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ENHANCING PERIPHERAL OPIOID ANALGESIA: DEVELOPMENT OF VIRAL VECTOR AND SMALL PEPTIDE THERAPIES

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DEDICATION

"For I know the plans I have for you", declares the Lord "plans to prosper you and not to harm you, plans to give you hope and a future"

Jeremiah 29:11

Mom and dad thank you for supporting me and believing in me. In memory of my grandmother Mary Nelson whose love of education inspired me to be the best student I could be. Thank you to both of my grandmothers, Pauline Smith and Mary Nelson, for being solid and strong foundations for our families.

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ABSTRACT

Chronic neuropathic pain often decreases quality of life, incurs high medical costs and is difficult to treat. Neuropathic pain can develop as a result of nerve damage induced by trauma or diseases such as diabetes and cancer. This type of pain is often resistant to standard treatments for chronic pain. Effective and safe treatments for persistent neuropathic pain remain an unmet therapeutic need. The primary objective of this work was to test the hypotheses that peripheral opioid analgesia can be enhanced using a herpes simplex virus vector gene therapy approach and peripheral opioid tolerance can be prevented by blocking opioid receptor heterodimerization using a small peptide inhibitor in neuropathic pain conditions in rodents. L5 spinal nerve transection was used to model chronic neuropathic pain.

Utilization of an injury specific promoter to overexpress mu opioid receptors demonstrated a greater degree and an earlier onset of antinociception in animals infected with the GAL-MOR virus compared to those with the CMV-MOR virus. In addition, immunohistochemistry analysis showed a decrease in MOR expression in the dorsal horn of spinal cords in injured animals and this decrease was reversed with CMV and Gal promoter viruses. Overall, these results suggest that an injury and population specific promoter can drive gene expression in a specific population of neurons in the dorsal horn of the spinal cord.

By using a small peptide inhibitor of mu- and delta-opioid receptor heterodimerization, it was shown that an increase in heterodimer formation in HEK293 cells after chronictreatment with a MOR agonist can be prevented using a peptide inhibitor of MOR-DOR. *In vivo* results showed that by using a peptide inhibitor of MOR-DOR, peripheral tolerance can be prevented. Additionally, it was found that chronic opioid treatment in a rat model of neuropathic pain appears to cause changes in downstream mediators that can be augmented by the use of a peptide inhibitor of MOR-DOR heterodimer formation. These results suggest that MOR-mediated peripheral tolerance can be prevented by inhibiting MOR-DOR heterodimerization and that inhibiting MOR-DOR heterodimerization potentially has effects on downstream signaling mediators and pathways. Taken together these studies have developed both viral vector approaches to attenuate neuropathic pain and enhance opioid analgesia and a small peptide approach to prevent the development of opioid tolerance.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

CMV ..... Cytomegalovirus

CNS ..... Central nervous system

DAMGO ..... [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin

DOR ..... Delta opioid receptor

DRG ..... Dorsal root ganglia

GIRK2 ..... G protein-activated inward rectifier potassium channel 2

HEK ..... Human embryonic kidney

HSV ..... Herpes Simplex Virus Type 1

IHC ..... Immunohistochemistry

KOR ..... Kappa opioid receptor

MOR ..... Mu opioid receptor

MPE ..... Maximum possible effect

NP ..... Neuropathic Pain

SNT ..... Spinal nerve transection

CHAPTER 1

INTRODUCTION

Chronic pain is a life-altering condition associated with disabling physical and emotional symptoms. Chronic pain is defined as lasting longer than 3 months in duration. It is estimated that over 75 million Americans suffer from chronic pain. Direct and indirect costs associated with treating chronic pain conditions are estimated to be $150 billion annually. Four major forms of chronic pain are migraine/facial pain, arthritis, back pain, and neuropathic pain. About 25% of chronic pain cases are neuropathic in origin and are by far the most difficult to manage therapeutically. A large number of neuropathic pain patients still have moderate to severe pain even after treatment. As a result, there remains an unmet therapeutic need for the treatment of neuropathic pain. In the following sections an overview of neuropathic pain, including etiology, potential mechanisms, current treatment options, and opioid tolerance will be presented. In addition, the rationale for the animal model, gene therapy approach, and inhibition of opioid receptor heterodimerization will be discussed.

1.1 Neuropathic Pain

Neuropathic pain is described as pain that results from an injury or a disease affecting the somatosensory system (Treede et al., 2008). Common pain disorders that are classified as NP conditions include post herpetic neuralgia, diabetic neuropathy, and cancer-related neuropathies. People with these conditions often experience allodynia, pain in response to non-noxious stimuli, and hyperalgesia, a heightened response to noxious stimuli. The estimated number of people with a neuropathic pain condition varies depending on the specific disorder. In one community population in the United States, however, it was estimated that around 10% of the population suffered from some type of neuropathic pain (Yawn et al., 2009). Chronic neuropathic pain results in a

decrease in quality of life, high medical costs (Berger et al., 2004; Jensen et al., 2007) and is often times difficult to treat. The efficacy of the current treatments varies for individuals and can result in adverse side effects. Currently, alternative therapies are being explored for the treatment of neuropathic pain with one being gene therapy.

1.1.1 Etiology of Neuropathic pain:

1.1.1.a Types of Pain

The term pain involves many factors and the sense of pain is highly subjective and is different for each individual. There are two broad categories of pain: acute and chronic pain. Acute pain occurs in response to an injury or a disease process and is typically beneficial in nature because it signals the body of potential tissue damage. It is usually short in duration and typically ends when healing has occurred or when it has responded to treatment (Rougeot et al., 2010). Chronic pain, however, does not signal the body of potential tissue damage but is rather pathological in nature and typically does not respond immediately to treatment. The duration of chronic pain is often longer than that for acute pain and involves the emotional and psychological aspects of pain (Morgan et al., 2006; Rougeot et al., 2010).

The feeling of acute pain almost always begins in specialized sensory neurons called nociceptors that respond to noxious stimuli (Morgan et al., 2006). There are two different types of nociceptors that convey different types of pain that are referred to as first and second pain. Aδ fibers are myelinated free nerve endings that send a fairly rapid signal to the spinal cord that encodes a sharp sense of pain and are referred to as first pain. C fibers are unmyelinated free nerve endings that are the most abundant and send signals to the spinal cord from the periphery that is interpreted as a slow and burning sense of pain and are referred to as second pain (Moffat and Rae, 2011). As a result of

their slow signal transmission, C fibers are responsible for the feeling of 'hurt' and opiates work by influencing second pain that is caused by C fibers (Pasternak, 1988).

Pain can be further characterized as nociceptive (usually acute in duration) or non-nociceptive (chronic in duration) and can occur within any system in the body. For example, somatic pain, which occurs in skin, muscles, joints, and the connective tissues surrounding them and is often times localized to a specific area, and visceral pain, which originates in organs of the body and is often times not localized to a specific area, are both types of nociceptive pain (Morgan et al., 2006). Neuropathic pain is a type of non-nociceptive pain and it develops either due to a result of injury to the neurons themselves or an induction of peripheral or central sensitization that is caused by a transition from acute to chronic pain. Sensitization as a result of pain is similar to long term potentiation in the hippocampus and, in addition to neuropathic pain, can be induced by inflammatory pain which is caused by a release of chemicals from inflamed tissues that is in response to either a noxious or non-noxious stimulus (Kidd and Urban, 2001).

1.1.1.b Sources of Neuropathic Pain

Neuropathic pain can arise from disease states, injuries, or unknown sources. It is pathological in nature because pain still occurs in the absence of injury or damage. The sources of neuropathic pain are typically categorized by whether it is of peripheral or central origin and is also divided into major categories some of which are: metabolic, viral, cancer, and trauma. Diabetes can be a metabolic cause of neuropathic pain. Painful diabetic neuropathy is a common occurrence in patients with diabetes, particularly those with chronically high blood glucose levels. It is postulated that free radicals or advanced glycosylation endproducts resulting from poor blood glucose control

lead to vascular damage in the extremities (Vincent et al., 2004; Obrosova, 2009). These damaged vessels are then not able to properly supply nerve fibers resulting in nerve damage and causing chronic neuropathic pain.

Re-activation of the herpes zoster virus causes shingles which can lead to post-herpetic neuralgia (PHN). It is thought that PHN is a result of damage to the nerve fibers in which the virus was re-activated. Neuropathic pain can also develop in patients with cancer. This pain can either be directly associated with the tumor or as a result of chemotherapy. Tumors can press on nerves or chemicals released by tumors or the body's immune system can sensitize nerves eliciting neuropathic pain. In addition, chemotherapy can directly damage sensory neurons leading to pain. Neuropathic pain also often results from traumatic injury such as amputation of a limb can lead to phantom limb pain which is thought to result from both peripheral and central mechanisms.

1.1.2 Proposed mechanisms of neuropathic pain:

1.1.2.a Pain pathway: Introduction

The perception of pain begins with transduction of the external noxious stimulus along the pain pathway and ends with an integration of signals from the thalamus and higher cortical areas that actually encode for the perception of and response to pain (Moffat and Rae, 2011). The ascending pain pathway begins with transduction of peripheral nociceptors that terminate on second order neurons in the dorsal horn of the spinal cord (McDougall, 2011). The axons of these neurons then ascend as the spinothalamic tract and synapse on neurons in the brainstem and thalamus. Neurons in the thalamus either project to areas of the brain concerning discriminative aspects of pain, like the primary somatosensory cortex, or areas concerning the emotional aspect of pain

like the anterior cingulate cortex (Morgan et al., 2006; Chen et al., 2008). The modulation of pain occurs in the descending pain pathway and primarily involves areas at the level of spinal cord and the brainstem, particularly the hypothalamus, periaqueductal gray (PAG), and raphe nuclei (Beaumont and Hughes, 1979; Fields and Levine, 1984).

1.1.2.b Neuropathic pain mechanisms

The cellular mechanisms in the development and maintenance of neuropathic pain have begun to be elucidated with the development of animal models of neuropathic pain. The major cellular mechanisms leading to neuropathic pain include hyperexcitability of sensory neurons, upregulation of sodium channels in nociceptors, cross talk of nerve fibers, demyelination of sensory nerves, and loss of inhibitory tone in the spinal cord (Woolf and Mannion, 1999; Pasero, 2004; Ueda, 2006). It is likely that in combination with each other, these mechanisms are responsible for the development and maintenance of neuropathic pain as there is no single mechanism that is responsible for this phenomenon.

1.1.3 Animal models of neuropathic pain

To better understand neuropathic pain, researchers have made great strides in developing animal models that mirror the symptoms of neuropathic pain commonly seen in humans. Peripheral nerve injury models allow scientists to study the pain associated with damage to peripheral nerves. These models resemble human neuropathic pain syndromes in that they all provoke spontaneous pain, hypersensitivity to noxious stimuli, and sensitivity to non-noxious stimuli to varying degrees. The oldest peripheral nerve injury model is the complete sciatic nerve transection in which a small segment of the sciatic nerve is removed (Jaggi et al., 2011). This type of injury causes pain associated

with no sensory input which in animals can lead to autotomy behavior which is self-attack of the denervated limb. Because this model requires the complete transection of the nerve, it is often unrealistic to what happens in humans other than in phantom limb pain. To better model what happens in humans, the most common NP models later developed involved either a partial ligation or transection of the sciatic nerve or a section of the nerve that is proximal to the dorsal root ganglia. These models include the Bennett model (chronic constriction injury model), Seltzer model (Partial sciatic nerve ligation model), and Chung model (L5 /L6 spinal nerve ligation model). Bennett and Xie developed a chronic constriction model that involves the loose ligation of the sciatic nerve (1988). Approximately two days after injury, spontaneous pain and hypersensitivity to mechanical and thermal stimulation develops which lasts for about two months (Bennett and Xie, 1988; Attal et al., 1990). While the chronic constriction model has been the gold standard for drug development, its major limitation is operator variability regarding the tightness of the ligatures.

To avoid complete ligation of the sciatic nerve, the Seltzer model was developed in which only a portion of the sciatic nerve is ligated (Seltzer et al., 1990). Increased pain-like behaviors develop within one week of surgery and persist for about 6 weeks after injury (Jaggi et al., 2011). In a similar fashion, Decosterd and Woolf developed the spared nerve injury model in which the common peroneal and tibial nerves are ligated while the sural nerve is spared (2000). This type of injury results in early and prolonged neuropathic pain-like behaviors that are produced in all animals that undergo the procedure.

1.1.3.a Chung model

The spinal nerve ligation model of neuropathic pain developed by Kim and Chung involves the ligation of the distal portions of the L5 and L6 nerves to the dorsal root ganglia (1992). The procedure involves making an incision at the level of the illiac crest, separating the underlying muscles, and removing the transverse processes from the lumbar vertebrate to expose the L4-L6 spinal nerves then placing a unilateral tight ligation of silk suture around L5 and L6 spinal nerves being careful to avoid the L4 nerve. The L4 nerve is left intact due to its large number of motor fibers which if ligated results in severe motor deficits making pain behavioral assessments impossible. The benefit of using this model is the rapid onset of neuropathic pain behaviors (within 24 hours) and these behaviors typically last for 10-16 weeks (Jaggi et al., 2011). For this thesis, a modified Chung model was used in which the L5 spinal nerve was transected, leaving intact both the L4 and L6 nerves (Fig 1.1). This model has proven to be highly reproducible in both rats and mice.

1.1.4 Manifestations of neuropathic pain after nerve injury

The most common presentations of neuropathic pain are spontaneous pain, hyperalgesia, and allodynia. The spontaneous pain that occurs in many neuropathic pain conditions is often described as shooting, burning, or lancinating. Hyperalgesia and allodynia are thought of as the hallmark symptoms of neuropathic pain. When a noxious stimulus becomes more noxious, this type of sensation is referred to as hyperalgesia. One common example of hyperalgesia is a hot shower being painful for someone with sunburn. Hyperalgesia can occur as a result of a chemical, thermal, or mechanical stimulus. When a non-noxious stimulus becomes noxious, this is known as allodynia.

One common example of allodynia is pain caused by contact of clothing to the skin. Allodynia can occur as a result of disorders like postherpetic neuralgia or fibromyalgia which is thought to have central sensitization as a key component.

1.1.4.a Peripheral and central sensitization

Spontaneous pain is thought to occur as a result of the spontaneous activity of nociceptor C-fibers, particularly the burning sensation (Woolf and Mannion, 1999). As a result of nerve damage, neuromas develop which are sites of regenerative nerve sprouts. It is through these neuromas as well as other sites along the primary sensory afferents that peripheral sensitization occurs. Peripheral sensitization is mediated through nociceptor C-fibers and is thought to be the primary mechanism responsible for hyperalgesia. In addition, damage to the peripheral nervous system results in the release of a plethora of pro-nociceptive chemicals that result in the sensitization of nociceptors (Pasero, 2004). The sensitization of nociceptors is most likely due to insertion of more ion channels, particularly sodium channels, into the axon at both the site of neuroma and along the length of the axon, resulting in a lowered activation threshold (Woolf and Mannion, 1999; Pasero, 2004).

Because peripheral sensitization cannot account for all the characteristics of neuropathic pain, central mechanisms have been proposed to further explain the pain symptoms of neuropathic origin. Central sensitization takes place at the level of the spinal cord. In the spinal cord this phenomenon is thought to be due to wind-up in which primary afferents develop a lower activation threshold, a larger receptive field, and an increased response to stimuli whether noxious or non-noxious (Pasero, 2004). It is the dysfunction at the level of the central nervous system that changes the way the CNS

responds to somatosensory input (Latremoliere and Woolf, 2009). Normally, nociceptors have a high threshold. As a result of central sensitization, however, there is a switch to low threshold primary afferents that results in pain hypersensitivity (Latremoliere and Woolf, 2009).

1.1.5 Current treatment of neuropathic pain

Antidepressants, gabapentin and pregabalin, and lidocaine are first line treatments for neuropathic pain (O'Connor and Dworkin, 2009). The antidepressants used include selective serotonin-reuptake inhibitors, serotonin-norepinephrine reuptake inhibitors, and tricyclic anti-depressants. Gabapentin and pregabalin interact with the α2-δ subunit of voltage-gated calcium channels which results in a decrease in neurotransmitter release (Baron et al., 2010). Gabapentin and pregabalin have produced positive results in the treatment of several peripheral neuropathic pain conditions. Topical lidocaine has been found to be efficacious in the treatment of some NP conditions, particularly postherpetic neuralgia. The lidocaine patch works by blocking sodium channels to prevent pain transmission in the skin.

Second line treatments include opioids and tramadol, an opioid agonist and serotonin and norepinephrine reuptake inhibitor (O'Connor and Dworkin, 2009) (Fig 1.2). Opioids bind to opioid receptors that are located both pre- and post-synaptically. Pre-synaptically, the binding of opioids to opioid receptors results in the inhibition of voltage sensitive calcium channels which prevents excitatory neurotransmitter release. This block on calcium channel opening is downstream of G protein coupled inhibition of G-protein coupled inwardly rectifying potassium (GIRK) channels. Postsynaptic opioid receptors also inhibit GIRK channels, leading to hyperpolarization of the postsynaptic membrane

reducing the likelihood of action potential propagation. Thus, both pre-synaptic and post-synaptic opioid receptor binding inhibits neurotransmission from primary afferent neurons to ascending spinothalamic tract neurons.

1.1.6 Opioids in the treatment of neuropathic pain

The use of opioids in neuropathic pain is controversial. Early clinical trials of opioids in neuropathic pain showed little pain reduction with normal doses of opioids (Arner and Meyerson, 1988; Arner et al., 1988; Max, 1988; Eide et al., 1994; Park et al., 1995). Later studies using higher doses of opioids demonstrated greater pain reduction with opioids compared to placebo in neuropathic pain patients (Harke et al., 2001; Leung et al., 2001; Raja et al., 2002; Jorum et al., 2003; Rowbotham et al., 2003; Gilron et al., 2005). Use of high dose opioids to manage neuropathic pain results in significant side effects, including sedation, cognition impairment, and constipation (Fig 1.3). These side effects increase treatment drop out and decrease patient satisfaction (Morley et al., 2003; Stein et al., 2003b; Watson et al., 2003; Gimbel et al., 2005).

1.2 Endogenous opioid system

The analgesic and euphoric properties of opium have been known and used medicinally and recreationally since 4000 B.C. However, the identification of opioid receptors in the mammalian central nervous system was not identified until the 1970's (Pert and Snyder, 1973b, a; Terenius, 1973; Law et al., 2000). Over the last 45 years, numerous functions of opioids in the nervous system have been identified and are active areas of research and discovery. One of the active areas of opioid research (Hughes et al., 1975) is the role of these endogenous opioid peptides and opioid receptor systems in the pain pathway. The endogenous opioid system consists primarily of 3 classes of

opioid peptides: β-endorphin, enkephalins, and dynorphins; and 3 classes of opioid receptors: mu-, delta-, and kappa-opioid receptors (Table 1.1). These peptides and receptors are widely distributed throughout the central and peripheral nervous systems and modulate a variety of different physiologic functions including pain modulation, the stress response, and addiction (Benarroch, 2012). The endogenous opioid system modulates pain in the periphery as well as at the spinal and supraspinal levels.

1.2.1 Endogenous opioid peptides

Opioid peptides are the body's analgesic response to pain. It had been known for a long time that opiates like morphine relieve pain by producing analgesia via the CNS. As a result, researchers speculated that there were endogenous opioid receptors and therefore, there must be endogenous opioids as well. Since the discovery of morphine over 200 years ago (Brownstein, 1993), researchers have discovered a number of endogenous opioids and their endogenous receptors. The first of these endogenous opioids discovered was the enkephalins (ENK) by Hughes et al (1975). By using an opioid receptor antagonist to block the analgesic effects of enkephalins, researchers were able to prove that enkephalins were indeed endogenous opioids. Since then, a wide array of research has been done to elucidate the role of these peptides in the pain experience as well as in other body systems. Three classes of endogenous opioid peptides have been identified: enkephalins, endorphins, and dynorphins. These peptides are synthesized as three precursor peptides: preproenkephalin (PENK), preproopiomelanocortin (POMC), and preprodynorphins, respectively. All of the opioid peptides share a common amino terminus sequence of Tyr-Gly-Gly-Phe. The three peptides demonstrate different receptor selectivity but not specificity for the opioid receptors.

Preproenkephalin and active enkephalin peptides have been identified in the peripheral and central nervous system as well as non-neuronal tissues including immune cells (Howells et al., 1986; Merchenthaler et al., 1986; Garrett et al., 1990; Kuis et al., 1991; Linner et al., 1991). Met-enkephalin, leu-enkephalin, and several enkephalin-containing peptides have been identified as active peptides (Hughes et al., 1975). Preproenkephalin knockout mice have decreased pain responses (Yaksh and Noueihed, 1985; Ji et al., 1995; Mansour et al., 1995b; Konig et al., 1996). Similarly, endorphin opioid peptides have demonstrated antinociception. During inflammation, β-endorphin has been shown to produce peripheral analgesia (Stein et al., 1990a; Cabot et al., 1997; Mousa et al., 2001). In contrast to enkephalins and endorphins, dynorphin does not produce antinociception and actually may even be pronociceptive (Vanderah et al., 1996; Wang et al., 2001). More recently, the endomorphins have been identified as a fourth group of endogenous opioid peptides (Zadina et al., 1997). Endomorphins have a distinct amino terminal sequence and exhibit high selectivity to the MOR. Currently, the endomorphin precursor remains unidentified (Fichna et al., 2007).

1.2.2 Endogenous opioid receptors

Prior to the discovery of the endogenous opioids, in 1973, researchers discovered opioid binding sites in the CNS that were later determined to be mu-opioid receptors (Pert and Snyder, 1973b). Of the known opioid receptors, three were successfully cloned in the early 1990s with the first being DOR followed by MOR and KOR (Satoh and Minami, 1995; Przewlocki and Przewlocka, 2005). Opioid receptors belong to the rhodopsin subfamily of G-protein coupled receptors superfamily. They have the typical G-protein coupled receptor 7α-helical transmembrane domains and an extracellular N-

terminus with numerous glycosylation sites (Law et al., 2000). Opioid receptors are approximately 400 amino acids with high sequence homology particularly in the transmembrane domains and intracellular loops (Knapp et al., 1995; Akil et al., 1996; Connor and Christie, 1999; Pil and Tytgat, 2003). The unique pharmacological selectivity of opioid receptors result from small changes in sequences at the N- and C-termini and extracellular loops (Knapp et al., 1995; Akil et al., 1996; Connor and Christie, 1999; Pil and Tytgat, 2003).

Opioid receptors couple to pertussis toxin-sensitive and insensitive G-proteins with slight differences between receptor types (Jordan et al., 2000). Upon agonist binding, opioid receptors activate inhibitory intracellular G proteins ($G_{i/o}$) that target multiple downstream effectors, including adenylyl cyclase, inwardly rectifying K channels, and voltage-dependent calcium channels. Generally, in the nervous system this signaling pathway inhibits neurotransmitter and neuropeptide release, and decreases neuronal excitability and firing rate (Mansour et al., 1995a; Williams et al., 2001).

Enkephalins bind DOR with the greatest affinity (K_d, 2.8 nM) and are considered endogenous DOR ligands (Satoh and Minami, 1995; Przewlocki and Przewlocka, 2005). Both morphine and the enkephalins bind the μ_1 subtype of MOR with similar affinities (K_d, 0.4nM and 0.5nM respectively) while the enkephalins have a lower affinity for the μ_2 subtype of MOR (K_d, 50-75nM) and an even lower affinity for the kappa opioid receptors (K_d, 514nM) (Wolozin and Pasternak, 1981; Satoh and Minami, 1995). In addition to their well-studied roles in pain processing, opioid receptors also modulate a variety of important physiological processes including reward, neuroendocrine activities such as food intake, respiratory function, and gastrointestinal motility (De Luca and

Coupar, 1996; Schultz and Gross, 2001; Yeomans and Gray, 2002). The physiological function of opioid receptor activation is driven by the anatomical location and the expression levels of opioid subtypes (Mansour et al., 1995a). For example, expression of MOR in the medullary respiratory center regulates respiration, and expression of MOR in the intestines regulates gastrointestinal motility.

1.2.3 Opioid receptor expression and function in the central and peripheral nervous systems

In both laboratory animals (Lamotte et al., 1976; Stein et al., 1990b; Hassan et al., 1993; Coggeshall et al., 1997) and in humans (Stein et al., 1996), opioid receptors are located on neuronal cell bodies in dorsal root ganglia (Ji et al., 1995; Mansour et al., 1995c), and on the central and peripheral terminals of those same primary afferent neurons. Centrally and peripherally expressed opioid receptors demonstrate similar pharmacological characteristics (Hassan et al., 1993). Activation of peripheral opioid receptors increase potassium currents (Rodrigues and Duarte, 2000), and decrease calcium currents (Xie et al., 1999). This leads to a decrease in the release of pronociceptive compounds from peripheral nociceptive terminals (Acosta and Lopez, 1999). Decreased neurotransmitter release from afferent neurons coupled with increased potassium currents in central nervous system neurons produces an overall decrease in action potential generation and propagation in the central nervous system.

In addition, opioid receptors have been identified on peripheral nervous system neurons which innervate peripheral organs such as skin, mucosal tissues, and GI tract (Bagnol et al., 1997; Fickel et al., 1997; Samoriski and Gross, 2000; Bigliardi-Qi et al., 2004). In these sites, opioid receptors modulate GI motility, visceral pain, and regulation

of mucosal transport of fluids and electrolytes (Bagnol et al., 1997; Eastwood and Grundy, 2000; Bigliardi-Qi et al., 2004; Holzer, 2004).

Opioid receptors are located throughout the CNS but there is anatomical specificity for the different classes of receptors. KORs are located in the spinal cord, the nucleus accumbens, hypothalamus, amygdala, and in the caudate putamen (Mansour et al., 1995b; Mansour et al., 1995a). KORs modulate feeding, nociception, neuroendocrine functions and postulated functions in motor and cognitive processing. DORs are located in the neocortex, caudate-putamen, nucleus accumbens and amygdala (Mansour et al., 1995b; Mansour et al., 1995a). MOR is more widely expressed in the brain including in the forebrain, midbrain, and hindbrain. The highest levels of MOR expression are in the thalamus, amygdala, hippocampus, nucleus accumbens, and periaqueductal gray (Sessle and Henry, 1985; Mansour et al., 1995b). This localization of MOR supports the importance of this receptor system in pain perception and sensory and motor integration.

The widespread expression of opioid receptors in the central nervous system underlies the importance of these signaling pathways in many homeostatic physiological processes. This leads to the significant side effect profile in the clinical use of opioid receptor agonists for the treatment of pain. For example, the dose-limiting respiratory depression caused by systemic opioid administration is a result of MOR and DOR activation in the medullary respiratory centers (Sessle and Henry, 1985). In addition, systemic opioid administration produces drowsiness and a "mental fog" as a result of a general inhibition of cognitive processing across multiple brain regions.

Opioids modulate both ascending and descending pain pathways (Vaughan et al., 1997; Pan et al., 2004). DORs and MORs are expressed in dorsal root ganglia, spinal

cord, trigeminal nucleus, pontine, intermediate reticular nuclei, periaqueductal gray and locus coeruleus (Mansour et al., 1995b). These anatomical sites are crucial to both ascending and descending pain pathways. For example, the periaqueductal gray matter receives input from both nociceptive fibers that can influence descending control mechanisms and from higher order brain regions that can result in the release of endogenous opioids. Studies have shown that by electrically stimulating the periaqueductal gray area, analgesia can be produced (Gray and Dostrovsky, 1983). This effect can be blocked using naloxone an opioid receptor antagonist (Hosobuchi et al., 1977). These results suggest that the opioid system plays an important role along the ascending and descending pain pathways.

1.2.3 Non-neuronal opioid receptor expression and function

Opioid receptors and endogenous peptides are also expressed in various immune cells including, lymphocytes and macrophages (Carr et al., 1989; Cabot et al., 1997). Clinically, administration of opioid agonists can be immunosuppressive (Halford et al., 1995; McCarthy et al., 2001; Sacerdote, 2006). Release of endogenous opioid peptides from immune cells has been found to produce potent peripheral opioid analgesia (Parsons et al., 1990; Parsons and Herz, 1990; Stein et al., 1990b; Przewlocki et al., 1992). This peripheral opioid analgesia is most pronounced in inflammatory pain models (Bidlack, 2000; Stein et al., 2001). The role of endogenous opioid peptides in modulating the immune system is an area of current study.

1.2.4 Exogenously administered peripheral opioid agonists

The main pharmacological effect of opiates like morphine is providing analgesia through binding to its particular receptors in the central nervous system. As result of its

actions in the CNS, side effects such as respiratory depression and addiction can develop. As these two major opioid-mediated side effects result from the activation of opioid receptors in the CNS, peripheral opioid analgesia represents an attractive alternative. Peripheral opioid analgesia has been demonstrated in experimental models of inflammatory pain (Nozaki-Taguchi and Yaksh, 1999; Shannon and Lutz, 2002; Janson and Stein, 2003), and neuropathic pain (Pertovaara and Wei, 2001; Martinez et al., 2002; Truong et al., 2003; Obara et al., 2004; Guan et al., 2008), and in clinical settings (Stein, 1995; Gu et al., 2005).

Peripheral opioid analgesia is targeted by local administration and by using pharmacophores which do not cross the blood brain barrier (Rogers et al., 1992; Stein et al., 1993; Giardina et al., 1995). As with systemic opioid analgesia, peripheral opioid analgesia demonstrates stereospecificity, dose-dependency, and is naloxone reversible. This suggests that peripheral opioid analgesia is mediated by peripheral opioid receptors (Stein et al., 1993). Clinically, peripherally administered morphine in postoperative pain following knee surgery (Stein et al., 1991; Khoury et al., 1992) demonstrated comparable potency to local anesthetics (Khoury et al., 1992). In theory, this approach will limit centrally mediated respiratory and cognitive side effects of opioid analgesics. It will not prevent or lessen peripherally mediated side effects such as immunosuppression and constipation. One way to overcome the limitations of both systemic and peripherally restricted opioid agonists is to use viral gene delivery approaches to increase peripheral opioid receptors and peptide expression to enhance peripheral opioid analgesia and decrease neuropathic pain (Table 1.2). The value of this approach is to produce peripheral analgesia without the off-target peripheral and centrally mediated side effects.

Peripherally acting opioids have been shown to be effective in attenuating mechanical allodynia and thermal hyperalgesia in a rodent model of neuropathic pain (Guan et al., 2008; Chung et al., 2012). Loperamide is a MOR agonist that does not readily cross the blood brain barrier and is mainly used to treat acute diarrhea. Loperamide has been shown in previous studies to produce analgesia in animal models of inflammatory and neuropathic pain (Stein et al., 2003b; Sevostianova et al., 2005; Shinoda et al., 2007; Guan et al., 2008) and is thought to produce antihyperalgesia through DOR in the periphery (Shinoda et al., 2007).

1.3 Gene therapy for the treatment of pain using viral vectors

The crux of gene therapy is the idea of focal delivery. As a result of the overlap of the interactions of neuronal factors (neurotransmitters, ion channels, etc.) with other systems, off-target effects often occur in the current treatments for chronic pain. Gene therapy offers a more targeted approach to treat chronic pain conditions, therefore bypassing unwanted and often times serious side effects. Gene therapy was originally developed as an approach which sought to replace defective genes with desired ones (Friedmann and Roblin, 1972). In order to accomplish this, viral mediated gene transfer was developed and is the most used of the gene therapy techniques (Fig 1.4). This strategy dates back to the 1970s and within the last 15 years has been explored as a treatment option for chronic pain (Friedmann and Roblin, 1972). The goal for the use of vectors in the treatment of chronic pain is not to replace defective genes but rather to introduce genes that will produce a certain protein or peptide locally to avoid side effects seen with drugs given systemically (Hao et al., 2007). While viral vectors are used most often, non-viral vectors such as plasmids introduced by electroporation (Lee et al., 2003;

Chuang et al., 2004) and non-viral, non-plasmid minimalistic immunologically defined gene expression (MIDGE) vectors encoding proopiomelanocortin (POMC), the precursor for endorphins, have also been used in the treatment of chronic pain (Machelska et al., 2009). There are multiple viruses that are used as vectors in pain research using animal models with one of the most common being the herpes simplex virus (HSV) due to its natural propensity for sensory neurons.

1.3.1 Herpes simplex virus-mediated gene transfer

Herpes simplex virus- based vectors have been used as a therapeutic strategy in a number of pain models, both acute and chronic, and the gene of interest has ranged from anti-inflammatory cytokines and ion channels (Yeomans et al., 2005; Hao et al., 2006; Chattopadhyay et al., 2008; Zhou et al., 2008) to endogenous opioids and their receptors (Jones et al., 2003; Zhang et al., 2008; Fink et al., 2011) (Fig 1.4). HSV-1 is an enveloped double stranded DNA virus that has a large capacity for exogenous transgenes. The use of recombinant herpes simplex virus as a means to deliver genetic material to a targeted region in the nervous system is particularly beneficial because HSV specifically targets neurons and can therefore be used to directly infect primary afferent neurons. Infection begins in epithelial cells and eventually takes up residency in neuronal cell bodies via retrograde transport. Once established in the cell bodies, the virus can either begin a lytic cycle or can enter a latent state. When in the latent state, a portion of the HSV genome remains transcriptionally active through the production of latency-associated transcripts. These transcripts may regulate the host genome in a way to prevent the cell from undergoing apoptosis (Fink et al., 1996). The ability of the virus to enter a latent state while still retaining the ability to produce proteins is advantageous in

the use of this virus as a vector for gene therapy. In order to prevent the virus from re-entering the lytic cycle, vectors constructed for use in the treatment of neuropathic pain are rendered replication defective by eliminating immediate early genes essential for the viral replication thereby preventing the viral lytic cycle.

1.4 Development of opioid tolerance

Long term use of opioids can lead to the development of tolerance, a decrease in efficacy using the same dosage amounts. There is no consensus as to whether tolerance develops in the periphery with the chronic use of peripheral opioids. It has been shown that when morphine is administered locally in the hindpaw, tolerance occurs while central administration results in a significant reduction in morphine analgesia (Tokuyama et al., 1998). These results suggest that tolerance develops in a centrally-mediated way that most likely involves synaptic plasticity in the central nervous system rather than receptor desensitization at the cellular level. Similarly, it has been found that chronic morphine treatment does not lead to the development of tolerance in a rodent model of inflammatory pain and only when endogenous opioids were depleted was tolerance restored (Zollner et al., 2008). Based on these results, it appears that the use of peripherally acting opioids in the treatment of chronic pain would not lead to tolerance. Other groups, however, have found that peripheral tolerance can be induced with repeat peripheral injections of opioids. Aley and Levine examined DAMGO-induced tolerance in the periphery and found that nitric oxide and Ca^{2+} dependent mechanisms are involved in the development and expression of tolerance, respectively (1997). In addition, others have shown that tolerance can be prevented using Ca^{2+} blockers which further suggests

that Ca^{2+} plays an important role in the expression of tolerance (Bongianni et al., 1986; Ruiz et al., 1993; Diaz et al., 1995; Garaulet et al., 1996).

1.4.1 Role of Delta opioid receptors in opioid tolerance

Delta opioid receptors have been implicated in the development and maintenance of opioid tolerance. In mice pretreated with naltrindole, a highly selective DOR antagonist, the development of morphine tolerance was significantly hindered (Abdelhamid et al., 1991). Similarly, in studies using DOR knockout mice or an antisense oligodeoxynucleotide to knock down DOR expression, morphine tolerance could be prevented (Kest et al., 1996; Zhu et al., 1999). Researchers have found that while DOR is not required for the development and maintenance of MOR analgesic activity, it does appear to play an important role in the development of opioid tolerance.

In an opioid naïve state, the majority of delta opioid receptors are not trafficked to the cell membrane but rather are found either in cytoplasmic compartments or large dense core vesicles (Cheng et al., 1995; Petaja-Repo et al., 2000; Guan et al., 2005). In a persistent opioid exposed state, however, an increase in the density of delta opioid receptors at the plasma membrane has been found (Guan et al., 2005). Agonist binding of DOR results in internalization of the receptors where they undergo either long-term sequestration or lysosomal degradation (Pradhan et al., 2009). Chronic morphine treatment has been shown to result in a greater degree of translocation of DOR to the cell membrane (Cahill et al., 2001; Chieng and Christie, 2009). The increase in cell surface DOR leads to a change in MOR function (Milan-Lobo et al., 2013).

1.4.2 Mu and delta opioid receptor heterodimerization and opioid tolerance

As a result of the affects that delta opioid receptors have on MOR-mediated analgesia, it has been proposed that mu- and delta- opioid receptors interact with each other to form heterodimers (Gomes et al., 2000). MOR and DOR can be found in the same population of neurons and chronic treatment with MOR agonists have been shown to result in the translocation of DOR to the plasma membrane (Cahill et al., 2001; Morinville et al., 2003; Bailey and Connor, 2005). The interaction of the two receptors leads to changes in pharmacological properties of the heterodimer complex (Milan-Lobo and Whistler, 2011). The development of a mixed MOR agonist/DOR antagonist has shown that greater analgesia and less acute tolerance can be produced compared to morphine (Schiller et al., 1999). These results suggest that there is an interaction of mu and delta opioid receptors and that the interaction of these receptors plays an important role in both analgesia and tolerance.

1.5 Thesis Design

Sufficient neuropathic pain relief with high opioid doses is accompanied by an increase in side effects, including sedation and cognition impairment, which leads to poor medication adherence (Morley et al., 2003; Stein et al., 2003a; Watson et al., 2003; Gimbel et al., 2005). Most opioid side effects are attributed to their actions in the central nervous system. Accordingly, peripheral opioid agonists, such as loperamide, have been postulated to have therapeutic potential as an alternative to centrally active opioid agonists (Stein, 2003; Stein et al., 2003a). The goal of developing a peripherally restricted opioid analgesic would be to achieve similar analgesia as a centrally available opioid analgesic while avoiding centrally mediated side effects such as sedation and

addiction. With the recognition that tolerance also develops in peripherally restricted opioids any attempt to enhance peripheral opioid analgesia must also include prevention of tolerance development. **The ultimate goal of this dissertation is to enhance peripheral opioid analgesia and to prevent peripheral opioid tolerance in neuropathic pain conditions.**

To this end, alternative treatments such as gene therapy have been explored in animal models of neuropathic pain (Hao et al., 2003; Meunier et al., 2005; Wolfe et al., 2007; Hao et al., 2009a; Hao et al., 2009b) and in human cancer pain (Goss et al., 2002; Fink et al., 2011). The use of recombinant HSV-1 as a means to deliver genetic material to a targeted region in the peripheral nervous system is particularly beneficial because of its natural propensity for afferent neurons. My lab has previously shown that an overexpression of mu opioid receptor in primary afferent neurons using HSV-1 viral vectors containing the human cytomegalovirus (hCMV) promoter is antinociceptive in the L5 nerve transected rodent model of neuropathic pain (Zhang et al., 2008). The promoter influences the duration of changes in gene expression as well as the afferent population expressing the transgene allowing for targeted gene expression to enhance peripheral opioid analgesia while avoiding CNS mediated side effects. In addition, therapeutic approaches which block peripheral opioid tolerance may increase the effectiveness of opioids in the treatment of chronic neuropathic pain. Studies suggest that mu- and delta-opioid receptor heterodimers are involved in the development of opioid tolerance and that blocking heterodimerization may be a viable therapeutic target to enhance peripheral opioid analgesia.

The following specific aims will be used to test the hypotheses that **injury specific expression of mu opioid receptors can enhance herpes simplex virus-mediated anti-nociception and peripheral opioid tolerance can be prevented by blocking mu- and delta-opioid receptor heterodimers in the periphery in a rodent model of neuropathic pain**

The use of recombinant herpes simplex virus type-1 (HSV-1) as a means to deliver genetic material to a targeted region in the peripheral nervous system is particularly beneficial because of its natural propensity for afferent neurons. The specific afferent neuron populations infected have not been characterized. The first aim of this dissertation was to characterize afferent populations infected with HSV-1 viral vectors using track tracing techniques (Chapter 3).

Previous studies have shown that the use of HSV-1 as a viral vector has produced changes in gene expression in the overall afferent neuron population with antinociceptive effects. The promoter influences the duration of changes in gene expression as well as the afferent population that expresses the transgene. The use of tissue specific promoters in gene therapy has been explored to treat numerous disorders. Using tissue and injury specific targeting of transgenes to distinct afferent populations for the treatment of pain, however, has not been explored. The second aim was to determine whether HSV driven MOR expression can be optimized by using an injury specific promoter (Chapter 4). Galanin, a neuropeptide expressed in the central nervous system, is normally present in low levels in dorsal root ganglia and the spinal cord. After nerve injury that results in neuropathic pain-like behavior, galanin is highly upregulated and is used as a marker of nerve injury (Zhang et al., 1998). I investigated the use of HSV-1 and an injury-specific

galanin promoter to express mu opioid receptors specifically in injured and neighboring primary afferent neurons. To determine whether an injury specific promoter can enhance antinociception and drive expression of MOR in specific populations of afferent neurons, nociceptive behaviors of animals infected with a galanin promoter virus were compared with those of animals infected with a control virus that contains a constitutively active promoter. In addition, MOR expression in the dorsal horn of spinal cords from animals given the galanin promoter virus was compared with that in animals given the control virus.

The third aim was to determine whether peripheral opioid tolerance can be prevented and reversed by blocking MOR-DOR heterodimers in the periphery. Despite the effectiveness of opioids in the treatment of pain, the use of opioids in the treatment of neuropathic pain is controversial due to the development of serious central nervous system side effects. Peripheral opioid analgesics are currently being developed to avoid CNS side effects. As with other opioids, however, tolerance still develops with peripherally restricted opioids. My lab has previously shown that MOR and DOR are co-localized in dorsal root ganglia in rats. In addition to these findings, it has previously been shown that mu and delta opioid receptors can form heteromers with each other and delta receptors can negatively regulate mu opioid receptor mediated analgesia in the periphery. As a result, I hypothesized that peripheral tolerance can be prevented by blocking the formation of mu and delta opioid receptor heterodimerization. In order to block MOR-DOR heterodimers formation, a novel small peptide attached to a TAT carrier protein for delivery into cells was developed. Once inside cells, the TAT protein is cleaved and the peptide inhibitor becomes biologically active. There are three

experiments in this specific aim: the first examines the role of delta opioid receptors in peripheral opioid tolerance, the second evaluates a novel MOR-DOR heterodimers blocking peptide in an *in vitro* model of opioid tolerance, and the third evaluates this novel blocking peptide in an *in vivo* model of peripheral opioid tolerance.

Table 1.1: Opioid receptors, endogenous ligands, precursors for the endogenous ligands, and the exogenous ligands that are most commonly associated with the respective receptors.

Types of receptors	Endogenous ligands	Precursors for endogenous ligands	Exogenous ligands
μ (MOR)	endorphins	Pre-POMC	morphine, loperamide, DAMGO
δ (DOR)	enkephalins	Pre-pENK	naltrindole (ant.), deltorphin II
κ (KOR)	dynorphins	Pre-pDYN	ketazocine

Table 1.2: Gene therapy in the treatment of pain with the associated key findings. Genes of interest include endogenous opioids, precursor proteins, or opioid receptors in the sense or antisense direction. ↑=increase; ↓= decrease; nocicep.= nociceptive; SNL= spinal nerve ligation; CFA= Complete Freund's Adjuvant; perip.= peripheral

Vector	Gene of Interest - Ligand	Pain Model	Key Findings	References
HSV	Enkephalin	Human cancer pain patients	Well tolerated Highest doses: pain relief	(Fink et al., 2011)
HSV	Enkephalin	Bone cancer pain	↓ nocicep. behaviors	(Goss et al., 2002)
HSV	Enkephalin	L5 SNL	↑ antinociception, morphine efficacy ↓ morphine withdrawal symptoms Tolerance: no effect on analgesia	(Hao et al., 2003; Hao et al., 2009a)
HSV	Enkephalin	Trigeminal neuropathic pain	↑ met-ENK ↓ mech. hypersensitivity	(Meunier et al., 2005)
HSV	Enkephalin	Capsaicin, DMSO	Blockade of hyperalgesia Localized antihyperalgesic and analgesic effect	(Wilson et al., 1999; Wilson and Yeomans, 2002; Yeomans et al., 2006)
Plasmid	POMC	Inflammatory (CFA, formalin)	↑ endorphin levels ↓ paw swelling, therm. hyperalgesia, nocicep. behaviors (formalin test)	(Lee et al., 2003; Chuang et al., 2004)
MIDGE (non-viral vector)	POMC	Inflammatory (CFA)	Slight ↓ mech. allodynia	(Machelska et al., 2009)

Vector	Gene of Interest-Receptor	Pain Model	Key Findings	References
HSV	MOR	N/A	↑ MOR immunoreactivity Enhancement of perip. opioid analgesia	(Zhang et al., 2008)
HSV	AMOR (antisense)	N/A	↓ MOR immunoreactivity, DAMGO potency (C-fiber)	(Jones et al., 2003)
AAV	MOR	Inflammatory (CFA)	↑ MOR expression, morphine potency	(Xu et al., 2003a; Xu et al., 2003b; Gu et al., 2005)
AAV	MORS196A	MOR-KO mice	↑ antinociception No tolerance w/ naloxone treatment	(Chen et al., 2010)

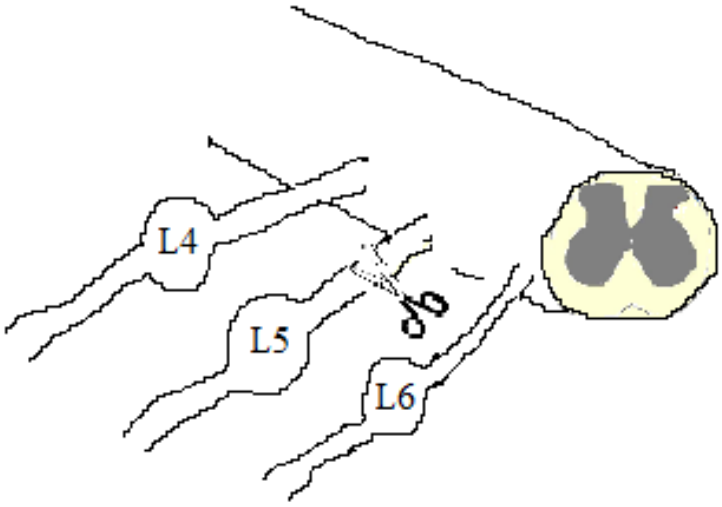

FIGURE 1.1: Schematic for the modified Chung model in which the L5 nerve is transected leaving intact the L4 and L6 nerves.

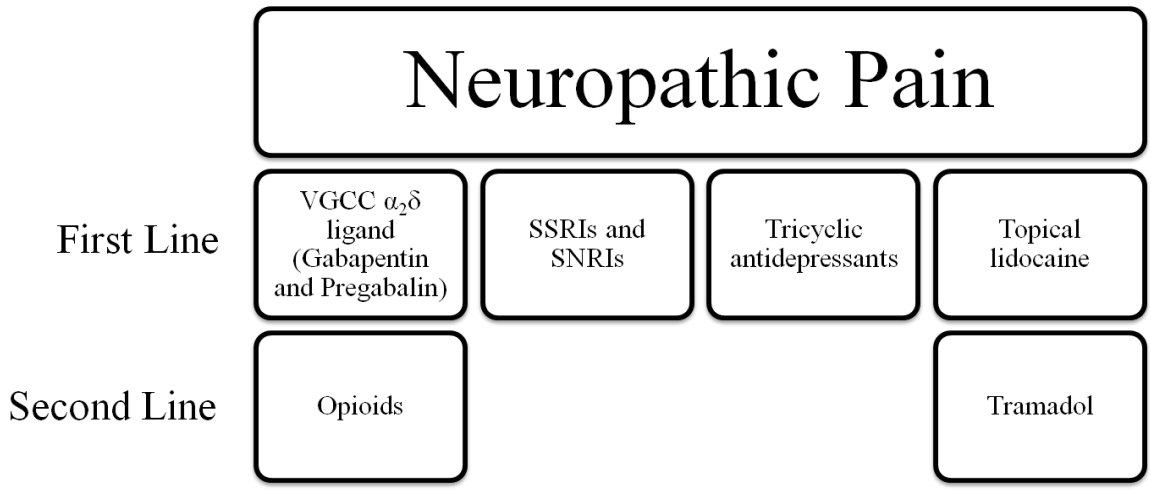

FIGURE 1.2: Schematic for the common treatment regimens for neuropathic pain. VGCC= voltage gated calcium channel; SSRIs= selective serotonin reuptake inhibitors; SNRIs= serotonin-norepinephrine reuptake inhibitors.

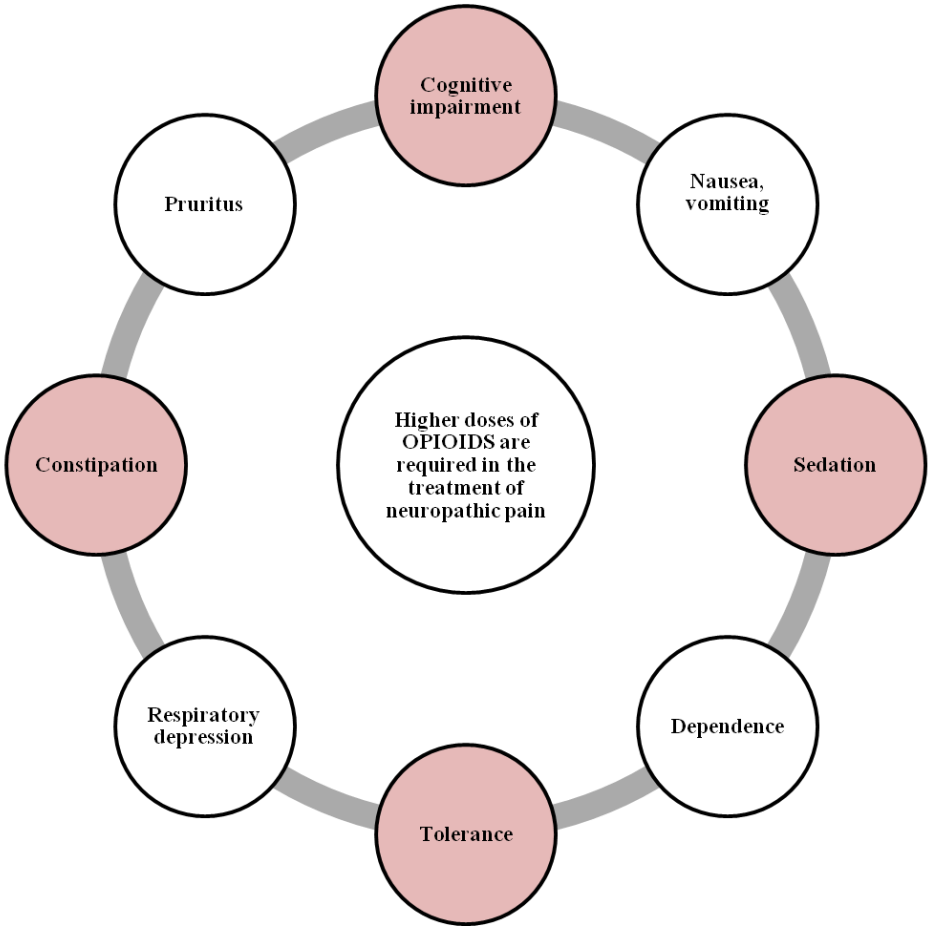

FIGURE 1.3: Schematic of the side effects most associated with high dose opioid treatment.

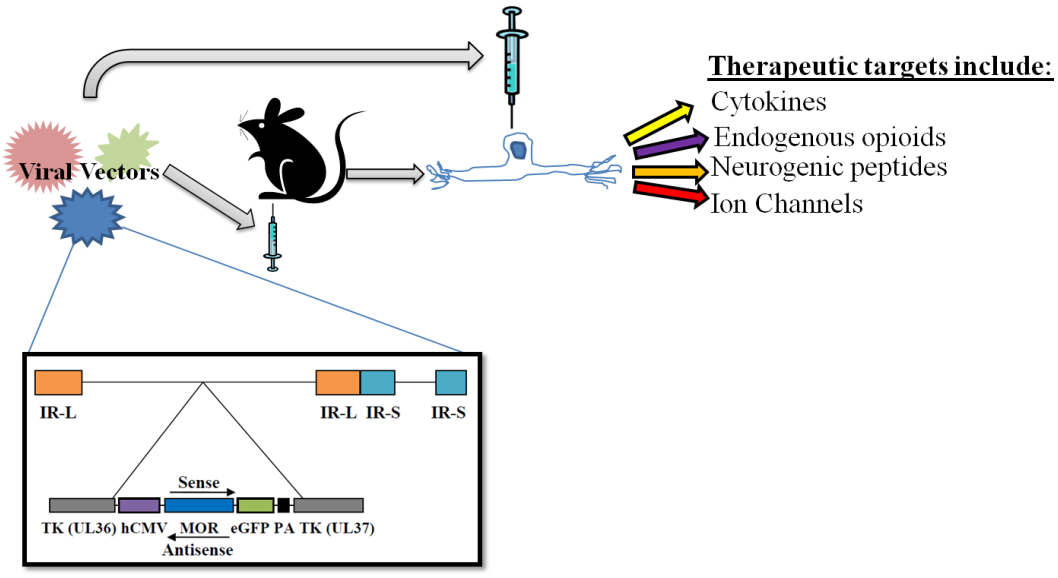

FIGURE 1.4: Schematic showing the routes of virus infection (subcutaneous hindpaw injection or direct injection in DRG) as well as a diagram of an example of a vector. Numerous therapeutic targets are shown that include cytokines, endogenous opioids and receptors, neurogenic peptides, and ion channels.

CHAPTER 2

GENERAL METHODS

2.1 Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of South Carolina. Efforts were made to reduce the number of animals used and to minimize discomfort. Swiss Webster mice (5-6 weeks old, 20-25g, Harlan) were group housed and Holtzman rats (175-225 grams, Harlan) were single housed on a 12-h light/dark cycle with food and water available *ad libitum*.

2.2 Recombinant HSV-1 Vector Infection

The following viral vectors have been previously described (Zhang et al., 2008): hsvCON, the control virus expresses β-galactosidase (*E. coli* lacZ) that is driven by the CMV immediate-early enhancer promoter, previously called SGZ, cmvMOR expresses MOR and is driven by the CMV immediate-early enhancer promoter, previously called SGMOR. A newly constructed virus, galMOR expresses MOR under the control of the galanin promoter. The full-length rat DOR cDNA (HSV-DOR) was cloned into a herpes shuttle plasmid under control of the hCMV promoter in either the sense (HSV-sDOR) or anti-sense orientation (HSV-αDOR) direction. Animals were anesthetized with isoflurane and 10µl of the HSV-1 constructs ($1x10^7$tu/µl) were administered subcutaneously into the left plantar hindpaw.

2.3 Neuropathic Pain Model

For L5 transection surgeries, animals were anesthetized with isoflurane and all surgical procedures were performed under sterile conditions. After maintenance of anesthesia, the fur overlying the lower back (L3-S3) was shaven and cleaned with betadine. An incision over L5-S1 was made and the underlying muscle was teased apart to expose the vertebral transverse processes. The L6 transverse process was partially

removed to reveal the L5 nerve. The L5 nerve was isolated and transected. In sham animals, an identical procedure was followed but the L5 nerve was not transected. After appropriate hemostasis, the fascia and underlying muscle was closed with 3.0 polyester sutures and the skin closed with metal clips for rats and skin adhesive for mice.

2.4 Behavioral Testing

Animals were acclimated to the experimenter and testing environment prior to testing as previously described (McKelvy and Sweitzer, 2009). Animals were placed in plastic boxes over a mesh floor and their responses to mechanical stimuli were quantified as the total number of paw withdrawals to an ascending series of calibrated Von Frey filaments (VF) (0.16, 0.4, 1.0, 2.0, and 4.0g for mice; 2.0, 4.0, 6.0, 10.0, 15.0, 26.0g for rats) applied to the plantar surface of the left hind paw. A positive response was withdrawal of the hindpaw from the filament. Each VF was applied 5 consecutive times at approximately 3 second intervals and the maximum possible withdrawal was 25 responses. Paw withdrawal latency (PWL) to radiant heat stimuli was measured using a plantar stimulator analgesia meter as the time it took until the animal removed its paw. Rodents were placed in Plexiglas enclosures on a heated glass surface. Radiant heat was applied from below to the plantar surface of the left hindpaw with a cutoff time of 20 seconds to prevent tissue damage.

2.5 Immunohistochemistry

Animals were deeply anesthetized with isoflurane then transcardially perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS. Vertebral columns were removed and post-fixed in 4% paraformaldehyde solution for 24 hours followed by isolation of lumber spinal cords and DRGs then stored in 30%

sucrose solution in 0.1M phosphate buffer. Lumber spinal cords (20µm) and DRGs (12µm) were serially sectioned. DRG sections were thaw-mounted onto slides for staining. IHC was performed using the avidin-biotin method for MOR expression and immunofluorescence was used to examine galanin expression. Free-floating spinal cord sections and slide mounted DRGs were washed in Tris-buffered saline (TBS) then quenched for endogenous peroxidase activity. Spinal cord and DRG sections were rinsed 3 times (10 min each) in TBS then blocked in TBS with 0.2% Triton X100 (TBS+) and normal horse serum for 20 min. Primary antibody incubation was done in TBS+ overnight (4°C for spinal cords and room temperature in a humidifier box for DRGs). For immunofluorescence, samples were incubated in primary antibody overnight followed by 3 washes in TBS then incubation in secondary antibody (donkey anti-goat Alexa Flor 594, Invitrogen, 1:200). Spinal cord sections were then mounted onto slides and both DRGs and spinal cords were coverslipped. MOR and galanin expression was examined in both spinal cord and DRG sections. The following primary antibodies were used: MOR (Neuromics, RA10104, 1:1000) and galanin (Santa Cruz, N20, sc-16411, 1:500).

2.5.1 Image Analysis

Digital images were taken and density and area analysis was performed on the spinal cord sections using ImageJ software (National Institutes of Health, Bethesda, MD). To examine MOR expression levels after L5 transection and virus infection, the L5 transected groups were normalized to the uninjured groups to get the percent MOR positive area and density. In dorsal root ganglia, the number of MOR immunoreactive or galanin immunoreactive cell bodies were counted and categorized by size (small,

medium, or large). The total number of small, medium, and large cell bodies was counted and the percentages of MOR positive cells and galanin positive cells were calculated.

2.6 Small Peptide Inhibitor of MOR-DOR heterodimerization

A novel cell permeable small peptide inhibitor of heterdimerization has been developed. Efficient heterodimerization of DOR to MOR requires the 15 amino acids at the C-terminus of the DOR (Val357-Ala372) (Cvejic and Devi, 1997; Fan et al., 2005; Walwyn et al., 2009). The small peptide inhibitor (synthesized by American Peptide) is composed of 15 amino acids at the C-terminus of the DOR. It is conjugated by a di-sulfide bond to the Tat carrier sequence that is derived from HIV Tat. The Tat carrier allows for delivery into the cell. Once inside the cell the di-sulfide bond is cleaved releasing the competitive inhibitor sequence. This cell permeable small peptide delivery system has been previously used to selectively target specific isozymes of protein kinase C (Shumilla et al., 2004; Sweitzer et al., 2004b; Shumilla et al., 2005).

2.7 Osmotic Pump Implantation and Tolerance Induction

Osmotic pumps (Alzet, Model 1003D) were filled with the small cell permeable peptide inhibitor (custom made by American Peptide). The pumps were primed by placing in a 37°C water bath for 6 hours. Pumps delivered 250ng of the peptide per day throughout the experiment. Animals were anesthetized with isoflurane and the implantation site was shaved and cleaned with betadine. A mid-scalpular incision was made and the underlying subcutaneous tissue was separated using a hemostat to create a pocket for the pump. The pumps were subcutaneously implanted between the scapulas. In rats not implanted with the pump (sham group), only a mid-scalpular incision was

made and separation of the subcutaneous tissue was done. The incision was closed with wound clips. Tolerance was induced by injecting the animals subcutaneously with 10mg/kg of morphine or 3mg/kg of loperamide twice a day for 3 consecutive days.

2.8 Human Embryonic Kidney (HEK) 293 Cells:

Mammalian expression vectors encoding full-length cDNAs of MOR and DOR-FLAG, were transfected into human embryonic kidney (HEK) 293T cells (GenHunter, Nashville, TN) using calcium phosphate precipitation as previously described (Lee et al., 2011). DNA was added to cells in a 1:1 ratio (MOR:DOR) for a total of 4 µg. 4 to 6 h later, the cells were treated with a 15% glycerol solution in buffer for 30 s then feeding media (Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin) was added.

2.8.1 Dose Response for HEK293 cells

24h after transfection, cells were incubated with 250ng, 625ng, or 1250ng of peptide inhibitor or vehicle (sterile nanopure water). After 1h of incubation, cells were collected and resuspended in 100ul of chilled lysis buffer [50 mM Tris, 150 nM NaCl, 1% NP-40, 1 mM EDTA, 1% Triton X-100 and Protease Inhibitor Cocktail at 1:100 (Sigma P8340)]. After homogenization, the samples were centrifuged at 14,000g for 10 min at 4 C. The supernatants were collected for co-immunoprecipitation and western blot analysis as described below.

2.9 Co-Immunoprecipitations

Cells were collected and lysed. Briefly, culture media was removed from the cells and the cells were rinsed with 1X phosphate buffered saline (PBS). Using a cell

scraper to detach the cells from the culture plates, the cells were collected into microcentrifuge tubes and maintained on ice. To make sure no culture media remained, cells were pelleted by low speed centrifugation (500g, 5 min), the supernatant was removed, and lysis buffer with protease inhibitor was added to the cells. The cells were incubated at 4°C for 60 min. The cells were then centrifuged at 4°C (14,000g, 10min). Cell lysate supernatants were transferred to a new tube and a BSA protein assay was performed to determine protein concentrations. Resulting cell lysate was incubated with ANTI-FLAG M2 affinity gel (Sigma Aldrich). Elution fractions were collected using low pH buffer and SDS-PAGE was performed.

2.10 Western Blot Analysis

For Co-immunoprecipitations, samples were resolved on a 4-15% reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (35µl of sample was loaded per well). Proteins were transferred to a polyvinylidene difluoride membrane with a Trans-Blot Transfer Cell system (Bio-Rad, Hercules, CA). Membranes were incubated overnight with primary antibody at 4°C. Immunoreactivity was detected using enhanced chemiluminescence (ECL, Pierce Thermo Scientific). The following primary antibodies were used: anti-MOR (Neuromics 1:2000; anti-FLAG M2 (Sigma Aldrich 1:5000). For analysis, a ratio of MOR to FLAG was calculated.

2.11 Chemicals

Morphine sulfate (Baxter Healthcare Corporation, Deerfield, IL), DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin), loperamide HCl (Tocris Bioscience, Minneapolis, MN). All stock solutions were made per the manufacturers' instructions. Morphine and DAMGO solutions were dissolved in distilled water followed by 0.9% saline to achieve

the final concentrations. Loperamide was prepared in 20% CDEX, made by diluting the 40% CDEX/water solution (isotonic) with saline.

2.12 Statistical Analysis

Significant differences between groups were assessed using Student's t tests or one way ANOVA with Bonferroni post test. Results are presented as mean ± SEM. All statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). A $P<0.05$ was considered significant.

CHAPTER 3

CHARACTERIZATIONS OF HSV VECTOR INFECTION PATTERNS

3.1 Introduction

While my laboratory has been using HSV vectors to study pain processing in a variety of preclinical settings, there were still key questions that remained unanswered regarding the characterization of infection and expression of downstream intracellular regulators of opioid function. This first set of studies was important for the general characterization of HSV infection in our neuropathic pain model in mice. These studies filled in holes in my lab's previous research and were important key experiments to determine the feasibility of electrophysiology studies with the virus in the laboratories of our collaborator. This chapter will discuss findings regarding the percentage of primary afferent neurons that innervate a particular area of skin that incorporate the HSV vector following inoculation, the comparison of HSV vector expression in male versus female mice, and the expression of downstream GIRK channels in mouse DRG and spinal cord.

3.1.1. Transduction Efficacy

While it is known that HSV vectors have high transduction efficiency in primary afferent neurons, it is not well documented which afferent populations take up the virus *in vivo* and the levels of gene expression within infected neurons. In this experiment, I wanted to determine the percentage and populations of primary sensory afferents which take up the HSV virus following topical hindpaw infection of a given region of skin. Neuronal tracers were used to elucidate the population of neurons that take up the HSV vector. Cholera toxin B subunit (CTb) and Wheat germ agglutinin (WGA) were used as retrograde tracers in conjunction with an HSV vector that over-expresses β-galactosidase to label neurons that were infected within a specific region of skin. Neuronal tracers can either be transported in a retrograde (axon to soma) or anterograde (soma to axon) fashion. CTb and WGA were used as the neuronal tracers (Neumann et al., 2008). CTb

is typically carried by medium and large-diameter myelinated afferents. CTb binds to a surface ganglioside found on medium and large-diameter DRGs (Neumann et al., 2008). WGA primarily traces small diameter unmyelinated afferents (LaMotte et al., 1991). Research has shown that CTb and WGA label separate populations of neurons as colocalization of CTb and WGA is not seen in dorsal root fibers in the presence or absence of nerve injury (El Maarouf et al., 2005). This characterization was critical to determine if single fiber teased electrophysiology experiments could be carried out in mice infected with HSV vectors. The findings with the track tracing experiment allowed our collaborators to begin single fiber electrophysiology experiments with the HSV vectors.

3.1.2 Sex Differences in Transduction

While sex has been shown to influence transduction efficiency of an adeno-associated viral vector in the liver (Davidoff et al., 2003), expression of gene products has not been compared for male and female mice using an HSV viral vector. The purpose of this experiment was to determine if expression levels of MOR are comparable in male and female mice after infection with a viral vector that overexpresses MOR (SGMOR). Historically my laboratory has only used female mice as older studies with the wild type HSV virus suggested better infection patterns with female mice as compared to male mice. There is a growing literature demonstrating sex differences in pain and analgesia in both rodents and humans (Berkley, 1997; Dixon et al., 2004). In rodents, sex differences observed in different pain models may be strain specific. For instance, in rats it has been shown that female rats display greater mechanical allodynia compared to male rats after L5 spinal nerve transection and this effect is dependent on

strain (DeLeo and Rutkowski, 2000). To avoid any potential confounds of menstrual hormones on electrophysiology teased fiber recordings from one day or recording to the next our collaborators wanted to record from male mice. Thus, this characterization of HSV vector expression in males versus females was critical.

3.1.2 GIRK channel expression

In rats, research has shown that 4 subtypes of GIRK channel subunits are found in DRGs, including GIRK2 (Gao et al., 2007). In contrast, GIRK2-like immunostaining has not been found in mouse DRG neurons and it has been suggested that subunits other than GIRK2 comprise functional GIRK channels in mouse DRGs (Mitrovic et al., 2003; Nockemann et al., 2013). Nockemann *et al.* suggest that GIRK 2 is required for peripheral opioid analgesia and that peripheral opioid analgesia does not exist in mice because they lack the GIRK 2 subunit (2013). In light of my laboratory's previous findings that demonstrated peripheral opioid analgesia in female mice and that peripheral opioid analgesia could be enhanced with HSV vectors encoding for mu opioid receptor, this report on the necessity for GIRK 2 raised an important question about the expression of GIRK 2 in our specific strain of mice. In this experiment, DRGs from female Swiss Webster mice were evaluated by western blot analysis for the presence of GIRK2.

3.2 Specific Methods

3.2.1 Track Tracing

Swiss Webster mice (Harlan, n=3) were infected with SGZ viral vector, 10µl in each hindpaw, under isoflurane anesthesia. Two days after virus infection, mice were injected with 1% WGA (WGA-biotin), 10µl in each hindpaw. Two days after WGA injection, mice were injected with 0.1% CTb (CTb-biotin), 10µl in each hindpaw. Three

days after injection of CTb-biotin, the mice were perfused and DRGs were removed following laminectomy. For laminectomy, the spinal column was exposed by cutting away skin and underlying muscles. The top of the spinal column was cut away as to expose the DRGs for extraction. DRGs were then prepared for immunohistochemistry. DRGs were serially sectioned (16μm) and thaw-mounted onto slides for staining.

Immunohistochemistry was performed using the avidin-biotin method for track tracer (WGA-biotin and CTb-biotin) expression. β-galactosidase (β-Gal) staining was detected using a β-Gal primary antibody (Cortex Biochem Anti β-Gal, 1:500) followed by the avidin-biotin method. A peroxidase substrate (Vector SG Peroxidase Substrate kit) was used to produce a blue/gray reaction to distinguish staining for β-Gal from that of the track tracers. Slides were then dehydrated.

Light microscopy was used to analyze the DRG slices. DRG cell bodies were counted based on general size (small, medium, or large). The shapes of the cell bodies were distinguishable enough that experimenter discretion was used to divide the cells into small, medium, and large sizes. Small sized cell bodies correspond to C fibers, medium cell bodies correspond to Aδ fibers, and large sized cell bodies correspond to Aβ fibers. Cells were further divided in to whether they were stained for the virus (blue/gray), the tracers (brown), or both colors.

3.2.2 MOR Expression in Male Compared to Female Mice

Eight week old male and female Swiss Webster mice (Charles River) were used for this experiment. Some mice were infected with the SGMOR virus, 10μl in the left hindpaw (control males, n=2, SGMOR males, n=3; control females, n=2, SGMOR females, n=3). On days 1, 3, 5, and 7, the mice underwent behavioral testing (von Frey

and Hargreaves plantar tests). On day 7, the animals were perfused for IHC. L4-L6 spinal cord sections were examined for MOR expression.

For statistical analysis, two way ANOVA with Bonferroni post test to compare all groups was used.

3.2.3 GIRK2 in Mouse DRG

Western blot analysis was performed on DRG cell lysates from both L5 transected and control (uninjured) Swiss Webster mice. As controls, samples from rat DRG were included in the analysis (Alomone Labs, Anti-K_{ir}3.2 APC-006, 1:250). β-actin (Sigma Aldrich, 1:75,000) was used as a loading control. Protein bands were densitized using Scion Image. Data is presented as a ratio of the optical density of GIRK2 to β-actin.

For statistical analysis, one way ANOVA with Bonferroni post test to compare all columns was used. Data is presented as mean ± SEM.

3.3 Results

3.3.1 Track Tracing

Immunohistochemical analysis of the tract tracer expression in L3 and L4 DRGs was assessed following dorsal (hairy surface) administration of track tracers and HSV vectors. The number of small, medium, and large diameter neurons in the L3 and L4 DRG with tract tracer was counted by general visualization of the sizes of the cells. Approximately 11-14% of small diameter neurons in the L3 and L4 DRG arise from the dorsal hairy surface of the hindpaw (Fig 3.1A). For the medium and larger neurons there was differential innervation in L3 and L4 DRGs such that a larger percentage of medium (12% versus 4%, respectively) and large (17% versus 5%, respectively) neurons that innervate the dorsal hairy surface reside in the L4 DRG as compared to the L3 DRG.

Analysis of HSV mediated expression of β-galactocidase showed afferent population specificity for infection and expression of the transgene (Fig 3.1B). A larger percentage of small diameter afferent neurons expressed β-galactocidase (16-24%) compared to medium (4-17%) and large (2-6%) diameter afferent neurons. There was no difference in the percentage of afferent populations that express transgene in L3 versus L4 DRG.

Similar percentages of small, medium, and large diameter afferent neurons were co-labeled with track tracer and β-galactosidase in the L3 and L4 DRG (Fig 3.1C). There was a size difference in the percentage of double labeled neurons with an increasing percentage of the population being double labeled with increasing cell size. Approximately 28-37% of small diameter neurons, 33-45% of medium diameter neurons, and 51-58% of large diameter neurons were double labeled with track tracer and β-galactosidase (Fig 3.1C). A two way ANOVA analysis of the data showed that size was a statistically significant predictor of co-labeling ($p=0.0026$) with increased relative percentages in larger diameter afferent neurons. The increased percentages in large diameter neurons could be due to differences in the total number of the different sized cells as there are few large diameter neurons in DRG at the level of L3-L5 compared to small and medium diameter neurons.

3.3.2 MOR Expression in Male Compared to Female Mice

Expression of MOR in the dorsal horn of male and female mice was evaluated using immunohistochemistry. Seven days after infection with SGMOR, the ipsilateral area of staining was significantly larger for both male and female mice compared to the contralateral side (Fig 3.2A, B). There were no significant differences for the density of

MOR immunostaining between male and female mice in either the control or virus treated animals (Fig 3.2C).

3.3.3 Nociceptive Behaviors in Male Compared to Female Mice after SGMOR infection

Nociceptive testing was performed on days 1, 3, 5, and 7 after virus infection (Fig 3.3A). Behaviorally, female mice had a significantly greater number of paw withdrawals compared to male mice on days 3 and 7 after virus infection (Fig 3.3B). There were no significant differences in withdrawal latency between female and male mice (Fig 3.3C).

3.3.4 GIRK2 in Mouse DRG

Western blotting of mouse DRG and spinal cord showed that both contained GIRK2 but at lower expression levels than that in rat DRG (Fig 3.4). In mouse DRG, there is 2-fold less GIRK2 expression compared to rat DRG (0.7577 ± 0.08477, 0.3837 ± 0.06287). In mouse spinal cord, there is a 4-fold less GIRK2 expression compared to rat DRG (0.7577 ± 0.08477, 0.1940 ± 0.04376).

3.4 Discussion

This chapter characterized the specific populations of primary afferent neurons that take up and express HSV vectors within a particular area of skin, compared the expression of HSV vector expression in male versus female mice, and confirmed the expression of downstream GIRK channels in mouse DRG and spinal cord. These studies form the foundation for both our laboratories previous published studies and for the current electrophysiology experiments in our collaborators' laboratory.

3.4.1 Track Tracing

As both CTb and WGA were used as track tracers, these studies were able to count small, medium, and large diameter primary afferent populations. The track tracing

experiment found increased innervation of L4 DRG with medium and large neurons compared to L3 DRG which suggests larger role of L4 DRG in sensory sensation and motor function from the dorsal hairy hindpaw when compared to L3 DRG. At a neuropathic pain modeling level this confirms what researchers have observed which is that an intact L4 spinal nerve is crucial for generating neuropathic pain-associated behaviors in models of L5 and L6 transection or constriction (Kim and Chung, 1991, 1992; Yoon et al., 1996). Furthermore, accidental transection of L4 spinal nerve leads to motor weakness and thus confounds analysis of pain-related behaviors which is supported by the increased innervation from larger afferent motor fibers. Interestingly, HSV induced expression of the β-galactosidase gene was highest in small diameter afferents and lowest in large diameter afferents. The higher percentage of small diameter cells expressing transgene compared to that being track traced from the skin suggests that there is either some spread of viral infection beyond the initial area of infection, that track tracer for small diameter afferents is not efficiently taken up and transported, or that this variability is a result of relatively small animal numbers (n=3/group). Overall, 30-60% of the afferent neurons which innervate a specific area of skin are infected and produce transgene. This suggests a high level of transduction efficacy with HSV vectors in our model and is in line with our previous reported findings of mu opioid receptor expression using an HSV vector encoding mu opioid receptor (Zhang et al., 2008).

3.4.2 MOR Expression in Males versus Females

Sex-linked differences have been found in KOR distribution in the dorsal horn of the spinal cord in rodents and differences in opioid receptor density have been found in brain regions like the periaqueductal gray (Harris et al., 2004; Loyd et al., 2008). To my

knowledge, sex differences in MOR density and area in an overexpression system have not been explored. The results show that while there is a greater area of MOR immunoreactivity on the ipsilateral side compared to the contralateral side, there are no significant differences between male and female mice for either side. Similarly, no differences were found in the density of immunoreactivity between the sexes. These results suggest that sex-related hormones have no effect on MOR expression after virus infection. As such, it was concluded that the use of male mice in electrophysiology teased fiber recordings would not have confounding consequences in the interpretation of results from HSV vector experiments using female mice.

Results for sex differences in nociceptive sensitivity have been mixed depending on the strain and species of animal. In many of the studies that evaluated sex differences in pain assays, female rodents were shown to be more sensitive to nociceptive stimuli while in others males displayed a greater sensitivity to noxious stimuli (Mogil et al., 2000). Experiments looking at opioid sensitivity in rodents, however, have yielded more consistent results that males are more sensitive to morphine or other opioids than females (Mogil et al., 2000). Female mice in the experiment presented here had a greater number of paw withdrawals compared to males on days 3 and 7 after virus infection and in the absence of L5 spinal nerve transection. While there were no significant differences in thermal paw withdrawal latency, female mice appeared to be slightly more sensitive on day 5 after virus infection. These findings suggest that virus injection into the dorsal hindpaw of female mice particularly may have a sensitizing effect on the animals and this may be something to take into account when interpreting results from experiments that use male rather than female rodents.

3.4.3 GIRK2 Expression

GIRK channels are opened by interaction of the channel with the $G_{\beta\gamma}$ dimeric protein of the G protein complex. Once the channel opens, the cell becomes permeable to potassium and hyperpolarization occurs. There are 4 subunits that can make up GIRK channels, 3 of which (GIRK1-GIRK3) are widely expressed in the CNS. GIRK channels are tetramers that can either be homo- or heterotetrameric. GIRK2-containing channels are thought to play an important role in opioid signaling in the nervous system (Mitrovic et al., 2003; Nockemann et al., 2013). When opioids bind to postsynaptic opioid receptors, these receptors are linked to GIRK channels and hyperpolarization of the cell can occur. GIRK2 channels are found in rat DRG but have been speculated to not be found in mouse DRG (Mitrovic et al., 2003; Nockemann et al., 2013). The results from this experiment showed that while significantly less than in rat DRG, GIRK2 is expressed in mouse DRG. These results likely suggest a strain difference in GIRK2 expression in DRG as the mice used here are Swiss Webster while the mice exhibiting a lack of GIRK2 in DRG were C57Bl6-J mice. Surprisingly, the results from this experiment show less GIRK2 expression in mouse spinal cord than that in the DRG. Others have shown GIRK2 expression in the superficial layers of the dorsal horn (Marker et al., 2004). Since whole spinal sections were collected and not just the dorsal horns, this may explain that low levels of expression in the spinal cord that were seen in this experiment.

In summary, HSV transduction of primary sensory afferents is efficient and transgene expression is comparable in male and female mice. Additionally, it was proven that GIRK2 is present in DRG of mice providing evidence that peripheral opioid analgesia is possible in mice as GIRK2 is necessary for this to occur. Overall, these

results provided the necessary information to proceed with experiments using HSV vectors to further explore gene therapy in the enhancement of opioid analgesia in the treatment of neuropathic pain.

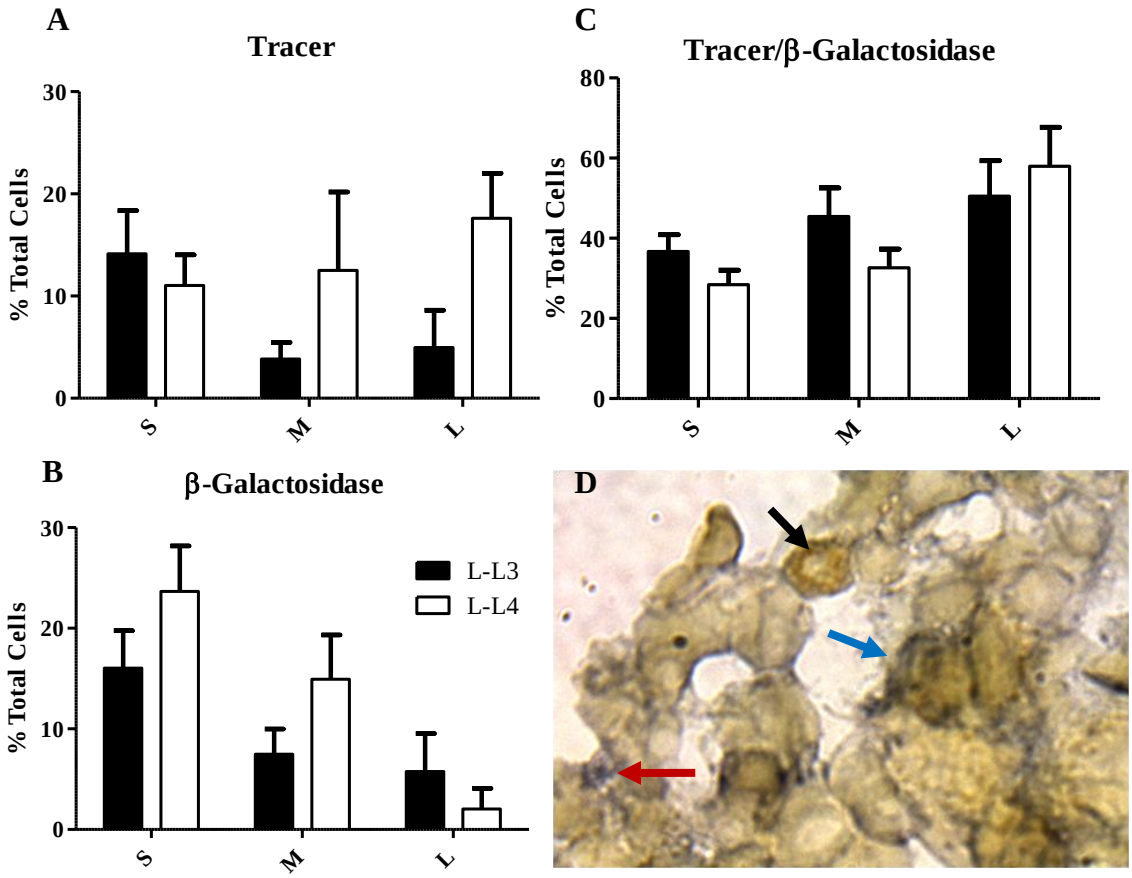

FIGURE 3.1: The percentage of small, medium, and large neurons that took up track tracer was calculated (%total cells = number of positive small, medium, or large cells/the total number of small, medium, or large cells, respectively in a section x 100). Results show that approximately 11-14% of small diameter neurons in the L3 and L4 DRG originate from the dorsal hairy surface of the hindpaw (A). For the medium and large diameter neurons, a greater percentage of medium (12% versus 4%, respectively) and large (17% versus 5%, respectively) neurons reside in the L4 DRG than the L3 DRG (A). HSV mediated expression of β-galactosidase showed that a larger percentage of small diameter afferent neurons expressed β-galactocidase (16-24%) compared to medium (4-17%) and large (2-6%) diameter afferent neurons (B). There were no significant differences in the percentage of afferent populations that express β-galactosidase in L3 versus L4 DRG. In doubled labeled neurons, approximately 28-37% of small diameter neurons, 33-45% of medium diameter neurons, and 51-58% of large diameter neurons were labeled for both track tracer and β-galactosidase (C). No difference in the percentage of neurons double labeled for track tracer and β-galactosidase in L3 versus L4 DRG was found. Panel D shows a β-galactosidase labeled cell body (red arrow), track tracer labeled cell body (black arrow), and a double labeled cell body (blue arrow).

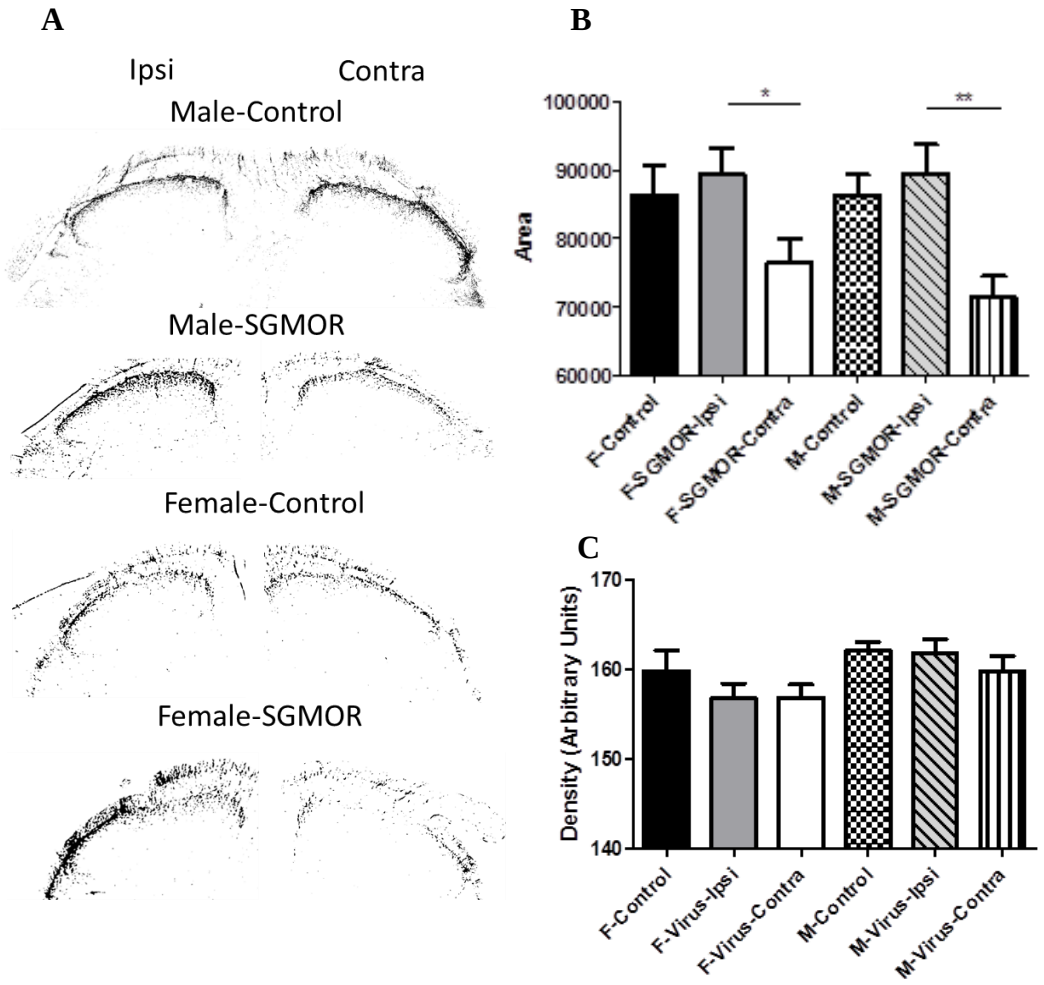

FIGURE 3.2: Representative images of MOR expression in male control, male SGMOR virus, female SGMOR virus, and female control mice (A). Ipsi (Ipsilateral) represents the side of virus injection and contra (contralateral) is the opposite side. The area of staining for MOR expression (B) shows a significantly greater area of staining in the ipsilateral side compared to the contralateral side in virus treated animals. The density of MOR positive staining reveals no significant changes between either virus and control mice or ipsilateral and contralateral side of infection with virus (C). (*P<0.05, **P<0.01)

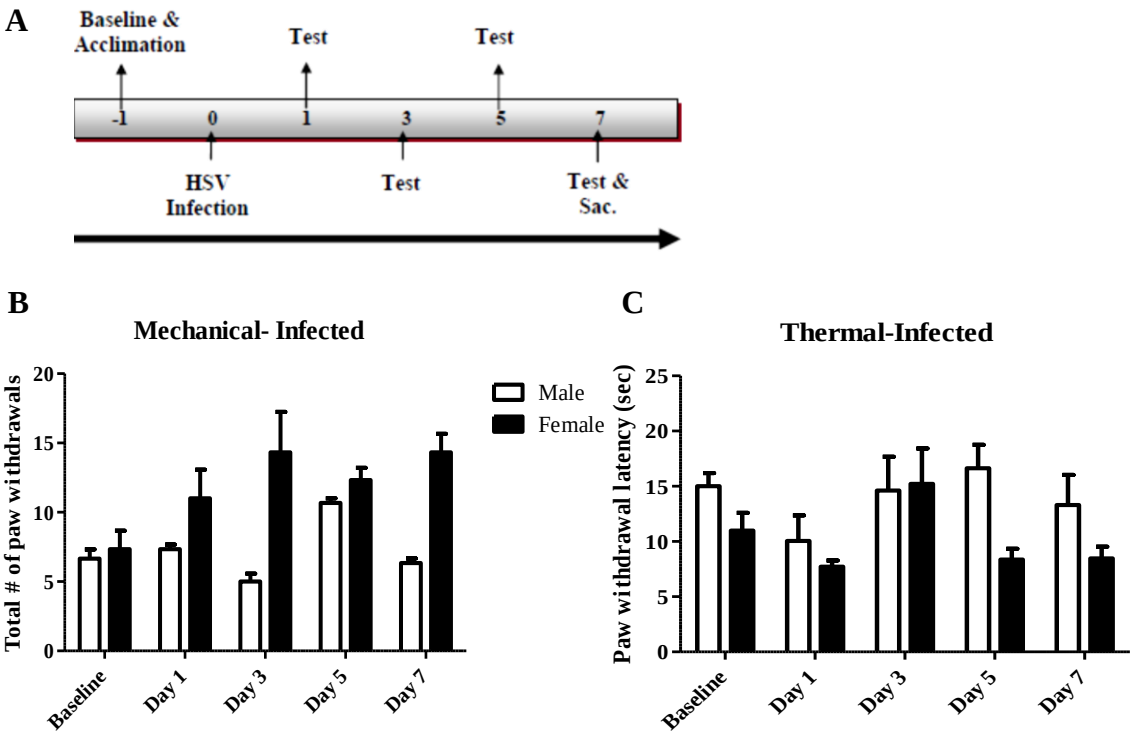

FIGURE 3.3: The timeline for behavioral portion of experiment (A). Mechanical behaviors after virus infection show significantly a higher number of total paw withdrawals in female mice compared to male mice on days 3 and 7 ($p<0.05$) while no significant differences were observed on days 1 and 5 (B). Thermal behaviors reveal no significant differences between male and female mice on any days of testing (C).

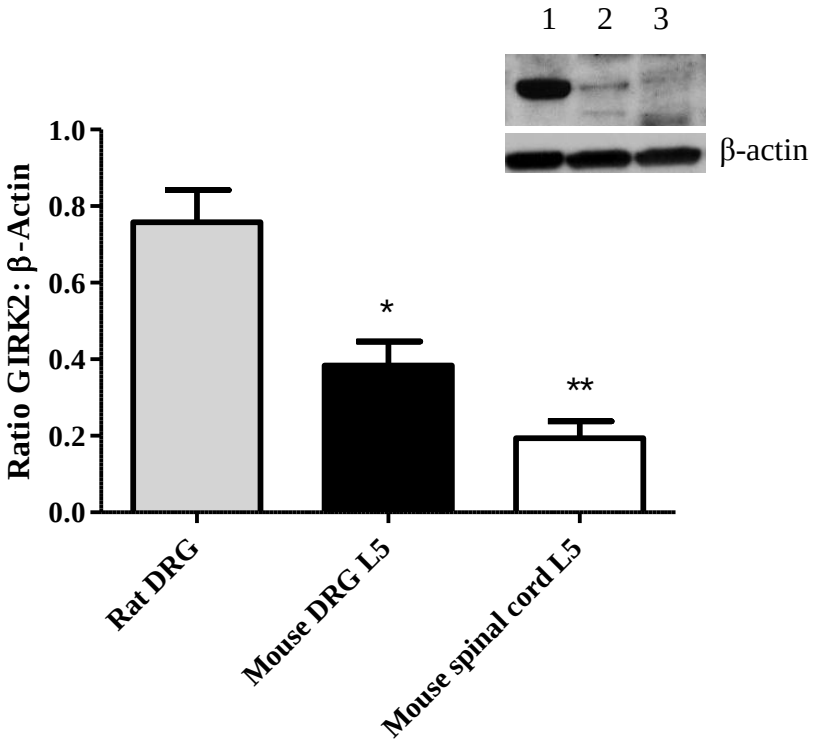

FIGURE 3.4: Western blot analysis for GIRK2 expression revealed lower levels of expression in mouse DRG and spinal cord compared to rat DRG. There are two-folds less GIRK2 expression in mouse DRG ($p<0.05$) and four-folds less expression in mouse spinal cord compared to rat DRG ($p<0.01$). Inset image are representative bands from blotting membrane (1: rat DRG, 2: mouse DRG, 3: mouse spinal cord)..

CHAPTER 4

INJURY-SPECIFIC PROMOTERS ENHANCE HERPES SIMPLEX VIRUS MEDIATED GENE THERAPY FOR TREATING NEUROPATHIC PAIN IN RODENTS[1]

[1] S.N. Smith, C.Paige, K.T. Velazquez, T.P. Smith, S. N. Raja, S.P. Wilson, S.M. Sweitzer submitted to *Journal of Pain*, 04/09/2014.

4.1 Introduction

Chronic neuropathic pain often decreases quality of life, incurs high medical costs (Berger et al., 2004; Jensen et al., 2007) and is difficult to treat. The efficacy of current treatments varies across individuals and is associated with adverse side effects. Alternative treatments such as gene therapy are being explored in animal models of neuropathic pain (Hao et al., 2003; Meunier et al., 2005; Wolfe et al., 2007; Hao et al., 2009a; Hao et al., 2009b) and in human cancer pain (Goss et al., 2002; Fink et al., 2011). The use of recombinant herpes simplex virus type-1 (HSV-1) as a means to deliver genetic material to a targeted region in the peripheral nervous system is particularly beneficial because of its natural propensity for afferent neurons. Studies to date using HSV-1 as a gene therapy vector have produced changes in gene expression in the overall afferent neuron population with antinociceptive effects. The promoter influences the duration of changes in gene expression as well as the afferent population expressing the transgene. The use of tissue specific promoters in gene therapy has been explored to treat liver carcinomas (Foka et al., 2010; Xu et al., 2011), cardiac ischemia (Wei et al., 2011), and neurological disorders (Bockstael et al., 2008). However, using tissue and injury specific targeting of transgenes to distinct afferent populations for the treatment of pain has not been explored.

Galanin is normally present in low levels in dorsal root ganglia and the spinal cord. After injury that results in neuropathic pain-like behavior, galanin is highly upregulated proportionate to the extent of constriction injury and is used as a marker of nerve injury (Merchenthaler et al., 1993; Carlton and Coggeshall, 1996; Rydh-Rinder et al., 1996; Coronel et al., 2008). In the present study, I investigated the use of HSV-1 and

an injury-specific galanin promoter to express mu opioid receptors specifically in injured primary afferent neurons. To determine whether an injury specific promoter can enhance antinociception and drive expression of MOR in specific populations of afferent neurons, I compared animals infected with the galanin-promoter virus (galMOR) to those infected with the CMV-promoter virus (cmvMOR).

4.2 Specific Methods

4.2.1 Animals

Female Swiss Webster mice (5-6 weeks old, 20-25g, Harlan) were group housed on 12-h light/dark cycle with food and water available *ad libitum*.

4.2.2 L5 Transection

For L5 transection surgeries, animals were anesthetized with isoflurane and all surgical procedures were performed under sterile conditions. An incision over L5-S1 was made, the L6 transverse process was partially removed and the L5 nerve was isolated and transected. In sham animals, an identical procedure was followed but the L5 nerve was not transected. The fascia was closed with 3.0 polyester sutures. Seven days after L5 transection surgery, the mice were infected with virus as previously described (Wilson et al., 1999).

4.2.3 Viral Constructs and Infection

The following viral vectors were used: hsvCON, cmvMOR, and galMOR (Fig 4.1). Animals were anesthetized with isoflurane and 10µl of the HSV-1 constructs ($1x10^7$tu/µl) were administered into the left plantar hindpaw.

4.2.4 Behavioral Testing

The responses to mechanical stimuli were quantified as the total number of paw withdrawals to the following Von Frey filaments (VF): 0.16, 0.4, 1.0, 2.0, and 4.0g. Each VF was applied 5 consecutive times at approximately 3 second intervals and the maximum possible withdrawal was 25 responses. Paw withdrawal latency (PWL) to radiant heat stimuli was measured using a plantar stimulator analgesia meter as the time it took until the animal removed its paw with 20 seconds being the cutoff time. Baseline behavioral responses were measured prior to surgery and prior to infection with HSV-1 vectors. On the last day of testing, mice were deeply anesthetized for transcardiac perfusion.

4.2.5 Immunohistochemistry

Lumber spinal cords (20µm) and DRGs (12µm) were serially sectioned. DRG sections were thaw-mounted onto slides for staining. IHC was performed using the avidin-biotin method for MOR expression and immunofluorescence was used to examine galanin expression. MOR and galanin expression was examined in both spinal cord and DRG sections. The following primary antibodies were used: MOR (Neuromics, RA10104, 1:1000) and galanin (Santa Cruz, N20, sc-16411, 1:500).

4.2.6 Image Analysis

Digital images were taken and optical density measurements were obtained and area analysis was performed on the spinal cord sections using ImageJ software National Institutes of Health, Bethesda, MD. The average pixel density of MOR positive staining was measured and the area from which this measurement was taken was calculated for each section and recorded. To examine MOR expression levels after L5 transection and

virus infection, the L5 transected groups were normalized to the uninjured groups to get the percent MOR positive area and density. In dorsal root ganglia, the number of MOR immunoreactive or galanin immunoreactive cell bodies were counted and categorized by size (small, medium, or large). The total number of small, medium, and large cell bodies was counted and the percentages of MOR positive cells and galanin positive cells were calculated.

4.2.7 Statistical Analysis

Significant differences between groups were assessed using one way ANOVA with Bonferroni post test or Student's t tests.

4.3 Results

Galanin was up-regulated in the ipsilateral dorsal horn of L5 transected but not sham surgery mice (Fig 4.2). Infection with cmvMOR or galMOR did not alter galanin expression in the ipsilateral or contralateral spinal cord in sham or nerve injured mice (Fig 4.2E).

4.3.1 Neuropathic Pain Behavior

L5 nerve transected animals displayed an increase in total number of paw withdrawals to mechanical stimuli and a decrease in paw withdrawal latency to heat on day 7 post-transection. Mice were randomized to 3 groups and infected on day 7 with hsvCON, cmvMOR, or galMOR. L5 nerve transected mice that received the control virus did not show a decrease in nociceptive behaviors. Those that received either the cmvMOR or galMOR virus, in contrast, exhibited a reduction in nociceptive behavior (a decrease in paw withdrawals and an increase in paw withdrawal latency after virus infection) compared to the control virus group (cmvMOR vs. hsvCON, $p<0.05$). The

mice that received the galMOR virus, however, displayed the greatest and most sustained reduction mechanical allodynia (galMOR vs hsvCON, $p< 0.01$, 0.001; galMOR vs cmvMOR, $p< 0.05$) and thermal hyperalgesia (galMOR vs hsvCON, $p<0.05$, 0.01, 0.001; galMOR vs cmvMOR, $p<0.05$) (Fig 4.3).

4.3.2 Spinal Cord and DRG Immunohistochemistry

Immunohistochemistry of spinal cords from the L5 transected control virus group revealed a significant decrease ($P<0.05$) in MOR positive staining in the dorsal horn of the lumbar spinal cord sections compared to the uninjured group (Fig 4.4A-D). Spinal cord sections from L5 transected cmvMOR treated animals showed an increase in the area and density of MOR positive staining throughout the dorsal horn (Fig. 4.4A,B,E), while sections from L5 transected galMOR treated mice revealed a significant increase in the density ($p<0.001$) but not the area of MOR positive staining in the superficial lamina of the dorsal horn compared to the control virus infected L5 transected mice (Fig 4.4A,B,F). These results suggest that the galanin promoter drives gene expression in a specific population of neurons, particularly those in the superficial lamina of the dorsal horn. In examining the dorsal root ganglia, no significant differences in galanin staining were observed in any of the different treatment groups or between sizes of the DRG cells (Fig 4.4G). Mice treated with the galMOR and cmvMOR viruses, however, exhibited a higher degree of MOR staining in small and medium sized DRG cells compared to the hsvCON group (Fig 4.4H)

4.4 Discussion

This study explored whether the use of an injury specific promoter to drive MOR expression would produce greater anti-nociception and greater specificity of expression

to nociceptive afferents than a non-specific global promoter. Behaviorally, use of the galanin promoter to drive MOR expression produced a significant and sustained reduction in nociceptive behaviors compared to the non-specific CMV promoter. Immunohistochemical analysis of MOR expression in the spinal cord suggests a more limited pattern of transgene expression across the lamina of the spinal cord with the galanin promoter compared to the CMV promoter. Coupled with the more limited anatomical profile of transgene expression the galanin promoter appears to drive higher levels of transgene expression compared to the CMV promoter. Galanin has been shown to be involved in pain modulation. Following peripheral nerve injury, galanin is upregulated and there is increased transport to the dorsal horn of the spinal cord (Colvin et al., 1997; Colvin and Duggan, 1998). In terms of future translation of these studies to humans, it has been reported that in monkey DRG neurons, galanin is the only neuropeptide that is found to be strongly up-regulated after nerve injury (Zhang et al., 1993). When increased, galanin potentiates the analgesic effects of endogenous (Zhang et al., 2000; Xiong et al., 2005; Jin et al., 2010) and exogenous opioids (Wiesenfeld-Hallin et al., 1990; Przewlocka et al., 1995).

HSV-1 infects both Aδ and C afferent fiber types and it appears that presynaptic mu-opioid receptors contribute more to C-fiber mediated behavioral responses rather than Aδ mediated responses (Jones et al., 2003). In a similar manner, galanin is primarily produced by C fibers. Our previous studies with the cmvMOR construct have shown up-regulation of expression of MOR in small, medium, and large diameter DRG neurons. This increased expression of MOR across all DRG neuronal populations underlies the expanded area of MOR expression into deeper dorsal horn lamina. In parallel with this

change in MOR afferent expression patterns there is a development of increased mechanical and thermal thresholds.

Since this study only examined MOR expression at a single time point it cannot be determined if the different time course and degree of pain reversal is due to only the differential distribution that is observed on the final day of the behavioral analysis. Alternatively, the differences in time course of reversal may be due to differential timing of virally induced MOR up-regulation. Similarly the increased degree of pain reversal in the galMOR infected animals may be due to increased upregulation of MOR expression in DRG neurons at both peripheral and central terminals. Future studies are needed to assess these possibilities.

Based on the involvement of galanin in opioid analgesia and its co-localization with endogenous opioids in the superficial layers of the dorsal horn of the spinal cord, it is likely that the galanin promoter drove MOR gene expression in a specific population of neurons since a reduced area and an increased density were observed. Morphine or naloxone application to spinal cord neurons have revealed that deeper wide dynamic range (WDR) neurons respond more to high frequency stimulation compared to more superficial neurons suggesting a greater role for deeper neurons in neuronal sensitization than the response to acute noxious inputs (Xu et al., 2013). Similarly, in MOR knockout mice, neuronal sensitization is greatly enhanced in deep WDR neurons while naloxone precipitation of a wind up response in wild type mice is significantly enhanced in both deep and superficial WDR neurons(Guan et al., 2006). These findings suggest that there are differential effects of MOR activation on spinal nociceptive transmission that is dependent on the depth of MOR containing neurons in the dorsal horn of the spinal cord.

The benefit of using specific promoters over more general promoters is that gene delivery can be restricted to a localized region. Currently, only tissue and cell type specific promoters have been studied extensively (Andersen et al., 1992; Kaplitt et al., 1994; Nettelbeck et al., 2000; Paterna et al., 2000). While effective, transgene expression is indiscriminately turned on, even in the absence of injury; overexpression of the transgene in the absence of injury would produce analgesia which could lead to an increased risk of damage. In conclusion, by using an injury specific promoter such as the galanin promoter, transgene expression will potentially only occur in neurons and tissues relevant to nociception and will be turned on in the presence of injuries that result in pain leading to more efficient gene therapy in the treatment of neuropathic pain.

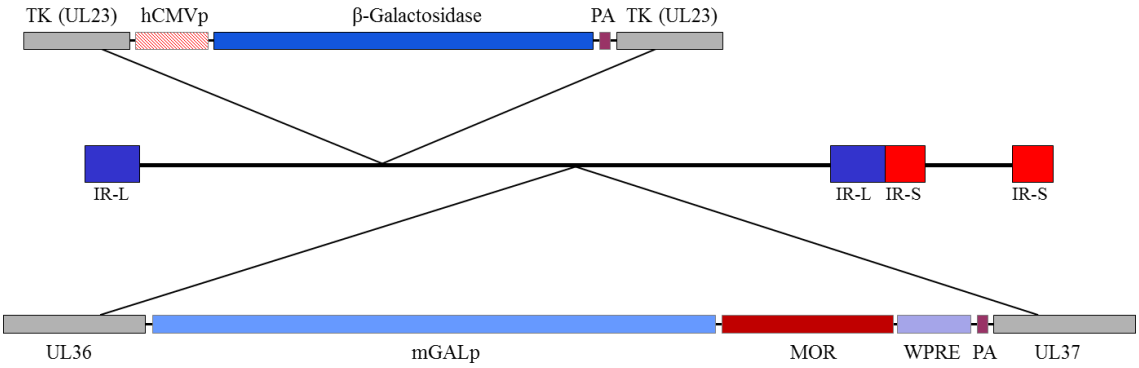

FIGURE 4.1: Schematic diagram of galMOR recombinant herpes vector. Expression of β-galactosidase (*E. coli* lacZ) is driven by the hCMV immediate-early enhancer-promoter. Expression of the rat mu opioid receptor is driven by the mouse 4.6-kb galanin promoter (Bacon et al., 2007). This transcription cassette contains a woodchuck hepatitis virus element (WPRE) to enhance RNA stability. PA, polyadenyation signal; IR, internal repeat; L, long; S, short.

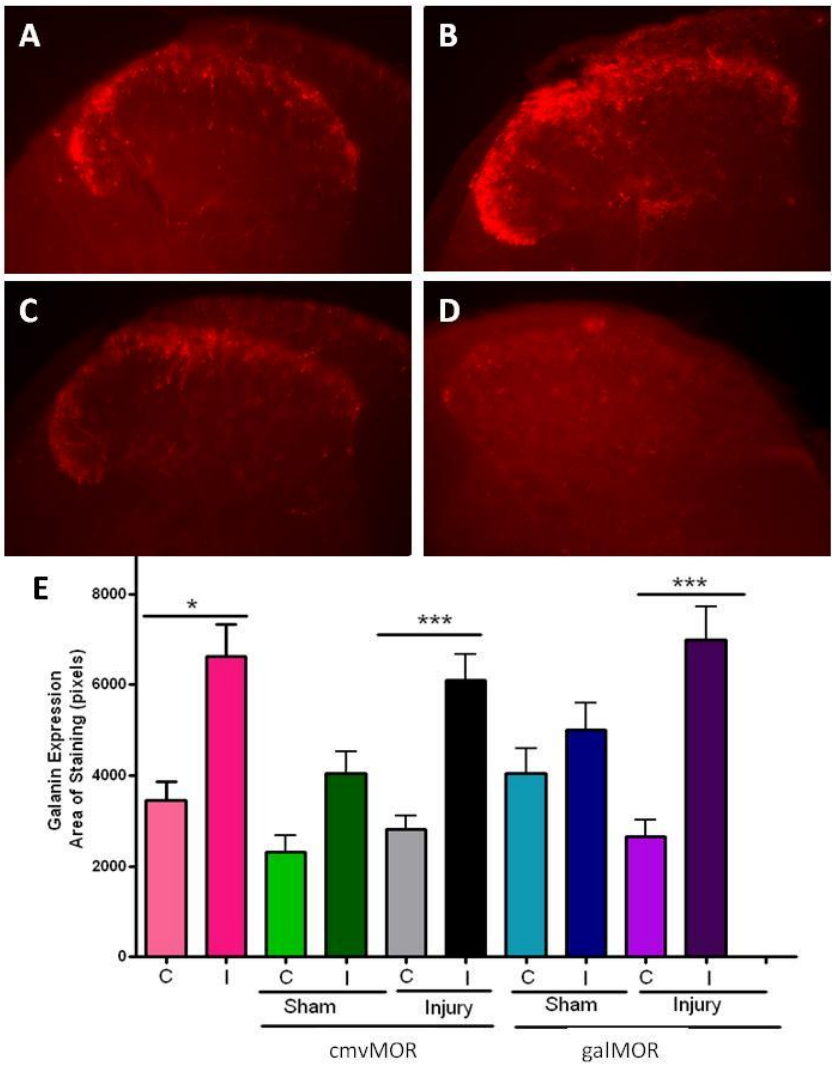

FIGURE 4.2: Galanin is similarly up-regulated in the ipsilateral (i) dorsal horn of the spinal cord in uninfected mice (A), galMOR infected mice (B), and cmvMOR infected mice (C) following L5 spinal nerve transection. Galanin is not upregulated in the contralateral (c) dorsal horn of L5 spinal nerve transected mice infected with cmvMOR (D). Galanin is up-regulated only in the ipsilateral dorsal horn of L5 spinal nerve transected mice and is not altered by viral infection (E). $^{*,***}p<0.05, 0.001$.

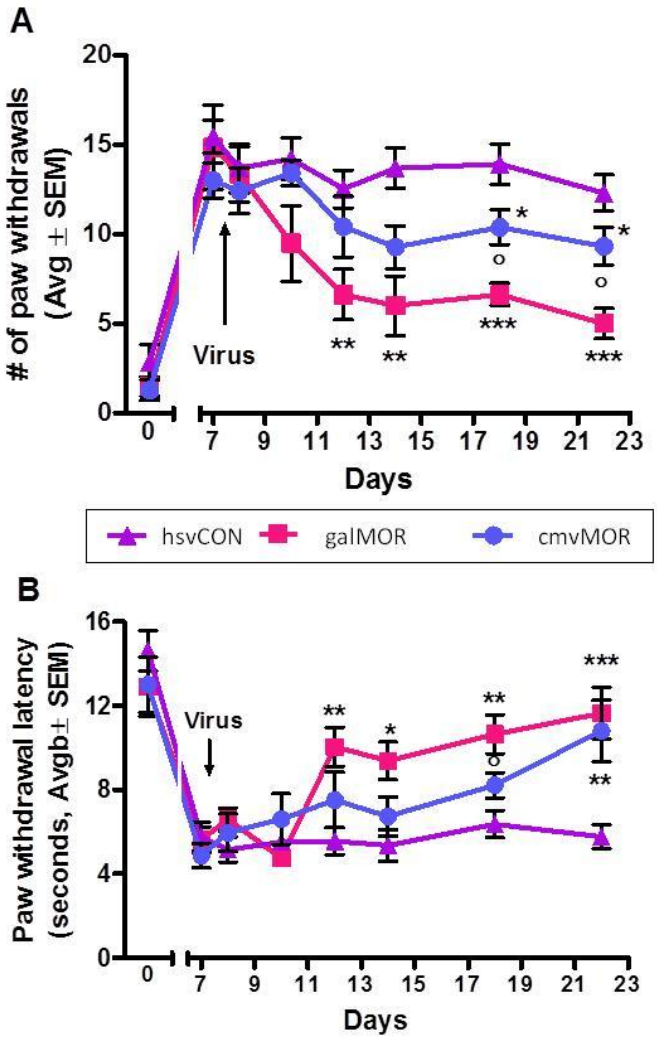

FIGURE 4.3: L5 spinal nerve transection produced mechanical allodynia (A) and thermal hyperalgesia (B) that was differentially reversed by infection with galMOR and cmvMOR viral constructs on day 7 post-injury. Using the galanin promoter to drive expression of the MOR (galMOR) produced a rapid and robust reversal of allodynia and hyperalgesia. In contrast, using the CMV promoter to drive expression of MOR produced a partial reversal of allodynia and hyperalgesia that was slower in on-set compared to the galMOR vector. *, **, *** $p<0.05$, 0.01, 0.001 versus hsvCON control. ◦$p<0.05$ between galMOR and cmvMOR.

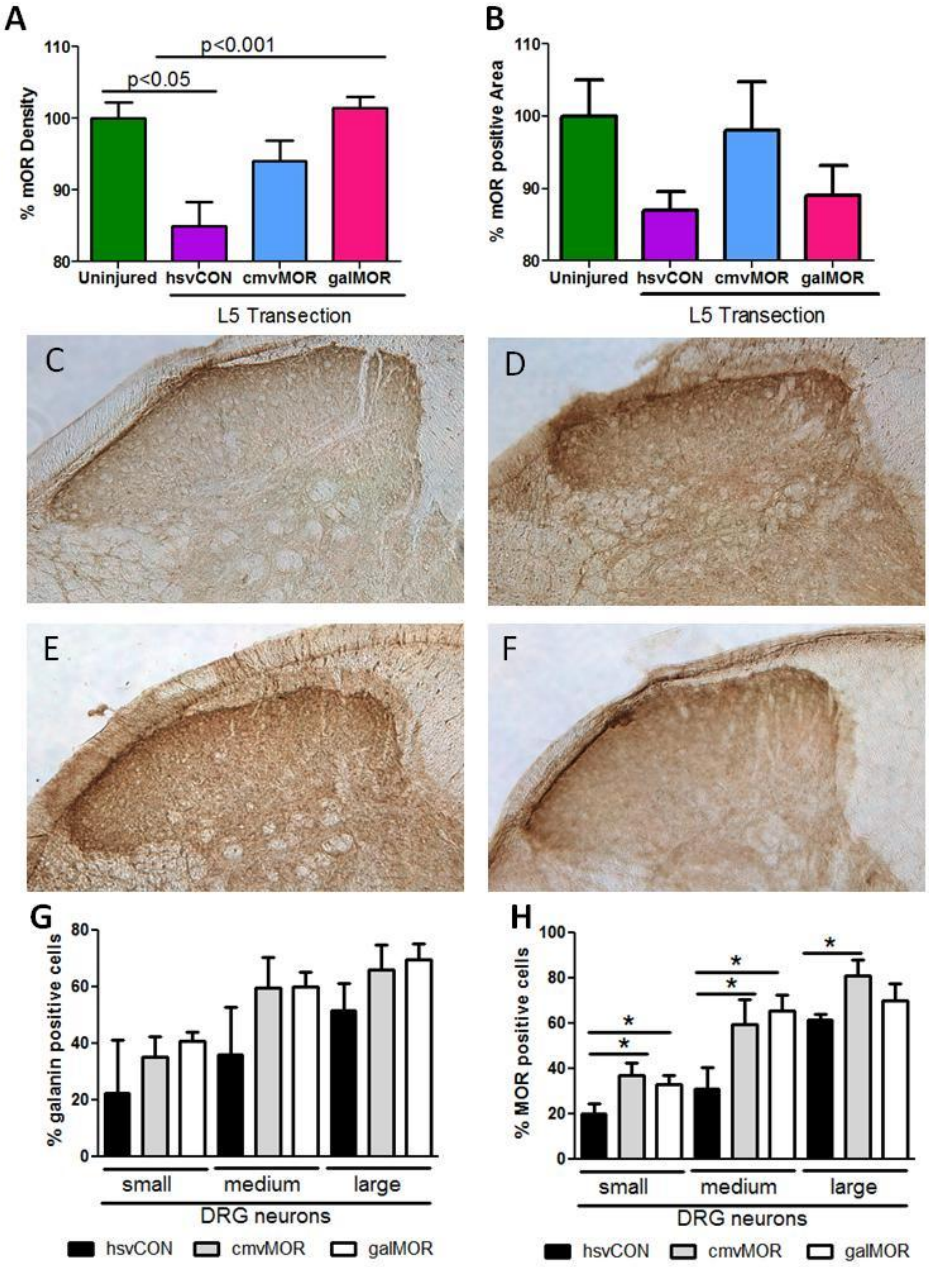

FIGURE 4.4: Decreased MOR positive density was observed in sectioned spinal cords from L5 transected mice given the control (hsvCON) virus compared to the uninjured controls while less of a decrease is seen in sections from the cmvMOR virus group and no decrease is observed in the galMOR group (A). Similarly, a decrease in MOR positive area was observed in all of the L5 transected animals compared to uninjured controls with less of a decrease seen in the cmvMOR virus group (B). Data is reported as %MOR positive density (A) or %MOR positive area (B) ± S.D. (n=2-4 mice/group). Statistical values represent differences between uninjured control and hsvCON L5 transected or hsvCON L5 transected and galMOR transected animals as indicated by the lines over the bars on the graph. The pictures below the graphs are representative images for the different treatment groups: hsvCON L5 transected (C), hsvCON not L5 transected (D), cmvMOR L5 transected (E), galMOR L5 transected (F). No significant changes in galanin expression were observed in the DRG of any of the treatment groups compared to the control animals (G). cmvMOR was shown to be significantly increased in all sizes of DRG neurons while galMOR was predominately increased in small and medium DRG neurons compared to the control virus treated animals (H) *, $p<0.05$.

CHAPTER 5

OPIOID RECEPTOR HETERODIMERIZATION IN THE DEVELOPMENT OF PERIPHERAL OPIOID TOLERANCE[2]

[2] **S.N. Smith**, J.L. Fisher, F.M. Perez, K.T. Velazquez, I. Mark, S.P. Wilson, S.N. Raja, S.M. Sweitzer to be submitted to *Pain*.

5.1 Introduction

Neuropathic pain conditions often cause debilitating pain and result in a decrease in quality of life. It is estimated that 6-8% of the general population suffers from neuropathic pain and costs related to treating these pain conditions are estimated to be over $100 billion in the United States alone (Magrinelli et al., 2013). Neuropathic pain is defined as pain originating as a direct consequence of a lesion or disease of the somatosensory system (Treede et al., 2008). It is characterized by allodynia, a non-noxious stimulus becoming noxious, and hyperalgesia, a noxious stimulus becoming more noxious.

The standard treatments for chronic neuropathic pain often times provide little relief for patients. The use of opioids to treat neuropathic pain is hindered by the usually high doses needed to achieve significant pain relief. As a result of the high doses needed, centrally-mediated side effects such as tolerance occur. As a therapeutic alternative, peripherally restricted opioids have been developed. As with centrally-mediated opioids, however, tolerance also develops (He et al., 2013). Mu and delta opioid receptors are G-protein coupled receptors that are primarily coupled to $G\alpha_{i/o}$. For opioid analgesia, these receptors work to inhibit voltage sensitive calcium channels presynaptically and to activate inwardly rectifying potassium channels postsynaptically with an overall action of reducing nerve impulses and neurotransmitter release. In an opioid naïve state, the majority of delta opioid receptors are not trafficked to the cell membrane but rather are found either in cytoplasmic compartments or large dense core vesicles (Cheng et al., 1995; Petaja-Repo et al., 2000; Guan et al., 2005). In a persistent opioid exposed state, however, an increase in the density of delta opioid receptors at the plasma membrane has

been found (Guan et al., 2005). In addition, results from several studies indicate that an interaction between mu (MOR) and delta (DOR) opioid receptors contributes to opioid tolerance (Qi et al., 1990; Kest et al., 1996; Riba et al., 2002). For instance, in morphine tolerance studies, in either DOR knockout mice (Zhu et al., 1999) or mice pre-treated with a delta antagonist (Abdelhamid et al., 1991), tolerance to morphine was prevented or significantly hindered.

The aim of this study was to investigate the use of a small peptide inhibitor of MOR-DOR heterodimerization to prevent peripheral opioid tolerance. Additionally, the knockdown of DOR was also evaluated at the spinal level using an HSV vector approach. A knockdown of DOR resulted in a faster return to baseline neuropathic pain behaviors and a greater analgesic response in an opioid exposed state compared to animals overexpressing DOR. Based on these results that suggest an important role for DOR in MOR-mediated analgesia, a novel peptide inhibitor was developed to block the interaction of MOR and DOR. Results from *in vitro* and *in vivo* paradigms suggest that the interaction between mu- and delta-opioid receptors can be blocked using a TAT linked small peptide and this blocking of the interaction can lead to a prevention and reversal of opioid tolerance in a rodent model of neuropathic pain. Therefore, using a small peptide blocker of MOR-DOR heterodimerization could serve as a novel therapeutic avenue to enhance peripheral opioid analgesia while preventing opioid tolerance in the treatment of chronic neuropathic pain.

5.2 Specific Methods

5.2.1 Primary Dorsal Root Ganglia Cell Culture

For DRG primary cultures, adult male rats were asphyxiated with CO_2 and decapitated. DRGs were isolated from the spinal columns and collected in ice cold Tyrode's buffer (132mM NaCl, 4.8mM KCl, 1mM $CaCl_2$, 5mM dextrose, 5mM HEPES). The DRGs were digested for 45 minutes in an enzymatic solution consisting of 1mg/mL Dispase II (Gibco 17105-041) and 2mg/mL collagenase (Roche 1088831) in warm Tyrode's buffer at 37°C in a shaking water bath. After digestion, the ganglia were triturated with a fire polished Pasteur pipet for several seconds. The neuronal cells were isolated using a gradient Shake OptiPrep (Sigma D1556). After centrifugation (1900rpm for 20min), the bottom two layers were collected and cells were counted. Approximately 60,000 cells were plated in a 96 well plate (Costar 3603) that was previously coated with poly-D-lysine (Sigma P4707) in MEM media (Sigma M0643; with 3mL of 20% glucose, 10% fetal bovine serum (Invitrogen 16000-036), 1% penicillin-streptomycin solution (Invitrogen 15070-063), and 10ng/mL of NGF (Millipore 01-125). For immunofluorescence, cells were plated on coverslips at approximately 41,000 cells per coverslip.

Calcium imaging was performed as previously described (He et al., 2013). Briefly, DRG neurons were exposed to loperamide (3 μM) or DAMGO (10 nM) for 72 hours to model *in vitro* tolerance. For acute exposure, DRG neurons were exposed to loperamide (3 μM) or DAMGO (10 nM) only on the day of calcium imaging. DRG cells were loaded with Fluo4-AM. Individual wells were pre-treated for 5 minutes with the DOR antagonist, naltrindole (0.1, 1, 5, 10 μM) or vehicle. This was followed by a 5

minute incubation with loperamide (1, 3, 5, 10 µg) or DAMGO (1, 5, 10, 20 nmoles). KCl (30 mM) was added and Ca^{2+} transients were measured using a BioTek Synergy 2 plate reader using a 200 µl/well total volume. The plates were read every 3-6 seconds for 6 minutes.

For examination of the small peptide inhibitor of dimerization in tolerant DRG neurons, cells were exposed to DAMGO (10 nM) for 72 hours to produce *in vitro* opioid tolerance. DRG neurons were loaded with Fluo4-AM. Individual wells were pre-treated for 30 minutes with a MOR-DOR heterodimer specific antibody (1:200, courtesy of Dr. Lakshmi Devi, Mount Sinai School of Medicine, NY) (Gupta et al., 2010)) or the peptide inhibitor of dimerization (250 ng). This was followed by a 5 minute incubation with DAMGO (8nM) prior to addition of KCl (30 mM final concentration).

F-F0 was calculated using the following formula: Fluorescence (KCl stimulated) – Fluorescence (no KCl control). Area Under the Curve (AUC) was calculated from the Ca^{2+} transients. AUC was normalized to the AUC of KCl stimulated vehicle treated cells and analyzed by non-parametric or parametric ANOVA where appropriate. A minimum of 6 wells of cells was used for each condition. All Ca^{2+} transients compared were analyzed on the same day and from the same plate.

5.2.2 DRG Immunofluorescence

DRG cells were fixed with ice cold methanol for 10min followed by 4 rinses (3 quick rinses and one 10min rinse) with 1X phosphate buffered saline (PBS). Cells were made permeable by incubating in PBS with 0.1% Triton X for 5min. After permeabilization, cells were rinsed then blocked with 4% normal horse serum in PBS. The following antibodies were used: primary antibody: MOR-DOR heterodimer selective

antibody ([1:1000],; secondary antibody, Alexa Fluor 488 donkey anti-mouse ([1:200], Invitrogen, Carlsbad, CA). Coverslips were mounted with Vectashield (Vector laboratories, Burlingame, CA).

5.2.3 Animals

Male Holtzman (Harlan) rats 175-225 grams at time of surgery (n=13) and female Swiss Webster mice (n=37) were used in this study.

5.2.4 Tolerance Induction

For virus studies, tolerance was induced with twice a day administration of loperamide (6 mg/kg) for 3 days from day 5-8 post-SNT. A challenge dose of loperamide (2 mg/kg) was administered on day 9 post-SNT. Infection with $10x10^7$pfu was on day 3 post-SNT. For peptide studies, tolerance was induced after SNT by injecting the animals subcutaneously with 10mg/kg of morphine or 3mg/kg of loperamide twice a day for 3 consecutive days starting on day 3 post-L5 spinal nerve transection. Percentage of maximum possible effect (%MPE): [1 − (cut-off PWT − post−drug PWT)/(cut-off PWT − pre−drug baseline PWT)] × 100]. PWT, paw withdrawal threshold, is the smallest filament at which the animal withdraws its hindpaw 3 out of the 5 times the filament is applied.

5.2.5 Viral Vector Construction and Infection

The full-length rat DOR cDNA (HSV-DOR) was cloned into a herpes shuttle plasmid under control of the hCMV promoter in either the sense (HSV-sDOR, NPDOR) or anti-sense orientation (HSV-αDOR, NPADOR) direction. A control virus contained the β-galactosidase gene under control of the hCMV promoter. Under isoflurane

anesthesia, mice were injected subcutaneously ($10x10^7$pfu in 10µl in left hindpaw) with either HSV-sDOR (n=9), HSV-αDOR (n=8), or control virus (n=20).

5.2.6 Behavioral Testing

Behavioral testing was performed after an initial dose of morphine (pre-tolerance dose; 3mg/kg) or loperamide (1.5mg/kg) and after a challenge dose of morphine (3mg/kg) or loperamide (1.5mg/kg) following the tolerance induction (post tolerance: with or without peptide).

5.2.7 Immunohistochemistry

Mice were perfused and the spinal columns and hindpaws were post fixed in 4% paraformaldehyde. Skin from the plantar surface of the virus infected hindpaw was collected and spinal cords were frozen for cryostat sectioning. Spinal cords were sectioned at 30 microns for free floating IHC. Skin was sectioned at 10 microns for slide mounted IHC. Sections were immunostained using avidin-biotin methods as previously detailed (Zhang et al., 2008). The primary antibody was anti-DOR (Santa Cruz, 1:250) with an overnight incubation at 4°C. Slides were evaluated using light microscopy. For image analysis, DOR+ nerve endings were counted and the dorsal horns of the spinal cord were densitized for DOR expression using NIH Image J.

5.2.8 Western Blot Analysis

Fresh frozen DRG or lumbar spinal cord sections were used for western blot analysis. The same amounts of total protein were resolved on reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Proteins were transferred to a polyvinylidene difluoride membrane. The following antibodies were used: anti- β-Actin (Sigma Aldrich 1:75,000); $G\alpha_o$ (Santa Cruz 1:1000); $G\alpha_s$ (Santa Cruz 1:1000).

5.2.9 Statistical Analysis

Data are presented as mean ±SEM. *,**,*** $p<0.05$, 0.01, 0.001 compared to control or vehicle; ∞,∞∞,∞∞∞ $p<0.05$, 0.01, 0.001 significance within experimental groups.

5.3 Results

5.3.1 HSV induced changes in DOR expression

HSV viral vectors encoding the DOR gene in the sense or antisense direction behind the CMV promoter were used to examine the role of DORs in neuropathic pain, peripheral opioid analgesia, and central opioid analgesia. An L5 spinal nerve transection model was used to induce neuropathic-pain associated mechanical allodynia and thermal hyperalgesia. Seven days following nerve transection, mice were infected by intraplantar administration with HSV vectors to increase DOR expression (HSV-sDOR) or knock-down DOR expression (HSV-αDOR) in primary afferent neurons. On day 15, post-infection mice were perfused and skin and spinal cords were collected for immunohistochemical analysis of DOR and MOR expression. Infection with HSV-sDOR increased the number of DOR positive nerve endings in the skin (Fig 5.1A) while infection with HSV-αDOR decreased the number of DOR positive nerve endings (***, $p<0.001$ compared to control). There were significantly more DOR positive nerve endings in HSV-sDOR infected mice compared to HSV-αDOR mice (∞∞∞, $p<0.001$). Similarly, there was increased immunoreactivity for DOR in the superficial dorsal horn of the spinal cord following infection with HSV-sDOR (*, $p<0.05$) compared to control (Fig 5.1B). HSV-αDOR infection decreased the immunoreactivity for DOR in the superficial dorsal horn of the spinal cord (***, $p<0.001$) compared to control. This

demonstrates that the HSV-sDOR and HSV-αDOR viral vectors increase and decrease DOR expression in afferent neurons, respectively.

5.3.2 HSV induced changes in neuropathic pain-associated behaviors

Paw withdrawal thresholds decreased on day 1 following L5 spinal nerve transection and remained low on day 7 post-transection (Fig 5.1C). Following infection with HSV-sDOR, HSV-αDOR, or control on day 7 post-transection, a statistically significant increase in paw withdrawal threshold back to pre-transection levels was observed in HSV-αDOR infected mice. In contrast, mice infected with HSV-sDOR had similar paw withdrawal thresholds as control mice. Paw withdrawal latencies decreased on day 1 following nerve transection and remained decreased on day 7 post-transection (Fig 5.1D). Following infection with HSV-αDOR on day 7, paw withdrawal latency times increased to pre-transection times by day 10 post-transection. Mice infected with HSV-sDOR demonstrated a longer duration of thermal hyperalgesia compared to control infected mice. This demonstrates a surprising decrease in neuropathic pain-associated behaviors with DOR knockdown in primary afferents and retention of pain behaviors with virally mediated increase in DOR expression in primary afferents.

5.3.3 HSV induced changes in opioid analgesia

On day 22 post-transection (day 15 post-infection) cumulative dose-response curves were generated for the peripherally restricted opioid agonist, loperamide, and systemic morphine. HSV-αDOR infected mice had a leftward shift in the loperamide dose-response curve compared to control and HSV-sDOR (Fig 5.1E). HSV-sDOR infected mice had similar loperamide analgesia compared to control. The median effective dose (ED50) for loperamide was 0.22 grams/kg in HSV-αDOR mice compared

to 1.15 in control mice (Table 5.1). A smaller left-ward shift in HSV-αDOR infected mice, and a small rightward shift in HSV-sDOR infected mice was observed for systemic morphine (Fig 5.1F). The ED50 for morphine was 1.25 mg/kg in control mice, 1.94 in HSV-sDOR, and 0.48 in HSV-αDOR (Table 5.1). This demonstrates increased peripheral and systemic MOR-mediated analgesia with virally mediated knockdown of DOR in afferent neurons suggesting that DOR expression is negatively correlated with MOR-mediated analgesia in neuropathic pain states.

5.5.4 HSV induced changes in peripheral opioid analgesia and opioid tolerance

In L5 spinal nerve transected mice, virally driven over-expression of DOR with HSV-sDOR increased mechanical thresholds to a single dose of loperamide (2 mg/kg, sc) compared to control ($p<0.01$, Fig 5.2A). In contrast, knock-down of DOR expression with HSV-αDOR decreased acute loperamide analgesia compared to control ($p<0.01$). In contrast, loperamide analgesia is restored by knockdown of DOR expression with HSV-αDOR in a loperamide tolerant state (Fig 5.2B). Reduced loperamide analgesia was present in HSV-DOR and control tolerant mice, compared to control non-tolerant mice. This suggests a state dependent role of DOR in opioid analgesia and tolerance such that in opioid naive states DOR knockdown decreases MOR-mediated analgesia and in opioid tolerant states DOR knock-down prevents MOR tolerance.

5.5.5 State dependent role for DOR in opioid analgesia

Experiments were carried out in a 96 well plate using a plate reader and thus, the results represent the average fluorescence change in a heterogenous population of DRG neurons. Calcium transients were stimulated with KCl (30 μM) and readings were made every 3 seconds. The DOR antagonist naltrindole (5 μM) reversed DAMGO (10 nM, Fig

5.3A, B) and loperamide (3 μM, Fig 5.3B) mediated inhibition of KCl-stimulated Ca^{2+} influx in opioid naïve cultured dorsal root ganglion cells. In contrast, following induction of tolerance with a 72 hour exposure to DAMGO (10 nM), the DOR antagonist naltrindole (1 μM) enhanced DAMGO (10 nM, Fig 5.3C,D) and loperamide (3 μM, Fig 5.3D) mediated inhibition of KCl-stimulated Ca^{2+} influx. Tolerance was demonstrated by a larger area under the curve following application of loperamide (3 μM) or DAMGO in 3 day DAMGO exposed cells (Fig 5.4D) compared to opioid naive cells (Fig 5.4B). These calcium imaging experiments along with the behavioral results with the HSV vectors to modulate DOR expression suggest that DOR regulate peripheral mu opioid analgesia in a state dependent manner with agonism in opioid naive states and antagonism in opioid tolerant states. A state dependent role of DOR in MOR-mediated analgesia was confirmed in primary cultured afferent neurons such that DOR increased MOR-mediated analgesia in opioid naive states and DOR antagonizes MOR-mediated analgesia in opioid tolerant states.

5.5.6 Development of a peptide inhibitor of mu-delta opioid receptor heterodimerization

A small cell permeable peptide was developed to inhibit MOR-DOR dimerization. Prior to evaluating the peptide inhibitor in co-immunoprecipitation experiments, specificity of MOR and FLAG antibodies was confirmed using transient transfection of MOR and FLAG-tagged DOR in HEK 293 cells. Western blot analysis of untransfected (control) HEK293 cells or cells transfected with MOR, DOR-FLAG, or DOR, or protein samples from brain and spinal cord of MOR knock-out mice demonstrated specificity of both the MOR and FLAG antibodies (Fig 5.4A).

HEK 293 cells were transfected with MOR and FLAG-tagged DOR for immunoprecipitation to analyze heterodimer formation. Cells were treated with different concentrations of the heterodimer inhibiting peptide for 1 hour. Immunoprecipitation with FLAG and immunoblotting with MOR showed that control cells treated with vehicle had greater heterodimer formation compared to peptide treated cells. A significantly higher ($p<0.05$) level of MOR-DOR was found in control cells compared to cells given 625ng of the peptide inhibitor (Fig 5.4B). This demonstrates that the peptide inhibitor blocks heterodimer formation in HEK cells.

DRG primary cultured neurons were chronically treated with DAMGO (10nM, 72hr) in the presence or absence of the heterodimer inhibiting peptide. Using an antibody that is specific for MOR-DOR heterodimers, an increase in the number of cells and staining intensity of cells was observed in DAMGO treated cells compared to control opioid naïve cells. In contrast, cells that were chronically exposed to both DAMGO and the peptide inhibitor of heterodimerization showed a reduction in MOR-DOR formation (Fig 5.5A) compared to vehicle treated and DAMGO exposed cells. This demonstrates that the peptide inhibitor blocks the upregulation of heterodimer formation following chronic exposure to DAMGO.

In calcium imaging using DRG primary neurons, pretreatment of opioid tolerant cells with a MOR-DOR heterodimer antibody was able to block naltrindole, from increasing DAMGO-mediated inhibition of KCl stimulated Ca^{2+} transients. It did not, however, prevent tolerance. In contrast, administration of the peptide inhibitor (250ng/well, 72hr) in conjunction with DAMGO (10nm, 72 hr) prevented DAMGO tolerance. Administering peptide without DAMGO had no effect on KCl-stimulated Ca^{2+}

flux (Fig 5.5B). This demonstrates that the peptide inhibitor blocks the downstream functional changes in calcium signaling that occur with the development of tolerance *in vitro*.

5.5.7 Prevention of peripheral opioid tolerance with a peptide inhibitor of dimerization

In rats post-SNT, the peptide (250 ng/day) was delivered in a 3 day mini osmotic pump placed s.c. between the scapula 3 hours after the first exposure to loperamide (1.5 mg/kg, pre-tolerance testing) or morphine (3 mg/kg, pre-tolerance testing). Tolerance was then induced with loperamide (3 mg/kg, b.i.d., s.c.) or morphine (10 mg/kg, b.i.d., s.c.). In animals that received the peptide inhibitor during tolerance induction, a greater reduction in the total number of paw flinches was observed following a challenge dose of loperamide or morphine compared to animals that did not receive the peptide inhibitor (Fig 5.6A,B). In the Hargreave's plantar test, a greater increase in paw withdrawal latency was observed upon a challenge dose of opioid in animals that received the peptide inhibitor during tolerance induction compared to the animals that did not receive the peptide inhibitor (Fig 5.6C). This demonstrates a role of MOR-DOR heterodimers in the development of both peripheral and systemic opioid tolerance and that these heterodimers are potential therapeutic targets to reduce the development of tolerance.

5.5.8 Prevention of tolerance alters inhibitory intracellular signaling

Opioid receptors are coupled to G proteins, predominately with the inhibitory G proteins $G\alpha_{i/o}$. Western blot analysis was performed on rat DRGs from the loperamide peripheral tolerance study. Analysis determined the expression level of $G\alpha_o$ and $G\alpha_s$ in the presence and absence of treatment with the peptide inhibitor. Results show that levels of $G\alpha_o$ are significantly higher ($p<0,05$) in the nerve injured DRG in animals treated with

the peptide compared to the control animals (no peptide) (Fig5.7A). No significant differences were seen in $G\alpha_s$ expression in DRG of control or peptide treated rats (Fig 5.7B). This suggests that treatment with the heterodimer blocking peptide may increase inhibitory signaling by upregulating $G\alpha_o$ and not $G\alpha_s$ and thus, potentially shifting the balance between these two signaling pathways.

5.4 Discussion

In this chapter pharmacological, behavioral, and biochemical evidence was presented for a role of MOR-DOR heterodimers in peripheral opioid tolerance. Both *in vitro* systems of opioid tolerance and an *in vivo* system of neuropathic pain and opioid tolerance were used to provide evidence that a disruption in MOR-DOR interactions can result in functional and behavioral consequences regarding opioid tolerance.

DOR agonists have been shown to enhance central morphine analgesia in the absence of tolerance and neuropathic injury (Chen et al., 2007). It is believed that only a low amount of DOR are present in the cell membrane and are located on large dense core vesicles. With chronic opioid exposure, however DOR is trafficked to the cell membrane where there is a greater chance of interaction with MOR. In the first set of experiments the knocking down of DOR in an opioid exposed state, evidenced by the decrease in DOR immunostaining in nerve endings and spinal cord, results in a faster return to baseline pain behaviors and enhanced opioid analgesia and less tolerance. As further confirmation of DOR's role in MOR-mediated analgesia, in DRG primary cultures it was found that in an opioid naïve state, DAMGO and loperamide mediated inhibition of calcium influx is blocked by the DOR antagonist naltrindole. In an opioid exposed state, however, naltrindole actually enhances DAMGO and loperamide induced inhibition of

calcium influx. Taken together, these results provided us with evidence that there is an interaction between MOR and DOR that DOR appears to be a negative modulator of MOR in a tolerant state.

It is speculated that MOR and DOR homodimers are antinociceptive while MOR-DOR heterodimers are pro-nociceptive (Franco et al., 2008). Using a specific antibody that only recognizes MOR-DOR heterodimers, in cultured DRG neurons in an opioid naïve state there are low levels of MOR-DOR whereas in an opioid tolerant state there is an increase in heterodimers suggesting that chronic exposure to opioids leads to a greater interaction between the two receptors. In addition to what was found in the *in vitro* models of opioid tolerance, others have shown that chronic morphine treatment increases the abundance of MOR-DOR heterodimers in cultured neurons (Gupta et al., 2010), and the development and maintenance of opioid tolerance may involve the dimerization of MOR and DOR. While there is substantial evidence that MOR-DOR form heterodimers in *in vitro* systems, *in vivo* evidence of MOR-DOR heterodimers in a NP and opioid exposed state has been lacking.

To this end, a novel peptide inhibitor of MOR-DOR was developed using the information that the 15 amino acids at the C-terminus of the DOR (Val357-Ala372) is required for efficient heterodimerization of MOR with DOR. With the experiments in this chapter it was shown that in L5 SNT rats, a continuous infusion of the novel peptide inhibitor of MOR-DOR heterodimerization during tolerance induction with either morphine or loperamide results in an attenuation of peripheral opioid tolerance. When I looked at potential intracellular signaling mechanisms that are influenced by MOR-DOR

heterodimers, I found that there is an increase in $G\alpha_o$ expression in the DRG of rats treated with the peptide inhibitor compared to those that were not.

These results suggest that potentially there is a change in G-protein coupling with MOR-DOR heterodimers and when the dimers are disrupted there is a shift back to a coupling with $G\alpha_o$. Indeed research has shown that MOR-DOR formation leads to a switch from $G\alpha_o$ to $G\alpha_s$ which would result in stimulatory intracellular signaling rather than inhibitory signaling. While further validation of the peptide inhibitor in the disruption of heterodimer formation is needed, these results strongly suggest that MOR-DOR heterodimers form *in vivo* and that a disruption in this interaction leads to a prevention of opioid tolerance.

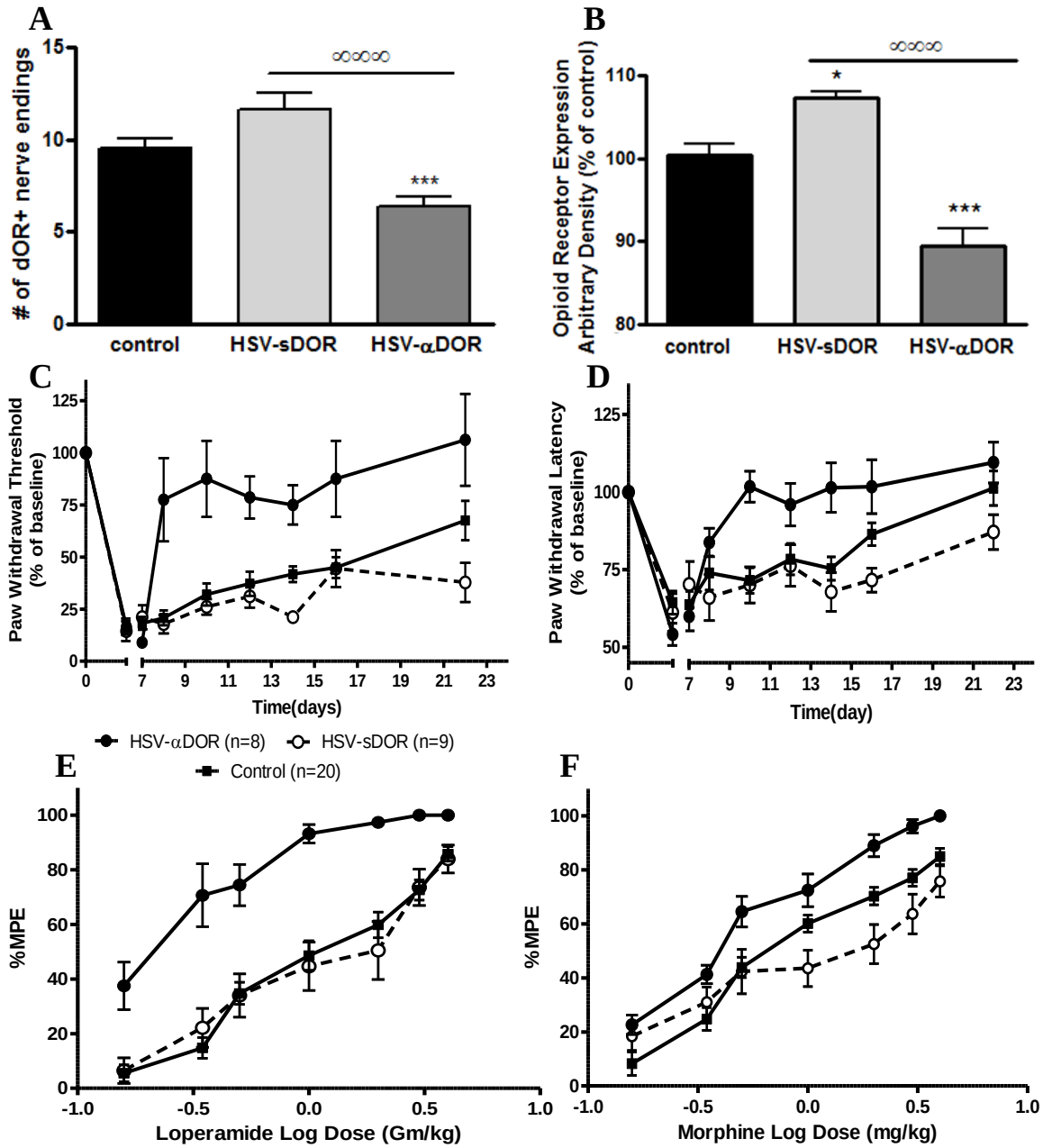

FIGURE 5.1: IHC of nerve endings in the skin of virus treated animals revealed significantly more DOR+ nerve endings in HSV-sDOR treated animals compared to HSV-αDOR treated animals (∞∞∞∞, $p<0.001$). Additionally, HSV-αDOR infected mice had significantly less DOR+ nerve endings compared to control mice (***, $p<0.001$) (A). In the dorsal horn of the spinal cord, there was significantly more DOR expression in mice treated with HSV-sDOR compared to animals given HSV-αDOR (∞∞∞∞, $p<0.001$) and control mice (*, $p<0.05$). Similarly, there was significantly less DOR expression in mice treated with HSV-αDOR compared to control mice (***, $p<0.001$) (B). Knocking down DOR expression using HSV-αDOR resulted in a faster return to baseline after nerve injury (C,D) and a greater increase in opioid-induced analgesia with both loperamide (E) and morphine (F) compared to control animals and animals treated with HSV-sDOR.

Table 5.1: ED_{50} for loperamide and morphine in control, HSV-sDOR, and HSV-αDOR treated mice

Virus	ED50 Loperamide (95% CI)	Morphine
Control	1.15 (1.00, 1.32)	1.25 (1.09, 1.41)
HSV-sDOR	1.24 (0.94, 1.64)	1.94 (1.54, 2.33)
HSV-αDOR	0.22 (0.17, 0.27)	0.48 (0.41, 0.54)

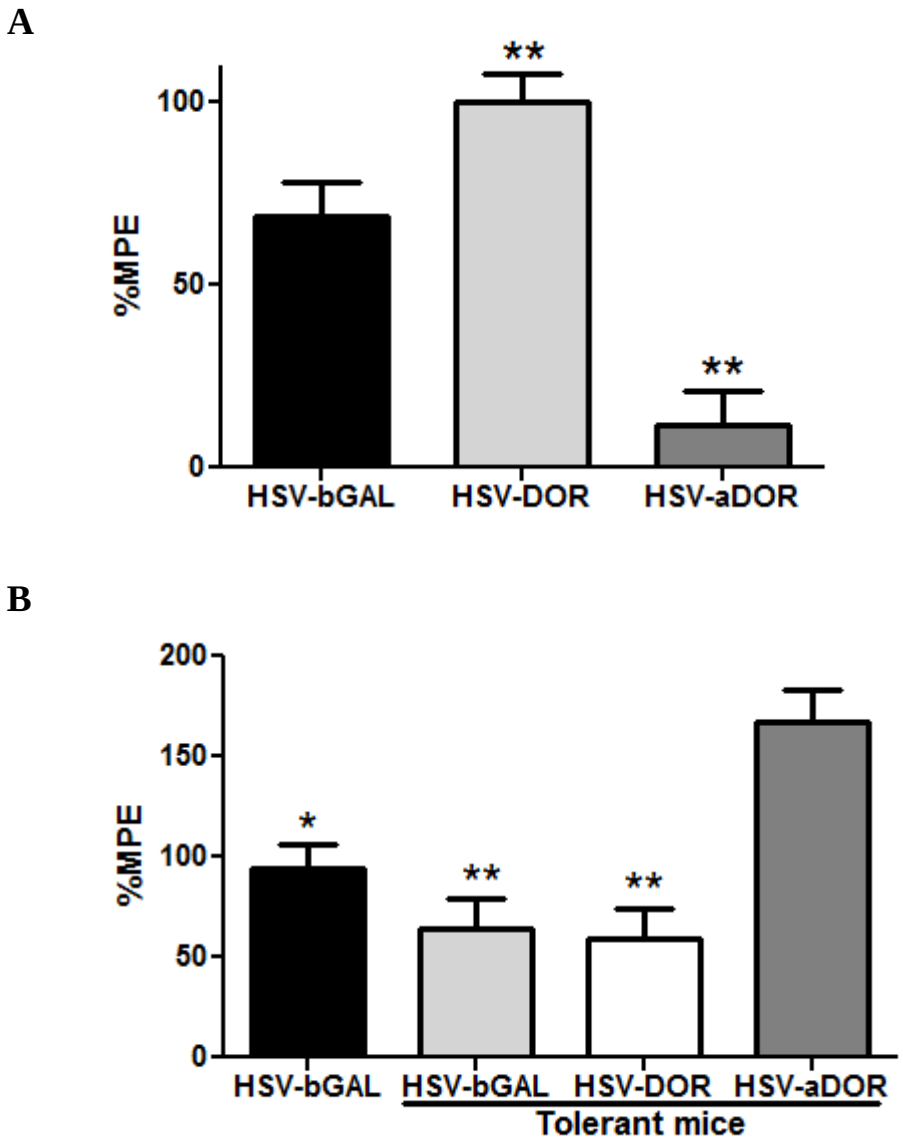

FIGURE 5.2: In an opioid naïve state (A), treatment with HSV-DOR results in greater %MPE compared to control virus (HSV-bGAL) and HSV-αDOR treated mice. In a tolerant-state (B), a knockdown of DOR (HSV-αDOR) results in the greatest %MPE compared to animals given the control or HSV-DOR viruses. **P<0.01

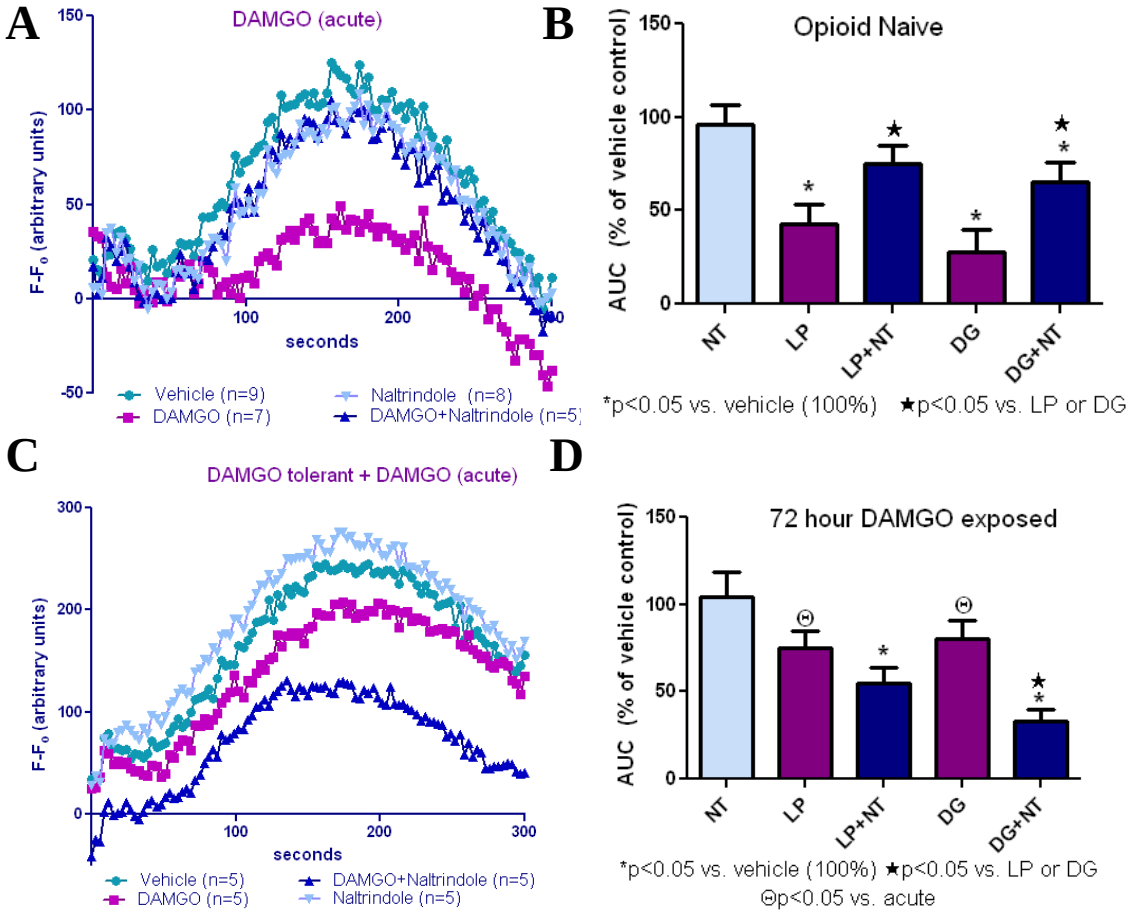

FIGURE 5.3: The DOR antagonist naltrindole (5 μM) reverses DAMGO (10 nM, Panel A) inhibition of KCl-stimulated Ca^{2+} influx in opioid naïve cultured dorsal root ganglion cells. In contrast, following induction of tolerance with a 72 hour exposure to DAMGO (10 nM), the DOR antagonist naltrindole (1 μM) enhances DAMGO (10 nM, Panel C) inhibition of KCl-stimulated Ca^{2+} influx. For Panels B and D, Area under the curve was generated for each data set to allow for comparison across experiments. Tolerance was demonstrated by a significant reduction in AUC following application of loperamide (3 μM) or DAMGO in acute (Panel B) but not DAMGO exposed cells (Panel D). Similarly, naltrindole (1 μM) partially reverses DAMGO and loperamide induced inhibition in opioid naïve cells (Panel B). In contrast, naltrindole (1 μM) enhances loperamide and DAMGO induced inhibition in DAMGO exposed cells (Panel D). Experiments were carried out in a 96 well plate using a plate reader and thus, the results represent the average fluorescence change in a heterogenous population of DRG neurons. KCl (30 μM) was added in 10 ul to 190 ul in each well and readings were made every 3 seconds.

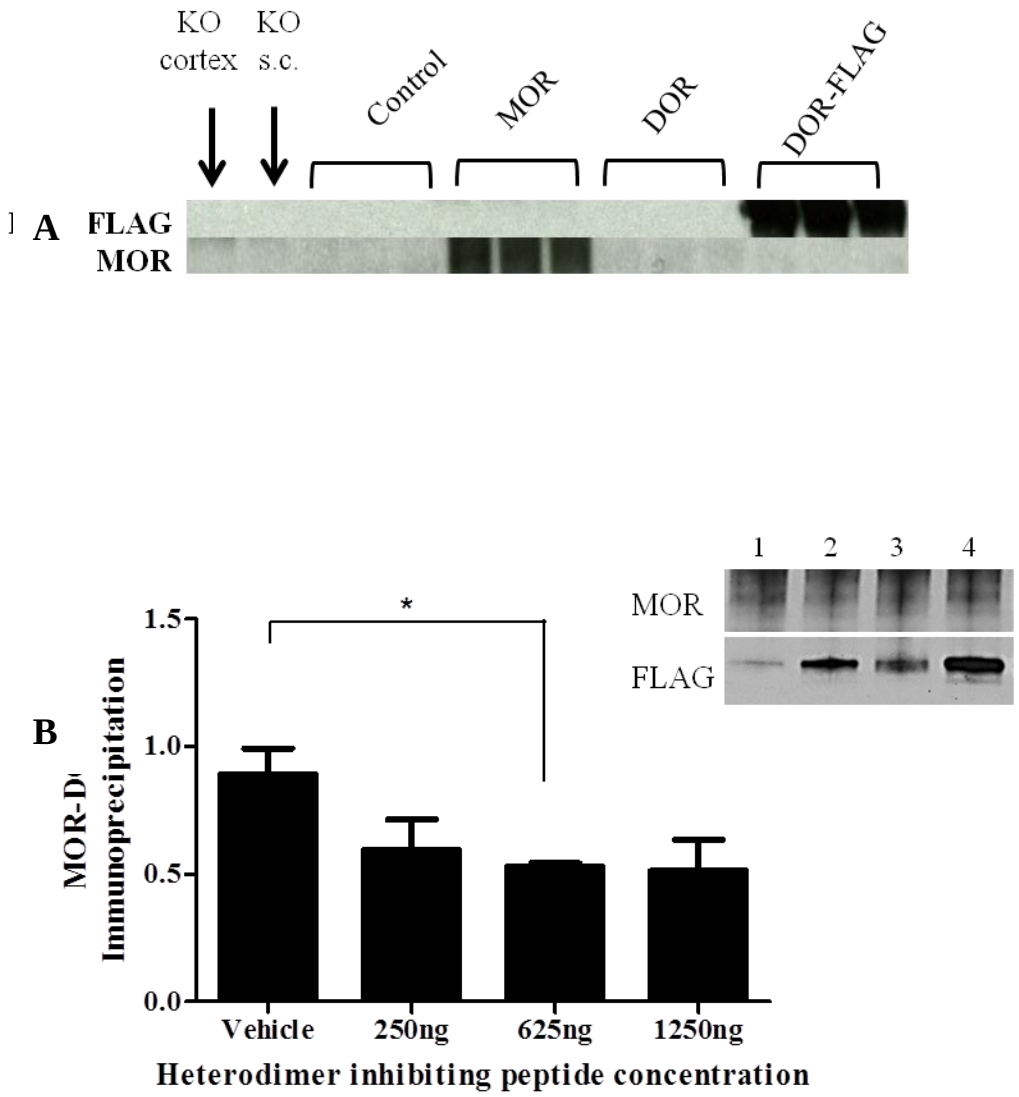

FIGURE 5.4: (A) Specificity of antibody was evaluated in MOR or DOR-FLAG transfected HEK293 cells in addition to MOR knockout mice brain and spinal cord tissue. For the MOR antibody, only in the MOR lanes is the protein detected. Similarly, only in the DOR-FLAG lanes in the protein detected. (B) A dose response for the heterodimer inhibiting peptide revealed a higher presence of heterodimer formation in vehicle-treated cells than in cells treated with any concentration of the peptide inhibitor (*$p<0.05$). Inset is a representative Western blot image (lane 1=vehicle; lane 2=250ng; lane 3=625ng; lane 4=1250ng).

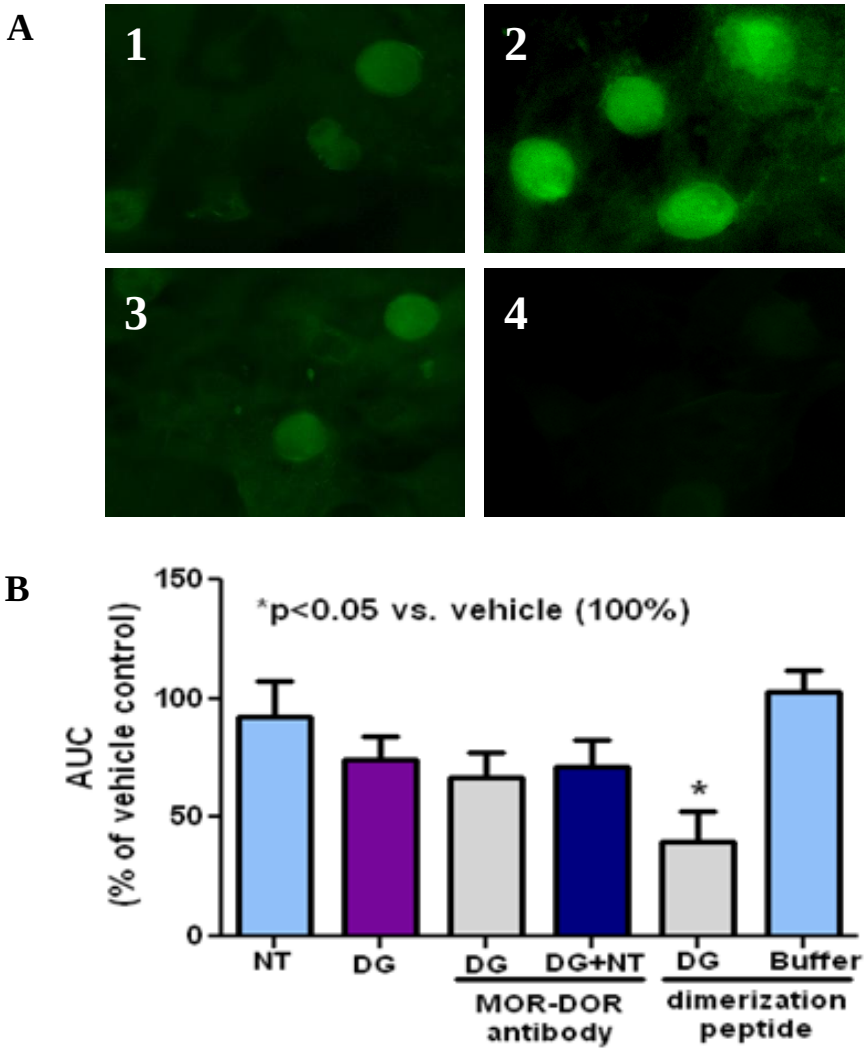

FIGURE 5.5: (A): Immunoflurescence of DRG cells using a MOR-DOR antibody. 1. Control opioid-naïve primary DRG cultures show minimal staining for MOR-DOR heterodimers. 2. Cells exposed to MOR agonist DAMGO (10 nM, 72 hrs) show increased MOR-DOR heterodimerization. 3. Decreased DAMGO-induced dimer formation with application of a MOR-DOR peptide inhibitor (250 ng/well, 72 hrs) . 4. No primary antibody control. (B): A small cell permeable peptide inhibitor of MOR-DOR dimerization prevents DAMGO tolerance *in vitro*. Pretreatment of DAMGO tolerant cells (10 nM, 72 h) with a MOR-DOR heterodimer antibody (30 min) prevents naltrindole from increasing DAMGO-mediated inhibition of KCl stimulated Ca^{2+} transients, and does not reverse tolerance. In contrast, administration of the peptide inhibitor of heterodimerization (250 ng/well for 72 h) during induction of tolerance prevented DAMGO tolerance. The peptide alone had no effect on KCl-stimulated Ca^{2+} transients.

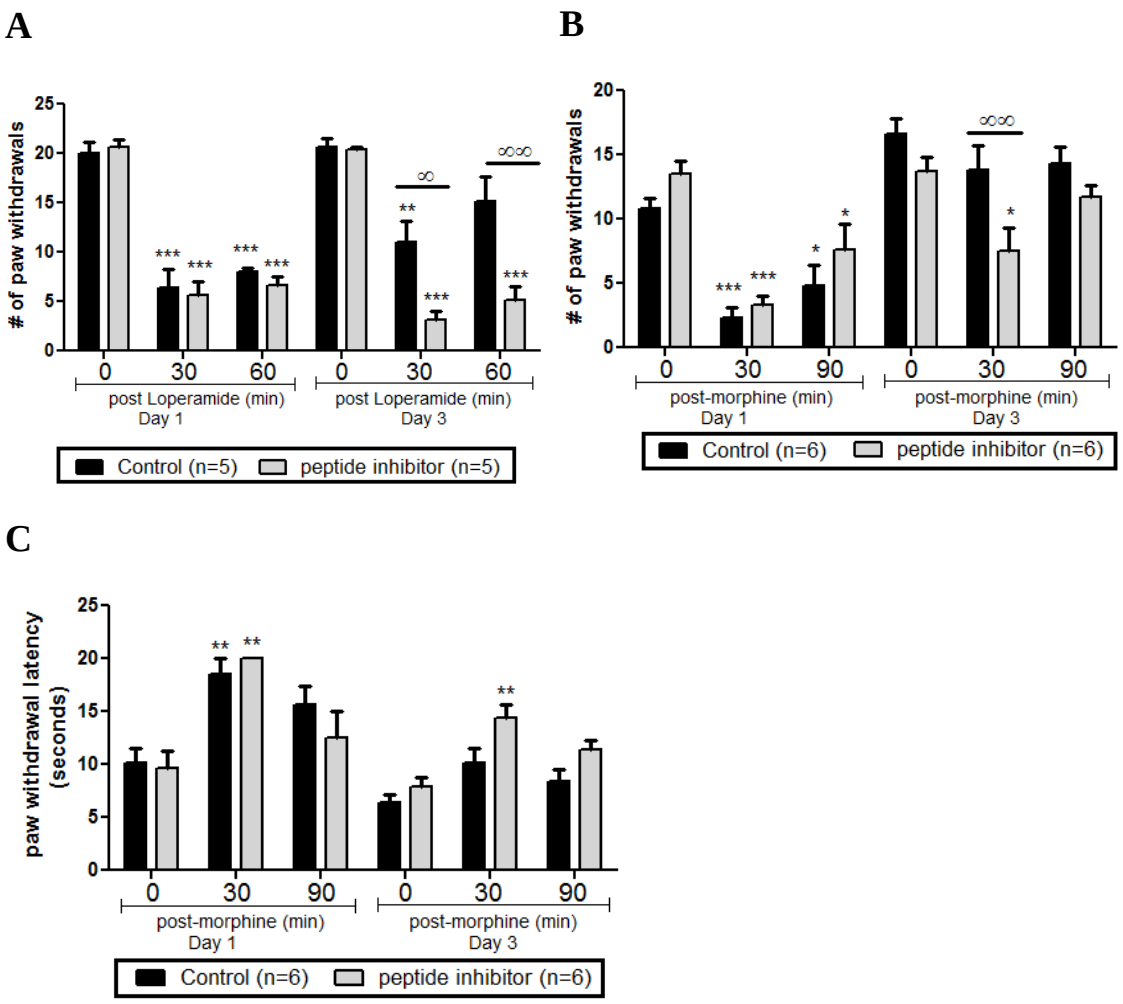

FIGURE 5.6: (A) Peptide treated rats (n=5) demonstrated partial analgesia upon challenge with 1.5 mg/kg loperamide in the post-tolerant condition (reduced tolerance) as compared to control vehicle treated rats (n=5). *, **, ***P<0.05, 0.01, 0.001 vs. control; ∞, ∞∞ P<0.05, 0.01. Similarly, in morphine treated rats (B), both groups of animals experienced potent analgesia after morphine was given on day 1. After tolerance induction, only the peptide inhibitor group had a significant reduction in paw withdrawals 30 min after morphine was given on day 3. In evaluating thermal hyperalgesia (C), a greater increase in paw withdrawal latency was observed in the peptide treated animals 30 min post morphine injection on day 3 compared to the vehicle treated controls.

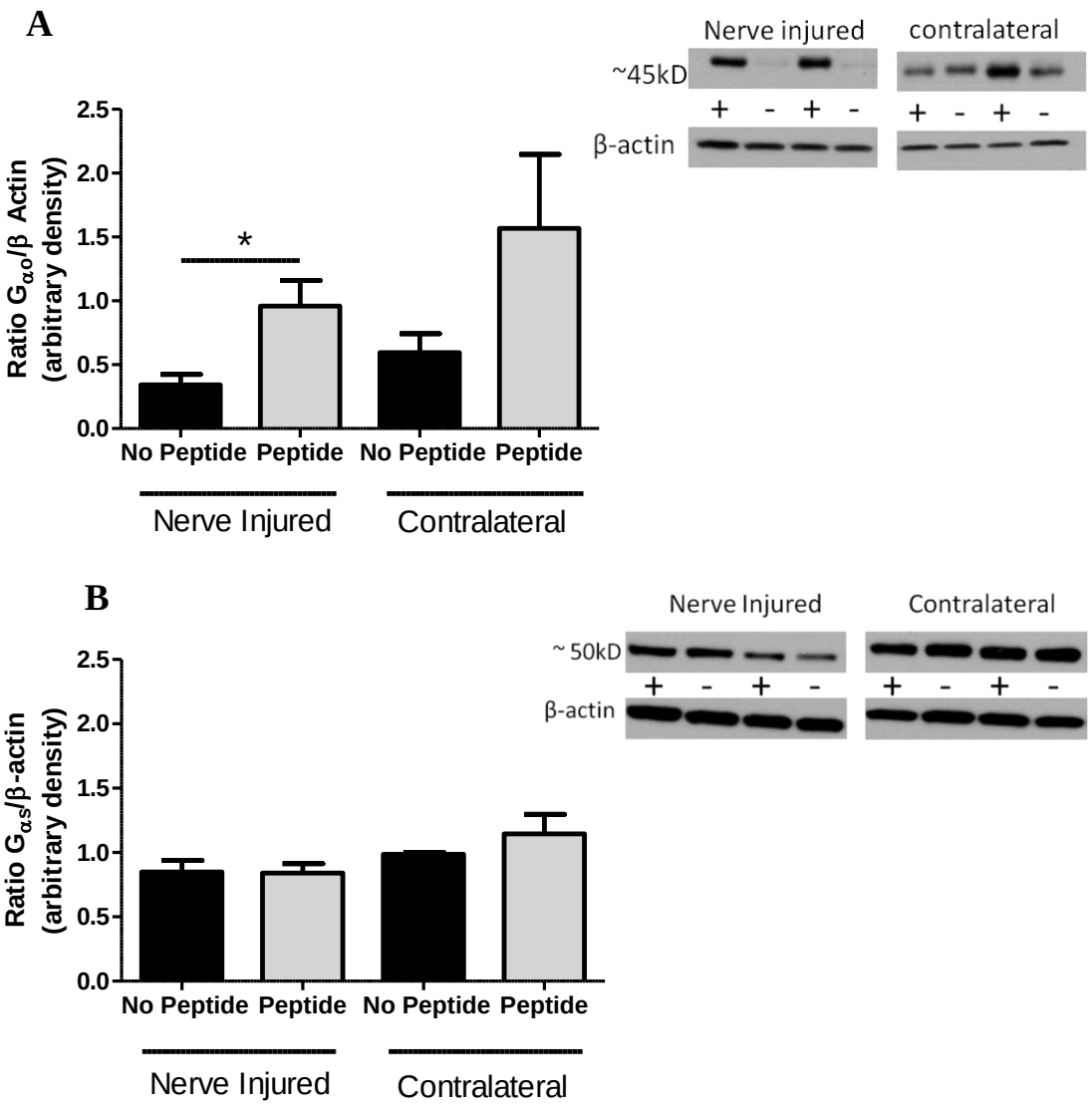

FIGURE 5.7: Western blot analysis of DRG from peptide and vehicle treated animals show a significant increase in $G_{\alpha o}$ expression (A) in the nerve injured dorsal root ganglia of animals treated with the peptide inhibitor compared to animals not given the peptide inhibitor (*P <0.05). In contrast, no significant differences were found in $G_{\alpha s}$ expression (B) in peptide treated or control rats for either the nerve injured or contralateral DRGs. Beside the graphs are representative protein bands for the respective blots.

CHAPTER 6

GENERAL DISCUSSION

6.1 Summary of Findings

6.1.1 Characterization of HSV transfection patterns

HSV vectors are a promising therapeutic tool in the treatment of neuropathic pain. While the vectors themselves have been characterized, transduction efficiency and sex differences in transgene expression have not been fully explored. In this aim I sought to characterize HSV infection patterns as well as GIRK2 expression in mouse DRG and spinal cord. In order to examine which primary sensory afferents are transduced by HSV viral vectors, I used track tracers to label small, medium, and large neurons and counted the cell bodies that were labeled with track tracer and β-galactosidase from the HSV vector. I found that approximately one third of small and medium primary sensory afferents are transduced by the virus while over half of large diameter neurons take up the virus. Based on these results our collaborators were able to conduct electrophysiological recordings of single fibers with the HSV vectors.

While my lab more commonly uses female mice for HSV vector experiments, I wanted to see if MOR expression is comparable in male and female mice as our collaborators most commonly use male mice. Male and female mice were infected with SGMOR. Behavioral testing was performed to see if any differences would be observed after virus infection and immunohistochemistry to examine MOR expression in the dorsal horn of the spinal cord. Behavioral results showed differences in mechanical nociception with females being more sensitive to mechanical stimuli days after virus infection but no significant differences were observed between male and female mice to thermal stimuli. Immunohistochemically, the pattern of MOR expression is similar in both males and females which led us to conclude that sex hormones do not play a significant role in HSV mediated MOR expression.

GIRK2 is thought to play an important role in opioid analgesia and tolerance. It has been speculated that mice DRG lack GIRK2. Mouse DRG and spinal cord were examined for GIRK2 using western blotting and it was found that GIRK2 is indeed present in DRG albeit at much lower expression levels than rat DRG. As a result of the discrepancies between these results and those of others, I postulate that GIRK2 expression is strain dependent.

6.1.2 Using an injury specific promoter results in greater antinociception

Chronic neuropathic pain is often difficult to treat with current pain medications. Gene therapy is currently being explored as a therapeutic approach for the treatment of neuropathic and cancer pain. In this aim I sought to use an injury-specific promoter to deliver the MOR transgene such that expression would only occur during the injured state in response to release of injury-specific galanin. To determine whether an injury specific promoter can produce neuron-specific MOR expression and enhanced antinociception I compared animals infected with a galanin promoter virus (galMOR) or a human cytomegalovirus (CMV) promoter virus. (cmvMOR). In behavioral assays, I found an earlier onset and a larger magnitude of antinociception in animals infected with galMOR compared to cmvMOR. Immunohistochemistry of DRG neurons revealed a significant increase in MOR positive staining in cmvMOR and galMOR treated mice. Spinal cord sections from galMOR treated mice showed a greater increase in density but not area of MOR positive staining. These results suggest that using injury-specific promoters to drive gene expression in primary afferent neurons can influence the onset and magnitude of antinociception in a rodent model of neuropathic pain and can be used to upregulate MOR expression in populations of neurons that are potentially injury specific.

6.1.3 A peptide inhibitor of MOR-DOR heterodimers reduces tolerance

The treatment of neuropathic pain with opioid analgesics is limited by centrally-mediated side effects thus, leading to the development of peripherally restricted opioids as therapeutic alternatives. My lab has recently shown that as with centrally active opioids, tolerance also develops following repeated administration of peripherally restricted opioids (He et al., 2013). It is postulated that heterodimerization of MOR and DOR plays a role in the development of tolerance. The purpose of this aim was to determine if blocking the MOR-DOR heterodimerization would prevent and reverse peripheral opioid tolerance. To block heterodimerization a novel small peptide blocker was linked to a TAT carrier peptide for intracellular delivery following systemic administration. The peptide blocker was tested in *in vitro* and *in vivo* models of peripheral opioid tolerance. *In vitro,* the peptide blocker reduced mu- and delta- opioid receptor heterodimerization in opioid exposed dorsal root ganglia cells or human embryonic kidney cells. In L5 spinal nerve transected rats, continuous treatment with the peptide blocker prevented the development of peripheral opioid tolerance. These results suggest that peripheral tolerance can be prevented with a peptide blocker of mu- and delta- opioid receptor heterodimerization providing a novel therapeutic target to enhance peripheral opioid analgesia in the treatment of chronic neuropathic pain.

6.2 Limitations

While great strides have been made for translational success in pain research from rodents to humans, there is still a gap. Galanin has been shown to be upregulated in human DRG in patients with neuropathic pain conditions (Landry et al., 2003) but to what extent remains to be seen. As such, the use of the galanin promoter for humans

would have to be further validated as to its effectiveness as an injury specific promoter. There has, however, been early clinical success in the use of a HSV-adapted vector in treating pain in bone cancer patients (Fink et al., 2011). Fink *et al.* showed that an HSV-based vector that overexpressed enkephalins was safe in humans with the highest dose producing the greatest reduction in pain (2011).

A limitation in the HSV DOR knockdown studies is the use of a DOR antibody that has been shown to recognize MOR and KOR as well. In order to avoid non-specificity for the HEK293 peptide inhibitor studies, I used a FLAG-tagged DOR. Regarding the peptide inhibitor of MOR-DOR, while immunohistochemical and cellular signaling changes *in vitro* and behavioral changes *in vivo* were seen, further characterization needs to be carried out to ensure specificity of the peptide inhibitor for blocking MOR-DOR and not other heterodimers that form with DOR and potentially with DOR homodimers if they use the same binding motif. To address this limitation, IP studies with other known DOR heterodimer partners should be investigated in a cell type specific manner. It is also possible that the peptide may have off target effects on other body systems in which MOR and DOR are expressed in the same cell. These would represent potential side effects for this therapeutic effect. Currently little is known about MOR-DOR heterodimerization outside of the nervous system.

6.3 Clinical Implications

The experiments in chapter four provide some of the first evidence that an injury specific promoter can be used to enhance transgene expression in a subset of primary afferent neurons. As HSV is tissue specific in that it primarily infects primary sensory afferents, the idea of viral vector specificity by choosing a promoter for a peptide that is

highly upregulated after nerve injury was able to be expanded upon. As such, constitutively active transgene expression, which has high potential to be turned off over time (Papadakis et al., 2004) and to potentially dampen physiological pain, could be replaced with a vector system in which a transgene is only expressed in an injured state and in the affected neurons. As there has already been clinical success using a HSV-based vector (Fink et al., 2011), the findings presented here have the potential to lead to greater efficiency and better design of viral vectors in the treatment of neuropathic pain.

Chapter five explores the development of the MOR-DOR heterodimer as a novel therapeutic target for the prevention of peripheral opioid tolerance. A novel HSV vector strategy to increase or decrease the expression of DOR was used as well as a small cell permeable peptide inhibitor of MOR-DOR heterodimers was used to prevent MOR-mediated tolerance. Pre-clinically, small TAT peptides have been developed using the same platform to inhibit PKC isozymes in the study of formalin induced nociception (Sweitzer et al., 2004a). Similar small TAT linked peptides are being developed and utilized in early stage clinical trials for the treatment of neuropathic pain (Cousins et al., 2013). Understanding the mechanisms of MOR-DOR interactions has significant clinical relevance as this information can potentially lead to the development of opioid pain relievers at doses lower than that which is normally required and therefore limiting adverse side effects.

6.4 Future Directions

More studies are needed to further elucidate the molecular mechanisms of MOR-DOR heterodimerization after chronic opioid treatment. Several changes in signaling pathways and downstream mediators have been observed after chronic opioid treatment

that potentially play a role in tolerance. These changes include a switch from $G\alpha_{i/o}$ coupling to $G\alpha_s$ coupling (Wang et al., 2005), β-arrestin recruitment and targeting for the lysosomal pathway (Rozenfeld and Devi, 2007; He et al., 2011; Milan-Lobo and Whistler, 2011), changes in ERK phosphorylation (Rozenfeld and Devi, 2007), increased protein kinase C (PKC) (Bailey et al., 2006) and G protein coupled receptor kinases (GRK) activity (Whistler and von Zastrow, 1998), and changes in regulator of G protein signaling (RGS) protein modulation of opioid receptor signaling (Garnier et al., 2003; Traynor, 2012) (Fig 6.1). Now that more information about MOR-DOR heterodimerization is emerging and a peptide inhibitor of this heterodimerization has been developed, these changes in signaling pathways and downstream mediators should be evaluated with a focus on MOR-DOR interactions. As a result of the findings in chapter five, it is hypothesized that blocking MOR-DOR heterodimerization will lead to a return to normal signaling through $G\alpha_{i/o}$ and recycling of receptors rather than degradation. Since an increase in $G\alpha_o$ expression was seen in DRG of opioid exposed rats, it is predicted that during opioid tolerance there is a switch to stimulatory signaling pathways. Rather than a decrease in adenylyl cyclase and cAMP there would now be an increase which, for example, would cause increased PKA activity. Therefore, in the peptide inhibitor strategy, it is predicted that there would be a decrease in PKA expression with the use of the peptide inhibitor of MOR-DOR in animals that are chronically treated with opioids compared to those that are not given the inhibitor.

Molecular approaches could include Western blot analysis to further look for changes in protein expression of signaling mediators, receptor binding assays to evaluate potential changes in receptor binding properties, cAMP and β-arrestin recruitment assays

to measure cAMP levels and β-arrestin in the presence or absence of the peptide inhibitor, and pharmacological approaches such as inhibitors of PKC, GRKs to further understand the underlying mechanisms of peripheral opioid tolerance.

6.5 Conclusions

More pre-clinical research needs to be conducted in order to further elucidate the mechanisms of neuropathic pain in order to lead to the development of better treatment options. The ideal opioid treatment for NP conditions would be one that relieves pain while also having a low risk for tolerance development and unwanted side effects. The work presented in this thesis demonstrates that HSV-mediated expression of a transgene can be driven in an injury specific manner to enhance analgesia and a peptide inhibitor of MOR-DOR heterodimerization can lead to a decrease or prevention of peripheral opioid tolerance. Together, these two strategies may serve as the basis for more safe and effective treatments for neuropathic pain.

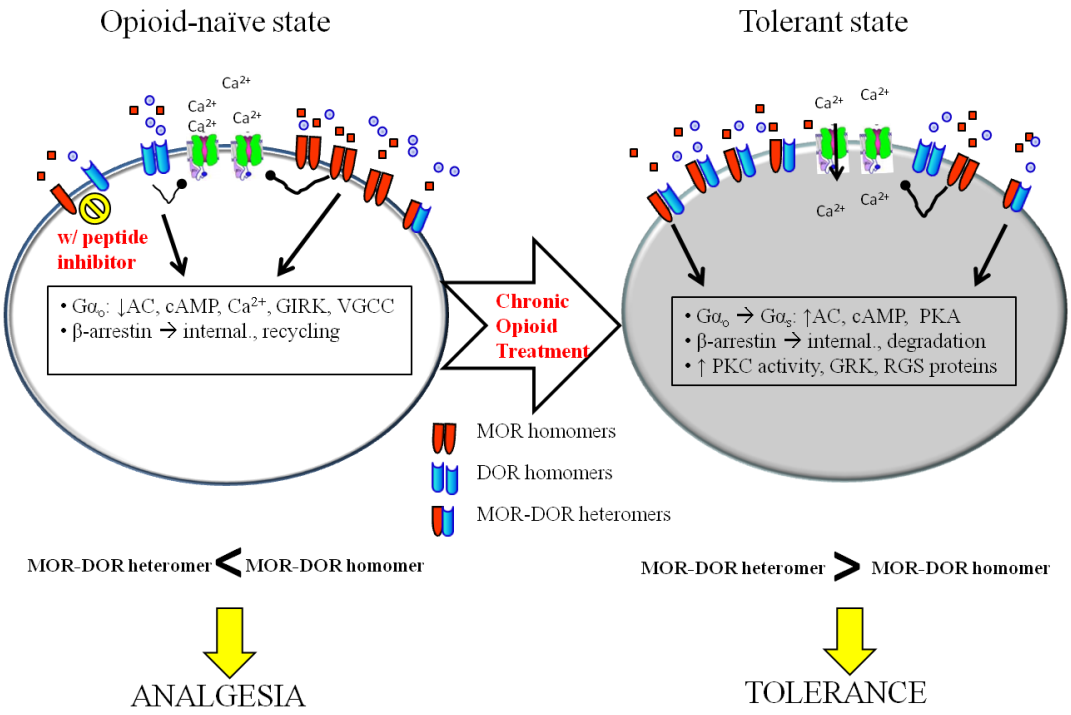

FIGURE 6.1: Schematic of proposed changes in downstream effects of MOR-DOR heterodimerization in an opioid tolerant state versus that in an opioid naïve state and after treatment with a peptide inhibitor of MOR-DOR heterodimerization. AC= adenylyl cyclase; GIRK= G-protein coupled inwardly rectifying potassium channel; VGCC= voltage gated calcium channel; PKA= Protein kinase A

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