Pharmacological dissection of the actions of the Mu opioid receptor in the Rostroventral medial medulla

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PHARMACOLOGICAL DISSECTION OF THE ACTIONS OF THE MU OPIOID RECEPTOR IN THE ROSTROVENTRAL MEDIAL MEDULLA

by

Marlene Cano

An Abstract

Of a thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Neuroscience in the Graduate College of The University of Iowa

May 2013

Thesis Supervisor: Professor Donna L. Hammond
Chronic pain is a significant healthcare problem. It is disabling and diminishes quality of life. Opioids, such as morphine, remain a primary pharmacologic management for chronic pain. Opioids act at mu opioid receptors (MOPr) in the rostroventral medial medulla (RVM) to produce their analgesic effect. The RVM is a critical relay in pain inhibitory and facilitatory pathways of pain modulation. Furthermore, chronic inflammatory pain, produced by CFA hindpaw injection, leads to adaptive changes in the RVM that change the balance of these pathways and increase the potency of opioids.

MOPr are known to produce their effects via G_{i/o} proteins. Pretreatment of several pain modulatory regions with pertussis toxin (PTX) effectively attenuates the antinociceptive effects of MOPr agonists, such as DAMGO. In the RVM, PTX effectively reduced DAMGO stimulated GTPγS binding in uninjured rats. However, despite their effective inactivation of G_{i/o} proteins, PTX did not diminish the antinociceptive effects of DAMGO in the RVM of uninjured rats. In contrast, in rats with a chronic inflammatory injury, PTX completely abolished the antinociceptive effects of DAMGO. These results suggest a transition from G_{i/o} independent to G_{i/o} dependent mechanisms following CFA treatment. In addition, the anti-hyperalgesic effects of DAMGO were not inhibited by PTX, suggesting that DAMGO produces anti-hyperalgesia and antinociception by different mechanisms.

In the RVM, MOPr are present both postsynaptically and presynaptically. Postsynaptic MOPr are thought to produce antinociception by activating GIRK channels, resulting in hyperpolarization and inhibition of pain facilitatory neurons. Indeed, inhibition of GIRK channels in the RVM, via microinjection of tertiapin-Q, attenuated the antinociceptive effects of DAMGO in uninjured rats, providing the first behavioral evidence that MOPr agonists produce analgesia via this proposed mechanism. Interestingly, however, tertiapin-Q did not block the anti-hyperalgesic effects of DAMGO, nor did it diminish the antinociceptive effects of DAMGO in the contralateral hindpaw of CFA treated rats. Furthermore, these differential effects of tertiapin-Q in the
uninjured and injured rats are not the result of transcriptional down regulation of GIRK channels in the RVM. Finally, tertiapin-Q alone in the RVM produced a modest antinociception in uninjured rats, providing the first evidence of constitutive GIRK channel activity in the RVM and demonstrating a role for these in pain modulation.

Presynaptic MOPr are thought to produce antinociception by decreasing GABA release onto pain inhibitory neurons. Indeed, microdialysis studies demonstrated that levels of GABA release were decreased in response to DAMGO perfused into the RVM, as well as to high potassium after perfusion of DAMGO. However, they were not decreased in rats after CFA treatment. This suggests that chronic inflammatory injury alters the presynaptic actions of MOPr agonists in the RVM. Interestingly, levels of GLU release where not altered by DAMGO in uninjured or injured rats. Moreover, basal levels of GLU and GABA were also unaltered by CFA treatment.

In conclusion, although MOPr mediate their antinociceptive effects in other pain modulatory regions via Gi/o proteins, this is not the case in the RVM during an uninjured state. However, MOPr-induced antinociception transitions from Gi/o independent to Gi/o dependent mechanisms after CFA treatment. Additionally, these results support both the presynaptic and the postsynaptic postulates by which MOPr agonists are thought to produce their analgesic effects. However, although CFA treatment alters the activity of neurons in the RVM and promotes changes that result in an enhanced anti-hyperalgesic and antinociceptive response to DAMGO in the RVM, neither the postsynaptic nor the presynaptic mechanism, in isolation, seem to account for this enhancement.
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by

Marlene Cano

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Neuroscience in the Graduate College of The University of Iowa

May 2013

Thesis Supervisor: Professor Donna L. Hammond
CERTIFICATE OF APPROVAL

PH.D. THESIS

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

Marlene Cano

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Neuroscience at the May 2013 graduation.

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To the ups! To the downs… To your presence during these. Great strength was gained, grave stress dampened, and comfort sought.

To the future! To the past… To all your hard work, to all your advice, to all your teachings, to all your love, and sacrifice.

To new beginnings! To the little beings… to the anxious, the sweet, the energetic, the stout; but ultimately, to the reciprocal, the unconditional.

To Margarita Cano Rangel y Jesus Cano Gutierrez
ABSTRACT

Chronic pain is a significant healthcare problem. It is disabling and diminishes quality of life. Opioids, such as morphine, remain a primary pharmacologic management for chronic pain. Opioids act at mu opioid receptors (MOPr) in the rostroventral medial medulla (RVM) to produce their analgesic effect. The RVM is a critical relay in pain inhibitory and facilitatory pathways of pain modulation. Furthermore, chronic inflammatory pain, produced by CFA hindpaw injection, leads to adaptive changes in the RVM that change the balance of these pathways and increase the potency of opioids.

MOPr are known to produce their effects via $G_{i/o}$ proteins. Pretreatment of several pain modulatory regions with pertussis toxin (PTX) effectively attenuates the antinociceptive effects of MOPr agonists, such as DAMGO. In the RVM, PTX effectively reduced DAMGO stimulated GTPγS binding in uninjured rats. However, despite their effective inactivation of $G_{i/o}$ proteins, PTX did not diminish the antinociceptive effects of DAMGO in the RVM of uninjured rats. In contrast, in rats with a chronic inflammatory injury, PTX completely abolished the antinociceptive effects of DAMGO. These results suggest a transition from $G_{i/o}$ independent to $G_{i/o}$ dependent mechanisms following CFA treatment. In addition, the anti-hyperalgesic effects of DAMGO were not inhibited by PTX, suggesting that DAMGO produces anti-hyperalgesia and antinociception by different mechanisms.

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LIST OF ABBREVIATIONS

1. RVM: Rostroventral medial medulla
2. CFA: Complete Freund’s Adjuvant, inactivated mycobacteria
3. SAL: Saline injection into the hindpaw
4. MOPr: mu opioid receptor
5. DAMGO: [D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin acetate salt – selective agonist at μ-opioid receptors
6. PAG: Periaqueductal grey
7. GIRK: G protein inward rectifying Potassium Channels
8. VGCC: Voltage gated calcium channels
CHAPTER I
INTRODUCTION

Introduction

Health Care Significance of Chronic Pain

Chronic pain is a significant public health problem experienced by at least 116 million American adults (Institute of Medicine, 2011). It is disabling and diminishes the quality of life. In fact, approximately 25% of people suffer from chronic pain categorized as moderate to severe, and 10% have a disabling chronic pain condition (Institute of Medicine, 2011). Chronic pain also leads to adjustments in lifestyle, including unemployment. It is the second highest reason for workplace absenteeism and more than 50 million workdays are lost each year due to pain (American Pain Foundation, 2009). A conservative estimate from 2008 places the economic burden of chronic pain to the U.S. at ~$600 billion in terms of healthcare expenses, lost income and lost productivity (Institute of Medicine, 2011). Additionally, the prevalence of chronic pain is increasing, due in part to the aging of the U.S population, the prevalence of obesity and increasing surgical procedures (Institute of Medicine, 2011).

Mu Opioid Receptor (MOPr) Agonists are Used for the Clinical Pharmacologic Management of Chronic Pain

Opioids remain a primary clinical approach to the pharmacologic management of acute and chronic pain (Beaulieu, Lussier, Porreca, & Dickenson, 2010; Marvizon, Ma, Charles, Walwyn, & Evans, 2010). Furthermore, the clinical use of opioids for management of pain is increasing with time (Marvizon et al., 2010). The most common clinically used analgesics for moderate to severe pain management are agonists at the MOPr. These include morphine, fentanyl, hydromorphone, oxymorphone, and buprenorphine among others (Marvizon et al., 2010). Although the existence of delta
(DOPr) and kappa (KOPr) receptors has been recognized nearly as long as the MOPr, very few clinically used opioids act at the DOPr or KOPr. It is also thought that the direct analgesic effect of these is milder and often requires an indirect pathway in which MOPr are activated as well (Marvizon et al., 2010). Therefore, given the current and future clinical importance of opioid analgesics that act at the MOPr, this work primarily focuses on the mechanisms of action by the MOPr.

**Clinical Significance of This Work**

Although MOPr agonists are clearly an important pharmacologic therapy for clinical pain management, the mechanisms by which MOPr agonists produce their analgesic effects are still not completely understood. In addition, their use for effective long-term pain management is hindered by a variety of adverse effects including constipation, urinary retention, tolerance, sedation and respiratory depression. Moreover, these adverse effects appear to be produced by direct actions at the MOPr (Marvizon et al., 2010). Clearly, there is a need for improved opioid analgesics that effectively manage chronic pain without an attendant constellation of adverse side effects. Achieving this goal will require a more complete and detailed understanding of the mechanisms by which MOPr agonists produce their analgesic effects. To this end, this body of work focused on, dissection of the different mechanisms and pathways by which MOPr agonists produce their analgesic effects in anticipation that this information will aid in the identification of new, non-opioid pharmacologic therapies for pain management without the adverse effects of opioids.

**Definitions of Pain**

Table 1.1 defines the common terminology that will be used throughout this work. Table 1.1A provides the definitions of the terminology, as accepted by the International Association of the Study of Pain (IASP, 1994). These terminologies are well recognized
and used with ease in a clinical setting. However, Table 1.1B provides the definitions as they apply to an animal laboratory setting and therefore will be used to describe the findings of this work. Figure 1.1 illustrates these terminologies.

Briefly, pain is defined as “an unpleasant sensory and emotional experience…” (IASP, 1994). By definition, pain is subjective. Analgesia is defined as “the absence of pain”. Therefore, analgesia is also subjective. Because pain is subjective, it cannot be measured in animals. It is instead inferred from their motor responses (e.g. withdrawal or avoidance to noxious stimuli). In laboratory animals, it is termed “nociception.” Opioid agonists such as morphine increase the nociceptive threshold above normal values so that the animal takes longer to withdraw from noxious stimuli. This effect is termed “antinociception”. Injury decreases nociceptive threshold below normal values, producing enhanced sensitivity to noxious stimuli and therefore decreasing withdrawal latency. This effect is termed “hyperalgesia.” Agents like aspirin produce “anti-hyperalgesia” because they return the decreased nociceptive threshold to normal values. However, after injury, opioids not only return the decreased nociceptive threshold to normal values (anti-hyperalgesic effects) but they also increase the nociceptive threshold above normal values (antinociceptive effects). Therefore, opioids produce both anti-hyperalgesic and antinociceptive effects after injury. The terms antinociception and anti-hyperalgesia are not interchangeable. They describe two different phenomena that could perhaps be mechanistically different.

Describing the results of the paw withdrawal latency (PWL) test as increases and decreases in PWL will be difficult to follow. For ease of communication, the terminology on Table 1.1B will be applied to all studies regarding the PWL test.
Opioid Signaling Mechanisms: Mu Opioid Receptor
(MOPr)

\[ \text{MOPr is a GPCR} \]

The MOPr is a seven transmembrane G protein coupled receptor (GPCR) (Childers, 1991; Law, Wong, & Loh, 2000). G proteins are heterotrimeric proteins consisting of three subunits, one \( \alpha \), one \( \beta \), and one \( \gamma \). In an inactive state, a GDP is bound to the G proteins. However, when an agonist binds to the MOPr, the heterotrimeric G protein is activated, couples to the receptor and the GDP is exchanged for a GTP. The G\( \alpha \) subunit then dissociates from the G\( \beta\gamma \) subunits, and each can function as an intracellular effector.

The G\( \alpha \) subunit inhibits adenyl cyclase and decreases intracellular levels of cAMP. Postsynaptically, the G\( \beta\gamma \) dimer activates G protein inward rectifying potassium (GIRK) channels resulting in hyperpolarization and inhibition of neuronal excitability. Presynaptically, the G\( \beta\gamma \) dimer inhibits voltage gated calcium channels (VGCCs) resulting in an inhibition of neurotransmitter release.

MOPr Effects Are Mediated Primarily Via G\( \text{i/o} \) Proteins

The MOPr couples principally to G\( i \) and G\( o \) proteins (Connor & Christie, 1999). Evidence that MOPr effects are mediated by G\( i/o \) proteins comes primarily from the use of pertussis toxin (PTX) in biochemical, electrophysiological and behavioral studies.

PTX is produced from the bacterium \textit{Bordetella pertussis}. It consists of two components, an A and a B component. The A component is the enzymatic part of PTX, a ribosylase. The B component is the receptor binding component and therefore binds the toxin to the target cells (Kaslow, Lim, Moss, & Lesikar, 1987; Lim, Sekura, & Kaslow, 1985). PTX irreversibly ribosylates ADP to the \( \alpha \) subunits of G\( i \) and G\( o \) proteins. Ribosylated G proteins are no longer available to interact with cell membrane GPCRs, and thus intracellular signaling is interrupted.
Biochemical and Electrophysiology Studies

Studies first demonstrated that the primary second messenger system associated with opioid receptors involved an inhibition of adenylate cyclase (Bodnar, Paul, Rosenblum, Liu, & Pasternak, 1990; Costa, Aktories, Schultz, & Wuster, 1983; Parolaro et al., 1990). Inhibition of adenylate cyclase is typically the subcellular effect of $G_{i/o}$ proteins.

In addition, activation of the opioid receptor resulted in the opening of potassium channels in CNS neurons and inhibition of VGCCs in primary cultures of dorsal root ganglion (DRG), effects that were inhibited by in vitro pretreatment with PTX (Costa et al., 1983). PTX also blocked the depressant effect of morphine in cultured spinal cord-DRG explants (Crain, Crain, & Makman, 1987). Increasing the concentration of morphine to $>100$ times failed to overcome the blockade produced by PTX.

Similarly, in vivo pretreatment with PTX 1-3 days before slice electrophysiology blocked the hyperpolarization, and therefore the inhibition of neuronal firing typically produced by morphine in the locus ceruleus (LC) (Aghajanian & Wang, 1986). The maximal outward currents evoked by morphine were reduced by $\sim90\%$ (Aghajanian & Wang, 1986).

Behavioral Studies

Many studies have examined the effect of in vivo pretreatment with PTX on the antinociceptive effects of opioid agonists (Bodnar et al., 1990; Hoehn, Reid, & Sawynok, 1988; Parenti, Tirone, Giagnoni, Pecora, & Parolaro, 1986; Parolaro et al., 1990; Przewlocki, Costa, Lang, & Herz, 1987; Sanchez-Blazquez & Garzon, 1988; Sanchez-Blazquez & Garzon, 1991; Shah, Duttaroy, Davis, & Yoburn, 1994)(Hoehn et al., 1988; Parenti et al., 1986; Przewlocka et al., 2002; Przewlocki et al., 1987). All of these studies have demonstrated that PTX attenuates the antinociception produced by MOPr agonists. However, all of these studies vary in several respects including the region and dose at which PTX is administered as well as the time lag between the administration of PTX
and behavior testing.

Intracerebroventricular (i.c.v.) microinjection of PTX reduced the antinociceptive effects of i.c.v. morphine in a tail flick test. These effects were not reduced 24 hrs after PTX but were robustly diminished 6 days after PTX (Parenti et al., 1986). Intrathecal (i.t.) delivery of PTX also inhibited the antinociceptive effects of i.t. morphine in both a tail flick and a hot plate test (Hoehn et al., 1988). This attenuation was noted 2 days, but peaked at 5 and 7 days after PTX treatment (Hoehn et al., 1988). Intrathecal PTX also attenuates the antinociceptive effects of other i.t. opioid agonists such as DAMGO, a MOPr selective agonist.

The antinociception produced by systemic morphine (i.p.) was also inhibited by PTX administered either i.c.v. or i.t. alone (Parolaro et al., 1990; Shah et al., 1994). However, dual injection of both i.c.v. and i.t. PTX in the same rat resulted in an additive inhibition (Shah et al., 1994).

A few of these studies observed that the rats receiving the PTX treatment were slightly more ‘generally ill’ than their control rats (Bodnar et al., 1990; Hoehn et al., 1988). To address the possibility that administering PTX i.c.v. or i.t. produced more ‘systemic’ effects including weight loss that might make the animals more generally ill and the results more difficult to interpret, Bodnar et al (1990), microinjected PTX directly into discrete nuclei, the LC and the PAG. PTX microinjected directly into these nuclei also reduced the antinociceptive effects of morphine (Bodnar et al., 1990).

Time Dependent Effects of PTX

The interval between the administration of PTX and the behavior testing varied in the studies mentioned above. PTX administered at various time points 2 - 24 days before behavior testing resulted in an attenuation of antinociception (Parenti et al., 1986; Sanchez-Blazquez & Garzon, 1988; Sanchez-Blazquez & Garzon, 1991). However, the most common pretreatment interval for PTX was 3-10 days. In addition, the peak reduction in antinociception was measured 5-7 days after PTX administration (Hoehn et
al., 1988; Parenti et al., 1986; Sanchez-Blazquez & Garzon, 1988; Sanchez-Blazquez & Garzon, 1991). It is thought that this pretreatment interval is necessary because this effect requires 1) the gradual insertion and penetration of PTX into the cell membrane, 2) PTX has to reach the ultimate site of action on the intracellular membrane surface, and 3) ADP-ribosylation must take place.

**Dose Dependent Effects of PTX**

A range of PTX doses were effective in reducing the antinociceptive effects of opioids. The range was from 5 ng to 1 µg (Bodnar et al., 1990; Przewlocki et al., 1987). The 5 ng dose was microinjected into discrete nuclei, whereas the rest were administered i.c.v. or i.t, and perhaps this accounts for the difference. The most common effective doses were 0.1 µg and 0.5 µg (Hoehn et al., 1988; Parenti et al., 1986; Sanchez-Blazquez & Garzon, 1988; Self, Terwilliger, Nestler, & Stein, 1994; Shah et al., 1994).

**ADP-ribosylation by PTX**

ADP ribosylation studies have been performed on the PTX treated tissue to demonstrate that in vivo PTX treatment effectively inactivates Gi/o proteins. In lumbar-sacral spinal cord, LC and paraventricular tissue, PTX treatment resulted in a ~50-60% reduction of [³²P] NAD labeling. This translates to a 50-60% reduction in the number of sites available for ADP ribosylation in the tissue after PTX treatment, therefore a reduction in the number of Gi/o protein sites available for activation and promoting subcellular activity after MOPr agonists are administered (Correa-Sales, Reid, & Maze, 1992; Hayashi, Rabin, Guo, & Maze, 1995; Przewlocki et al., 1987). In the nucleus accumbens, PTX maximally reduced [³²P] NAD labeling 7 days after PTX treatment and this result remained steady for over 28 days (Self et al., 1994). However, in the VTA, there was almost complete recovery of ADP ribosylation by 30 days after PTX (Steketee, Striplin, Murray, & Kalivas, 1992). Therefore, it is possible that different regions have different G protein turnover rates. In addition, the partial (~50-60%) ADP ribosylation might explain the partial reduction in antinociception seen in all the behavioral studies.
PTX leads to changes in G proteins

It is not expressly clear whether PTX alters the levels of G proteins in the tissue treated with PTX. Seven days after PTX treatment in the LC, Correa-Sales et al (1992) did not see any changes in Ga protein levels. Interestingly, in the nucleus accumbens, PTX decreased Ga_i and Ga_o but not Ga_s or Gβ protein levels 14 days after PTX treatment. This decrease lasted until 28 days and was an unexpected finding (Self et al., 1994). In fact, the expectation was that Gi/o proteins would be increased in order to compensate for the inactivation of other Gi/o proteins by PTX. Since the decrease in protein seems to be a slower effect of PTX, it is possible that PTX first reduces functional Gi/o proteins by ribosylation and then follows with a secondary effect, a reduction in Gi/o proteins.

It is also not certain whether this decrease in Gi/o proteins is due to a decrease in mRNA expression or due to an increase in the protein breakdown (Self et al., 1994). However, one study has demonstrated a decrease in the levels of Gi/o mRNA 15-20 days after PTX treatment (Ramkumar & Stiles, 1990).

MOPr Mediates Some Effects Via Gs Proteins

There is some evidence that MOPr may have direct actions on G_s proteins, although this is not widely accepted. In spinal cord-DRG explants, PTX inhibited the depressive effects of opioids in extracellular recordings (Crain et al., 1987). Therefore, DRG evoked spinal cord responses remained stably maintained during the 10-30 min testing periods. However, in a few of these explants, responses were not only stably maintained but instead showed an unusual enhancement in potential amplitude during exposure to morphine (Crain et al., 1987). This suggests an excitatory effect mediated by the MOPr.

In addition, although PTX typically decreases the duration of the perikaryal action potential, in a few PTX treated neurons, opioids instead induced a prolongation of the action potential (Crain, Crain, & Makman, 1986). Since these neurons do not possess presynaptic components, these excitatory effects must be directly mediated (Crain et al.,
Therefore, these neurons must possess MOPr that can positively couple to adenylate cyclase, via Gs proteins. If this is the case, perhaps PTX treatment ‘unmasks’ these excitatory effects (Crain et al., 1987). In addition, under conditions in which Gs is dephosphorylated, MOPr is known to couple to Gs proteins (Chakrabarti & Gintzler, 2007). Finally, there’s support for bimodal effects of most opioid agonists such that at very low doses, opioids produce excitatory effects but at higher doses opioids, they produce their well-known depressive effects (Crain & Shen, 1990).

Cholera toxin was thought to selectively ADP-ribosylate Gs proteins. It has therefore been previously used to examine whether Gs proteins mediate the antinociception produced by opioids (Bodnar et al., 1990; Sanchez-Blazquez & Garzon, 1991). However, these results are controversial. In one study, microinjection of cholera toxin into the PAG and LC reduced the antinociceptive effects of morphine (Bodnar et al., 1990), indicating that Gs proteins are necessary for MOPr agonists to produce antinociception. However, in another study cholera toxin actually increased opioid-induced antinociception (Sanchez-Blazquez & Garzon, 1991). Presently, cholera toxin is unfortunately thought to have some ‘dirty’ or nonspecific effects and is therefore not a good tool towards assessing this aim.

**Opioid Effectors**

**Postsynaptic Effects of MOPr Via GIRK Channels**

GIRK channels are thought to mediate the postsynaptic inhibitory effects of MOPr agonists (North & Uchimura, 1989). Several lines of evidence support this. First, GIRK channels are expressed in regions of the CNS where MOPr agonists are known to produce antinociception, including the PAG, the LC and the spinal cord. Second, mRNA expression of the GIRK channels coexists with the mRNA expression of MOPr in various CNS regions (Ikeda et al., 1996). In addition, GIRK subunits co-localize with MOPr at postsynaptic sites in the dorsal horn of the spinal cord (Marker, Lujan, Loh, & Wickman,
Third, recombinant MOPr can activate recombinant GIRK channels in a heterologous expression system (Ikeda et al., 1996). Fourth, MOPr agonists activate outward potassium currents exhibiting GIRK-like characteristics, including 1) activation by hyperpolarization negative to the reversal potential for K$^+$ and 2) blockage by Cs$^+$ and Ba$^{2+}$ (Lesage et al., 1995). Finally, the outward currents produced by opioids in the spinal cord and the LC are eliminated in GIRK knockout mice (Marker et al., 2006; Torrecilla et al., 2002).

**GIRK Channels**

GIRK channels are involved in maintaining the resting membrane potential as well as regulating cellular hyperexcitability. They are gated by the G$_{\beta\gamma}$ subunit of G$_{i/o}$ coupled receptors (Krapivinsky, Krapivinsky, Wickman, & Clapham, 1995; Mark & Herlitze, 2000; North & Uchimura, 1989). GIRK channels are homo and heterotetrameric channels composed of GIRK subunits 1-4 (Corey, Krapivinsky, Krapivinsky, & Clapham, 1998; Duprat et al., 1995; Kobayashi et al., 1995; Lesage et al., 1995). GIRK1 and GIRK4 are primarily found in the heart where they regulate heart rate while GIRK1-3 subunits are found throughout the CNS (Karschin, Dissmann, Stuhmer, & Karschin, 1996; Koyrakh et al., 2005; Krapivinsky et al., 1995). Although most regions in the CNS express GIRK1-3, there are regions where only GIRK2 and GIRK3 are expressed, or where GIRK1 expression is very low (Jelacic, Kennedy, Wickman, & Clapham, 2000). GIRK4 expression has been noted in the CNS but primarily in more discrete regions (Karschin et al., 1996; Wickman, Karschin, Karschin, Picciotto, & Clapham, 2000).

The relevance of the GIRK channel subunit composition is not yet fully appreciated. However, GIRK subunits have unique characteristics, and therefore, their anatomical diversity implies a functional diversity. For example, there are no functional GIRK1 homotetramers (Ma et al., 2002). GIRK1 contains an ER retention signal that requires its expression with GIRK2-4 to reach the cell membrane (Aguado et al., 2008;
Hedin, Lim, & Clapham, 1996; Jelacic et al., 2000). There is evidence that GIRK3 also does not form functional homotetramers (Luscher & Slesinger, 2010; Ma et al., 2002). However, GIRK3 contains a lysosomal targeting signal and can direct functional GIRK channels towards lysosomal degradation (Ma et al., 2002). Conversely, GIRK2 and GIRK4 homotetramers are thought to be efficiently directed to the cell surface (Ma et al., 2002). In addition, GIRK1 has an increased affinity to $G_{\beta\gamma}$. Therefore, GIRK2/GIRK3 channels are 5 fold less sensitive to activation by $G_{\beta\gamma}$ than GIRK1/GIRKx heterotetramers (Jelacic et al., 2000). Finally, there is some evidence that channels formed by different GIRK subunits exhibit distinct single-channel profiles and whole cell current kinetics.

**GIRK Channels Mediate the Antinociceptive Effects Produced by MOPr Agonists**

Despite the anatomical and electrophysiological support for the postulate that GIRK channels mediate the antinociceptive effects of MOPr, there is little behavioral evidence to support this. More recently, the use of GIRK subunit knockout mice has provided the first behavioral evidence that GIRK channels, specifically GIRK1 and GIRK2, indeed mediate the antinociceptive effects of opioids. However, this work has been largely confined to the spinal cord.

These studies revealed that GIRK1 and GIRK2 knockout mice, but not GIRK3 knockout mice exhibited a blunted response to intrathecal morphine, indicating that in the spinal cord, GIRK1 and GIRK2 subunits mediate the antinociception produced by morphine (Marker, Stoffel, & Wickman, 2004). In addition, these results were replicated in wild type mice to rule out developmental differences or compensations in the knockout mice. Intrathecal co-administration of tertiapin-Q, a selective GIRK channel antagonist, blunted the antinociception produced by morphine in wild-type mice (Marker et al., 2004).
The finding that GIRK4 knockout mice did not show blunted antinociception (Marker, Cintora, Roman, Stoffel, & Wickman, 2002) coupled with the limited anatomical distribution of GIRK4 in the CNS suggests that GIRK4 does not play a substantial role in morphine induced analgesia (Marker et al., 2002).

Presynaptic Effects of MOPr Via VGCC

Presynaptic MOPr Inhibit Neurotransmitter Release and Synaptic Transmission

Presynaptic MOPr are thought to mediate their effects by acting directly on presynaptic GABAergic terminals to decrease the release of GABA onto the neurons on which they synapse (Pan, Williams, & Osborne, 1990). There is strong supportive evidence for this postulate. Briefly, opioids produce excitation, or increased firing of some neurons in various regions involved in pain modulation. However, the direct action of opioids is inhibitory. Therefore, these excitatory actions must be indirect. Opioids must be acting directly on GABAergic interneurons to inhibit neurotransmitter release and thereby disinhibit, or excite the opposing neurons (Basbaum & Fields, 1984; Chieng & Christie, 1994; Finnegan, Li, Chen, & Pan, 2004; Vaughan & Christie, 1997). In support of this, various studies have determined that opioids diminish the inhibitory postsynaptic potentials (IPSPs) mediated by GABA (Chieng & Christie, 1994; Finnegan et al., 2004; Vaughan & Christie, 1997). However, opioids also diminish the excitatory postsynaptic potentials (EPSPs) mediated by glutamate (Chieng & Christie, 1994; Finnegan et al., 2004; Vaughan & Christie, 1997). Therefore, the neuronal circuitry is much more complex but the signaling of opioids involves presynaptic inhibition of both glutamatergic and GABAergic transmission.
The Rostroventral Medial Medulla (RVM)

*The RVM is a Central Relay in the Modulation of Pain*

Pain is a complex sensory process. It is a process best represented by a loop of intricate circuitry including ascending and descending pathways (Heinricher & Ingram, 2009). Nociceptive information is relayed from the periphery, through the spinal cord to various regions in the central nervous system. However, this information is filtered and modulated at the level of the spinal cord by supraspinal nuclei that project directly or indirectly to the dorsal horn. These descending pathways convene and relay via the RVM. Therefore, the RVM and its projections to the dorsal horn are considered a common output pathway and a critical region for descending modulation of nociceptive information (K. Ren & Dubner, 2009).

The RVM is a region in the brainstem including the nucleus raphe magnus and the nucleus gigantocellularis pars α. It consists of a heterogenous population of neurons and functions as a central relay center for the bulbospinal modulation of pain information. The RVM receives projections from more rostral nuclei implicated in pain modulation including the periaqueductal gray (PAG), amygdala, and the hypothalamus (K. Ren & Dubner, 2009). In fact, chemical or electrical inactivation of the RVM interferes with the ability of these other nuclei to modulate nociception. In turn, RVM neurons project directly to the spinal cord dorsal horn where they can either inhibit or facilitate the transmission of nociceptive information. *Via* its projections to the A7 catecholamine nucleus, the RVM can also recruit a parallel noradrenergic bulbospinal pain modulatory pathway (D. L. Hammond & Proudfit, 2007; D. L. Hammond, 1986; Jones, 1992; Millan, 2002). The RVM is therefore a central relay in the modulation of pain. Alterations in synaptic transmission in the RVM will impact not only the function of the RVM, but also influence the function of more rostral pain modulatory nuclei that exert their actions via their projections to the RVM. Given its central role in bulbospinal modulation of nociception, further investigation of the mechanisms by which this region modulates
nociception in uninjured as well as in injured conditions is warranted and can provide insight into better treatment options for chronic pain.

The RVM is an Important Site of Opioid Action

The RVM is also an important site at which opioids act to produce their analgesic effects. The RVM contains all three subtypes of the opioid receptor, μ: MOPr, δ: DOPr and κ: KOPr (Mansour, Khachaturian, Lewis, Akil, & Watson, 1987; Mansour, Fox, Akil, & Watson, 1995). Microinjection of MOPr, DOPr or KOPr agonists in the RVM produces antinociception in acute models of nociception (Ackley, Hurley, Virnich, & Hammond, 2001; Tershner, Mitchell, & Fields, 2000; Thorat & Hammond, 1997), as well as anti-hyperalgesia in models of persistent inflammatory nociception (Hurley & Hammond, 2000; Schepers, Mahoney, Gehrke, & Shippenberg, 2008). In addition, microinjection of a MOPr antagonist into the RVM is sufficient to antagonize the antinociception produced by systemic morphine (Dickenson, Oliveras, & Besson, 1979). Therefore, the RVM is both necessary and sufficient for opioids to produce antinociception.

Two Complementary/Competing Hypotheses of Opioid Action in the RVM

Early studies indicated that electrical or chemical stimulation of RVM neurons suppressed the responses of dorsal horn neurons to noxious stimuli, resulting in antinociception (K. Ren & Dubner, 2009). In addition, an intact and functional RVM is necessary for the production of antinociception. Later studies indicated that activation of RVM neurons could also enhance the responses of dorsal horn neurons to noxious stimuli, resulting in hyperalgesia (K. Ren & Dubner, 2009). In addition, an intact and functional RVM is necessary for the development of generalized hyperalgesia resulting from prolonged nociceptive input such as in inflammation (H. Fields, 2004). Thus, modulation of nociception by the RVM is a bidirectional process. Nociceptive threshold is determined by the net sum of activity in the pain facilitatory and pain inhibitory

MOPr are distributed both postsynaptically (Marinelli, Vaughan, Schnell, Wessendorf, & Christie, 2002; Zhang & Hammond, 2010) and presynaptically in the RVM (Ackley et al., 2001; Bie & Pan, 2003). There are currently two different mechanisms by which MOPr agonists are postulated to produce antinociception in the RVM (Figure 1), one mediated by the postsynaptic and the other by the presynaptic MOPr. These are thought to occur simultaneously in the RVM.

The first mechanism is direct and involves postsynaptic inhibition of spinally-projecting pain facilitatory neurons. Agonists act at postsynaptic MOPr to open GIRK channels, resulting in hyperpolarization and inhibition of pain facilitatory neurons. Inhibition of the pain facilitatory neurons in the RVM in turn produces antinociception (D. L. Hammond & Proudfit, 2007).

The second mechanism is indirect and involves presynaptic inhibition of GABAergic neurons and concomitant disinhibition (activation) of the spinally-projecting pain inhibitory neurons on which they synapse. GABAergic neurons are tonically active and maintain pain inhibitory neurons in a hyperpolarized state. Agonists act at presynaptic MOPr on GABAergic neurons to inhibit VGCC, resulting in decreased neurotransmitter release and subsequent disinhibition of the pain inhibitory neurons on which they synapse. Increased firing of the pain inhibitory neurons in the RVM in turn produces antinociception.

**Postsynaptic Inhibition Via GIRK Channels in the RVM**

Opioids acting at postsynaptic MOPr result in an outward current mediated by GIRK channels in spinally projecting RVM neurons (Marinelli et al., 2002; Zhang & Hammond, 2010). However, no behavioral data exists in the RVM to support the postulate that GIRK channels mediate the antinociceptive effects produced by MOPr agonists in the RVM.
Presynaptic Inhibition Via VGCC in the RVM

Substantial data support the postulate that presynaptic MOPr inhibit neurotransmitter release at GABAergic but also at glutamatergic terminals in the RVM. In support of this, the effects of GABA and GLU agonists and antagonists have been studied extensively in the RVM. In addition, the roles of GABA and GLU agonists and antagonists on the effects produced by MOPr agonists in the RVM have also been studied extensively. However, few studies have directly measured whether MOPr agonists indeed decrease GLU or GABA release in the RVM.

The Role of GABA in the RVM

Effects of GABA Agonists and Antagonists in the RVM

There is an abundance of GABA containing cell bodies and terminals, as well as GABA_A binding sites in the RVM (Bowery, Hudson, & Price, 1987; H. L. Fields, Heinricher, & Mason, 1991; Millhorn, Hokfelt, Seroogy, & Verhofstad, 1988). Microinjection of GABA_A receptor antagonists into the RVM produces antinociception, while microinjection of GABA_A receptor agonists in the RVM produces hyperalgesia (Drower & Hammond, 1988; H. L. Fields et al., 1991; Gilbert & Franklin, 2001; Heinricher & Kaplan, 1991). This maintains that inhibiting GABA transmission is a mechanism by which antinociception is produced in the RVM.

Effects of GABA Agonists and Antagonists on MOPr-
induced Antinociception

Opioids are thought to produce antinociception by inhibiting GABAergic transmission onto pain inhibitory neurons in the RVM {87 Fields,H.L. 1983}). In support of this, morphine increases the firing rates of pain inhibitory neurons (H. L. Fields et al., 1991; Heinricher, Morgan, Tortorici, & Fields, 1994). Increased firing of these pain inhibitory neurons is sufficient to produce antinociception. In addition, local infusion of GABA_A receptor antagonists into the RVM replicates these effects (Heinricher & Tortorici, 1994). In contrast, administration of GABA_A receptor agonists
in the RVM blocks the antinociceptive effects produced by morphine {208 Gilbert,A.K. 2002}. Finally, MOPr agonists decrease the inhibitory synaptic transmission onto spinally projecting neurons in the RVM (H. L. Fields et al., 1991; Finnegan et al., 2004; Pan et al., 1990).

Dialysis of GABA in the RVM in Response to MOPr Agonists

The advantage of microdialysis is that collection of the samples is done in vivo, in freely moving rats. Perfusion of DAMGO into the RVM decreased levels of GABA release in a dose dependent manner (Schepers, Mahoney, Zapata, Chefer, & Shippenberg, 2008). DAMGO only decreased GABA release at doses that also produced an antinociceptive response in a paw flick task (Schepers, Mahoney, Zapata et al., 2008). However, this study perfused DAMGO continuously for 1 hour. This is of course, a different approach than the more common methods in which a single microinjection produces antinociception.

The Role of Glutamate in the RVM

Effects of GLU Agonists and Antagonists in the RVM

Administration of L-glutamate or NMDA into the RVM produces antinociception (Jensen & Yaksh, 1984; McGowan & Hammond, 1993; Satoh, Oku, & Akaike, 1983). However, administration of AMPA or NMDA receptor antagonists alone is not sufficient to alter basal nociceptive threshold, suggesting that there is no tonic release of glutamate in the RVM (Spinella, Cooper, & Bodnar, 1996).

Effects of GLU Agonists and Antagonists on MOPr-induced Antinociception

Microinjection of AMPA or NMDA receptor antagonists into the RVM inhibit the antinociceptive response produced by opioids (Javanmardi et al., 2005; Spinella et al., 1996). Infusion of excitatory amino acid antagonists into the RVM also inhibit the activation and the increased firing of pain inhibitory neurons induced by opioids.
(Heinricher, McGaraughty, & Farr, 1999; Heinricher, Schouten, & Jobst, 2001). These data suggest that an increase in glutamate is necessary for the production of antinociception. One theory is that MOPr agonists disinhibit (activate) glutamate containing neurons projecting from the PAG onto pain inhibitory neurons in the RVM (Schepers, Mahoney, Zapata et al., 2008). This would result in increased firing by these pain inhibitory neurons, producing antinociception. However, previous electrophysiology data suggest that MOPr agonists primarily inhibit, not stimulate, glutamatergic transmission in many regions of the CNS, including the PAG and RVM (Chieng & Christie, 1994; Finnegan et al., 2004; Vaughan & Christie, 1997; Zhu & Pan, 2005).

Clearly, glutamate transmission plays an important role in opioid mediated antinociception. Additional research will be required to resolve the conundrum of electrophysiology data that indicate that opioids inhibit glutamatergic transmission in the RVM and behavioral data that suggest that glutamatergic transmission is necessary for the production of the antinociceptive effects of opioids in the RVM.

Dialysis of GLU in the RVM in Response to MOPr Agonists

Perfusion of DAMGO into the RVM increased glutamate release in a dose-dependent manner (Schepers, Mahoney, Zapata et al., 2008). Interestingly, glutamate increases could not be detected in response to DAMGO or to potassium evoked depolarization without the use of tPDC (L-trans-Pyrrolidine-2,4-dicarboxylic acid), a glutamate reuptake transport inhibitor. These results support the hypothesis that MOPr agonists disinhibit glutamate containing neurons projecting from the PAG onto pain inhibitory neurons in the RVM. However, these results were unexpected given extensive electrophysiology data suggesting that opioids instead inhibit glutamate transmission.
**Ramifications of Persistent Inflammatory Pain for Opioid Action in the RVM**

**Defining the CFA Rat Model of Persistent Inflammatory Injury**

Injection of Complete Freund’s adjuvant (CFA), an agent consisting of inactivated mycobacteria, is commonly used as a model of chronic pain (Mager, Pillman, & Heidelberg, 2007). Specifically, injection of CFA in the hindpaw produces an inflammatory pain state that persists for at least 2 weeks in the rat. In addition, injection of CFA alters the activity and responsiveness of neurons in the RVM (Guan, Terayama, Dubner, & Ren, 2002; Miki et al., 2002; K. Ren & Dubner, 2002; Zhang & Hammond, 2009). Injection of CFA also results in time-dependent changes in the balance of activity in both the *pain inhibitory* and the *pain facilitatory pathways* (Hurley & Hammond, 2000; K. Ren & Dubner, 2002; Vanegas & Schaible, 2004). One consequence is an enhancement of the anti-hyperalgesic and the antinociceptive effects of systemic MOPr agonists (D. L. Hammond, 2004; Stein, 1995). In addition, the anti-hyperalgesic and the antinociceptive effects of MOPr agonists microinjected directly into the RVM are also enhanced (Hurley & Hammond, 2000; Schepers, Mahoney, & Shippenberg, 2008).

**G proteins in Persistent Inflammation**

The effect of persistent inflammation on G proteins in the RVM and the subsequent effects on the anti-hyperalgesic and the antinociceptive effects of MOPr agonists in the RVM have not been examined.

**GIRK Channels in Persistent Inflammation**

An increased number of RVM neurons respond to DAMGO with an outward, GIRK mediated current after CFA treatment (Zhang & Hammond, 2010). The subsequent effects of this on the anti-hyperalgesic and the antinociceptive effects of MOPr agonists in the RVM have not been examined. However, these data suggest that
the postsynaptic mechanisms of MOPr-induced antinociception are potentiated in inflammatory conditions.

**Neurotransmitter Release in Persistent Inflammation**

**GABA Release during Persistent Inflammatory Conditions**

Direct evidence for the effects of persistent inflammatory pain on the release of GABA at baseline in the RVM, as well as in response to MOPr agonists in the RVM is not available.

However, one study provided indirect pharmacological evidence suggesting that there are increased levels of GABA release in the RVM after inflammatory injury (Gilbert & Franklin, 2001). This study demonstrated that under highly noxious stimuli, such as after a 2-4% formalin injection, microinjection of a GABA<sub>A</sub> agonist did not further increase pain scores, presumably due to a saturation of GABA<sub>A</sub> receptors (Gilbert & Franklin, 2001). In addition, microinjection of a GABA<sub>A</sub> antagonist into the RVM produced an enhanced antinociception (Gilbert & Franklin, 2001).

**Glutamate Release during Persistent Inflammatory Conditions**

To date, the effect of persistent inflammation on glutamate release in the RVM and the subsequent effects on the anti-hyperalgesic and the antinociceptive effects of MOPr agonists in the RVM have not been examined.

However, levels of glutamate release in the RVM transiently increased in a pre-clinical model of non-inflammatory chronic widespread muscle pain (Radhakrishnan & Sluka, 2009). This model consists of two injections of acidic saline into the gastrocnemius muscle. Hyperalgesia develops within hours after the second injection and lasts for weeks. However, the levels of glutamate release were only increased during the second injection but not throughout the rest of the 2 hrs in which samples were collected (Radhakrishnan & Sluka, 2009).
Goal of the Thesis: How do Opioids Act in the RVM to Modulate Nociception: Naïve and Injured

In summary, opioids continue to be a primary clinical pharmacologic management for moderate to severe pain. Most clinically used opioids act at the MOPr. However, the mechanisms by which the MOPr subsequently produces their analgesic effects are still not completely understood. Additionally, a variety of unwanted side effects hinders the use of opioids for effective long-term pain management. Therefore, the goal of this thesis is to dissect the different mechanisms by which MOPr produce analgesia, and thereby advance the understanding of the contribution of these different mechanisms, with the ultimate clinical goal that this might lead to the identification of new, non-opioid pharmacologic therapies for pain management without the adverse effects of opioids.

Given that most clinically used opioids act at the MOPr rather than the DOPr or KOPr, this thesis focuses primarily on the mechanisms by which agonists at the MOPr produce analgesia. Additionally, given that the RVM is an important site at which opioids produce their analgesic effects, that it is both necessary and sufficient for opioids to produce analgesia and that it is such a critical, central relay through which the CNS modulates all incoming nociceptive information, this thesis will focus on the mechanisms by which MOPr agonists produce analgesia in the RVM. Finally, given that persistent inflammatory injury alters the physiology of neurons in the RVM, and results in an enhancement of the anti-hyperalgesic and antinociceptive effects of MOPr agonists in the RVM, this thesis also seeks to understand how the mechanisms by which MOPr produce analgesia are altered in the RVM after persistent inflammatory injury.

The ultimate scientific goal of this work is to increase the understanding of the downstream mechanisms by which MOPr produce their analgesic effects and how these mechanisms differ after persistent inflammatory injury. The ultimate clinical goal of this work is that elucidation of these mechanisms may lay the groundwork for identifying new pharmacologic therapies for improved management of chronic pain. These new
pharmacologic therapies could perhaps bypass the MOPr and instead target downstream mechanisms elucidated by this work. The goal, is that these may be more effective, in particular in addressing pain during a state of persistent inflammatory injury. Additionally, perhaps these new therapies would, by bypassing the MOPr, also bypass the various adverse effects that currently hinder the use of opioids for long-term pain management.

To reiterate, MOPr are G protein coupled receptors known to mediate their effects primarily via $G_{i/o}$ proteins. MOPr are present both postsynaptically and presynaptically in the RVM. Postsynaptic MOPr are thought to act by opening GIRK channels to hyperpolarize and inhibit pain facilitatory neurons, whereas presynaptic MOPr are thought to act by inhibiting VGCC to decrease neurotransmitter release onto pain inhibitory neurons. It is well accepted that both of these mechanisms occur simultaneously within the RVM, and that both of these mechanisms contribute to the production of antinociception in the RVM. However, the relative contribution of the postsynaptic and the presynaptic MOPr to the antinociception produced by MOPr agonists in the RVM has not been established. Additionally, how persistent inflammatory pain alters the actions of agonists at the postsynaptic and the presynaptic MOPr in the RVM has not been examined.

This thesis therefore, sought to confirm that $G_{i/o}$ proteins mediate the antinociceptive effects of MOPr agonists in the RVM in uninjured rats and in rats with a persistent inflammatory injury. In addition, this thesis examined the contribution of the postsynaptic MOPr and the presynaptic MOPr to opioid-induced antinociception, as well as determined whether their contribution changes under conditions of persistent inflammatory pain.
What are the Hypotheses and How Will They Be Tested?

Chapter II: $G_{i/o}$ Proteins

**Hypothesis**

Microinjection of PTX into the RVM attenuates the antinociception of the MOPr agonist DAMGO, in the RVM of uninjured rats. In addition, these effects are enhanced in rats after persistent inflammatory injury.

**Approach**

The anti-hyperalgesic and the antinociceptive effects of DAMGO were determined after a prior treatment of the RVM with PTX in rats that received prior treatment of either SAL or CFA in one hindpaw. In addition, GTP$_\gamma$S assays were performed to examine whether the treatment with PTX in the RVM effectively inactivated $G_{i/o}$ proteins.

Chapter III: Postsynaptic MOPr

**Hypothesis**

Inhibition of GIRK channels in the RVM attenuates the antinociception of the MOPr agonist DAMGO, in the RVM of uninjured rats. In addition, these effects are enhanced in rats after a persistent inflammatory injury.

**Approach**

The response to thermal noxious stimuli was determined after administration of a selective GIRK channel inhibitor, tertiapin-Q, into the RVM of rats that received prior treatment of either SAL or CFA in one hindpaw. In addition, the anti-hyperalgesic and antinociceptive effects of DAMGO were determined after a prior treatment of the RVM with tertiapin-Q in both SAL and CFA hindpaw-treated rats. Finally, qRT-PCR analysis were performed to determine the levels of mRNA of the GIRK1-4 subunits in the RVM and to assess whether CFA treatment upregulates the expression of these GIRK subunits.
Chapter IV: Presynaptic MOPr

Hypothesis

Levels of GLU and GABA release in the RVM decrease in response to microinjection of the MOPr agonist DAMGO into the RVM. In addition, these effects are diminished in rats with a persistent inflammatory injury.

Approach

In vivo microdialysis was used to quantitate the levels of GLU or GABA release in the RVM of freely moving rats. The first aspect of these studies examined whether basal release is altered as a consequence of persistent inflammatory nociception. The second aspect assessed the levels of GLU and GABA release in response to DAMGO microinjected into the RVM of SAL and CFA hindpaw-treated rats. Finally, this study determined whether perfusion of DAMGO into the RVM altered levels of GLU or GABA differently than microinjection of DAMGO into the RVM.
### A. Terminology

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Pain</td>
<td>An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.</td>
</tr>
<tr>
<td>Nociception</td>
<td>The neural process of encoding noxious stimuli.</td>
</tr>
<tr>
<td>Hyperalgesia</td>
<td>Increased pain from a stimulus that normally provokes pain.</td>
</tr>
<tr>
<td>Analgesia</td>
<td>Absence of pain in response to stimulation which would normally be painful.</td>
</tr>
</tbody>
</table>

### B. Terminology

<table>
<thead>
<tr>
<th>Term</th>
<th>Effect on Paw Withdrawal Latency (PWL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperalgesia</td>
<td>Decreased PWL below baseline nociceptive threshold</td>
</tr>
<tr>
<td>Anti-hyperalgesia</td>
<td>Return of PWL back to baseline nociceptive threshold</td>
</tr>
<tr>
<td>Antinociception</td>
<td>Increased PWL above baseline nociceptive threshold</td>
</tr>
</tbody>
</table>

Note: This terminology will be used throughout the thesis. The terminology in A) is the currently accepted terminology by the International Association of the Society of Pain (IASP, 1994). The terminology in B) focuses on the effects on the paw withdrawal latency that will be used during the results and discussion.

Table 1.1: Definitions of Pain and Nociception.
Figure 1.1: Representation of the terminology in Table 1.1. A) Normal nociception, B) Hyperalgesia, decreased PWL below normal nociceptive threshold. C) Anti-hyperalgesia, return of PWL back to normal nociceptive threshold, D) Antinociception, an increased PWL above normal nociceptive threshold.
Figure 1.2: Two Complementary/Competing Hypotheses of Opioid Action in the RVM.  

Top: In the uninjured state, pain facilitatory neurons (left) are relatively inactive, while pain inhibitory neurons (right) are tonically inhibited by GABA acting at GABA_A receptors. Antinociception is predominantly produced by presynaptic inhibition of GABAergic inputs and disinhibition of pain inhibitory neurons (right).

Bottom: After an inflammatory injury, the balance of activity in the pain inhibitory and the pain facilitatory pathways changes. There is an increase in the activity of both populations. However, hyperalgesia is thought to develop from a net increase in the activity of pain facilitatory neurons. Therefore, MOPr agonists in the RVM produce anti-hyperalgesia and perhaps enhanced antinociception by direct postsynaptic inhibition of pain facilitatory neurons (left).
CHAPTER II
DIFFERENTIAL ROLE OF G_{i/o} PROTEINS IN THE ANTINOCICEPTIVE EFFECTS OF A MU OPIOID RECEPTOR (MOPr) AGONIST IN THE ROSTRAL VENTROMEDIAL MEDULLA (RVM) OF UNINJURED RATS AND RATS AFTER PERSISTENT INFLAMMATORY INJURY

Abstract

The RVM is an important site of opioid action. It is also a critical, central relay center of pain modulation through which more rostral pain modulatory nuclei exert their actions. Persistent pain leads to adaptive changes in the RVM. One effect of this is an increase in the potency of opioid analgesics. Most clinically used opioids act at the MOPr in the RVM. MOPr are known to produce their effects via G_{i/o} proteins, and PTX, a G_{i/o} inactivator, attenuates the antinociceptive effects of MOPr agonists in many pain modulatory regions. However, given the importance of the RVM to pain modulation and opioid analgesia, it is surprising that this has not been examined in the RVM.

This study sought to confirm that in the RVM, G_{i/o} proteins mediate the antinociception produced by MOPr agonists in uninjured as well as in CFA treated rats. To this end, rats received a microinjection of PTX in the RVM 5 days before a microinjection of DAMGO, a MOPr agonist, in the same site. 50 ng PTX effectively reduced DAMGO stimulated GTPγS binding in uninjured rats. However, despite effective inactivation of G_{i/o} proteins, 50 ng PTX did not diminish the antinociceptive effects of DAMGO in the RVM of uninjured rats. In contrast, 50 ng PTX abolished the antinociceptive effects of DAMGO in the RVM of rats after an inflammatory injury. These results suggest a transition from G_{i/o} independent to G_{i/o} dependent mechanisms following CFA treatment. In addition, the anti-hyperalgesic effects of DAMGO were not inhibited by PTX, suggesting DAMGO produces anti-hyperalgesia and antinociception.
by different mechanisms. Finally, the higher, 500 ng PTX dose did not reduce DAMGO stimulated GTPγS binding in the RVM in the absence of injury, suggesting a G protein switch to Gs proteins or increased recycling of Gi/o proteins after this robust inactivation of Gi/o proteins. This same dose of PTX did reduce DAMGO stimulated GTPγS binding in CFA treated rats, suggesting that CFA treatment alters G proteins mechanisms in the RVM.

Collectively, this study provides the first evidence of a differential role of Gi/o proteins in MOPr-induced antinociception in the RVM of SAL and CFA treated rats.

Introduction

The mu opioid receptor (MOPr) is a seven transmembrane G protein coupled receptor (GPCR) (Childers, 1991; Law, Wong, & Loh, 2000). G proteins are heterotrimeric proteins consisting of three subunits, one α, one β, and one γ. In an inactive state, a GDP is bound to the G proteins. However, when an agonist binds to the MOPr, the heterotrimeric G protein couples to the receptor and the GDP is exchanged for a GTP. The Gα subunit then dissociates from the Gβγ subunits, and each can function as an intracellular effector.

The Gαi/o subunit inhibits adenyl cyclase and decreases intracellular levels of cAMP. Postsynaptically, the Gβγ dimer activates G protein inward rectifying potassium (GIRK) channels resulting in hyperpolarization and inhibition of neuronal excitability. Presynaptically, the Gβγ dimer inhibits voltage gated calcium channels (VGCCs) resulting in an inhibition of neurotransmitter release. The MOPr couples principally to Gi/o proteins (Connor & Christie, 1999). However, under conditions in which Gi and Go are absent or Gs is dephosphorylated, MOPr may also couple to Gs (Chakrabarti & Gintzler, 2007).

Pertussis toxin (PTX) selectively ribosylates and therefore inactivates Gi/o proteins.
As such, it is a functional antagonist of Gi/o-coupled GPCRs. For example, PTX pretreatment inhibits the ability of MOPr agonists to inhibit adenylate cyclase (Bodnar, Paul, Rosenblum, Liu, & Pasternak, 1990; Costa, Aktories, Schultz, & Wuster, 1983). In the presence of PTX, MOPr agonists are unable to inhibit VGCCs or activate GIRK channels (Aghajanian & Wang, 1986; Costa et al., 1983; Crain, Crain, & Makman, 1986; Parolaro et al., 1990). Finally, intracerebroventricular (i.c.v.) or intrathecal (i.t.) microinjection of PTX attenuates the antinociception produced by MOPr agonists administered systemically, i.c.v. or i.t (Bodnar et al., 1990; Hoehn, Reid, & Sawynok, 1988; Parenti, Tirone, Giagnoni, Pecora, & Parolaro, 1986; Parolaro et al., 1990; Przewlocki, Costa, Lang, & Herz, 1987; Sanchez-Blazquez & Garzon, 1988; Sanchez-Blazquez & Garzon, 1991; Shah, Duttaroy, Davis, & Yoburn, 1994). In addition, direct microinjection of PTX into the periaqueductal grey (PAG) or the locus ceruleus (LC) nuclei, known to play a role in pain modulation, also attenuates the antinociceptive effects of morphine (Bodnar et al., 1990). Surprisingly, although the role of Gi/o proteins in these regions has been extensively studied, this is not the case in the rostroventral medial medulla (RVM).

The RVM is a brainstem region consisting of the nucleus raphe magnus and the nucleus gigantocellularis pars α. It is a critical, central relay center of pain modulation through which the more rostral pain modulatory nuclei exert their actions of pain transmission through to the level of the spinal cord (Millan, 2002). The RVM is also an important site at which opioids act to produce their effects, via MOPr. In fact, microinjection of MOPr agonists in the RVM alone, is sufficient to produce antinociception (Ackley, Hurley, Virnich, & Hammond, 2001; Tershner, Mitchell, & Fields, 2000; Thorat & Hammond, 1997). Additionally, persistent inflammatory injury leads to adaptive changes in the RVM neurons (Guan, Terayama, Dubner, & Ren, 2002; Miki et al., 2002; Ren & Dubner, 2002; Zhang & Hammond, 2009). Indeed, under
conditions of persistent inflammatory nociception, such as that induced by intraplantar injection of complete Freund’s adjuvant (CFA), the anti-hyperalgesic and the antinociceptive effects of the MOPr agonist DAMGO are enhanced (Hurley & Hammond, 2000; Schepers, Mahoney, Gehrke, & Shippenberg, 2008). However, little is known about the mechanisms by which persistent inflammatory injury enhances the actions of MOPr agonists in the RVM, or whether these effects are related to differential actions by G\textsubscript{i/o} proteins under conditions of persistent pain.

Given the central role of the RVM in the modulation of nociception, and the analgesic effects mediated by opioids used clinically for management of chronic pain, further investigation of the role of G\textsubscript{i/o} proteins as a mechanism by which opioids transmit their analgesic effects in this region is warranted. Therefore, the work in this chapter sought to confirm that in the RVM, G\textsubscript{i/o} proteins mediate the antinociception produced by MOPr agonists in uninjured rats, and to determine whether the role of G\textsubscript{i/o} proteins is altered during conditions of persistent inflammatory pain.

This study first determined whether microinjection of PTX into the RVM attenuates the antinociception produced by microinjection of the MOPr agonist DAMGO at the same site. It then determined whether inactivation of G\textsubscript{i/o} proteins, via PTX microinjection, alters the enhanced anti-hyperalgesic and antinociceptive effects of DAMGO in the RVM of CFA treated rats. Finally, GTP\textgamma{}S assays were performed to demonstrate that the PTX microinjections effectively inactivated the G\textsubscript{i/o} proteins in the RVM.

Experimental Procedures

**Animals and the Experimental Model**

Male Charles River Sprague-Dawley rats (Charles River, Raleigh, NC, USA) were housed on a 12 hr light/dark cycle with water and food *ad libitum*. All studies were
conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health. The experiments were approved by the University of Iowa Animal Care and Use Committee, and care was taken to minimize the number of animals used and their suffering.

Intraplantar (ipl) injection of complete Freund’s adjuvant (CFA) in one hindpaw was used to model persistent inflammatory injury. The rats were lightly anesthetized with isofluorane, and the thickness of the hindpaw was measured with digital calipers. The plantar surface of the left hindpaw was then injected with 150 µL of CFA (150 µg of Mycobacterium butyricum, Calbiochem, La Jolla, CA, USA) or sterile-filtered saline at pH 7.4. The rats were returned to their cages for 4 days. After the rats were euthanized, the thickness of the hindpaw was measured once again to verify the presence of inflammation.

**Behavior Studies**

The first set of studies was designed to confirm that the antinociception produced by DAMGO is mediated via \( G_{i/o} \) proteins in the RVM, in the absence of injury. In addition, these studies determined whether the anti-hyperalgesia and the antinociception produced by DAMGO are mediated by \( G_{i/o} \) proteins in the presence of persistent inflammatory injury. To this end, rats were prepared with an intracerebral (i.c.) guide cannula aimed at the RVM for drug administration.

**Surgical Preparation**

Rats were anesthetized with a mixture of ketamine hydrochloride (70 mg/kg i.p.) and xylazine (13 mg/kg i.p.), and an i.c. guide cannula (26 gauge; Plastics One Inc., Roanoke, VA) was stereotaxically implanted such that it terminated 3 mm dorsal to the RVM. The cannulae were affixed to the skull with sterile, stainless steel screws and dental acrylic. A stainless steel stylet was inserted into the guide cannula to maintain
patency. After surgery, the animals were housed separately on corncob bedding. The cannulae were implanted 7 days before behavior testing.

Heat Nociceptive Testing

Heat nociceptive testing was assessed by focusing a high intensity beam of light on the plantar surface of the hindpaw. The time required for the rat to withdraw its hindpaw from the thermal stimulus was termed the paw withdrawal latency (PWL). The lamp was adjusted to elicit a baseline PWL of 8-12 s. A maximum of 20 s were allowed to prevent tissue injury, and this latency was assigned to a rat meeting the maximum. On the day of testing, the rats were acclimated to the testing environment for 30 min and then placed in individual Plexiglas chambers on a glass surface maintained at 25°C. After an additional 30 min period of acclimation to the chamber, the PWL was determined.

Microinjection Procedure and Experimental Design

To confirm that the antinociception produced by DAMGO is mediated via G\textsubscript{i/o} proteins in the RVM, rats received a microinjection of 50 ng PTX, 500 ng PTX or saline in the RVM 5 days before testing. Previous studies have demonstrated that \textit{in vivo} microinjection of PTX effectively and maximally inactivates G\textsubscript{i/o} proteins 2-7 days after its administration (Bodnar et al., 1990; Hoehn et al., 1988; Parenti et al., 1986; Sanchez-Blazquez & Garzon, 1988; Sanchez-Blazquez & Garzon, 1991). In addition, preliminary studies determined that a prior microinjection of saline 5 days before DAMGO into the RVM did not attenuate DAMGO’s well-known antinociception. The baseline PWL was determined four days before behavior testing, immediately before ipl injection of CFA or saline in the left hindpaw. On the day of testing, the baseline PWL was re-determined. Rats that did not exhibit hyperalgesia after injection of CFA were excluded from the study. Rats then received a microinjection of saline or DAMGO into the RVM and the PWL was determined 15, 30, 45 and 60 min later.
Drugs were injected via a 33 gauge stainless steel injector needle that extended 3 mm beyond the guide cannula tip and targeted the RVM. PTX 50 ng and its saline control as well as DAMGO and its saline control were administered in a volume of 0.25 µL. PTX 500 ng and its saline control were administered in a 0.5 µL volume because it could not be diluted otherwise. The drug delivery was monitored by following the movement of an air bubble in the tubing that connected the injector to the syringe pump. The needle was left in place for 45 s after the injection to minimize diffusion of the drug up the injection tract. The investigator was blinded to all the drugs administered. Each rat was used for only one experiment but received two microinjections, 5 days apart. To assess the overall well-being, the weight of the rats was measured at the time of ipl injection of CFA or saline and then again after they were euthanized. In addition, gross observations of changes in behavior (catatonia, agitation, ataxia, sedation), as well as levels of alertness and orientation to a cotton applicator were recorded throughout the period of testing. PTX (List Biologicals, Campbell, CA) and DAMGO (Sigma, St. Louis, MO) were dissolved in sterile-filtered saline at pH 7.0. Sterile-filtered saline at pH 7 served as the control for both drugs.

**Histology**

After testing, the rats were euthanized by CO₂ inhalation. Their brains were removed, fixed in 10% formalin containing 30% sucrose, and transverse sections were cut using a cryostat microtome. These were then stained with Cresyl Violet. The injection sites were identified, and then verified by a person blinded to the behavioral outcome. The sites were then plotted on an atlas of the rat brain. Figure 2.1 illustrates the distribution of the microinjection sites into the RVM and represents the distribution across all of these studies. Sites outside of the RVM fell rostral to the RVM within the trapezoid body, caudal to the RVM in the longitudinal faciculus, and ventral to the RVM in the pyramids. Results gained from these out sites are discussed in the results section.
Statistical Analysis

Data were expressed as the mean and standard error of the mean (S.E.M.). A two-way repeated measures analysis of variance was used to compare the PWL among the different groups. One factor was treatment in the RVM (PTX or Saline), the other factor was treatment in the hindpaw (SAL or CFA), and the repeated factor was time. Post-hoc comparisons among mean values for the individual treatment groups were made using the Student Newman test. A $P \leq 0.05$ was considered significant.

GTPγS Binding Assays

GTPγS binding studies were performed to determine whether 50 ng and 500 ng PTX effectively inactivated Gi/o proteins in the RVM. Rats in these experiments were prepared as above. They received an i.c. cannula implantation and a microinjection of 50 ng, 500 ng PTX or saline into the RVM 5 days before obtaining the RVM tissue. The next day, they received an ipl injection of saline or CFA into one hindpaw. Four days later, the rats were euthanized by CO$_2$ inhalation and one 1.5 mm RVM punch was obtained from each rat. Three RVM punches were pooled into one sample. Samples were collected on ice and stored at -20°C until use.

Membrane homogenates of the RVM were prepared and GTPγS binding assay performed as previously described (Engle, Gassman, Sykes, Bettler, & Hammond, 2006; Sykes et al., 2007). Briefly, 25 µg of membrane homogenates were incubated at 25°C for 2 hr in 1 mL of assay buffer with (0.03-10 µM) DAMGO, 30 µM GDP and 50 pM [$^{35}$S]GTPγS (specific activity, 1000 Ci/mmole; Amersham, Piscataway, NJ, USA). Adenosine deaminase (10.52 U/mL) was added to eliminate adenosine-mediated stimulation of GTPγS binding. The reaction was terminated by filtration using a Brandel cell harvester and presoaked Whatman GF/B glass filters. The filters were washed and transferred to scintillation vials with Scintisafe Econo1 scintillation fluid. They were
allowed to equilibrate overnight. The radioactivity was measured via a liquid scintillation analyzer. Non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. Tissue samples were analyzed in triplicate and the investigator was blinded to the treatment condition of the RVM tissue membrane preparations.

**Statistical Analysis**

Binding isotherms were fit with Graphpad Prism (San Diego, CA, USA). Interestingly, groups pretreated with PTX demonstrated lower baseline GTPγS binding in both SAL and CFA treated rats (Figure 2.4). However, this effect only reached significance in the CFA treated group. Regardless, it is sufficient to suggest that expressing data as % DAMGO stimulation would not be appropriate. Therefore, data are expressed as the difference from baseline, in counts per minute (cpm). Data are shown as the mean ± S.E.M. A two way ANOVA was used to compare E_max values. One factor was treatment in the RVM (50 ng PTX, 500 ng PTX, saline) and the other was treatment in the hindpaw (SAL, CFA). Post-hoc comparisons among mean values for the individual treatment groups were made using the Student Newman test. A \( P \leq 0.05 \) was considered significant.

**Results**

**In the Absence of Injury: The Role of \( G_{i/o} \) Proteins in the RVM in the Modulation of Nociceptive Threshold and DAMGO-induced Antinociception**

\( G_{i/o} \) Proteins Do Not Mediate DAMGO-induced Antinociception

This first set of experiments determined whether \( G_{i/o} \) proteins in the RVM play a role in the modulation of nociceptive threshold, as well as DAMGO induced antinociception in the RVM in the absence of injury. To assess this, PTX, which
selectively inactivates $G_{i/o}$ proteins, was microinjected into the RVM 5 days before microinjection of DAMGO into the same site in the RVM. Figure 2.2 (inset) illustrates that microinjection of 50 ng or 500 ng PTX in the RVM 5 days earlier does not alter responsiveness to a noxious heat stimulus. In addition, microinjection of saline into the RVM 5 days earlier does not alter the well-known antinociceptive effects of DAMGO in the RVM. Surprisingly, microinjection of 50 ng or 500 ng PTX does not diminish the antinociceptive effects of DAMGO in uninjured rats (Figure 2.2). In addition, microinjection of DAMGO in regions outside of the RVM also produced antinociception. These microinjection sites fell in various regions including the rostral trapezoid body, one up in the longitudinal fasciculus and the others were distributed in the pyramids, ventral to the RVM. The magnitude of the effects of DAMGO in these out sites was larger than expected. However, it is likely that microinjection of DAMGO into some of these sites diffused into the RVM, resulting in the production of antinociception. Interestingly, PTX also did not attenuate this antinociceptive effect produced by microinjection in these sites outside of the RVM (Figure 2.5A).

**In Persistent Inflammatory Injury: The Role of $G_{i/o}$ Proteins in the RVM in CFA-induced Heat Hyperalgesia, as well as DAMGO-induced Anti-hyperalgesia and Antinociception**

This set of experiments determined whether $G_{i/o}$ proteins in the RVM mediate the well-known hyperalgesia that develops after CFA treatment. In addition, these experiments examined whether $G_{i/o}$ proteins mediate the anti-hyperalgesic and the antinociceptive effects of DAMGO in the RVM under conditions of persistent inflammatory injury.
**Gi/o Proteins Do Not Mediate Hyperalgesia Or Heat Nociception**

Figure 2.3 (insert) illustrates that the baseline, B2, PWL of rats microinjected with either Saline or 50 ng PTX in the RVM do not differ, demonstrating that in the RVM, PTX does not enhance or suppress the heat hyperalgesia that develops in the ipsilateral hindpaw four days after the injection of CFA. Microinjection of PTX also does not alter the heat nociceptive threshold of the uninjured, contralateral hindpaw of CFA treated rats (Figure 2.4, insert).

**Gi/o Proteins Do Not Mediate DAMGO-induced Anti-hyperalgesia**

As expected, microinjection of DAMGO into the RVM of rats pretreated with saline in the same site 5 days earlier produced anti-hyperalgesia in the ipsilateral hindpaw (Figure 2.3; lower bracket). Interestingly, 50 ng PTX did not attenuate the anti-hyperalgesic effect of DAMGO. Microinjection of DAMGO in sites outside the RVM also produced an anti-hyperalgesic effect at the 15 min time point (Figure 2.5B). Two sites were in the rostral trapezoid body and two sites were ventral to the RVM. However, there are not enough microinjection sites outside the RVM to conclusively state the effects of PTX on this anti-hyperalgesic effect (Figure 2.5B).

**Gi/o Proteins Mediate DAMGO-induced Antinociception**

As expected, microinjection of DAMGO into the RVM of rats pretreated with saline produced antinociception in both the ipsilateral (Figure 2.3; upper bracket) and the contralateral (Figure 2.4) hindpaw of CFA treated rats. Additionally, microinjection of 50 ng PTX completely abolished the antinociceptive effects of DAMGO in the ipsilateral hindpaw (Figure 2.3), and nearly completely diminished its antinociceptive effect in the uninjured, contralateral hindpaw (Figure 2.4). Microinjection of DAMGO in sites outside the RVM produced a modest antinociceptive effect in the ipsilateral and the
contralateral hindpaw. In addition, the data is too variable and the sample size too small to make a conclusive statement about the effects of PTX in sites outside the RVM (Figure 2.5B). Finally, in the contralateral hindpaw, the antinociceptive effect produced by DAMGO microinjected into sites outside of the RVM is milder than that produced by DAMGO microinjected into the RVM. In addition, contrary to the effects of PTX in the RVM, PTX microinjected outside of the RVM does not decrease this antinociceptive effect (Figure 2.5C).

**PTX Effectively Inactivates G\textsubscript{i/o} Proteins in the RVM**

Given that the PTX pretreatment did not attenuate the antinociception produced by DAMGO in the uninjured rats, this set of studies examined whether these doses of PTX effectively inactivated G\textsubscript{i/o} proteins in the RVM. To assess this, GTP\textgamma S binding was conducted in the absence and presence of DAMGO using RVM tissue obtained from SAL and CFA treated rats 5 days after microinjection of saline or PTX in the RVM. Basal binding of GTP\textgamma S is not significantly decreased by either 50 or 500 ng PTX in uninjured rats (Figure 2.6). However, both doses significantly reduce basal GTP\textgamma S binding in CFA-treated rats.

In SAL treated rats, treatment with 50 ng PTX significantly abolishes the ability of DAMGO to stimulate GTP\textgamma S binding (Figure 2.7). Surprisingly, treatment with 500 ng PTX does not reduce DAMGO stimulated GTP\textgamma S binding. This finding suggests that this higher dose perhaps results in a switch to G\textsubscript{s} proteins or quicker recycling of G\textsubscript{i/o} proteins. In contrast, in CFA treated rats, both 50 ng and 500 ng PTX significantly diminish DAMGO stimulated GTP\textgamma S binding in the RVM (Figure 2.8). Figure 2.9 is an alternate presentation of these same data. It compares the effects of each different RVM treatment in saline and CFA treated rats.
DAMGO stimulated GTPγS binding was slightly higher in CFA treated rats than in SAL treated rats. However, this was not statistically significant (Figure 2.9A, D). Both SAL and CFA treated rats pretreated with 50 ng PTX in the RVM demonstrated similar counts of DAMGO stimulated GTPγS binding (Figure 2.9B, D). However, CFA treated rats pretreated with 500 ng PTX demonstrated much lower DAMGO stimulated GTPγS counts than the counterpart, SAL treated rats also pretreated with 500 ng PTX (Figure 2.9C, D).

Discussion

**MOPr Mechanisms of Action in the Absence of Injury**

This study tested the hypothesis that the antinociceptive effects of MOPr agonists in the RVM are mediated by G_{i/o} proteins. This hypothesis was based on abundant evidence that the postsynaptic and the presynaptic inhibitory effects of MOPr agonists are mediated by G_{i/o} proteins (Childers, 1991; Law et al., 2000), as well as prior reports that \textit{in vivo} administration of PTX attenuates the antinociception produced by MOPr agonists in various pain modulatory regions (Bodnar et al., 1990; Hoehn et al., 1988; Parenti et al., 1986; Parolaro et al., 1990; Sanchez-Blazquez & Garzon, 1988; Sanchez-Blazquez & Garzon, 1991). Previous studies effectively used doses of PTX ranging from 5 ng to 1 µg (Bodnar et al., 1990; Przewlocki et al., 1987). Typically, 100 ng or 500 ng PTX were administered i.c.v. and i.t, where perhaps higher doses of PTX are necessary to appropriately penetrate into the target tissue. However, one study microinjected PTX into discrete nuclei, the PAG or the LC (Bodnar et al., 1990). In this case, 5 ng PTX effectively attenuated the effects of morphine microinjected into the same region (Hoehn et al., 1988; Parenti et al., 1986; Parolaro et al., 1990; Sanchez-Blazquez & Garzon, 1988; Self, Terwilliger, Nestler, & Stein, 1994; Shah et al., 1994). Additionally, an appropriate interval from the time PTX is administered to the time of testing is necessary
for PTX to reach the target cells and appropriately inactivate $G_{i/o}$ proteins. In previous studies, this interval ranged from 2-24 days. However, the peak reduction was measured 5-7 days after the administration of PTX (Hoehn et al., 1988; Parenti et al., 1986; Sanchez-Blazquez & Garzon, 1988; Sanchez-Blazquez & Garzon, 1991). Given this, the failure of 50 or 500 ng PTX microinjected 5 days before testing, to attenuate the antinociception produced by DAMGO in the RVM of uninjured rats in this study was entirely unexpected.

The first inclination is that perhaps PTX did not effectively inactivate $G_{i/o}$ proteins in the RVM. However, these data cannot be dismissed on the basis of inadequate ribosylation and inactivation of $G_{i/o}$ because 50 ng PTX effectively prevented DAMGO-stimulated GTPγS binding in the RVM. Although PTX treatment only decreased DAMGO-stimulated GTPγS binding ~50%, this is consistent with previous studies. Several studies previously demonstrated that pretreatment with PTX that effectively reduced the antinociception produced by MOPr agonists, only reduced $[^{32}\text{P}]$ NAD labeling by ~50%. This translates to a 50-60% reduction in the number of sites available for ADP ribosylation in the tissue after PTX treatment, therefore a reduction in the number of $G_{i/o}$ protein sites available for activation and promoting subcellular activity after MOPr agonists are administered (Correa-Sales, Reid, & Maze, 1992; Hayashi, Rabin, Guo, & Maze, 1995; Przewlocki et al., 1987). It is thought that a limiting factor for complete inactivation of $G_{i/o}$ proteins is that PTX microinjected in vivo must penetrate the tissue, whereas in vitro pretreatment of PTX can completely abolish all $G_{i/o}$ activity. Regardless, given that ~50% inactivation of $G_{i/o}$ proteins was sufficient to reduce the antinociception of MOPr agonists in other pain modulatory regions and that the conditions of this experiment were closely mirrored to those in other studies in which PTX effectively reduced the antinociceptive effects of various MOPr agonists, the results in this study indicate that the RVM is different from these other pain modulatory regions.
These data indicate that, in an uninjured state, the antinociceptive effects of MOPr agonists in the RVM do not involve coupling of MOPr to G_{i/o} proteins.

Under certain conditions, MOPr agonists can couple to G_s proteins. The earliest evidence for direct excitatory actions of MOPr agonists presumably mediated by G_s proteins was obtained in spinal cord-dorsal root ganglion explants. Pretreatment with PTX inhibited the depressive effects of MOPr agonists and prevented the opioid-induced shortening of perikaryal action potentials in these explants. However, in a few PTX treated explants, not only were the depressive effects of morphine inhibited but some excitatory effects resulted. For example, morphine increased the amplitude of the action potentials and also prolonged their duration (Crain et al., 1986; Crain, Crain, & Makman, 1987; Crain & Shen, 1990a; Crain & Shen, 1990b). Until recently, biochemical evidence for the interaction between MOPr and G_{s} had been lacking. However, recently, Chakrabarti et al (2005) demonstrated that MOPr is present in immunoprecipitates obtained using anti-G_{s} antibodies in CHO cells but also in rat spinal cord tissue (Chakrabarti, Regec, & Gintzler, 2005). This interaction is enhanced after administration of morphine. Additional studies demonstrated that the interaction of MOPr and G_{s} proteins is enhanced when G_{s} is dephosphorylated (Chakrabarti & Gintzler, 2007). Together, these results indicate that opioids indeed have direct excitatory effects on G_{s} proteins, and that these effects are perhaps ‘unmasked’ by PTX pretreatment.

Furthermore, although G protein switching has not been demonstrated for the MOPr, it has been for other receptors such as the β adrenoreceptor, which was formerly considered to couple exclusively to G_s proteins but has more recently demonstrated switch to G_i signaling under certain circumstances (Magocsi, Vizi, Selmeczy, Brozik, & Szelenyi, 2007). The hypothesis that in an uninjured state, the antinociceptive effects of MOPr agonists in the RVM are instead mediated via G_s proteins remains to be tested, perhaps
by selective knock-down of Gs proteins since, unfortunately, cholera toxin is no longer a suitable selective Gs protein inhibitor due to recently recognized nonspecific effects.

A role for Gz proteins in mediating the antinociceptive effects of DAMGO in the RVM of uninjured rats must also be considered. Gz proteins are part of the subfamily of Gs proteins. Their subcellular effectors include inhibition of adenylyl cyclase and VGCC, as well as activation of GIRK channels (Tso & Wong, 2000; Tso, Yung, & Wong, 2000). They also stimulate MAPK as well as ERK1/2 (Tso & Wong, 2000; Tso et al., 2000).

However, Gz proteins lack the site for ADP ribosylation and are therefore insensitive to inactivation by PTX (Jeong & Ikeda, 1998). These proteins have been shown to couple to MOPr in heterologous systems, as well as in vivo in response to MOPr agonists including DAMGO (Garzon, Castro, & Sanchez-Blazquez, 1998; Jeong & Ikeda, 1998; Sanchez-Blazquez, Juarros, Martinez-Pena, Castro, & Garzon, 1993). Gz proteins are thought to contribute towards the production of antinociception in pain modulatory regions such as the PAG. Indeed, selective knockdown of Gz proteins, by icv administration of antisense oligodeoxynucleotides reduces the antinociception produced by several MOPr agonists (Sanchez-Blazquez, Gomez-Serranillos, & Garzon, 2001). Although their role has not been examined in the RVM, it is foreseeable that after PTX inactivation of Gvo proteins, Gz proteins mediate the antinociceptive effects of DAMGO.

Additionally, a possible involvement of mitogen-activated protein (MAP) kinase signaling pathways, such as extracellular signaling-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, may also merit consideration. Although there is extensive in vitro evidence for MOPr engagement of multiple kinases in heterologous cell systems, the corresponding in vivo evidence is not nearly as well developed. For example, acute systemic administration of morphine increases phosphorylation of p42/p44 MAPK in the locus coeruleus and anterior cingulate nucleus and decreases it in the nucleus accumbens. However, it does not affect levels of phosphorylated p42/p44
MAPK in the RVM (Eitan et al., 2003). Also, with the exception of the locus coeruleus, the changes in p42/p44 MAPK phosphorylation were confined to neurons that did not express the MOPr and were subsequently determined to be dependent on NMDA receptor activation. The extent to which MAPK signaling pathways mediate the antinociceptive effects of MOPr agonists in the RVM of uninjured rats remains to be directly tested. Additionally, although MAPK signaling is typically recognized as a downstream effect of G protein activation, there is a G protein independent, β-arrestin related pathway that can activate MAPK signaling (Zheng, Loh, & Law, 2010). It is thought that there is a competition between β-arrestin and the G protein and although typically, the G protein-dependent pathway is activated. If the β-arrestin binds the MOPr before G protein activation, then G protein-independent pathways will be activated (Zheng et al., 2010). It is possible then, that if the G protein binds but is not activated because PTX has inactivated it, the β-arrestin pathway may still be activated.

The finding that a ten-fold higher dose of PTX did not significantly decrease DAMGO-stimulated GTPγS binding may initially be difficult to accept. However, it is possible that the robust inactivation of G_{i/o} proteins by 500 ng PTX triggered an increased recycling rate of G_{i/o} proteins as a compensatory mechanism. An argument against this, however, is that previous studies indicate that G_{i/o} proteins remain ADP-ribosylated as long as 28 days after PTX pretreatment (Correa-Sales et al., 1992; Przewlocki et al., 1987; Self et al., 1994), suggesting that the turnover rate for G_{i/o} proteins is much longer than 5 days after PTX treatment. It should be noted that the GTPγS binding assay is a functional measure of G protein activation. It is typically performed for G_{i/o} coupled receptors because G_{i/o} proteins are more abundant, which results in a higher signal-to-noise ratio. However, this assay has also been performed with receptors that couple to G_s or G_q proteins (Harrison & Traynor, 2003). Essentially, the agonist drives the G protein activation. Since MOPr agonists are known to act via G_{i/o}, the G protein activation in the
GTPγS assay is presumed to be Gi/o. However, it is possible that in the complete absence or unavailability of Gi/o proteins, as with a high dose of PTX pretreatment, the observed stimulation is instead mediated by Gs or other G proteins insensitive to PTX.

**MOPr Mechanisms of Action after Inflammatory Injury**

This study also tested the hypothesis that the antinociceptive and the antihyperalgesic effects of DAMGO are mediated by Gi/o proteins after inflammatory injury. Interestingly, in CFA treated rats, 50 ng PTX completely abolished the antinociceptive effects of DAMGO in both the ipsilateral, inflamed hindpaw and the contralateral, uninflamed hindpaw. This dose of PTX also prevented DAMGO-stimulated GTPγS binding in CFA treated rats. These data provide strong evidence for a role of Gi/o proteins in the RVM in the antinociceptive actions of a MOPr agonist in the RVM under conditions of persistent inflammatory nociception. Thus, the mechanisms responsible for the antinociceptive effects have transitioned from Gi/o protein independent in uninjured rats to Gi/o protein dependent after inflammatory injury. Whether this reflects the recruitment of Gi/o proteins as an additional signaling pathway or replacement of another pathway remains to be determined.

Whereas 500 ng PTX did not inhibit DAMGO-stimulated GTPγS binding in the RVM of uninjured rats, it did so in CFA treated rats. This interesting finding suggests that CFA treatment either alters G protein mechanisms in such a way that impairs the ability for MOPr to switch to Gs proteins or impairs the ability to increase the recycling rate of Gi/o proteins. A prior report, confirmed here, determined that CFA treatment does not increase DAMGO-stimulated GTPγS binding (Sykes et al., 2007). However, both doses of PTX impaired constitutive GTPγS binding in the absence of an agonist in CFA
treated rats suggesting that persistent inflammatory pain state has increased the sensitivity of $G_{i/o}$ proteins to ribosylation.

Finally, despite evidence for an enhanced sensitivity of $G_{i/o}$ proteins to the effects of PTX, the anti-hyperalgesic effects of DAMGO in the RVM were not inhibited. The finding that the antinociceptive effects but not the anti-hyperalgesic effects of DAMGO in the RVM were inhibited by PTX suggests that there are different mechanisms for MOPr mediated antinociception and anti-hyperalgesia, and that only antinociception is $G_{i/o}$ dependent. These data are the first to suggest that the mechanisms by which MOPr agonists produce antinociception and anti-hyperalgesia are fundamentally different. Interestingly, Senba and colleagues have provided evidence for an increase in phosphorylation of both p38 MAPK and ERK in the RVM of rats with acute inflammatory injury induced by ipl injection of CFA (Imbe et al., 2005; Imbe et al., 2007). They attribute the development of hyperalgesia in this model to phosphorylation of ERK (Imbe et al., 2011). In fact, microinjection of MAPK kinase inhibitors into the RVM reduced the phosphorylation of ERK and reversed the hyperalgesia. Thus, it will be of interest to determine whether longer term inflammatory injury also increases the phosphorylation of the various MAPKs in the RVM. In addition, it will be of interest to determine whether microinjection of DAMGO into the RVM also suppresses MAPK phosphorylation in the RVM of CFA treated rats, and if so, to determine in which subpopulations of RVM neurons these changes occur.

Final Comments

This study uses primarily a pharmacological approach to determine whether the effects of DAMGO are mediated via $G_{i/o}$ proteins. The use of PTX is well-established and the extensive prior literature allowed us to choose, with confidence, an appropriate experimental design including PTX dose as well as pretreatment interval. Additionally,
we were able to confirm that the dose of PTX is effective by performing a G protein coupling assay which also mimics the results seen in the literature. However, it would of course be appropriate, given that these results are unexpected and inconsistent with what has been seen in the literature, that we could corroborate these data with either 1) another pharmacological agent, or 2) a molecular approach. Unfortunately, PTX is the only available \( G_{i/o} \) inhibitor. However, Sanchez-Blazquez et. al. has previously used antibodies against different \( G\alpha \) subunits and demonstrated successfully that these inhibit binding to specific G protein subunits (Sanchez-Blazquez & Garzon, 1994). This could be another pharmacologic and biochemical approach to corroborate these data. A second approach is to selectively downregulate the levels of \( G_{i/o} \) proteins via use of miRNA or siRNA. Although to date, no studies have taken this approach, prior studies have successfully used antisense oligodeoxynucleotides to selectively downregulate various \( G\alpha \) subunits (Sanchez-Blazquez, Garcia-Espana, & Garzon, 1995). The use of multiple of these modalities could certainly strengthen our results.
Figure 2.1: Distribution of the sites microinjected in the RVM. The microinjection sites in this panel belong to the group treated with 50 ng PTX 5 days before DAMGO into the same site, four days after CFA treatment (the data for this group is represented in figure 2.3 and 2.4 in blue filled circles). This panel is representative of the distribution of the microinjection sites of all other treatment groups. Numbers to the left of the section are the distance from the interaural line. Filled circles indicate sites in the RVM. Each filled circle indicates one site corresponding to one rat.
Figure 2.2: Effects of PTX on DAMGO induced antinociception in the RVM in the absence of injury. Saline, 50 ng PTX or 500 ng PTX was microinjected into the RVM 5 days before behavior testing. B1 is the PWL to heat stimulus at baseline. It is also represented in the inset. On the day of testing, 30 ng DAMGO was microinjected into the same site in the RVM. PWL was then measured 15, 30, 45, and 60 min after the microinjection of DAMGO. Data are expressed as the mean ± S.E.M. Numbers of animals are shown above in parentheses. *P<0.05, **P<0.01 compared to B1.
Figure 2.3: Effects of PTX on DAMGO induced anti-hyperalgesia and antinociception in the RVM. Saline or 50 ng PTX was microinjected into the RVM 5 days before behavior testing. B1 is the PWL at baseline one day after saline or PTX microinjection and right before the ipl injection of CFA. B2 is the baseline nociceptive threshold on the day of testing, 4 days after CFA injection. The development of hyperalgesia is also represented in the inset. On the day of testing, 30 ng DAMGO was microinjected into the same site in the RVM. The lower bracket denotes the anti-hyperalgesia; the upper bracket denotes the antinociception produced by microinjection of DAMGO in the RVM. Data are expressed as the mean ± S.E.M. Numbers of animals are shown above in parentheses. *P<0.05, **P<0.01 compared to B2. +P<0.05, ++P<0.01 compared to saline at the corresponding time point.
Figure 2.4: Effects of PTX on DAMGO induced antinociception in the RVM of rats that received an ipl injection of CFA in one hindpaw 4 days earlier. Saline or 50 ng PTX was microinjected into the RVM 5 days before behavior testing. B1 is the PWL of the contralateral hindpaw determined one day after saline or PTX microinjection and immediately before ipl injection of CFA. B2 is the baseline nociceptive threshold on the day of testing, 4 days after CFA injection. B2 is also represented in the inset. On the day of testing, 30 ng DAMGO was microinjected into the same site in the RVM. Data are expressed as the mean ± S.E.M. Numbers of animals are shown above in parentheses. *P<0.05, **P<0.01 compared to B2. +P<0.05, ++P<0.01 compared to saline at the corresponding time point.
Figure 2.5: Effects of PTX on DAMGO microinjected outside of the RVM in A) uninjured rats or B, C) rats that received an ipl injection of CFA in one hindpaw 4 days earlier. Data are expressed as the mean ± S.E.M. Numbers of animals are shown above in parentheses. In A) *P<0.05, **P<0.01 compared to B1. In B) *P<0.05, **P<0.01 compared to B2.
Figure 2.6: Effect of PTX on GTPγS binding in the absence of DAMGO determined in the RVM of rats that received an ipl injection of saline or CFA 4 days earlier. Saline, 50 ng or 500 ng PTX was microinjected into the RVM 5 days before the tissue was obtained. Data are expressed as the mean ± S.E.M. RVM punches from 3 rats were pooled per sample. Each sample was run in triplicate. Numbers of samples per group are shown above in parentheses. **P<0.01 compared to its saline control.
Figure 2.7: Effect of PTX on DAMGO stimulated GTPγS binding in the RVM of rats that received an ipl injection of saline 4 days earlier. Saline, 50 ng or 500 ng PTX was microinjected into the RVM 5 days before the RVM tissue was obtained. GTPγS assays were performed on membrane preparations in the presence of DAMGO. Data are expressed in as the mean ± S.E.M of the difference from binding in the absence of DAMGO, in counts per minute. **Fitted binding curves, B) E_{max} of the respective curves. RVM punches from 3 rats were pooled per sample. Each sample was run in triplicate. Numbers of samples per group are shown above in parentheses. *P<0.05 and **P<0.01
Figure 2.8: Effect of PTX on DAMGO stimulated GTPγS binding in the RVM of rats that received an ipl injection of CFA 4 days earlier. Saline, 50 ng or 500 ng PTX was microinjected into the RVM 5 days before the RVM tissue was obtained. GTPγS assays were performed on membrane preparations in the presence of DAMGO. Data are expressed as the mean ± S.E.M of the difference from binding in the absence of DAMGO, in counts per minute. A) Fitted binding curves, B) $E_{\text{max}}$ of the respective curves. RVM punches from 3 rats were pooled per sample. Each sample was run in triplicate. Numbers of samples per group are shown above in parentheses. **$P<0.01$ compared to the saline control.
Figure 2.9: Effect of CFA treatment on DAMGO stimulated GTPγS binding in the RVM after microinjection of A) Saline, B) 50 ng PTX, or C) 500 ng PTX in the RVM 5 days earlier. Data are expressed as the mean ± S.E.M of the difference from binding in the absence of DAMGO, in counts per minute. D) E_{max} of the respective curves. RVM punches from 3 rats were pooled per sample. Each sample was run in triplicate. Numbers of samples per group are shown above in parentheses. **P<0.01
CHAPTER III
DIFFERENTIAL ROLE OF G-PROTEIN INWARD RECTIFYING POTASSIUM
(GIRK) CHANNELS IN THE ANTI-HYPERALGESIC AND ANTINOCICEPTIVE
EFFECTS OF A MU OPIOID RECEPTOR (MOPr) AGONIST IN THE ROSTRAL
VENTROMEDIAL MEDULLA (RVM)

Abstract
The RVM is a critical, central relay of pain modulation through which more
rostral pain modulatory nuclei exert their actions through both pain inhibitory and pain
facilitatory pathways. It is also an important site at which opioids act to produce their
analgesic effects. Indeed, most clinically used opioids act at the MOPr in the RVM.
These MOPr are present both presynaptically and postsynaptically in the RVM.
Postsynaptic MOPr are thought to produce antinociception by activating GIRK channels,
resulting in hyperpolarization and inhibition of pain facilitatory neurons. However, the
data supporting this postulate is largely electrophysiology data and therefore has
limitations to clinical application. There is currently no behavioral, whole animal
evidence in the RVM supporting this postulate. Additionally, persistent inflammatory
injury, such as that induced by CFA leads to adaptive changes in the RVM. One known
effect of this is an increase in the potency of opioid analgesics. However, little is known
about the mechanisms by which persistent inflammatory injury enhances the actions of
MOPr agonists in the RVM, or whether these effects are mediated by enhanced actions
by the postsynaptic MOPr under conditions of persistent pain.

Therefore, this study sought to further characterize the postsynaptic actions of a
MOPr agonist, DAMGO, in the RVM in whole animal studies, and to determine whether
persistent inflammatory nociception enhances these postsynaptic actions. Microinjection
of a GIRK channel inhibitor, tertiapin-Q, in the RVM, produced a modest antinociception
in uninjured rats. However, it did not attenuate or enhance the hyperalgesia that develops 4 days after ipl injection of CFA. In addition, tertiapin-Q attenuated the antinociceptive effects of DAMGO in uninjured rats. However, tertiapin-Q did not block the anti-hyperalgesic effects of DAMGO, nor did it diminish the antinociceptive effects of DAMGO in the contralateral hindpaw of CFA treated rats. Finally, these differential effects of tertiapin-Q in the uninjured and injured rats are not the result of transcriptional downregulation of GIRK channels in the RVM. The levels of GIRK1-3 mRNA in the RVM or the relative ratio of expression $GIRK2 \geq GIRK3 > GIRK1$ was not altered after CFA treatment.

Prior to this study, support for the postulate that MOPr agonists mediate their analgesic effects via GIRK channels in the RVM was provided primarily by electrophysiology data. This has the limitation that analgesia cannot be assessed by electrophysiology alone. However, this study provides the first behavioral evidence in the RVM that postsynaptic MOPr indeed act via GIRK channels to produce their analgesic effects in the whole animal. Additionally, this study provides evidence of differential effects of GIRK channels after persistent inflammatory injury. However, it does not support the postulate that these postsynaptic mechanisms are responsible for the enhanced analgesic effects of opioids in the RVM after CFA treatment. Overall, these data advance the understanding of the contribution of postsynaptic MOPr and GIRK channels to the analgesic effects produced by opioids in the RVM. It is a clinical goal that this data may lead to the identification of new pharmacologic therapies for chronic pain management. Perhaps these therapies would target GIRK channels or other downstream mechanisms of this pathway while bypassing the MOPr.
Introduction

G protein inward rectifying potassium (GIRK) channels play a critical role in maintaining resting membrane potential as well as regulating neuronal excitability. They are homo or heterotetrameric channels composed of subunits 1-4 with tissue-specific distributions and subunit composition (Corey, Krapivinsky, Krapivinsky, & Clapham, 1998; Duprat et al., 1995; Kobayashi et al., 1995; Lesage et al., 1995). For example, GIRK1 and GIRK4 are primarily found in the heart where they regulate heart rate, whereas GIRK1-3 subunits are found throughout the central nervous system (CNS) (Karschin, Dissmann, Stuhmer, & Karschin, 1996; Koyrakh et al., 2005; Krapivinsky et al., 1995). Within the CNS, there are regions where only GIRK2 and GIRK3 are expressed, or where GIRK1 expression is very low (Jelacic, Kennedy, Wickman, & Clapham, 2000). GIRK4 expression has been noted in the CNS, but typically in discrete regions (Karschin et al., 1996; Wickman, Karschin, Karschin, Picciotto, & Clapham, 2000).

Although the relevance of GIRK channel subunit composition is not yet fully appreciated, the anatomical diversity of GIRK1-4 subunits implies a functional diversity. In support of this, different subunits of GIRK channels have unique characteristics. For example, there are no functional GIRK1 homotetramers (Ma et al., 2002). GIRK1 contains an ER retention signal that requires its expression with GIRK2-4 to reach the cell membrane (Aguado et al., 2008; Hedin, Lim, & Clapham, 1996; Jelacic et al., 2000). However, GIRK1 has an increased affinity to Gβγ. Therefore, GIRK2/GIRK3 heterotetramers are 5 fold less sensitive to activation by Gβγ than GIRK1/GIRKx heterotetramers (Jelacic et al., 2000). In addition, GIRK3 contains a lysosomal targeting signal that can direct functional GIRK channels towards lysosomes for degradation (Ma et al., 2002).

The mu opioid receptor (MOPr) is a G protein-coupled receptor (GPCR).
Following binding of an agonist at the MOPr, Gβγ subunits released from G_i/o associate with and open GIRK channels (Krapivinsky, Krapivinsky, Wickman, & Clapham, 1995; Mark & Herlitze, 2000; North & Uchimura, 1989). The resulting efflux of potassium causes hyperpolarization and neuronal inhibition. MOPr agonists act peripherally and centrally to produce antinociception and relieve hyperalgesia after injury. Within the CNS, the rostral ventromedial medulla (RVM) is a critical site of action for MOPr agonists. MOPr agonists are thought to directly inhibit spinally-projecting pain facilitatory neurons in the RVM by postsynaptically activating GIRK channels. This is thought to be one mechanism by which MOPr agonists produce antinociception and anti-hyperalgesia after injury (Hammond & Proudfit, 2007). However, despite the importance of the RVM in opioid-induced antinociception and anti-hyperalgesia, this postulate has never been directly tested. In addition, not much is known about the composition of GIRK channels in the RVM, or their role in modulating nociception.

Research in the last decade has revealed that persistent inflammatory pain states alter the activity and responsiveness of RVM neurons (Guan, Terayama, Dubner, & Ren, 2002; Miki et al., 2002; Ren & Dubner, 2002; Zhang & Hammond, 2009). In addition, persistent inflammatory injury results in time-dependent changes in the balance of activity in both pain inhibitory and pain facilitatory pathways in the RVM (Hurley & Hammond, 2000; Ren & Dubner, 2002; Vanegas & Schaible, 2004). Specifically, the anti-hyperalgesic and antinociceptive effects of MOPr agonists are enhanced in rats with persistent inflammatory injury induced by intraplantar injection of complete Freund’s adjuvant (CFA). The mechanisms by which persistent inflammatory pain enhances the effects of MOPr agonists in the RVM remain poorly understood. However, a recent study demonstrated that the percentage of RVM neurons that respond to the MOPr agonist DAMGO with an outward current was significantly increased in CFA treated rats (Zhang & Hammond, 2010). These results indicate that the postsynaptic mechanisms of
MOPr-induced antinociception are potentiated during inflammatory conditions. One mechanism by which the postsynaptic actions of DAMGO in the RVM are potentiated could involve alterations in GIRK channel composition or expression.

The overall goal of this study was to characterize the postsynaptic actions of a MOPr agonist in the RVM and to determine whether persistent inflammatory nociception enhances these postsynaptic actions. To this end, the response to thermal noxious stimuli was determined after administration of a GIRK channel inhibitor, tertiapin-Q, into the RVM of SAL and CFA treated rats. In addition, the anti-hyperalgesic and antinociceptive effects of a MOPr agonist, DAMGO, were determined after a prior treatment of the RVM with tertiapin-Q in both SAL and CFA treated rats. Finally, this study assessed which GIRK subunits are predominantly expressed in the RVM, and whether their expression is altered as a result of persistent inflammatory pain.

Experimental Procedures

Animals and the Experimental Model

Male Charles River Sprague-Dawley rats (Charles River, Raleigh, NC, USA) weighing 300-400g were housed on a 12 hr light/dark cycle with water and food ad libitum. All studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health. The experiments were approved by the University of Iowa Animal Care and Use Committee, and care was taken to minimize the number of animals used and their suffering.

Intraplantar (ipl) injection of complete Freund’s adjuvant (CFA) in one hindpaw was used to model acute, subchronic, and chronic inflammatory injury. The rats were lightly anesthetized with isoflurane, and the thickness of the hindpaw was measured with digital calipers. The plantar surface of the left hindpaw was then injected with 150 µL of CFA (150 µg of Mycobacterium butyricum, Calbiochem, La Jolla, CA, USA) or
sterile-filtered saline at pH 7.4. The rats were returned to their cages for 4 hours, 4 days or 2 weeks. On the designated day, the rats were returned to the laboratory and the thickness of the hindpaw was measured once again to verify the presence of inflammation.

**Behavior Studies**

*Experimental Design*

The first set of studies was designed to determine whether there was constitutive activation of GIRK channels in the RVM. The GIRK channel inhibitor, tertiapin-Q (3-100 ng) or saline was microinjected into the RVM and the paw withdrawal latency (PWL) to a noxious heat stimulus was determined 15, 30, 45 and 60 min later. The second set of studies determined whether activation of GIRK channels mediates the antinociceptive or the anti-hyperalgesic effects of DAMGO in the absence or presence of inflammatory injury. Saline or tertiapin-Q was microinjected in the RVM 15 min before microinjection of 6-30 ng DAMGO at the same site. The PWL was then determined 15, 30, 45 and 60 min later. Each rat was used once. The investigator was blinded to the test drug.

*Implantation of Guide Cannulae*

Rats were anesthetized with a mixture of ketamine hydrochloride (70 mg/kg i.p.) and xylazine (13 mg/kg i.p.), and a guide cannula (26 gauge; Plastics One Inc., Roanoke, VA) was stereotaxically implanted such that it terminated 3 mm dorsal to the RVM. The cannulae were affixed to the skull with sterile, stainless steel screws and dental acrylic. A stainless steel stylet was inserted into the guide cannula to maintain patency. After surgery, the animals were housed separately on corncob bedding. The cannulae were implanted 7 days before behavior testing.
Heat Nociceptive Testing

Heat nociceptive testing was assessed by focusing a high intensity beam of light on the plantar surface of the hindpaw. The time required for the rat to withdraw its hindpaw from the thermal stimulus was termed the PWL. The lamp was adjusted to elicit a baseline PWL of 8-12 s. A maximum of 20 s were allowed to prevent tissue injury, and a rat meeting this was assigned this latency. On the day of testing, the rats were acclimated to the testing environment for 30 min and then placed in individual Plexiglas chambers on a glass surface maintained at 25°C. After an additional 30 min period of acclimation to the chamber, the PWL was determined.

Microinjection Procedure

The baseline PWL was determined four days before behavior testing, immediately before ipl injection of CFA or saline in the left hindpaw. On the designated day of testing, the baseline PWL was re-determined. Rats that did not exhibit hyperalgesia after injection of CFA were excluded from the study. Drugs were injected via a 33 gauge stainless steel injector needle that extended 3 mm beyond the guide cannula tip and targeted the RVM. All agents were microinjected in a volume of 0.25 µL. The drug delivery was monitored by following the movement of an air bubble in the tubing that connected the injector to the syringe pump. The needle was left in place for 45 s after the injection to minimize diffusion of the drug up the injection tract. DAMGO (Sigma, St. Louis, MO) was dissolved in sterile-filtered saline at pH 7.0. Tertiapin-Q (Tocris, Ellisville, MO) was adjusted to pH 7.0 in saline on day of use. Saline served as vehicle control for both drugs. Saline was sterile-filtered, and the pH adjusted to 7.0 on the day of use.

Histology

After testing, the rats were euthanized by CO₂ inhalation. Their brains were removed, fixed in 10% formalin containing 30% sucrose, and transverse sections were
cut using a cryostat microtome. These were then stained with Cresyl Violet. The injection sites were identified, and then verified by a person blinded to the behavioral outcome. The sites were then plotted on an atlas of the rat brain. Figure 2.1 illustrates the distribution of the microinjection sites into the RVM across all of these studies. Few microinjection sites fell outside of the RVM. Sites outside of the RVM were primarily midline but ventral to the RVM, in the pyramids. However, since very few microinjection sites fell outside of the RVM within treatment groups, conclusions cannot be drawn from these.

Statistical Analysis

Data were expressed as the mean and standard error of the mean (S.E.M.). A two-way repeated measures analysis of variance was used to compare the PWL among the different groups. One factor was treatment in the RVM (Tertiapin or Saline), the other factor was treatment in the hindpaw (SAL or CFA), and the repeated factor was time. Post-hoc comparisons among mean values for the individual treatment groups were made using the Student Newman test. A $P \leq 0.05$ was considered significant.

GIRK mRNA Expression

The second set of studies quantitated the levels of mRNA of the four GIRK channel subunits (GIRK1-4) in the RVM in the presence and absence of acute, subchronic and chronic inflammatory injury.

qPCR Procedures

Rats received an ipl injection of CFA or SAL as described above and returned to their cages for 4 hours, 4 days or 2 weeks. On the designated test day, the rats were euthanized and a 1.5-mm tissue punch was obtained from the RVM. Punches from two rats were pooled as a single sample. The tissue was placed in RNALater™ and stored at 4°C overnight. For longer periods, it was stored at -20°C. Total RNA was isolated from
the RVM using the Illustra RNAspin Mini RNA Isolation kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and was DNase treated in column. The RNA concentration was determined using a Nanodrop™ 2000 (Thermo scientific, Wilmington, DE, USA) and the purity was assessed by gel electrophoresis. Reverse transcription was performed using the Taqman® RT-PCR mix (Applied Biosystems, Foster City, CA) with 0.5 μg RNA, 1x RT buffer, 1.1 μL 50 mM MgCl₂, 0.2 μL of 100 mM dNTP, 0.5 μL random hexamers, 0.2 μL RNase inhibitors, 0.25 μL multiscrIBE reverse transcriptase (Applied Biosystems) in 10 μL volume at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Q-PCR was performed using 50 ng cDNA in 4 μL, primers, and 7.5 μL GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) in 15 μL. The cycle conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and annealing/extension at the appropriate temperature for each primer set for 1 min and at 72°C for 1 min. The annealing/extension temperature for GIRK2 was 52°C, for GIRK1/3/4, 57°C, and for β-actin, 60°C. Reactions were performed in triplicate and analyzed using a Bio-Rad thermocycler (Hercules, CA, USA). At the end of the amplification, a thermal melt curve was generated. The primers for GIRK1 were (Forward: 5’ATGTCGGCAACTACACTCCC-TGTG’3; Reverse: 5’CCTGCTCTTTCACACTGTA-CGGAG’3) (Gregerson et al., 2001), GIRK2 (Forward:5’CGTGAGTGAATTAC-TGAATCT’3; Reverse: 5’GTCATTTCTTTGTGCTTTT’3), GIRK3 (Forward: 5’CAGAGGGGACCTAGGGTATTG’3; Reverse: 5’GAGACGGGATGT-CCAGGGTTC’3), GIRK4 (Forward: 5’AAGTTAGCCCCAAGGGTTCA’3; Reverse: 5’CGTTACACTTGCGTCTTC’3), β-actin (Forward: 5’CATCCTCTCTCTCCCTGGA-GAAGA’3; Reverse: 5’ACAGGATTCCCATACCCAGGAAGGAAG’3). GIRK1-4 primers were used at 150 nM per reaction. β-actin was used at 250 nM per reaction. All primers were cloned
using StrataClone™ PCR cloning kit (Stratagene, La Jolla, CA, USA) and verified by DNA sequencing. Standard curves were generated for pSC-AGIRK1-4. Standard curve for β-actin was obtained from Blanca Marquez de Prado (University of Iowa, Iowa City, IA, USA). Standards were serial diluted in 10 ng/mL yeast tRNA and C<sub>T</sub> values were converted to absolute numbers using these standard curves.

**Statistical Analysis**

The number of GIRK transcripts were normalized to the number of β-actin transcripts and expressed as the mean and S.E.M. of this ratio. A two-way ANOVA was used to analyze the qRT-PCR data. One factor was treatment in the hindpaw (SAL or CFA) and the other factor was the GIRK channel subunit (GIRK1 v GIRK2 v GIRK3). Post-hoc comparisons among mean values for the individual treatment groups were made using the Student Newman test. A \( P \leq 0.05 \) was considered significant.

**Results**

**In the Absence of Injury: The Role of GIRK Channels in the RVM in the Modulation of Nociceptive Threshold, as well as DAMGO-induced Antinociception**

**GIRK Channels Mediate Heat Nociception**

This first set of experiments determined whether there are constitutively active GIRK channels in the RVM that mediate heat nociception in the absence of injury. To assess this, a selective GIRK channel inhibitor, tertiapin-Q, was microinjected into the RVM of uninjured rats. Figure 3.1 illustrates the effects of microinjection of tertiapin-Q or its vehicle control on PWL. Microinjection of 3 ng tertiapin-Q in the RVM did not produce effects different from the saline control. Microinjection of 30, 100, or 300 ng tertiapin-Q increased PWL to an equivalent extent at 15 min. The effect dissipated by 30 min. The ability of tertiapin-Q to increase response latency to a noxious heat stimulus
suggests that there are GIRK channels in the RVM that are constitutively active, and that they regulate nociceptive threshold.

**GIRK Channels Mediate DAMGO-induced Antinociception**

This second set of experiments examined whether GIRK channels mediate DAMGO-induced antinociception in the RVM in the absence of injury. Given that modest increases in PWL occurred 15 min after microinjection of tertiapin-Q, the GIRK channel inhibitor was microinjected 15 min before DAMGO so that its effects on PWL had dissipated by the time measurements of the effects of DAMGO were made. Figure 3.2 illustrates that prior microinjection of saline in the RVM did not alter the well-known antinociceptive effects of DAMGO in the RVM (Hurley & Hammond, 2000).

Microinjection of 100 ng tertiapin-Q attenuated the antinociception produced by microinjection of 30 ng DAMGO at the same site. Lower doses of tertiapin-Q did not significantly attenuate DAMGO-induced antinociception at any time point. Interestingly, microinjection of 300 ng tertiapin-Q also did not attenuate DAMGO-induced antinociception, suggesting a U-shaped dose-response curve. The ability of tertiapin-Q to attenuate DAMGO-induced antinociception suggests that in the absence of injury, the antinociceptive effects of DAMGO in the RVM are mediated by GIRK channels.

**In Persistent Inflammatory Injury: The Role of GIRK Channels in the RVM in CFA-induced Heat Hyperalgesia, as well as DAMGO-induced Anti-hyperalgesia**

**GIRK Channels Do Not Mediate Hyperalgesia**

This set of experiments determined whether there are constitutively active GIRK channels in the RVM that mediate the hyperalgesia that develops in the ipsilateral hindpaw after inflammatory injury. Figure 3.3 illustrates that, as expected, ipl injection
of CFA induced heat hyperalgesia in the ipsilateral hindpaws. Microinjection of saline in the RVM of these rats produced a slight increase in PWL and modestly alleviated heat hyperalgesia by itself. Microinjection of 3 or 100 ng tertiapin-Q had effects similar to that of saline. These data suggest that constitutively active GIRKs in the RVM play little or no role in the maintenance of heat hyperalgesia after inflammatory injury.

GIRK Channels Do Not Mediate DAMGO-induced Anti-hyperalgesia

This set of experiments examined whether GIRK channels mediate the anti-hyperalgesic effects of DAMGO in the RVM under conditions of persistent inflammatory injury. Microinjection of saline in the RVM 15 min before DAMGO did not interfere with its well-established anti-hyperalgesic (Figure 3.4A, lower bracket) and antinociceptive (Figure 3.4A, upper bracket) effects. Interestingly, microinjection of 3-100 ng tertiapin-Q did not attenuate the anti-hyperalgesic effect of DAMGO. The experiment was repeated using 12 ng DAMGO, a lower dose that produced only anti-hyperalgesia and not antinociception (Figure 3.4B, bracket). Microinjection of 100 ng tertiapin-Q was still unable to attenuate the anti-hyperalgesic effects of this lower dose of DAMGO. These data indicate that GIRK channels do not mediate the anti-hyperalgesic effects of DAMGO under conditions of persistent inflammatory injury.

In Persistent Inflammatory Injury: The Role of GIRK Channels in the RVM in the Modulation of Nociceptive Threshold, as well as DAMGO-induced Antinociception

GIRK Channels Mediate Heat Nociception

This set of experiments determined whether there are constitutively active GIRK channels in the RVM that mediate heat nociception under persistent inflammatory pain. As expected, figure 3.5 illustrates that at baseline, heat nociception of the contralateral
Microinjection of 3 ng tertiapin-Q did not produce effects different from the saline control. However, microinjection of 100 ng tertiapin-Q increased PWL at 15 min. This effect dissipated by 30 min. The ability of tertiapin-Q to increase response latency to noxious heat stimuli in conditions of persistent inflammatory pain suggests that in this pain state, GIRK channels in the RVM remain constitutively active and continue to mediate heat nociceptive threshold.

**GIRK Channels Mediate DAMGO-induced Antinociception**

*in the Ipsilateral, Inflamed Hindpaw but Not in the Contralateral, Uninflamed Hindpaw*

This set of experiments examined whether GIRK channels mediate DAMGO-induced antinociception in the RVM under conditions of persistent inflammatory injury. Figure 3.4A illustrates the PWL data for the ipsilateral hindpaw of CFA treated rats. It illustrates that, as previously noted, a saline microinjection prior to a microinjection of DAMGO into the RVM does not interfere with its well-established antinociceptive response (Figure 3.4A, upper bracket). However, microinjection of 3 ng tertiapin-Q attenuated the antinociception induced by DAMGO. Microinjection of 30 ng and 100 ng tertiapin-Q completely abolished the antinociception induced by DAMGO at 15 min. Furthermore, although not significant, 100 ng tertiapin-Q trended a decrease in PWL at later time points as well. The ability of tertiapin-Q to attenuate the antinociception produced by DAMGO in a persistent inflammatory state, suggests that in this pain state the antinociceptive effects of DAMGO in the RVM are mediated by GIRK channels.

Figure 3.6A illustrates that in the contralateral hindpaw of these same CFA treated rats, prior microinjection of saline into the RVM 15 min before DAMGO produces a strong antinociceptive response. Surprisingly, 3-100 ng tertiapin-Q did not diminish the antinociceptive effects of DAMGO in the contralateral hindpaw. Furthermore, 100 ng tertiapin-Q also did not attenuate the mild antinociception produced
by the lower, 12 ng dose of DAMGO (Figure 3.6B). These data indicate that unlike in the ipsilateral hindpaw, in the contralateral hindpaw of 4 day CFA treated rats, GIRK channels are not required for the induction of antinociception by DAMGO. Furthermore, these data suggest that the ipsilateral and contralateral hindpaw can be differentiated mechanistically, at the level of the RVM.

Expression Levels of GIRK1-4 in the RVM are Unchanged in Rats with Persistent Inflammatory Pain

Given that GIRK channel inhibition differentially attenuates DAMGO-induced antinociception in CFA treated rats, we proposed that persistent inflammatory injury likely alters GIRK channels in the RVM. Regulation of GIRK channel expression is a mechanism by which GIRK channel activity can be modulated (Nassirpour et al., 2010). Therefore, this set of experiments used qRT-PCR to determine whether persistent inflammatory injury alters the levels of mRNA of the GIRK channel subunits, GIRK1-4, in the RVM.

Levels of β-actin in the RVM were not altered 4 hrs, 4 days, or 2 wks after CFA treatment (Figure 3.7A). Therefore, the GIRK transcript numbers were normalized to β-actin transcript numbers for each sample. Congruent with reports that GIRK4 expression levels are low or non-detectable in most CNS regions (Karschin et al., 1996), GIRK4 mRNA was not detected in the RVM in either the uninjured or injured conditions (data not shown). Surprisingly, the levels of mRNA expression of the subunits GIRK1-3 in the RVM were not altered 4 hrs, 4 days, or 2 wks after CFA treatment (Figure 3.7B, C, D).

In addition, given that the mRNA levels of the subunits GIRK1-3 were not different in the 4 day and the 2 week SAL treated rats, they were grouped to provide an assessment of the relative amounts of each GIRK subunit in the RVM in an uninjured state (Figure 3.8A). Furthermore, since the GIRK subunit levels were not different
between SAL and CFA treated rats, these were grouped to provide an overall assessment of the relative amounts of each GIRK subunit in a larger sample size (Figure 3.8B). Interestingly, the subunits GIRK1-3 are expressed at different levels in the RVM. Overall, the rank order of expression is $\text{GIRK2} \geq \text{GIRK3} > \text{GIRK1}$ (Figure 3.8).

**Discussion**

This study represents the first direct test of the long-held hypothesis that MOPr agonists produce antinociception in the RVM by postsynaptically activating GIRK channels. As expected, and in support of the hypothesis, this study determined that microinjection of a GIRK channel inhibitor blunted the antinociceptive effect of DAMGO in the RVM of uninjured rats. However, contrary to expectations, pretreatment with a GIRK channel inhibitor did not blunt the anti-hyperalgesic effects of DAMGO, nor did it antagonize the antinociceptive effects of DAMGO in the contralateral, uninflamed hindpaw in rats after a persistent inflammatory injury. It did reduce the antinociceptive effect of DAMGO in the ipsilateral, inflamed hindpaw of these same rats. Overall, these observations suggest that after persistent inflammatory injury, the mechanisms by which MOPr agonists produce anti-hyperalgesia and antinociception in the RVM transition from GIRK-dependent, to largely GIRK-independent. Finally, this study provides the first evidence that the RVM contains constitutively active GIRK channels that play a role in the regulation of nociceptive threshold.

**Constitutive GIRK Channels in the RVM**

The principal role of GIRK channels is to mediate slow synaptic inhibition by agonists of $G_{i/o}$ coupled receptors, such as MOPr and GABA$_B$. The extent to which they additionally regulate resting membrane potential and excitability, and integrate synaptic inputs is a function of their location (soma vs dendrites), open probability and a variety of
cytosolic inhibitory and stimulatory factors (Luscher & Slesinger, 2010). Constitutively active GIRK channels of low open probability have been documented in hippocampal (Chen & Johnston, 2005) and cerebellar granule neurons (Han, Kang, & Kim, 2003). Therefore, it was of interest to determine whether constitutively active GIRK channels are also present in the RVM. This hypothesis was tested by microinjection of tertiapin-Q in the RVM. The functional ramification of inhibiting constitutively active GIRK channels is an enhanced excitability of the neuron on which they are present, due to a more depolarized resting membrane potential.

The observation that microinjection of 30, 100 or 300 ng tertiapin-Q in uninjured rats produced a modest antinociception is consistent with the expression of constitutively active GIRK channels by a population of pain inhibitory neurons in the RVM. Blockade of constitutively active GIRK channels on spinally-projecting pain facilitatory neurons or on GABAergic interneurons that in turn inhibit pain inhibitory neurons would lead to a facilitation of nociception and therefore hyperalgesia, rather than antinociception. The limited magnitude of the antinociceptive effect and the absence of a dose-related effect were not entirely unexpected. As is the case in the hippocampus and cerebellum, GIRK channels in the RVM most likely have a low open probability and minimal activity under quiescent conditions.

All neurons in the RVM must express GIRK channels. One expectation could be that widespread inhibition of GIRK channels would result in no net change in the balance of activity in pain inhibitory and pain facilitatory neurons. Yet, in uninjured rats, tertiapin-Q produced antinociception suggesting that (1) pain inhibitory neurons either express a higher density of GIRK channels or GIRK channels that are more sensitive to tertiapin-Q and (2) GIRK channels are not constitutively active in pain facilitatory neurons or are less sensitive to inhibition by tertiapin-Q. It is highly likely that subpopulations of neurons in the RVM differ with respect to the levels, subunit
composition, anatomical localization or open probability of their GIRK channels. Indeed, substantial heterogeneity of GIRK channel subunit composition and expression has been documented for different types of cerebellar neurons (Aguado et al., 2008). Also, the subunit composition of GIRK channels in dopaminergic neurons of the ventral tegmentum differs from that of dopaminergic neurons in the substantia nigra (Luscher & Slesinger, 2010). Surprisingly, there have been no systematic studies in recombinant systems of the sensitivity of the different types of GIRK channels to inhibition by tertiapin-Q or its analogs. However, tertiapin-Q does inhibit GIRK1/4 channels in immortalized atrial HL-1 cells with 100-fold greater potency than GIRK1/2 channels in pituitary AtT20 cells (Walsh, 2011), providing a precedent for differential potency of GIRK channels composed by different subunits and or in different cell types.

The heat hyperalgesia that develops after ipl injection of CFA is thought to result from an imbalance of the pain facilitatory and the pain inhibitory pathways. Specifically, it is thought to result from a net increase in the activity of the pain facilitatory pathways. It was therefore of interest to determine whether the constitutive activity of GIRK channels was altered as a result of persistent inflammatory injury. Microinjection of tertiapin-Q did not alter the heat hyperalgesia in the ipsilateral, inflamed hindpaw compared to the effect of saline. It is unclear whether inhibition of GIRK channels by tertiapin-Q would produce a further decrease in PWL if spinally-projecting pain facilitatory neurons or GABAergic interneurons were already maximally active after CFA treatment. Any modest inhibition of GIRK channels in pain inhibitory neurons, which would increase their firing rate and alleviate the hyperalgesia, may very well have been masked by the overriding activity in pain facilitatory pathways. Interestingly, tertiapin-Q increased the PWL in the contralateral, uninflamed hindpaw of CFA treated rats to the same extent as in the naïve rats. This observation suggests that there is no
change in the constitutive activity of GIRK channels in the pain inhibitory neurons following four days of persistent inflammatory nociception.

**The Role of GIRK Channels in DAMGO-induced Antinociception in the Absence of Injury**

In uninjured rats, pretreatment with 100 ng tertiapin-Q attenuated the antinociceptive effects of DAMGO microinjected in the RVM. This finding is consistent with the hypothesis that MOPr agonists produce antinociception by postsynaptic inhibition of neurons in the RVM, possibly spinally-projecting pain facilitatory neurons or GABAergic interneurons that inhibit spinally-projecting pain inhibitory neurons. It implies that suppression of pain facilitatory neuronal activity and/or disinhibition of tonically-active GABAergic neurons contribute to the production of antinociception. It is unclear why the highest dose of tertiapin-Q (300 ng) also did not attenuate the effects of DAMGO. However, this would not be the first observation of a complex relationship between MOPr and GIRK channels. For example, genetic deletion of GIRK1 and GIRK2 channels or intrathecal injection of tertiapin-Q attenuate the antinociceptive effects of high, but not low doses of intrathecal morphine in mice (Marker, Stoffel, & Wickman, 2004).

**The Role of GIRK Channels in DAMGO-induced Anti-hyperalgesia and Antinociception in CFA Treated Rats**

The hypothesis that the anti-hyperalgesic effects of DAMGO result from a direct inhibition of spinally-projecting pain facilitatory neurons is based on the assumption that heat hyperalgesia results from increased activity in bulbospinal pain facilitatory neurons (Kincaid, Neubert, Xu, Kim, & Heinricher, 2006; Neubert, Kincaid, & Heinricher, 2004; Pertovaara, 2000). The inability of tertiapin-Q to block the anti-hyperalgesic effects of
either a low or a high dose of DAMGO was therefore unexpected. The most straightforward interpretation of the finding is that the anti-hyperalgesic effect of DAMGO does not involve GIRK-mediated postsynaptic inhibition of pain facilitatory neurons. However, the possibility that persistent inflammatory injury changes the subunit composition of GIRK channels in pain facilitatory neurons to one that is less sensitive to inhibition by tertiapin-Q cannot be excluded (Gonzalez-Rodriguez, Hidalgo, Baamonde, & Menendez, 2010; Gonzalez-Rodriguez, Llames, Hidalgo, Baamonde, & Menendez, 2011).

Pretreatment with tertiapin-Q did attenuate the antinociceptive effects of DAMGO on the ipsilateral hindpaw of CFA treated rats. Moreover, 30 ng of tertiapin-Q was sufficient to do so suggesting a greater sensitivity of GIRK channels to inhibition. However, no dose of tertiapin-Q attenuated the antinociceptive effects of either a high or a low dose of DAMGO on the contralateral, uninflamed hindpaw in contrast to findings in the uninjured rats. These data suggest that following persistent inflammatory injury, DAMGO produces antinociception by a largely GIRK-independent mechanism.

The Transition to GIRK-independent Mechanisms is Not Transcriptionally Regulated

The spinal cord is another pain modulatory region at which MOPr agonists produce antinociception by postsynaptically inhibiting neurons, via the activation of GIRK channels. GIRK channels in the dorsal horn of the spinal cord are comprised only of GIRK1 and GIRK2 subunits. However, GIRK3 subunits are present in several supraspinal pain modulatory sites such as the locus coeruleus and the periaqueductal gray. Therefore, the presence of GIRK1, GIRK2, and GIRK3 subunits in the RVM was not unexpected. The absence of GIRK4 in the RVM was also not unexpected, given that GIRK4 expression levels are low or non-detectable in most CNS regions (Karschin et al.,
In addition, GIRK4 is not thought to play a major role in nociception (Marker, Cintora, Roman, Stoffel, & Wickman, 2002). Levels of transcript for the GIRK1-3 subunits in the RVM did not differ in SAL or CFA treated rats. Moreover, the relative ratios of the GIRK channel subunits 1-3 in the RVM also did not differ between SAL and CFA treated rats. Thus, these data suggest that the transition to a GIRK-independent mechanism is not the result of a transcriptional downregulation. However, there are other factors that modulate GIRK channel activity and could therefore contribute to the differential role of GIRK channels after CFA treatment. For example, tyrosine phosphorylation of GIRK channels suppresses basal channel conductance and accelerates deactivation of GIRK channels (Ippolito, Xu, Bruchas, Wickman, & Chavkin, 2005). Increased tyrosine phosphorylation has been demonstrated in the spinal cord of mice in response to inflammatory injury (Ippolito et al., 2005). In addition, although the relative ratios of the total GIRK channel subunits in the RVM do not differ after CFA treatment, it is plausible that the composition of these channels differs, perhaps only in a subpopulation of RVM neurons. This could lead to varying sensitivity of GIRK channels to tertiapin-Q or to the MOPr receptor. For example, the MOPr activates GIRK channels via the Gβγ subunit, and the Gβγ subunit has a 5-fold greater sensitivity to GIRK1 containing heterotetramers than to GIRK channels without a GIRK1 subunit (Jelacic et al., 2000).

Final Comments

This study was based on the assumption that GIRK channels are nearly exclusively postsynaptic in location, a conclusion reinforced by numerous reports. For example, MOPr agonist, DAMGO, and GABA_B receptor agonists like baclofen, retain their ability to presynaptically inhibit transmitter release in GIRK knockout mice (Luscher, Jan, Stoffel, Malenka, & Nicoll, 1997). Also, tertiapin-Q does not prevent the
inhibition of glutamate release by baclofen (Moldavan, Irwin, & Allen, 2006). Subsequent studies supported the idea that GABA_B receptor and MOPr agonists inhibit transmitter release by decreasing the conductance of voltage-gated Ca^{++} channels in axon terminals. Therefore, this study used tertiapin-Q to pharmacologically isolate the postsynaptic versus the presynaptic components of the actions of DAMGO in the RVM. However, recent reports have demonstrated an anatomical existence of GIRK channels in presynaptic terminals. In addition, one study has provided functional evidence for a role of GIRK channels in the modulation of neurotransmitter release, principally by hyperpolarization of the axon terminal. Using synaptosomes prepared from the cerebral cortex, Ladera et al. (2008) demonstrated that baclofen inhibited glutamate release in a pertussis toxin insensitive, but tertiapin-Q sensitive manner. This mechanism was functional only in a subpopulation of terminals. In line with that finding, some 40% and 70% of GABA_B-immunoreactive axon terminals in the cerebral cortex were also immunoreactive for GIRK2 or GIRK3 subunits, respectively (Ladera et al., 2008). In addition, the existence of GIRK2 and GIRK3 channels in mossy fiber terminals of the cerebellum (Aguado et al., 2008) and of GIRK3 channels in axon terminals of the hippocampus (Fernandez-Alacid, Watanabe, Molnar, Wickman, & Lujan, 2011) has also been reported. Whether tertiapin-Q sensitive presynaptic GIRK channels are present in the RVM and if so, whether they contribute significantly to the antinociceptive or the anti-hyperalgesic actions of DAMGO is unclear. However, it would be of interest to determine whether they are restricted to GABAergic interneurons that synapse on spinally-projecting pain inhibitory neurons. These new findings suggest that caution should be used in interpreting the effects of tertiapin-Q solely in the construct of the roles of postsynaptic MOPr and GIRK channels. They also highlighted the importance of a direct examination of the presynaptic actions of MOPr agonists.
Figure 3.1: Time course effects after microinjection of 3-300 ng tertiapin-Q in the RVM of uninjured rats. B1 is the paw withdrawal latency (PWL) to a noxious heat stimulus at baseline. Data are expressed as the mean ± S.E.M. Numbers of rats are shown above in parentheses. $^+P<0.05$, $^{++}P<0.01$ compared to saline at the corresponding time point.
Figure 3.2: Effects of tertiapin-Q on DAMGO induced antinociception in the RVM of rats in the absence of injury. Saline or 3-300 ng tertiapin-Q was microinjected into the RVM 15 min before 30 ng DAMGO. B1 is the PWL to heat stimulus before any microinjection. PWL was then measured 15, 30, 45, and 60 min after the microinjection of DAMGO. Data are expressed as the mean ± S.E.M. Numbers of animals are shown in parentheses. $^+P<0.05$, $^{++}P<0.01$ compared to saline at the corresponding time point.
Figure 3.3: Effect of microinjection of 3 or 100 ng tertiapin-Q in the RVM on the hyperalgesia that develops after CFA treatment in the ipsilateral hindpaw. B1 and B2 are the PWL before and 4 days after ipl injection of CFA in this hindpaw. Symbols are the mean ± S.E.M. Numbers of animals are shown in parentheses. *P<0.05, **P<0.01 compared to saline at the corresponding time point.
Figure 3.4: Effects of microinjection of tertiapin-Q on DAMGO induced anti-hyperalgesia and antinociception in the RVM of rats with persistent inflammatory nociception. B1 and B2 are the PWL determined before and 4 days after ipl injection of CFA in this hindpaw. A) Saline or 3-100 ng tertiapin-Q were microinjected 15 min before 30 ng DAMGO into the same site in the RVM. The lower bracket denotes the anti-hyperalgesia; the upper bracket denotes the antinociception of DAMGO in the RVM. B) Saline or 100 ng tertiapin-Q was microinjected 15 min before 12 ng DAMGO into the same site in the RVM. The bracket denotes that this dose of DAMGO produces only anti-hyperalgesia and not antinociception in the ipsilateral hindpaw of CFA treated rats. Data are expressed as the mean ± S.E.M. Numbers of animals are indicated in parentheses. *P<0.05, **P<0.01 compared to saline at the corresponding time point.
Figure 3.5: Effect of microinjection of 3 or 100 ng tertiapin-Q in the RVM on the nociceptive threshold of the contralateral hindpaw of CFA treated rats. B1 and B2 are the PWL of the contralateral hindpaw before and 4 days after ipl injection of CFA in the other hindpaw. Data are expressed as the mean ± S.E.M. Numbers of animals are shown above in parentheses. †P<0.05, ‡P<0.01 compared to saline at the corresponding time point.
Figure 3.6: Effects of microinjection of tertiapin-Q on DAMGO induced antinociception in the RVM of rats with persistent inflammatory nociception. B1 and B2 are the PWL of the contralateral hindpaw determined before and 4 days after ipl injection of CFA in the other hindpaw. A) Saline or 3-100 ng tertiapin-Q was microinjected 15 min before 30 ng DAMGO into the same site in the RVM. B) Saline or 100 ng tertiapin-Q was microinjected 15 min before the lower dose of 12 ng DAMGO into the same site in the RVM. Data are expressed as the mean ± S.E.M. Numbers of animals are indicated in parentheses. †P<0.05, ‡P<0.01 compared to saline at the corresponding time point.
Figure 3.7: Levels of GIRK1-3 subunit mRNA in the RVM of saline and CFA treated rats. Rats received an ipl injection of saline or CFA 4 hrs, 4 days, or 2 weeks before obtaining the RVM tissue. Levels of mRNA were measured by qPCR. One RVM punch was obtained per rat, two RVM punches were pooled per sample and samples were run in triplicate. A) Levels of \( \beta \)-actin were not altered 4 hr, 4 days or 2 weeks after CFA treatment and were therefore used as a normalization control. The copies of GIRK subunits were determined by a standard curve B) 4 hours, C) 4 days and D) 2 weeks after CFA treatment. The number in each group is shown above in parenthesis and the mean ± S.E.M. are given. *\( P<0.05 \), **\( P<0.01 \) compared to the saline control group. *\( P<0.05 \), **\( P<0.01 \) compared to other GIRKs.
Figure 3.8: Relative expression levels of GIRK1-3 in the RVM. 

A) GIRK subunit mRNA levels relative to each other in the RVM of SAL treated rats. Given that GIRK subunit levels were not different in 4 day and 2 week SAL treated rats, they were grouped to provide an overall assessment of the relative amounts of each GIRK subunit in an uninjured state.

B) Given that GIRK subunit levels were not different in 4 day and 2 week SAL or CFA treated rats, they were grouped to provide an overall assessment of the relative amounts of each GIRK subunit in a larger sample size. The number in each group is shown above in parenthesis and the mean ± S.E.M. are given. *P<0.05, **P<0.01
CHAPTER IV

PERFUSION OF A MOP\textsubscript{r} AGONIST IN THE ROSTRAL VENTROMEDIAL MEDULLA (RVM) DECREASES GABA RELEASE IN UNINJURED RATS BUT NOT AFTER PERSISTENT INFLAMMATORY INJURY

Abstract

The RVM is an important site of opioid action. Persistent pain leads to adaptive changes in the RVM that increase the potency of opioid analgesics. Presynaptic MOP\textsubscript{r} are thought to produce antinociception by decreasing GABA release onto pain inhibitory neurons. The role of GLU in MOP\textsubscript{r}-induced antinociception is less well understood.

This study used microdialysis to determine whether baseline levels of GLU or GABA release are altered by CFA treatment. In addition, it determined whether microinjection of DAMGO into the RVM alters GLU or GABA release in SAL or CFA treated rats. Finally, it determined whether perfusing DAMGO into the RVM alters GLU or GABA release in SAL or CFA treated rats. Addition of a GLU transport reuptake inhibitor is necessary to detect GLU changes in response to high potassium depolarization and does not affect GABA release in the RVM. Basal levels of GABA release but not GLU release in the RVM were slightly increased after CFA treatment, suggesting a shift to greater pain facilitation. In addition, levels of GLU or GABA release were not decreased in response to DAMGO microinjected into the RVM in SAL or CFA treated rats. Moreover, levels of GLU release were not decreased in response to DAMGO perfused into the RVM of SAL or CFA treated rats. Interestingly, levels of GABA were decreased in response to DAMGO perfused into the RVM, as well as in response to high potassium after perfusion of DAMGO. However, they were not decreased in rats after CFA treatment.
Collectively, this study provides evidence that DAMGO acts in the RVM to decrease GABA release. In addition, it provides evidence that CFA treatment alters the presynaptic actions of MOPr agonists in the RVM.

Introduction

The RVM is a critical relay center for both pain inhibitory and pain facilitatory pathways (Millan, 2002). It is also an important site at which opioids act to produce antinociception. It was originally hypothesized that mu opioid receptor (MOPr) agonists produce antinociception by acting at presynaptic receptors situated on GABAergic neurons in the RVM. This hypothesis was based on the ability of MOPr agonists to inhibit voltage gated calcium channels (VGCCs), decreasing the release of GABA and subsequently disinhibiting the spinally-projecting pain inhibitory neurons on which they synapse. Disinhibition, or activation, of pain inhibitory neurons in the RVM produces antinociception (Hammond & Proudfit, 2007). This hypothesis is supported by substantial data.

First, microinjection of GABA$_A$ receptor antagonists into the RVM produces antinociception (Drower & Hammond, 1988; Fields, Heinricher, & Mason, 1991; Gilbert & Franklin, 2001; Heinricher & Kaplan, 1991). Second, MOPr co-localize with a population of RVM neurons containing GABA (Kalyuzhny & Wessendorf, 1998). Moreover, microinjection of GABA$_A$ receptor agonists into the RVM inhibit the continuous firing of pain inhibitory neurons and the antinociception produced by MOPr agonists (Fields et al., 1991; Gilbert & Franklin, 2002). Finally, MOPr agonists inhibit GABA mediated evoked and miniature inhibitory postsynaptic potentials (IPSPs) in spinally projecting RVM neurons (Fields et al., 1991; Finnegan, Li, Chen, & Pan, 2004; Pan, Williams, & Osborne, 1990).

Neurons in the RVM also receive glutamatergic inputs (Fields et al., 1991).
Administration of glutamate (GLU) or NMDA into the RVM produces antinociception (Guan, Terayama, Dubner, & Ren, 2002; Guan, Guo, Robbins, Dubner, & Ren, 2004; Miki et al., 2002). Microinjection of AMPA or NMDA receptor antagonists into the RVM attenuates the antinociceptive response produced by opioids (Javanmardi et al., 2005; Spinella, Cooper, & Bodnar, 1996). In addition, infusion of excitatory amino acid (EAA) antagonists into the RVM inhibits the activation and increased firing of pain inhibitory neurons (Heinricher, McGaraughty, & Farr, 1999; Heinricher, Schouten, & Jobst, 2001). These data suggest that MOPr agonists increase GLU transmission in the RVM. One postulate is that MOPr activation may produce analgesia by disinhibiting excitatory GLU input from neurons originating in the PAG onto pain inhibitory RVM neurons (Schepers, Mahoney, Zapata, Chefer, & Shippenberg, 2008). In this scenario, MOPr agonists would increase glutamatergic transmission onto pain inhibitory neurons allowing them to fire continuously. However, substantial electrophysiological data suggest that MOPr agonists primarily inhibit, not stimulate, glutamatergic transmission in many regions of the CNS, including the RVM (Chieng & Christie, 1994; Finnegan et al., 2004; Vaughan & Christie, 1997; Zhu & Pan, 2005). Clearly, additional studies are necessary to better understand the role of GLU in MOPr induced antinociception in the RVM.

Even less is known about the actions of MOPr agonists on GABA and GLU release in the RVM under conditions of persistent inflammatory injury. Injection of complete Freund’s adjuvant (CFA) in the hindpaw produces an inflammatory pain state that alters the activity and responsiveness of RVM neurons (Guan et al., 2002; Miki et al., 2002; Ren & Dubner, 2002; Zhang & Hammond, 2009). It also enhances the anti-hyperalgesic and the antinociceptive effects of MOPr agonists (Hurley & Hammond, 2000). However, the mechanisms by which persistent inflammatory injury enhances the actions of opioids in the RVM are not completely understood.
These experiments were undertaken to characterize the presynaptic actions of a MOPr agonist in the RVM, and to determine whether persistent inflammatory nociception enhances the presynaptic actions of a MOPr agonist. In vivo microdialysis was used to measure the release of both GLU and GABA in the RVM of freely moving rats. The first aspect of these studies examined whether basal release is altered as a consequence of persistent inflammatory nociception. The second aspect assessed the levels of GLU and GABA release in response to DAMGO microinjected into the RVM of SAL and CFA treated rats. Finally, this study assessed the levels of GLU and GABA release in response to DAMGO perfused into the RVM of SAL and CFA treated rats.

Experimental Procedures

Animals and the Experimental Model

Male Sprague-Dawley rats (Charles River, Raleigh, NC, USA) weighing 275-375 g were housed on a 12 hr light/dark cycle with water and food ad libitum. All studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health. The experiments were approved by the University of Iowa Animal Care and Use Committee. Every effort was made to minimize the number of animals used and their suffering.

Intraplantar (ipl) injection of complete Freund’s adjuvant (CFA) in one hindpaw was used to model persistent inflammatory injury. The rats were lightly anesthetized with isofluorane, and the thickness of the hindpaw was measured with digital calipers. The plantar surface of the left hindpaw was then injected with 150 µL of CFA (150 µg of Mycobacterium butyricum, Calbiochem, La Jolla, CA, USA) or sterile-filtered saline at pH 7.4. The rats were returned to their cages. After testing, the thickness of the hindpaw was measured again to verify the presence of inflammation.
Microdialysis Studies

The aims of these studies were three-fold: (1) to determine whether basal release of glutamate (GLU) or GABA levels in the RVM differs in uninjured rats and in those with a persistent inflammatory injury, (2) to determine whether DAMGO decreases the release of both GLU and GABA in the RVM, and (3) to determine whether this ability is enhanced in rats with persistent inflammatory injury. To this end, the rats were prepared with an intracerebral (i.c.) guide cannula for the introduction of a microdialysis probe in the RVM.

Surgical Preparation

Rats were anesthetized with a mixture of ketamine hydrochloride (70 mg/kg i.p.) and xylazine (13 mg/kg i.p.), and an omega ring i.c. guide cannula (BASi, West Lafayette, IN) was stereotaxically implanted such that it terminated 2 mm dorsal to the RVM. The cannulae were affixed to the skull with sterile, stainless steel screws and dental acrylic. A stylet was inserted into the guide cannula to maintain patency. After surgery, the animals were housed separately on corncob bedding. The cannulae were implanted 6-8 days before undergoing dialysis.

Acclimation to Raturn™ and Experimental Design

Rats were acclimated to the Raturn™ dialysis system (BASi) before placement of the i.c. guide cannula, and again after the surgery before undergoing dialysis. At all times, they had free access to food and water. In addition, they were always acclimated to the room for 30 min before being placed in the Raturn™ system. The rats were acclimated to the Raturn™ system for 2 hrs and 4 hrs on two separate days before they were implanted with an i.c. guide cannula. After implantation of the cannula, they were allowed to recover for 1 day. Four days before dialysis, the rats received an ipl injection of either saline or CFA. They were also acclimated to the Raturn™ system for another 2 hr and 4 hr time period on separate days. On the day of dialysis, the lines and
combination dialysis probe (BASi) were flushed with 1:200 Proclin solution (BASi), then double distilled water followed by artificial cerebrospinal fluid (aCSF) at a 10 µL/min flow rate. The dialyzing rate was 1 µL/min. The rats were gently restrained and the probe was inserted into the guide cannula. The dialysis probe contained a 2 mm membrane (BASi, MD-2268) that extended past the guide cannula and targeted the RVM.

Preliminary experiments showed that efflux of GLU and GABA from the RVM stabilized after three hours of dialysis with aCSF. After three hours of dialysis, the baseline samples were collected every 15 min for 1 hr (B1-B4). The dialysate was then switched to aCSF containing 0.6 mM tPDC (Tocris, Minneapolis, MN), a glutamate reuptake transport inhibitor (Schepers et al., 2008). For the remainder of the experiment, all dialysates contained tPDC. Two additional samples were collected at 15 min intervals, which was sufficient to achieve a stable increase in GLU levels in response to the tPDC. The rats then received a microinjection of DAMGO or saline into the RVM and four 15 min samples were collected. In another set of experiments, DAMGO was added to the dialysate and perfused into the RVM. Finally, the dialysate was switched to aCSF containing 60 mM potassium and a final four 15 min samples were collected. Samples were collected on ice and stored at -80°C until analysis. Each rat was used once.

**Microinjection Procedure and Drugs**

Drugs were injected via a combination probe (BASi) that allows for injection of the drug while perfusing, without dissolving the drug in the perfusate. The injection probe extends 2 mm below the guide cannula tip and targets the RVM. All agents were microinjected in a volume of 0.25 µL. The drug delivery was monitored by following the movement of an air bubble in the tubing that connected from the syringe pump to the probe. The tubing was left in place for 30 s after the injection to minimize diffusion of the drug. DAMGO (Sigma, St. Louis, MO) was dissolved in saline. Saline served as vehicle control. Saline was sterile-filtered, and the pH adjusted to 7.0 on the day of use.
The aCSF used for microdialysis contained NaCl 145 mmol/L; KCl 2.8 mmol/L; CaCl₂ 1.2 mmol/L; MgCl₂ 1.2 mmol/L; ascorbic acid 0.25 mmol/L; D-glucose 5.4 mmol/L, adjusted to pH 7.4. The glutamate reuptake inhibitor, tPDC, was dissolved in aCSF to a concentration of 0.6 mM. The glutamic acid decarboxylase inhibitor, L-allylglycine (Sigma) was dissolved in aCSF to a concentration of 10.8 mM. DAMGO was dissolved in aCSF to a concentration of 500 µM. A high concentration of potassium (60 mM) was used as a positive control to evoke release. The “high potassium aCSF” contained NaCl 87.8 mmol/L; KCl 60.0 mmol/L; CaCl₂ 1.2 mmol/L; MgCl₂ 1.2 mmol/L; ascorbic acid 0.25 mmol/L; D-glucose 5.4 mmol/L, adjusted to pH 7.4. Both aCSF and high potassium aCSF were filtered with a 0.22 µm membrane before use.

Quantification of GLU and GABA

Samples were analyzed using high performance liquid chromatography (HPLC). The HPLC consists of an ASI-100 automatic sample injector with temperature control (Dionex, Bannockburn, IL), a P680 HPLC pump (Dionex), a fluorescence detector RF-10AXL (Shimadzu, Kyoto, Japan) and a UCI 50 Universal Chromatography Interphase (Dionex). The mobile phase for GLU consisted of 15 % (v/v) acetonitrile, 8.2 g/L sodium acetate, 85 % (v/v) HPLC grade water adjusted to pH 6. The samples for GLU were run at a 385 µL/min flow rate. The mobile phase for GABA consisted of 23 % (v/v) acetonitrile, 8.2 g/L sodium acetate, 77 % (v/v) HPLC water adjusted to pH 7.10. The samples for GABA were run at a 330 µL/min flow rate. Both mobile phases were filtered through a 0.22 µm membrane (Millipore, Billerica, MA). The column for both GABA and GLU was Acclaim™ 120, 3 µm, 2.1 x 150 mm (Dionex).

On the day of HPLC analysis, the samples were prepared in a 1.2 mL vial with a 250 µL pulled point glass vial insert with polymer feet (Agilent, Wilmington, DE), and topped with a silicone filter (SSP, Ballston Spa, NY). After the samples were thawed, 6 µL were placed in a vial insert with 6 µL HPLC water. These samples were then stored
in the refrigerated autosampler at 4°C. Pre-column derivatization was performed by the autosampler by adding 12 μL of a solution of 4 μL/mL β-mercaptoethanol in OPA (Sigma) right before injection. The injection volume was 21 μL. Samples were detected by fluorescence detector using 300-450 nm excitation and 400-500 nm emission filters. GLU and GABA were detected with different sensitivity and gain parameters. GLU was detected with Sensitivity 2, Gain 2 and GABA with Sensitivity 1, Gain 2. A lower sensitivity parameter produces a lower limit of detection, which facilitates detection of smaller amounts of the neurotransmitter. A standard curve was run with each sample set and used to analyze GLU and GABA peaks. The chromatograms were evaluated by Chromeleon software (Dionex). Under these conditions, the retention time for GLU was 3.5 min and for GABA was 6.5 min. The limits of detection were 0.01 μg/mL and 0.0001 μg/mL, respectively.

**Histology**

After dialysis, the rats were euthanized by CO₂ inhalation. Their brains were removed, fixed in 10% formalin containing 30% sucrose, and transverse sections were cut using a cryostat microtome. These were then stained with Cresyl Violet. The dialysis sites were identified, plotted on an atlas of the rat brain, and verified by a second individual blind to the treatment. Microinjection sites located in the nucleus reticularis gigantocellularis (NGC) pars α and those in the nucleus raphe magnus were grouped and collectively referred to as the RVM. Figure 2.1 illustrates the distribution of the microinjection sites into the RVM across all of these studies. Only a few microdialysis probe sites fell outside of the RVM across all treatment groups. My hit rate targeting the probe placement into the RVM is ~99%. Therefore, no analysis or conclusions can be drawn from dialysis outside of the RVM.
Statistical Analysis

Data were expressed as the mean and S.E.M of the % baseline change. The four baseline samples were averaged and set to zero. A two-way analysis of variance was used to compare % baseline changes among the different groups. The factors included the treatment in the RVM (DAMGO v Saline microinjection, DAMGO v aCSF perfusion) and the treatment group in the hindpaw (SAL or CFA). In addition, the area under the curve (AUC) was also analyzed. Post-hoc comparisons among mean values for the individual treatment groups were made using the Student Newman test. A $P \leq 0.05$ was considered significant.

Results

Verification of Analytes

The first set of experiments verified that the peaks in the chromatogram denoted as GLU and GABA after the HPLC analysis, indeed represented GLU and GABA. First, a standard curve was used to determine the retention time, the shape, and the linearity as well as the reproducibility of the peaks for GLU and GABA. GLU and GABA were analyzed separately under different conditions. Second, samples collected from in vivo microdialysis were analyzed to verify that the retention time and the peak resolution of GLU and GABA remained appropriate in the presence of other analytes in the cerebrospinal fluid (CSF). Finally, the levels of GLU release should increase in response to tPDC (Schepers et al., 2008), a glutamate reuptake inhibitor. The levels of GABA release should decrease in response to L-allylglycine (Herbison, Heavens, & Dyer, 1990), a glutamate decarboxylase inhibitor. In addition, the peaks for GLU and GABA should increase or decrease as expected for the respective treatment.

Therefore, the levels of GLU and GABA were determined in microdialysis samples collected from rats perfused with aCSF containing either tPDC or L-
allylglycine. Figure 4.1A illustrates the GLU peak in a sample collected from microdialysis at baseline and figure 4.1B illustrates the increased GLU peak in response to tPDC. This provided further verification of peak specificity, as well as determined that increases in GLU levels in the RVM could be detected. In addition, Figure 4.1C illustrates the GABA peak in a sample collected from microdialysis at baseline and figure 4.1D illustrates the decreased GABA peak in response to L-allylglycine. This provided further verification of the GABA peak specificity, as well as determined that decreases in GABA levels in the RVM could be detected.

**Inhibition of GLU Reuptake is Necessary to Detect Potassium-evoked Release**

As previously reported (Schepers et al., 2008), it was not possible to detect an increase in the release of GLU in response to 60 mM potassium in the absence of a glutamate uptake inhibitor (data not shown). Therefore, 0.6 mM tPDC was added to the aCSF after collecting the four baseline samples. Figure 4.2A illustrates that GLU efflux from the RVM is stable after three hours of dialysis with aCSF. Addition of 0.6 mM tPDC to the aCSF results in a significant increase in GLU release within 30 min that remains stable throughout a 4 hour period. Finally, GLU release increases further in response to high potassium aCSF in the presence of tPDC. Importantly, addition of tPDC does not alter the levels of GABA release in the RVM at the time points of interest (Figure 4.1B, area within the pink bars and inset). In addition, an increase in GABA release in response to 60 mM potassium could be detected without the addition of a GABA reuptake inhibitor (Figure 4.1B). On the basis of these findings, all further studies were conducted in the presence of tPDC. This experimental design afforded a significant strength in that GLU and GABA could be measured in the same sample.
Baseline GLU and GABA Release in the RVM of SAL and CFA Treated Rats

This set of experiments determined whether persistent inflammatory injury alters the baseline levels of GLU or GABA release in the RVM. To obtain an overall assessment, data across groups were compiled to increase the sample size. Figure 4.3A illustrates that levels of GLU release are stable during the four baseline collection samples from both treatment groups and that tPDC increases GLU release to a comparable extent in both SAL and CFA treatment groups. Levels of basal release of GLU do not differ between SAL and CFA treated rats (Figure 4.3A, bar graph). Similarly, levels of GABA release are also stable during the four baseline collection samples (Figure 4.3B). As noted earlier, the presence of tPDC in the aCSF does not alter GABA release from the RVM of either SAL or CFA treated rats (Figure 4.3B). Finally, the basal levels of GABA release from the RVM trend higher four days after CFA treatment, however, this increase does not reach statistical significance (Figure 4.3B, bar graph).

Microinjection of DAMGO into the RVM Produces Antinociception

It is well accepted that microinjection of 30 ng DAMGO into the RVM produces antinociception to heat noxious stimuli (Hurley & Hammond, 2000). However, previous studies have used more ‘standard’ microinjection techniques. In the microdialysis experiments, the drug would instead be administered via a combination perfusion/injection microdialysis probe. Therefore, PWL to thermal noxious stimuli was measured in response to 30 ng DAMGO microinjected via the microdialysis combination probe. Figure 4.4 illustrates that 30 ng DAMGO increased PWL 15 and 30 min after the
microinjection. This antinociceptive effect dissipated by 45 min. These results are consistent with results previously reported (Hurley & Hammond, 2000).

**GLU Release in the RVM in Response to Saline or DAMGO microinjection in SAL or CFA Treated Rats**

This set of experiments determined whether microinjection of DAMGO in the RVM altered GLU release in either SAL or CFA treated rats. Figure 4.5A illustrates that microinjection of 30 ng DAMGO into the RVM does not alter GLU release compared to the effects of saline in SAL treated rats. Although the levels of GLU release trended higher after microinjection of DAMGO, this increase did not achieve statistical significance (Figure 4.5A). In addition, microinjection of 30 ng DAMGO into the RVM does not alter GLU release 4 days after CFA treatment (Figure 4.5B). Finally, the response of GLU release to high potassium aCSF is not altered by DAMGO microinjection in either SAL (Figure 4.5A) or CFA (Figure 4.5B) treated rats.

**GABA Release in the RVM in Response to Saline or DAMGO microinjection in SAL or CFA Treated Rats**

This set of experiments determined whether microinjection of DAMGO in the RVM altered GABA release in the RVM of either SAL or CFA treated rats. Figure 4.6A illustrates that microinjection of 30 ng DAMGO into the RVM does not alter GABA release in the RVM in SAL treated rats. In addition, microinjection of 30 ng DAMGO into the RVM does not alter GABA release 4 days after CFA treatment (Figure 4.6B). Finally, the response of GABA release to high potassium aCSF is not altered by DAMGO microinjection in either SAL (Figure 4.6A) or CFA (Figure 4.6B) treated rats.
GLU Release in the RVM in Response to DAMGO

Perfusion in SAL or CFA Treated Rats

A previous study demonstrated that GLU release increased in the RVM in response to DAMGO (Schepers et al., 2008). However, in this study DAMGO was perfused rather than microinjected into the RVM. Therefore, this set of experiments determined whether perfusing DAMGO into the RVM alters the levels of GLU release in SAL or CFA treated rats. To assess this, DAMGO was dissolved to 500 µM DAMGO in aCSF containing 0.6 mM tPDC, and perfused into the RVM. DAMGO was perfused beginning 30 min after tPDC for a period of 1 hour, at which point the perfusate was switched to high potassium aCSF in the presence of tPDC. Only ~12% DAMGO crosses the membrane, therefore ~30 ng DAMGO reached the RVM during this perfusion (Schepers et al., 2008).

Figure 4.7A illustrates that perfusing DAMGO into the RVM does not alter the levels of GLU release in the RVM compared to perfusion of aCSF in SAL treated rats. In addition, perfusing DAMGO into the RVM does not alter levels of GLU release 4 days after CFA treatment (Figure 4.7B). Finally, the response of GLU release to high potassium aCSF is not altered by perfusing DAMGO into the RVM of either SAL (Figure 4.7A) or CFA (Figure 4.7B) treated rats.

GABA Release in the RVM in Response to DAMGO

Perfusion in SAL or CFA Treated Rats

This set of experiments determined whether perfusing DAMGO into the RVM alters levels of GABA release. Figure 4.8A,C illustrate that perfusing DAMGO into the RVM decreases the levels of GABA release in the RVM of SAL treated rats. In addition, the response of GABA release to high potassium aCSF is increased by perfusing DAMGO into the RVM of SAL treated rats (Figure 4.8E). In contrast, perfusing
DAMGO into the RVM does not alter levels of GABA release in the RVM 4 days after CFA treatment (Figure 4.8B, D). Finally, the response of GABA release to high potassium aCSF is also not altered by perfusing DAMGO into the RVM of CFA treated rats (Figure 4.8F).

Discussion

Both glutamate and GABA are recognized to play important roles in the modulation of nociception by the RVM, and to be involved in the mechanisms by which opioids act in the RVM to produce antinociception. However, our understanding of their roles remains incomplete and is particularly rudimentary under conditions of persistent inflammatory injury. The purpose of this study was two-fold. First, it determined whether basal or evoked release of glutamate or GABA was enhanced after persistent inflammatory injury. Second, it investigated whether the presynaptic actions of the MOPr agonist DAMGO on glutamate or GABA release were enhanced in the RVM of rats with persistent inflammatory nociception.

Basal Release: GABA

GABA is an important mediator in the RVM. It modulates descending pain pathways and therefore, behavioral pain states. Increasing GABA transmission in the RVM reduces the activity of pain inhibitory neurons and produces hyperalgesia. However, inhibiting GABA transmission increases the firing rate of pain inhibitory neurons and produces antinociception. Nociception is thought to be a function of the balance between pain facilitatory and pain inhibitory pathways; therefore it is the net activity under different conditions which determines the behavioral state. Inflammatory injury promotes plasticity in the RVM that alters both the descending inhibitory as well as facilitatory pathways. Indirect pharmacological evidence suggests that GABA release
in the RVM is increased in response to acute inflammation (Gilbert & Franklin, 2001). However, this had not been directly examined. This study is the first to examine release of GABA in the RVM in response to CFA treatment. Although there was a trend for the basal levels of GABA release in the RVM to be greater after CFA treatment, the difference from SAL treated rats was marginal. Increasing or inhibiting GABA transmission in the RVM does not seem to alter the activity of pain facilitatory neurons (Heinricher & Kaplan, 1991; Heinricher & Tortorici, 1994), therefore this increase in levels of GABA would impact primarily pain inhibitory neurons. This would result in a reduction in pain inhibitory activity and therefore a shift to net descending pain facilitation, as well as the development or the maintenance of hyperalgesia. This would be consistent with previous data demonstrating that treatment with CFA or mustard oil results in increased activity of pain facilitatory neurons and a large reduction in the activity of pain inhibitory neurons (Kincaid, Neubert, Xu, Kim, & Heinricher, 2006; Miki et al., 2002).

CFA treatment increases the levels of endogenous opioids [Met₅]enkephalin and [Leu₅]enkephalin in the RVM, presumably in an attempt to attenuate the hyperalgesia (Hurley & Hammond, 2001). These endogenous opioids would act by inhibiting GABA release to disinhibit pain inhibitory neurons. It is therefore possible that inhibiting the activity of the endogenous opioids, by perfusing an opioid antagonist into the RVM of CFA treated rats, might result in a further increase in GABA release. In addition, it would be of interest to examine the levels of basal GABA release in the RVM at other time points during the inflammation. CFA treatment results in time-dependent changes in the RVM. During the more acute inflammation phase, the activity of pain facilitatory neurons increases in order to promote hyperalgesia. It is therefore probable that during this phase, the basal levels of GABA release are further enhanced, to dampen the pain inhibitory neuron response while the hyperalgesia develops. As the inflammation is
prolonged, there is an increase in pain inhibitory pathways in an attempt to alleviate the hyperalgesia. It is therefore likely that during more prolonged inflammation, such as 2 weeks after CFA treatment, the levels of GABA release would be decreased, as the pain inhibitory pathways become more prominent.

**Basal Release: Glutamate**

In agreement with the report of (Schepers et al., 2008), potassium-evoked increases in GLU release could not be detected without the use of a GLU reuptake transport inhibitor, tPDC. This finding suggests that GLU metabolism is extremely tightly regulated in the RVM. Whether levels are regulated by astrocytic or neuronal uptake mechanisms remains to be established.

The increased plasticity in the RVM after inflammatory injury is mediated by enhanced EAA neurotransmission involving both NMDA and AMPA receptors (Guan et al., 2002; Miki et al., 2002; Zhang & Hammond, 2009). In addition, persistent inflammation results in an upregulation of the mRNA and protein expression of NMDA receptor subunits in the RVM starting at 5 hrs and remaining from 1-7 days (Guan et al., 2002; Miki et al., 2002). Finally, CFA treatment enhances AMPA mediated excitatory synaptic input to spinally-projecting RVM neurons (Zhang & Hammond, 2009). The enhancement is mediated by substance P, which functions to postsynaptically enhance the actions of GLU at AMPA and possibly NMDA receptors. Indeed, paired pulse experiments established that there was no enhanced release of GLU in CFA treated rats. Thus, the present finding that the basal levels of GLU release are not increased in CFA treated rats is concordant with the conclusions of that previous report.
**Presynaptic Mechanisms of MOPr Action in the RVM**

MOPr agonists are thought to produce antinociception by acting at presynaptic MOPr to inhibit GABA release onto pain inhibitory neurons. Microinjection of MOPr agonist, DAMGO, into the RVM using a combination perfusion/injection microdialysis probe produced antinociception similar to previous reports (Hurley & Hammond, 2000). Yet, it did not reduce the levels of basal or potassium-evoked GABA release in the RVM in SAL treated or CFA treated rats. These results are in conflict with a previous study that demonstrated decreased levels of GABA release in response to DAMGO in the RVM (Schepers et al., 2008). However, in that study, DAMGO was perfused continuously for 1 hr rather than microinjected. Therefore, it was of interest to measure the levels of GABA release after perfusion of DAMGO for 1 hr into the RVM of SAL and CFA treated rats. Interestingly, similar to the previously published study, the levels of GABA release were decreased in response to DAMGO perfusion. These results provide further direct support that MOPr agonists produce their effects by decreasing GABA release in the RVM, and they are consistent with several studies that have demonstrated the ability of MOPr agonists to inhibit evoked inhibitory postsynaptic currents mediated by GABA in the RVM (Fields et al., 1991; Finnegan et al., 2004; Pan et al., 1990). In addition, potassium-evoked release of GABA was increased, which most likely reflects the inhibition of basal release and aggregation of synaptic vesicles at the axon terminal during the perfusion of DAMGO. Clearly, perfusing DAMGO through the RVM at a steady state for 1 hour produces different results than a single bolus of DAMGO microinjected into the RVM. Although the amount of drug delivered to the RVM is similar, perfusing for 1 hr would result in continuous activation of MOPr, and therefore continually inhibit GABA release. This approach probably improves the ability of microdialysis to detect these changes.
Perfusion of DAMGO into the RVM of CFA treated rats did not produce a greater decrease in the levels of GABA release in the RVM. This finding was not entirely unexpected. A prior report demonstrated that the antinociceptive effects of i.t. morphine were potentiated after acute inflammation. In that study, the effects of the Gi/o protein activator mastoparan, were also potentiated, but the effects of the N-type calcium channel blocker omega-conotoxin GVIA, were not, suggesting that the presynaptic mechanisms did not contribute towards this enhancement (Gonzalez-Rodriguez, Hidalgo, Baamonde, & Menendez, 2010) in the spinal cord. The finding that DAMGO actually lost its ability to suppress GABA release was unexpected. It is possible that after CFA treatment, DAMGO does not decrease levels of GABA release because the MOPr are desensitized, or internalized. Levels of endogenous opioids are increased in CFA treated rats and therefore MOPr are potentially, continually stimulated to the point of desensitization, or internalization (Hurley & Hammond, 2001). If this is the case, the administration of DAMGO into the RVM would not activate MOPr and therefore not result in a decrease in GABA release. As the antinociceptive and anti-hyperalgesic effects of DAMGO are enhanced in CFA treated rats, these data are interpreted to indicate that suppression of GABA release does not play a role in the enhancement of these effects.

The role of GLU in MOPr-mediated antinociception in the RVM is less well understood. Indirect pharmacological evidence suggests that MOPr increases GLU transmission in the RVM. In support of this, a previous microdialysis study determined that the levels of GLU release were increased in response to DAMGO perfusion into the RVM (Schepers et al., 2008). They postulated that MOPr activation may produce analgesia by disinhibiting excitatory glutamate input from neurons originating in the PAG onto pain inhibitory RVM neurons (Schepers et al., 2008). However, these results are unexpected since substantial electrophysiological data suggest that MOPr agonists primarily inhibit, not stimulate, glutamatergic transmission in many regions of the CNS,
including the RVM (Chieng & Christie, 1994; Finnegan et al., 2004; Vaughan & Christie, 1997; Zhu & Pan, 2005). In an effort to corroborate these data, levels of GLU release in the RVM were measured in response to DAMGO in the RVM. Levels of GLU release in the RVM were not altered in response to either a microinjection or perfusion of DAMGO into the RVM. In addition, although CFA treatment increases GLU-mediated synaptic transmission and enhances the anti-hyperalgesic and antinociceptive effects of MOPr agonists, CFA treatment did not alter GLU levels in the RVM in response to either a microinjection or perfusion of DAMGO in the RVM. Therefore, these studies do not support the previously proposed postulate.

Conclusions

Collectively, these data provide further direct evidence for the long-standing postulate that MOPr agonists produce antinociception in the RVM by presynaptic inhibition of GABA release. However, these data also suggest that the enhanced effects of DAMGO in the RVM under conditions of persistent inflammatory nociception are not mediated by the presynaptic mechanisms of MOPr. A technical limitation of microdialysis that must be acknowledged in reaching this conclusion is that it is based on the absence of an effect. Microdialysis measures neurotransmitter efflux, or overflow, from the synapse, and as a result lacks neuronal resolution and is not a direct measure of synaptic activity. Thus, confirmation of this conclusion will benefit from whole-cell patch-clamp recordings from identified neurons that afford both neuronal resolution and “real-time” analysis of synaptic transmission.
Figure 4.1: Chromatograms demonstrating the glutamate (Left Column) and the GABA (Right Column) peaks in samples collected from in vivo microdialysis. Top row demonstrates A) glutamate and C) GABA peaks at baseline. Bottom row demonstrates B) an increased glutamate peak in response to tPDC and D) a decreased GABA peak in response to L-allylglycine. The glutamate chromatograms A, B) represent the same rat and are scaled equally. The GABA chromatograms C, D) represent the same rat and are scaled equally. The scale between GLU and GABA is different since they are analyzed using different detection parameters.
Figure 4.2: Effects of tPDC on the levels of A) glutamate and B) GABA release in the RVM at baseline, in response to tPDC, and to high potassium aCSF. aCSF was perfused for three hours before collecting the baseline levels (area to the left of the first pink vertical line). The perfusate was then switched to aCSF+tPDC (the first pink vertical line aligns with the switch of the perfusate). After four hours, the perfusate was switched to high potassium aCSF+tPDC for 1 hr (the last pink vertical line aligns with the switch of the perfusate). Samples were collected every 15 min. A) The area between the second and third vertical line is the tPDC data. It doesn’t include the data between the first and second vertical line because this is a transition point for glutamate. B) The area between the first and second vertical line is the tPDC data. Data area expressed as % change glutamate or GABA baseline levels and the mean ± S.E.M. are shown. Numbers of rats is shown above in parentheses. ***P<0.001
Figure 4.3: Levels of A) glutamate and B) GABA release at baseline and in response to tPDC in SAL and CFA treated rats. aCSF was perfused for three hours before collecting the baseline levels. Samples were collected every 15 min for 1 hr before switching the perfusate to tPDC+aCSF. The pink vertical line aligns with the switch of the perfusate. Samples across groups were combined to provide an overall assessment of baseline levels of glutamate and GABA in the RVM. Data are expressed as the mean ± S.E.M. Numbers of animals are shown above in parentheses. ***P<0.001 compared to baseline levels.
Figure 4.4: Microinjection of 30 ng DAMGO in the RVM produces antinociception.

DAMGO was microinjected using the combination perfusion/injection microdialysis probes that will be used during the microdialysis experiments. BSL is the PWL to heat stimulus before any microinjection. PWL was then measured 15, 30, 45, and 60 min after the microinjection of DAMGO. Data are expressed as the mean ± S.E.M. Numbers of animals are shown in parenthesis. ***P<0.001 compared to the BSL PWL.
Figure 4.5: Microinjection of 30 ng DAMGO into the RVM of A) 4 d SAL or B) 4 d CFA treated rats does not alter the levels of glutamate release in the RVM. aCSF was perfused for three hours before collecting the baseline levels. Samples were collected every 15 min. The perfusate was then switched to tPDC+aCSF and two samples were collected before injecting either saline or DAMGO into the RVM. Samples were collected for 1 hr before the perfusate was switched to high potassium aCSF+tPDC. The pink vertical lines align with each transition in the experiment. Data are expressed as % baseline change and are shown as mean ± S.E.M. Numbers of animals are shown above in parentheses.
Figure 4.6: Microinjection of 30 ng DAMGO into the RVM of A) 4 d SAL or B) 4 d CFA treated rats does not alter the levels of GABA release in the RVM. aCSF was perfused for three hours before collecting the baseline levels. Samples were collected every 15 min. The perfusate was then switched to tPDC+aCSF and two samples were collected before injecting either saline or DAMGO into the RVM. Samples were collected for 1 hr before the perfusate was switched to high potassium aCSF+tPDC. The pink vertical lines align with each transition in the experiment. Data are expressed as % baseline change and are shown as mean ± S.E.M. Numbers of animals are shown above in parentheses.
Figure 4.7: *Perfusion* of DAMGO into the RVM of **A**) 4 d SAL or **B**) 4 d CFA treated rats does not alter the levels of glutamate release in the RVM. aCSF was perfused for three hours before collecting the baseline levels. Samples were collected every 15 min. The perfusate was then switched to tPDC+aCSF and two samples were collected. After this, the perfusate of the DAMGO treated group was switched to DAMGO+tPDC+aCSF. Samples were collected for 1 hr before the perfusate was switched to high potassium aCSF+tPDC. The pink vertical lines align with each transition in the experiment. Data are expressed as % baseline change and are shown as mean ± S.E.M. Numbers of animals are shown above in parentheses.
Figure 4.8: *Perfusion of DAMGO into the RVM decreases the levels of GABA release in A) 4 d SAL but not in B) 4 d CFA treated rats. aCSF was perfused for three hours before collecting the baseline levels. Samples were collected every 15 min. Perfusate was then switched to tPDC+aCSF and two samples were collected. After this, the perfusate of the DAMGO treated group was switched to DAMGO+tPDC+aCSF. Samples were collected for 1 hr before the perfusate was switched to high potassium aCSF+tPDC. Data are expressed as A, B) % baseline change, C, D) Area under the curve of the treatment, the region within the pink bars, and E, F) Area under the curve of the last hour, the high potassium aCSF. Data are shown as mean ± S.E.M. Numbers of animals are shown above in parentheses. **P<0.01*
CHAPTER 5
SUMMARY, CONCLUSIONS, FUTURE DIRECTIONS

Introduction

Chronic pain is a significant public health problem (Institute of Medicine, 2011). It is disabling and diminishes the quality of life. Despite adverse effects, opioids remain a primary pharmacologic therapy for the clinical management of acute and chronic pain (Beaulieu, Lussier, Porreca, & Dickenson, 2010; Marvizon, Ma, Charles, Walwyn, & Evans, 2010). However, the mechanisms by which opioids produce their analgesic effects are still not completely understood.

The RVM is a critical, central relay center of pain modulation through which more rostral pain modulatory nuclei exert their actions (Millan, 2002). It is also an important site at which opioids act to produce their analgesic effects (Ackley, Hurley, Virnich, & Hammond, 2001; Tershner, Mitchell, & Fields, 2000; Thorat & Hammond, 1997). The most common clinically used opioids, such as morphine, fentanyl, and hydromorphone act at the MOPr to produce their analgesic effects (Marvizon et al., 2010). MOPr are G protein coupled receptors known to mediate their effects primarily via G_{i/o} proteins, and PTX, a G_{i/o} protein inactivator, attenuates the antinociceptive effects of MOPr agonists in many pain modulatory regions. However, the role of G_{i/o} proteins has not been confirmed in the RVM. Additionally, MOPr are present both postsynaptically and presynaptically in the RVM. Postsynaptic MOPr are thought to act by opening GIRK channels to hyperpolarize and inhibit pain facilitatory neurons (Krapivinsky, Krapivinsky, Wickman, & Clapham, 1995; Mark & Herlitze, 2000; North & Uchimura, 1989). However, prior to this work, the data supporting this postulate was largely electrophysiology data, which of course, has limitations to clinical application. Presynaptic MOPr are thought to act by inhibiting VGCC to decrease neurotransmitter release onto pain inhibitory neurons (Hammond & Proudfit, 2007). Both the postsynaptic
and the presynaptic mechanisms are thought to occur simultaneously within the RVM to produce antinociception. However, the contribution of each of these mechanisms to the analgesic effects mediated by MOPr agonists in the RVM has never been examined. Further understanding of the contribution of these mechanisms could advance the identification and development of new pharmacologic therapies for more effective clinical management of chronic pain. These new therapies could perhaps bypass the MOPr and target instead one or both of these downstream mechanisms, based at least in part, on their contribution to the production of analgesia.

Furthermore, persistent inflammatory injury, such as that induced by CFA leads to adaptive changes in the RVM neurons (Guan, Terayama, Dubner, & Ren, 2002; Miki et al., 2002; Ren & Dubner, 2002; Zhang & Hammond, 2009). One known effect of this is an increase in the potency of MOPr agonists in the RVM (Hurley & Hammond, 2000). However, little is known about the mechanisms by which persistent inflammatory injury enhances the actions of MOPr agonists in the RVM, or whether these are mediated by enhanced actions by G proteins, postsynaptic MOPr, presynaptic MOPr, or perhaps a combination of these.

To this end, this thesis sought to confirm that in the RVM, $G_{i/o}$ proteins mediate the antinociception produced by MOPr agonists in uninjured rats, and to determine whether the role of $G_{i/o}$ proteins is enhanced during conditions of persistent inflammatory pain. In addition, this thesis sought to characterize the contribution of the postsynaptic MOPr and the presynaptic MOPr to the analgesic effects produced by MOPr agonists in the RVM, as well as to determine whether either or both of these mechanisms are enhanced under conditions of persistent inflammatory pain.

**The Role of $G_{i/o}$ Proteins in the RVM**

Prior reports have demonstrated that *in vivo* administration of PTX, a selective $G_{i/o}$ inhibitor, attenuates the antinociception produced by various MOPr agonists, including
DAMGO, in various pain modulatory regions (Bodnar, Paul, Rosenblum, Liu, & Pasternak, 1990; Hoehn, Reid, & Sawynok, 1988; Parenti, Tirone, Giagnoni, Pecora, & Parolaro, 1986; Parolaro et al., 1990; Sanchez-Blazquez & Garzon, 1988; Sanchez-Blazquez & Garzon, 1991). However, this had not been confirmed in the RVM. Given the central role of the RVM to pain modulation, as well as to the production of the analgesic effects of opioids, this was very surprising. Therefore, Chapter 2 in this thesis sought to confirm that in the RVM, G\textsubscript{i/o} proteins mediate the antinociception produced by MOPr agonist, DAMGO, microinjected directly into the RVM of uninjured rats. Even more surprising, were the results of this work. Pretreatment of the RVM in uninjured rats did not attenuate the antinociception mediated by DAMGO. Given that this was so unexpected, it is important to consider certain aspects of the study, including 1) PTX dose, 2) pretreatment time period of PTX, and 3) adequate inhibition of G\textsubscript{i/o} proteins by PTX. Previous studies demonstrated that 100 ng or 500 ng PTX were effective when injected i.c.v. or i.t., however, a dose as low as 5 ng microinjected directly into discrete nuclei is sufficient to significantly attenuate the effects of a MOPr agonist into that same region (Bodnar et al., 1990). Therefore, two doses, 50 ng and 500 ng were examined in this thesis. Additionally, an appropriate interval from the time PTX is administered to the time of testing is necessary for PTX to reach the target cells and appropriately inactivate G\textsubscript{i/o} proteins. In previous studies, this interval ranged from 2-24 days. However, the peak reduction was measured 5-7 days after the administration of PTX (Hoehn et al., 1988; Parenti et al., 1986; Sanchez-Blazquez & Garzon, 1988; Sanchez-Blazquez & Garzon, 1991). Therefore, a pretreatment period of 5 days was examined in this thesis. Finally, these data cannot be dismissed on the basis of inadequate ribosylation and inactivation of G\textsubscript{i/o} because 50 ng PTX significantly prevented DAMGO-stimulated GTPγS binding in the RVM. As discussed in more detail in Chapter 2, although PTX treatment only decreased DAMGO-stimulated GTPγS binding ~50%, this is consistent with previous studies (Correa-Sales, Reid, & Maze, 1992; Hayashi, Rabin, Guo, & Maze,
Therefore, these results demonstrate that despite effective inactivation of G\textsubscript{i/o} proteins, the antinociception produced by DAMGO in the RVM was not attenuated. These data indicate that, in an uninjured state, the antinociceptive effects of MOPr agonists in the RVM are mediated via G\textsubscript{i/o} independent mechanisms (represented in Figure 5.1). This is an important advancement scientifically. Although a few prior studies have provided evidence that MOPr couple with other G proteins, including G\textsubscript{s} and G\textsubscript{z}, and that MOPr can also signal instead via MAPK, ERK and JNK via G protein independent mechanisms, this is the first behavioral, whole animal study to support this (Chakrabarti, Regec, & Gintzler, 2005; Chakrabarti & Gintzler, 2007; Crain, Crain, & Makman, 1986; Crain, Crain, & Makman, 1987; Crain & Shen, 1990a; Crain & Shen, 1990b; Zheng, Loh, & Law, 2010). In the forefront of G protein signaling is the concept of G protein switching, which, in this context describes the fact that MOPr mediate their primary actions via G\textsubscript{i/o} proteins but perhaps have the ability to switch to G\textsubscript{s} or G\textsubscript{z} proteins under certain environments. Although G protein switching has not been demonstrated for MOPr, it has been for other receptors such as the β-adrenoreceptor. This receptor was formerly considered to couple exclusively to G\textsubscript{s} proteins but has more recently demonstrated a switch to G\textsubscript{i} signaling under certain circumstances (Magocsi, Vizi, Selmeczy, Brozik, & Szelenyi, 2007). Obviously, further investigations are required to determine through which of these mechanisms MOPr agonists are producing their antinociceptive effects in the RVM, and more importantly, to determine what effect these will have on the clinical use of opioids.

Another interesting result was that after CFA treatment, PTX did significantly attenuate the antinociceptive effects produced by DAMGO in the RVM, providing strong evidence for a role of G\textsubscript{i/o} proteins in the RVM after a persistent inflammatory injury. It is well accepted that CFA treatment leads to adaptive changes in the RVM neurons (Guan et al., 2002; Miki et al., 2002; Ren & Dubner, 2002; Zhang & Hammond, 2009). Additionally, one effect of this is an enhancement in the antinociception and the anti-
hyperalgesia produced by MOPr agonists in the RVM. It is still not yet clear what the mechanisms that mediate this effect are. However, this study does suggest that the mechanisms responsible for the antinociceptive effects have transitioned from $G_{i/o}$ protein independent in uninjured rats, to $G_{i/o}$ protein dependent after inflammatory injury (represented in Figure 5.1). Therefore, it would be important to further characterize the effects of this paradigm shift after CFA treatment and to assess whether this change involving $G_{i/o}$ proteins contributes, significantly, to the increased potency of opioids in the RVM after CFA treatment.

**The Role of Postsynaptic MOPr in the RVM**

Postsynaptic MOPr in the RVM are thought to produce antinociception by activating GIRK channels, resulting in hyperpolarization and inhibition of spinally projecting, pain facilitatory neurons (Hammond & Proudfit, 2007; Krapivinsky et al., 1995; Mark & Herlitze, 2000; North & Uchimura, 1989). However, prior to this thesis work, the data supporting this postulate was largely electrophysiology data, which has limitations to clinical application given that analgesia cannot be assessed by electrophysiology alone but instead requires support from behavioral, whole animal evidence. Therefore, Chapter 3 of this thesis sought to further characterize the postsynaptic actions of a MOPr agonist, DAMGO, in the RVM in behavioral, whole animal studies. Indeed, microinjection of tertiapin-Q, a GIRK channel inhibitor, into the RVM attenuated the antinociceptive effects of DAMGO in the RVM of uninjured rats. This is an important scientific advancement as this thesis is the first to provide behavioral evidence in the RVM that MOPr indeed act via GIRK channels to produce their analgesic effects in the whole animal. Furthermore, this is an important advancement for clinical translation. Currently, opioids remain a primary pharmacologic therapy for the treatment of pain. However, their wide range of adverse side effects hinders their use for effective management of chronic pain. Certainly, this new insight can be used to lay the
groundwork for the identification and development of new, non-opioid pharmacologic therapies. These therapies could perhaps bypass the MOPr and instead target GIRK channel activation to promote their analgesic effects. To date, there are no biologic GIRK channel agonists available that could be directly used to test this postulate. Furthermore, additional investigations will be required to further characterize whether all GIRK channels mediate these antinociceptive effects or whether specific GIRK channel compositions are required.

Another interesting finding was that after CFA treatment, inhibition of GIRK channels in the RVM no longer attenuated the antinociceptive effects produced by DAMGO in the RVM, suggesting a shift in paradigm from a GIRK dependent mechanism in the uninjured state to a GIRK independent mechanism after a persistent inflammatory injury (represented in Figure 5.1). However, this transition is not due to transcriptional downregulation of GIRK channels in the RVM. Interestingly, this closely mirrors an opposite trend to that of the G\textsubscript{i/o} proteins discussed in Chapter 2. Additionally, these data provide further evidence that persistent inflammatory injury promotes adaptive changes in the RVM.

Finally, along with data from Chapter 2, these data provide support for the hypothesis that the anti-hyperalgesic and the antinociceptive effects of DAMGO in the RVM are mediated via different mechanisms, and that the anti-hyperalgesic effects are mediated by G\textsubscript{i/o} and GIRK channel independent mechanisms (represented in Figure 5.1). The scientific and clinical implications of this are yet to be deciphered. However, to date, the expectation was that anti-hyperalgiesia and antinociception were simply a part of a continuum and therefore regarded as being produced by the same mechanisms. It is possible that in the clinical future, the therapeutic pharmacological goal might shift from providing analgesia after an acute injury, or post surgery to instead providing only anti-hyperalgesia. If so, this might be accompanied by less adverse effects, including sedation which might be beneficial to patient recovery times.
**The Role of Presynaptic MOPr in the RVM**

Presynaptic MOPr are thought to act by inhibiting VGCC and thereby decreasing GABA release onto spinally-projecting, pain inhibitory neurons. Disinhibition, or activation, of pain inhibitory neurons in the RVM in turn produces antinociception (Hammond & Proudfit, 2007). There is an abundance of indirect evidence supporting this postulate (Drower & Hammond, 1988; Fields, Heinricher, & Mason, 1991; Gilbert & Franklin, 2001; Gilbert & Franklin, 2002; Heinricher & Kaplan, 1991; Kalyuzhny & Wessendorf, 1998). However, only one prior study has directly demonstrated that the levels of GABA release are decreased in response to DAMGO, a MOPr agonist in the RVM (Schepers, Mahoney, Zapata, Chefer, & Shippenberg, 2008). As previously discussed, it is well established that persistent inflammatory injury, such as that induced by an intraplantar injection of CFA, leads to adaptive changes in the RVM, and that one effect of this is an increase in the potency of MOPr agonists. However, no prior studies have examined whether this effect is mediated by an enhancement of these presynaptic mechanisms. To this end, Chapter 4 of this thesis used *in vivo* microdialysis to measure levels of GABA release in the RVM in response to perfusion of DAMGO into the RVM. Indeed, the levels of GABA release were decreased in response to perfusion of DAMGO into the RVM, providing further direct support that MOPr agonists in the RVM produce their analgesic effects by decreasing GABA release in the RVM. The clinical implications for this are impressive. These data suggests that seeking new pharmacological therapies targeting VGCC could result in effective analgesics. In support of this are two relatively newer clinical success stories, pregabalin and gabapentin. Although initially designed to mimic GABA, further research has identified that their mechanism of action is by selective binding at \( \alpha_2-\delta \) type 1 subunit of the VGCC, which in turn results in inhibition of GABA release (Taylor, 2009). Both of these medications have proven effective in the management of neuropathic pain.

Unexpectedly, perfusion of DAMGO did not decrease the levels of GABA release
in the RVM after a persistent inflammatory injury. Consistent with the results in Chapter 2 and Chapter 3, these results provide further evidence that persistent inflammatory injury promotes adaptive changes in the RVM. Additionally, mirroring the results of Chapter 3, there is once again a paradigm shift from what is likely VGCC dependent production of analgesia in an uninjured state to VGCC independent production of analgesia after persistent inflammatory injury (represented in Figure 5.1). The clinical implications of this are much more evasive. If indeed VGCC do not mediate the production of analgesia after a persistent inflammatory injury, then pharmacologic therapies targeting VGCC may become minimally effective during this state. However, if the inactivity of VGCC is limited to analgesia initiated by the MOPr, then this would provide even further support for seeking pharmacologic therapies that bypass the MOPr and instead directly target the VGCC.

**Conclusions**

The goal of this thesis was to further characterize the mechanisms by which the MOPr relays the production of analgesia in the RVM, including the G proteins, the postsynaptic MOPr via GIRK channels and the presynaptic MOPr via VGCC. Additionally, this thesis sought to characterize how persistent inflammatory injury modulates these mechanisms.

Figure 5.1 illustrates the key results and paradigm shifts of this thesis. In the absence of injury, the mechanisms by which MOPr agonists produce antinociception in the RVM are G\(_{i/o}\) independent, but GIRK and VGCC dependent. Although G\(_{i/o}\) proteins do not play a role in the antinociceptive effects of MOPr agonists in the RVM in the uninjured state, the fact that GIRK and VGCC are activated and inhibited, respectively, by the \(\beta\gamma\) subunit of G proteins, suggests that there is involvement of other G proteins. Likely mediators are G\(_{z}\) and G\(_{s}\) proteins. G\(_{z}\) proteins are part of the subfamily of G\(_{i}\) proteins, and therefore, their subcellular effectors include inhibition of adenylyl cyclase.
and VGCC, as well as activation of GIRK channels (Tso & Wong, 2000; Tso, Yung, & Wong, 2000). They also stimulate MAPK and ERK1/2 (Tso & Wong, 2000; Tso et al., 2000). Importantly, they lack the site for ADP ribosylation and therefore are insensitive to inactivation by PTX (Jeong & Ikeda, 1998). As discussed in more detail in Chapter 2, prior studies have demonstrated the ability of Gα proteins to couple to MOPr, and some studies have demonstrated that Gα proteins contribute towards the production of antinociception in pain modulatory regions such as the PAG (Garzon, Castro, & Sanchez-Blazquez, 1998; Jeong & Ikeda, 1998; Sanchez-Blazquez, Juarros, Martinez-Pena, Castro, & Garzon, 1993; Sanchez-Blazquez, Gomez-Serranillos, & Garzon, 2001). There is also recent evidence that Gα couples to MOPr, and that this interaction is enhanced under certain conditions, including when Gα is dephosphorylated (Chakrabarti & Gintzler, 2007). Although these are the likely mediators, there are also intracellular GIRK mediators, such as sodium and PIP2 which have demonstrated to activate or modulate GIRK channel activity without prior activation by the βγ subunit.

Persistent inflammatory injury is known to lead to adaptive changes in the RVM neurons. This thesis demonstrated that several paradigm shifts take place in the RVM after CFA treatment. These are illustrated in figure 5.1. After CFA treatment, MOPr induced antinociception transitions from Gι/o independent to Gι/o dependent mechanisms. Moreover, the mechanisms by which the MOPr produce their antinociceptive effects in the RVM after CFA treatment transition from GIRK and VGCC dependent mechanisms to GIRK and VGCC independent mechanisms. Given that the βγ subunit of Gι/o no longer plays a role in MOPr mediated antinociception after CFA treatment, it is likely then that it is the α subunit of the Gι/o proteins that mediate the antinociceptive effects. The α subunit inhibits adenylyl cyclase to decrease intracellular cAMP levels. Beyond that, this mechanism, although important, is beyond the scope of the goal of this thesis.

Finally, as illustrated in figure 5.1, this thesis is the first work to demonstrate that the antinociceptive effects and the anti-hyperalgesic effects of MOPr agonists in the
RVM after a persistent inflammatory injury are not simply a continuum but are instead separate entities, mediated by different mechanisms. Specifically, anti-hyperalgesia is mediated by \( G_{i/o} \) independent and GIRK independent mechanisms. Although this does not exclude a role for other G proteins, a more likely mechanism for the production of anti-hyperalgesia could instead be through pERK or other MAP kinases. Recent studies have demonstrated that an acute inflammatory injury leads to increased phosphorylation of both p38 MAPK and ERK in the RVM. Importantly, inhibition of this phosphorylation, via microinjection of MAP kinase inhibitor into the RVM reversed the hyperalgesia (Imbe et al., 2011).

Future Directions

I have already carefully included future directions within the discussion of each section. However, I can briefly reaffirm the multiple paths that this work can take towards increasing the understanding of the mechanisms by which opioids produce analgesia, all with the ultimate goal of providing knowledge that can result in the development of better pharmacotherapies for more effective management of chronic pain.

**G Proteins in the RVM**

It will be of interest to pursue further studies to determine:

- If \( G_{i/o} \) proteins do not mediate the antinociception in the RVM, then what G proteins are involved?
  - Perhaps MOPr in the RVM do couple directly to \( G_s \) proteins
  - Perhaps \( G_z \) proteins mediate most of the effects relayed by MOPr in the RVM
  - An approach could involve the use of miRNA or siRNA to downregulate specific G proteins or G protein subunits, given that specific pharmacological inhibitors for these are not available
- A role for MAP kinases in MOPr antinociception has more recently been
elucidated.

- Not a lot is known about the mechanisms by which MAP kinases interact with MOPr. Recent evidence demonstrated that although activation of MAP kinases by MOPr typically is a downstream factor of G protein, there is a G protein independent pathway, involving instead β-arrestin. However, very little is known about the conditions in which each pathway predominates.

- MAP kinases have recently been implicated in the development of hyperalgesia in the RVM. Inhibition of the phosphorylation of ERK in the RVM alleviated the hyperalgesia (Imbe et al., 2011).
  - Perhaps this is the mechanism by which MOPr agonists mediate their anti-hyperalgesic effects in the RVM.
  - Perhaps it is this pathway involving MAP kinases that contribute to the enhanced effects of MOPr agonists after inflammatory injury.

*Postsynaptic MOPr in the RVM*

It will be of interest to pursue further studies to:

- Determine whether different subpopulations of neurons in the RVM consist of GIRK channels with different characteristics.
  - This could include different levels of GIRK channels, GIRK subunit composition, anatomical localization, levels tyrosine phosphorylation
  - Perhaps these characteristics are altered after CFA treatment.

- Using electrophysiology in the RVM, determine:
  - Whether tertiapin-Q completely abolishes the postsynaptic actions of MOPr agonists.
  - Whether tertiapin-Q inhibits the postsynaptic actions of MOPr agonists with different sensitivities/potencies in different subpopulations of neurons in the RVM?
- Whether this inhibition is altered by CFA treatment?
  - Determine whether there is a role for presynaptic GIRK channels in the RVM
    - Are GIRK channels anatomically present presynaptically in the RVM?
    - If so, are these sensitive to inhibition by tertiapin-Q?
    - Do they mediate presynaptic actions by MOPr agonists?
    - What subpopulations of neurons are these present in?
    - Is their role altered after CFA treatment?

*Presynaptic MOPr in the RVM*

It will be of interest to pursue further studies to determine:

- The role of basal GABA release during different stages of inflammation.
  - Perhaps the increased levels of endogenous opioids in the RVM after CFA treatment dampen the basal release of GABA.
  - Perhaps the basal levels of GABA release undergo a transition at different time points after CFA treatment, as the balance of the pain facilitatory and the pain inhibitory pathways shifts from:
    - Acute, to subchronic, to chronic phases of inflammation

- The role of VGCC in antinociception.
  - Does direct inhibition of VGCC in the RVM produce antinociception?
  - Does direct inhibition of VGCC in the RVM produce enhanced antinociception after CFA treatment?
  - Does direct inhibition of VGCC alter the levels of GLU or GABA release in the RVM?
  - If so, is the release of GLU or GABA altered by CFA treatment?

- Whether there is a presynaptic role for GIRK channels in the RVM
  - Another way of addressing this role could be to perfuse tertiapin-Q into the RVM and measure whether this alters the levels of GLU or GABA release in the RVM.
• If so, does CFA treatment alter the levels of GLU or GABA release in the RVM in response to tertiapin-Q?
Figure 5.1: Synthesis of findings and future directions.
REFERENCES


