatment studies. The oxycodone experiments illustrate the significance of active drug metabolites when interpreting results on MOR regulation. The primary active metabolite, oxymorphone, has very different effects on acute MOR regulation than oxycodone. It is not possible to discern between the effects of one drug or the other, although the brain concentrations of oxycodone are significantly higher than those of oxymorphone. In order to circumvent this technical complication, alternative drug delivery routes warrant consideration, particularly intrtathecal, intracerebroventricular, or via canula to the region/nucleus of interest. The fentanyl studies demonstrate that multiple properties of drugs must be considered to achieve comparable dosing strategies. Though 2.8 mg/kg/day was considered an extremely high dose, it resulted in low brain concentrations. It is reasonable to hypothesize that brain concentrations were below the threshold to induce desensitization and internalization. However, the extreme potency of fentanyl proves to be a limiting factor in dose delivery. These considerations make the results from oxycodone- and fentanyl-treated animals difficult to compare directly to morphine- and methadone-treated animals. The primary active metabolite of morphine, m-6-g, has the same acute profile as the parent drug, while methadone has no active metabolites. Additionally, both drugs reach high concentrations in the brain. Despite these

experimental differences, the method used here to compare all tested drugs is most physiologically relevant when taken in the context of clinical applications.

The results obtained following chronic buprenorphine treatment were unexpected and illustrate how unique this agonist is among opioids. Importantly, the buprenorphine doses used here were in the range of those used to achieve maximum possible acute analgesia (Meert and Vermeirsch, 2005). Even at low doses a considerable concentration of this compound remained bound to MOR in prepared brain slices. Studies examining MOR trafficking in vivo and in vitro have reported receptor up-regulation, down-regulation and no change (Belcheva et al., 1993; Koch et al., 2005; Yu et al., 1997). These studies do not permit commentary on MOR expression, but suggest that buprenorphine occupies MOR in a dose dependent manner. Interestingly, ME-induced current amplitudes and the degree of MOR desensitization inhibition were nearly identical in the experiments with a 1 hour buprenorphine (5 nM) and following 6-7 days of treatment with the highest dose of buprenorphine (10 mg/kg/day). This suggests that buprenorphine concentrations were the same in both slice preparations and also that the effects on ME-induced MOR regulation were due to the presence of buprenorphine bound receptors rather than long-term adaptations. Finally, there is a clear and profound dose-dependent affect on desensitization and recovery of MOR. The implications patients using this drug as a treatment for opiate abuse or as an analgesic warrant follow-up.

Tolerance

Ultimately these experiments must be interpreted with respect to physiological tolerance. Chronic treatment with methadone shifted the dose response curve to the right and facilitated acute MOR desensitization when challenged with a saturating dose of the highly efficacious endogenous peptide ME. These findings suggest that a small, but significant, degree of cellular tolerance occurred. But these same findings held true for morphine-treated animals, suggesting the same degree of tolerance was produced. However, *any* acute stimulation of MOR with saturating concentrations of ME (1 or 10 min) caused MOR to lose sensitivity for over 45 minutes in neurons from morphine-treated animals. During this time period, MOR never regained its signaling potential. This adaptation could be interpreted as a more significant increase in tolerance. Specifically, if an animal or human that is receiving chronic morphine treatment gets a large opiate bolus, MOR sensitivity becomes more compromised than if that subject were being chronically treated with methadone. The physiological relevance of this comes into play when selecting an opiate for long-term therapy. Subjects on chronic opiate therapy for analgesia or post-operatively may still require an opiate bolus for breakthrough pain. If the bolus inhibits the sensitivity of the base line therapy, the subject will then require an increase, thus propagating tolerance. However, if MOR sensitivity is maintained, returning base line therapy should be sufficient.

The results following buprenorphine, on the other hand, are difficult to interpret. It is clear that a significantly smaller population of MORs are available for agonist binding. This alone suggests that tolerance is increased. However, prolonged exposures to ME did

not result in any desensitization. If desensitization is a significant upstream component of tolerance, then this has been effectively eliminated. Moreover, following prolonged exposure to ME, MOR mediated currents increased in amplitude. "Sensitization" is the opposite of tolerance, thus, these findings are contradictory and require more experimental evidence to interpret properly. It may be of particular interest to maintain a subject on chronic buprenorphine and investigate the tolerance resulting from bolus doses on top of this. These boluses could represent the need to manage break through pain or, in the case of an opiate abuser being managed with opiate maintenance therapy, intravenous heroin use or prescription narcotic abuse.

FUTURE DIRECTIONS

There are several experiments that would serve to follow-up and expand on the work presented here.

1) How does buprenorphine exert its effects on MOR signaling? Buprenorphine eliminates ME-induced desensitization, but the underlying mechanism is unclear. It is also unknown how buprenorphine pre-incubated slices respond to ME superfusion by becoming more sensitive to subsequent ME superfusion. This could be the result of signaling via buprenorphine-bound MORs that alters second messenger cascades required for desensitization and recovery. It could also be caused by an interaction between ligand-bound MORs despite the ligands having different identities. For example, buprenorphine bound MOR may remain on the cell surface. However when other MOR are exposed to ME, they are trafficked to clathrin-coated pits as currently excepted

mechanistic schemas suggest. Here they are internalized along with some fraction of the buprenorphine occupied MOR and enter the recycling pathway by which endosomes are acidified and agonists liberated from MOR. Free MOR are then inserted back into the plasma membrane in greater numbers and are ready to bind agonist again. This would facilitate subsequent MOR signaling. Thus, if internalization could be blocked the observations made in Chapter 3, Figs 3-5 would be eliminated.

An alternative possibility may be that β-CNA does not behave as an irreversible antagonist when slices are pre-incubated in buprenorphine. This would manifest as progressively larger ME-induced currents with repeated applications. A possible way to address this would be through binding studies with the various conditions described in Chapter 3.

2) The LC is an excellent neuronal model to investigate MOR regulation, particularly agonist-specific regulation where isolating the MOR component is desirable. However, it has become clear that the intracellular environment, specific molecular components, and endogenous stoichiometry are of paramount importance to the physiology of MOR regulation. Thus, it is important to have a neuronal model that is relevant to the physiological phenomena that is being studied. In order to accurately address tolerance in pain pathways and how MOR regulation contributes to this, a spinal preparation is important. The dorsal horn and superior cervical ganglia may make interesting alternative preparations to continue this work.

3) Imaging experiments would determine the contribution of MOR internalization to desensitization measured electrophysiologically. Using the FLAG-tagged MOR, it is possible to monitor MOR trafficking during exposure to all different agonists. These experiments are necessary in both acute MOR regulation under the influence of different agonists and following chronic treatment with different agonists. 1) While it would be interesting to perform these experiments in a spinal preparation, dissociated striatal neurons would be an excellent model. In these neurons, morphine is a full agonist, similar in efficacy to DAMGO, for triggering endocytosis (Haberstock-Debic et al., 2005). This is the only system that has demonstrated such an effect to date and is significant because it is a neuronal preparation. It is possible that the molecular components required for endocytosis are different and/or are present in a different stoichiometry, thus making these neurons a more sensitive model for studying endocytosis. These neurons may be an attractive model to study oxycodone- and buprenorphine-induced endocytosis because both are drugs that have never demonstrated measurable internalization. 2) It would also be valuable to study the effects of chronic morphine- and methadone-treatment as they pertain to MOR trafficking. By imaging MOR during the recovery period it would be possible to determine whether or not the inhibited MOR recovery following desensitization in morphine-treated animals was due to reduced MOR recycling.

4) In order to determine the effects of drugs without significant metabolites and in another physiologically important delivery method, intrathecal injections would be useful. First, it would be important to determine equianalgesic doses of morphine,

methadone, oxycodone and fentanyl. Drugs could then be delivered for 7 days and adaptations in MOR regulation could be studied in a spinal preparation. This would permit a more accurate testing of the RAVE hypothesis or to investigate the contribution of endocytosis-inducing agonists to cellular changes underlying tolerance.

5) Methadone has been shown to be a NMDA antagonist (Callahan et al., 2004; Chizh et al., 2000; Davis and Inturrisi, 1999; Ebert et al., 1995; Ebert et al., 1998; Gorman et al., 1997) and NMDA antagonists have been suggested to inhibit the development of tolerance. NMDA antagonism has not been shown in a neuronal preparation. In order to do this LC preparations could be used in conjunction with aspartate inontophoresis. NMDA currents as stimulated by iontophoretic applications could be measured in the presence and absence of methadone bath-application to determine whether or not this drug is indeed an NMDA antagonist. This may also have implications for the interpretations of results presented in Chapter 2.

REFERENCES

Belcheva MM, Barg J, McHale RJ, Dawn S, Ho MT, Ignatova E and Coscia CJ (1993) Differential down- and up-regulation of rat brain opioid receptor types and subtypes by buprenorphine. *Mol Pharmacol* **44**(1):173-179.

Boundy VA, Gold SJ, Messer CJ, Chen J, Son JH, Joh TH and Nestler EJ (1998) Regulation of tyrosine hydroxylase promoter activity by chronic morphine in TH9.0-LacZ transgenic mice. *J Neurosci* **18**(23):9989-9995.

Callahan RJ, Au JD, Paul M, Liu C and Yost CS (2004) Functional inhibition by methadone of N-methyl-D-aspartate receptors expressed in Xenopus oocytes: stereospecific and subunit effects. *Anesth Analg* **98**(3):653-659, table of contents.

Chizh BA, Schlutz H, Scheede M and Englberger W (2000) The N-methyl-D-aspartate antagonistic and opioid components of d-methadone antinociception in the rat spinal cord. *Neurosci Lett* **296**(2-3):117-120.

Davis AM and Inturrisi CE (1999) d-Methadone blocks morphine tolerance and N-methyl-D-aspartate-induced hyperalgesia. *J Pharmacol Exp Ther* **289**(2):1048-1053.

Ebert B, Andersen S and Krogsgaard-Larsen P (1995) Ketobemidone, methadone and pethidine are non-competitive N-methyl-D-aspartate (NMDA) antagonists in the rat cortex and spinal cord. *Neurosci Lett* **187**(3):165-168.

Ebert B, Thorkildsen C, Andersen S, Christrup LL and Hjeds H (1998) Opioid analgesics as noncompetitive N-methyl-D-aspartate (NMDA) antagonists. *Biochem Pharmacol* **56**(5):553-559.

Fan X, Zhang J, Zhang X, Yue W and Ma L (2002) Acute and chronic morphine treatments and morphine withdrawal differentially regulate GRK2 and GRK5 gene expression in rat brain. *Neuropharmacology* **43**(5):809-816.

Gorman AL, Elliott KJ and Inturrisi CE (1997) The d- and l-isomers of methadone bind to the non-competitive site on the N-methyl-D-aspartate (NMDA) receptor in rat forebrain and spinal cord. *Neurosci Lett* **223**(1):5-8.

Guitart X, Hayward M, Nisenbaum LK, Beitner-Johnson DB, Haycock JW and Nestler EJ (1990) Identification of MARPP-58, a morphine- and cyclic AMP-regulated phosphoprotein of 58 kDa, as tyrosine hydroxylase: evidence for regulation of its expression by chronic morphine in the rat locus coeruleus. *J Neurosci* **10**(8):2649-2659.

Haberstock-Debic H, Kim KA, Yu YJ and von Zastrow M (2005) Morphine promotes rapid, arrestin-dependent endocytosis of mu-opioid receptors in striatal neurons. *J Neurosci* **25**(34):7847-7857.

Koch T, Widera A, Bartzsch K, Schulz S, Brandenburg LO, Wundrack N, Beyer A, Grecksch G and Hollt V (2005) Receptor endocytosis counteracts the development of opioid tolerance. *Mol Pharmacol* **67**(1):280-287.

McClung CA, Nestler EJ and Zachariou V (2005) Regulation of gene expression by chronic morphine and morphine withdrawal in the locus ceruleus and ventral tegmental area. *J Neurosci* **25**(25):6005-6015.

Meert TF and Vermeirsch HA (2005) A preclinical comparison between different opioids: antinociceptive versus adverse effects. *Pharmacol Biochem Behav* **80**(2):309-326.

Terwilliger RZ, Ortiz J, Guitart X and Nestler EJ (1994) Chronic morphine administration increases beta-adrenergic receptor kinase (beta ARK) levels in the rat locus coeruleus. *J Neurochem* **63**(5):1983-1986.

Yu Y, Zhang L, Yin X, Sun H, Uhl GR and Wang JB (1997) Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. *J Biol Chem* **272**(46):28869-28874.