CHARACTERIZATION OF MORPHINE SELF-ADMINISTRATION

FOLLOWING SPINAL CORD INJURY

A Dissertation

by

SARAH ANN WOLLER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Michelle A. Hook
Committee Members,	James W. Grau
	Rajesh Miranda
	Jianrong Li
Intercollegiate Faculty Chair,	Jane Welsh

August 2013

Major Subject: Neuroscience

ABSTRACT

Approximately two-thirds of patients will experience pain following spinal cord injury (SCI). This pain can arise as an immediate consequence of SCI, or can develop over time into chronic, neuropathic pain. Individuals are frequently prescribed opioid analgesics, including morphine, for the treatment of pain in both the acute and chronic phases of SCI. Yet, despite the prevalence of opioid use, no studies have examined the addictive potential of opioids, or their secondary effects, following spinal injury.

These experiments used a clinically relevant self-administration paradigm to examine both addiction and functional recovery after morphine administration. To assess morphine administration in the acute phase of SCI, animals were placed in operant chambers 24-hours following spinal injury. In the chambers, depression of a reinforced lever resulted in an intravenous infusion of morphine (or vehicle). Animals were placed in the chambers for 7, 12-hour sessions and could administer up to 30 mg of morphine per session. Morphine self-administration was also examined in the chronic phase of injury. Animals were placed into operant chambers for 7, 12-hour sessions beginning 14 or 35 days after injury. The amount of morphine administered, as well as recovery of locomotor function and general health, was compared across subjects with SCI and sham (no injury) controls.

In the acute phase of injury, SCI significantly reduced self-administration of morphine, but administration led to decreased recovery of locomotor function and

ii

weight loss. In the chronic phase of injury, self-administration did not differ between contused and sham animals. All subjects administered the full amount of morphine available each day. In this phase of injury, morphine administration led to significant weight loss, but did not attenuate recovery of locomotor function.

These studies suggest that spinal injury reduced the addictive potential of morphine in the acute, but not the chronic, phase of SCI. However, acute administration of high doses of morphine decreased recovery of locomotor function. Morphine should not be used in this phase of injury for the clinical treatment of pain. In the chronic phase, opioid use must be closely monitored as use may result in addictive behavior.

DEDICATION

For all that you do, Khairi, this work is for you.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I[1]

INTRODUCTION AND LITERATURE REVIEW

Overview

Spinal cord injury (SCI) results in numerous consequences affecting both physical and mental health. The most significant physical deficits resulting from injury are loss of sensation and paralysis. Despite loss of sensation, however, the majority of people will experience pain as a result of injury. For most, this pain will develop into severe or excruciating neuropathic pain within five years of injury (Siddall et al., 2003). This pathological pain has been shown to significantly decrease quality of life (Celik et al., 2012), and has been linked to a lower self-rating of general health (Siddall et al., 2003), stress (Störmer et al., 1997), and depression (Harden & Cohen, 2003; Wetering et al., 2010; Cairns et al., 1996). Given the significant impact pain has in this population, it is important that it be properly managed. Nonetheless, neuropathic pain remains difficult to treat and often requires clinicians to take a trial-and-error approach to pain management. Moreover, one of the most effective treatments for this pain, opioids and specifically morphine, are highly addictive in uninjured populations. Yet, there have been no empirical studies addressing the addictive potential for morphine following SCI. Studies of this nature are important because morphine cannot simply be removed as a treatment option when patients experience intractable pain, and consequently the injured population is maintained on high doses of opioids for long periods of time. It is

---

[1] Reprinted with permission from "Opioid administration following spinal cord injury: implications for pain and locomotor recovery" by S.A. Woller & M.A. Hook, 2013. *Experimental Neurology*, in press. Copyright (2013) by Elsevier.

imperative that we understand how injury can affect the addictive potential of opioids, and effects secondary to pain relief.

The experiments in this dissertation will investigate the addictive potential of morphine following SCI in a rodent contusion model. Use of a rodent model of SCI paired with a self-administration paradigm will allow for the assessment of abuse liability in the presence of both nociceptive and neuropathic pain. In addition, the effects of morphine on recovery of locomotor function, the development of pathological pain, and general health will be studied. This introduction will provide an overview of SCI, and the treatment of pain following injury. Then, the consequences of opioid administration following SCI, specifically addiction, attenuation of locomotor recovery, and the development of pathological pain, will be discussed. Finally, the specific aims of this dissertation will be outlined.

Spinal Cord Injury and the Development of Pain

The development of pain is a significant consequence of SCI. It has been estimated that in the period of time immediately following the injury, 11-94% of people will experience pain not only as a result of the injury itself, but also from peripheral injuries sustained in unison, as in the case of a motor vehicle accident (Störmer et al., 1997; Fenollosa et al., 1993; Donnelly & Eng, 2005; Cardenas et al., 2004; Siddall et al., 2003; Finnerup et al., 2001; Ravenscroft et al., 2000; Siddall & Loeser, 2001). Unfortunately, this pain is known to develop through a number of pathways, and is yet to be fully explained. Pain can result from the loss of cells, and the secondary processes initiated as a result of the mechanical trauma. For instance, traumatic injury to the spinal

cord results in the immediate death of neurons, glia, and endothelial cells at the impact site. Over time, continued apoptosis is caused by a number of secondary processes all set in motion by the initial impact, including increased levels of excitatory amino acids, disruption of calcium concentration, production of nitric oxide (NO), oxidative stress, and continued immune activation. The loss of cells resulting from any of these processes can directly or indirectly influence the development of pain following injury. For instance, the loss of serotonergic fibers decreases descending inhibition, resulting in hyperexcitation of dorsal horn neurons, contributing to the development of pain (Hains et al., 2003).

Spinal cord injury results in cell loss occurring via necrosis, a primary effect of injury or via apoptosis, a secondary pathology. Necrosis is characterized by cellular swelling, membrane lysis, release of intracellular contents, and the launch of an inflammatory response (Cohen, 1993; Majno & Joris, 1995). Apoptosis, on the other hand, is a programmed cell death characterized by cell shrinkage and phagocytic engulfment of dead cells. Apoptosis, as a result of trauma, is initiated by the release of cytochrome c from the mitochondria and the activation of procaspase-9, which, together, lead to the activation of caspase-3 (Springer et al., 1999; Sekhon & Fehlings, 2001). Activation of this apoptotic pathway has been demonstrated following SCI (Springer et al., 1999), resulting in the loss of GABAergic (Bao & Liu, 2003; Rafati et al., 2008; Sah et al., 2002; Sharma & Sjoguist, 2002) and serotonergic fibers to induce the loss of motor function and the development of pain (Hama & Sagen, 2012; Gwak & Hulsebosch, 2011; Meisner et al., 2010; Costigan et al., 2009).

Similarly, apoptosis leads to the release of pro-inflammatory cytokines and induces migration of immune cells to the injury site (Kang et al., 1997; Seino et al., 1997; Desbarats et al., 2003; Letellier et al., 2010). The immune response begins with the infiltration of neutrophils, monocytes, macrophages, microglia, and lymphocytes. This immune response, while completely necessary following injury, is often detrimental (Schwartz, 2003). In fact, astrocytes and microglia express many of the same receptors and release many of the same factors as neurons, marking them as prime contributors to the development of neuropathic pain following SCI (Gwak & Hulsebosch, 2011; Pineau & Lacroix, 2007; Porter & McCarthy, 1997; Wang et al., 2009; Jarvis, 2010). Normally, microglia are present in a resting state, but in the presence of interleukin (IL) -6, adenosine triphosphate (ATP), substance P, or fractalkine, among others, microglia become activated (Gwak et al., 2012; Hulsebosch et al., 2009), resulting in a morphological and functional change (Soulet & Rivest, 2008). Microglia are activated within minutes of CNS injury and normally function to remove debris and damaged cells (Kreutzberg, 1996; David et al., 1990; Avellino et al., 1995; Fleming et al., 2006; Carlson et al., 1998). However, these cells can remain activated for weeks to months following injury and contribute to continued damage and cell death via the release of glutamate, ATP, calcitonin gene-related peptide (CGRP), pro-inflammatory cytokines, reactive oxygen species (ROS), NO, and proteases (Kreutzberg, 1996; Aloisi, 2001; Dong & Benveniste, 2001; Fleming et al., 2006; Carlson et al., 1998; Lieberman et al., 1989; Priller et al., 1995; Rischke & Krieglstein, 1991; Stanley et al., 1994; Svensson et al., 1993). Several of these factors (e.g. increased extracellular glutamate, pro-

inflammatory cytokines) have been associated with the development of pain via sensitization of sensory circuits (Bennett et al., 2000a, b; Detloff et al., 2008). This persistent, dysfunctional glial reaction, termed "gliopathy" (Hulsebosch, 2008), is thought to contribute to central pain following injury (Costigan et al., 2009).

Astrocytes have also been shown to contribute to "gliopathy." Activated within 24 hours of spinal cord injury, astrocytes can remain active for months to years (Schnell et al., 1999; Popovich et al., 1997). Under normal circumstances, astrocytes are responsible for maintaining glutamate homeostasis, and preventing excitotoxicity (Lepore et al., 2011). However, following injury, astrocytic activation leads to the activation of MAPK pathways. These pathways can activate the nuclear transcription factor, NF-κB (nuclear factor κB), which subsequently results in increased production of pro-inflammatory cytokines, chemokines, prostaglandins, NO, free radicals, neurotoxins, and excitatory amino acids (Hameed et al., 2010). These substances, as with those released from microglia, are pain-mediating and, in a cyclic manner, contribute to continued neuronal hyperexcitability following injury (Detloff et al., 2008; Hulsebosch et al., 2009; Keane et al., 2006; Scholz & Woolf, 2007; Vallejo et al., 2010).

The toll-like receptor (TLR) has also been found to play a role in the development of pain. TLRs are a collection of 13 single transmembrane receptors normally expressed on microglia and, in response to inflammation, on astrocytes. TLRs are believed to activate pathways similar to those activated by IL-1, eventually leading to the activation of NF-κB, and resulting in the production of pro-inflammatory cytokines (e.g. IL-1β and TNFα). A role for this pathway is well established in the

initiation and maintenance of chronic pain (Milligan & Watkins, 2009; Nicotra et al., 2012). In particular, the TLR4 pathway has been well defined (see Hutchinson et al., 2011; Hameed et al., 2010; Nicotra et al., 2012). Briefly, ligand binding to the TLR4 results in activation of phosphoinositide 3-kinase (PI3K) and Akt, which can lead to apoptosis via the caspase-3 pathway. In addition, downstream activation includes mitogen activated protein kinase (MAPK), NFκB, and tumor necrosis factor (TNFα), which lead to the release of pro-inflammatory cytokines and the development of allodynia. Like the IL-1 pathway, this pathway is relevant to the development of pain (Nicotra et al., 2012; DeLeo et al., 2004; Raghavendra et al., 2003; 2004; Tanga et al., 2005). In support of this, blocking the TLR4 is able to prevent (Bettoni et al., 2008) and reverse neuropathic pain in an experimental setting (Hutchinson et al., 2007, 2008, 2010). In an SCI model, TLRs activate a number of signaling cascades, which affect the pathophysiology of secondary injury processes (Kigerl & Popovich, 2009), but the role of TLR4 in producing pain following SCI remains to be determined.

Many pathways have been found to play a role in the development of neuropathic pain following spinal cord injury. While neuronal pathways were thought to primarily contribute to the development of pain, increasing evidence supports a role of glia. Further investigation of the underlying mechanisms will allow for better, more specific treatments (with fewer side effects) for pain following SCI.

Treatment of Pain Following Spinal Cord Injury

Clinicians are faced with the challenge of properly managing and treating the various types of pain that develop after SCI. Not surprisingly, relief of chronic pain is a

high priority to the spinally injured. In fact, when a quadriplegic population was surveyed, 15-20% indicated that the relief of chronic pain, which develops in up to two-thirds of people, would most improve their quality of life (Siddall et al., 2003; Anderson, 2004). This number is almost equal to the percentage indicating restoration of locomotor function as a priority (Anderson, 2004). Although a considerable amount of research has focused on preventing the development of pain, and developing effective treatments, it remains difficult to treat (Heutink et al., 2012). Currently, chronic pain is treated with a variety of pharmaceuticals including opioids, antidepressants, anticonvulsants (e.g. gabapentin), non-steroidal anti-inflammatory drugs (NSAIDs), and N-methyl-D-aspartate (NMDA) antagonists (Xu & Yaksh, 2011; Cruccu, 2007; Teasell et al., 2010; Finnerup et al., 2010; Finnerup & Baastrup, 2012). While all show varying efficacy, morphine has long been considered a standard treatment in pain relief. Indeed, opioids are among the most effective treatments for neuropathic pain, and are commonly trialed for analgesic efficacy (Clark, 2002; Liang et al., 2008; O'Connor & Dworkin, 2009; Przwlocki & Przewlocki, 2005; Sindrup & Jensen, 1999; Widerstron-Noga & Turk, 2003; Warms et al., 2002).

For many years, opioid analgesics were considered to be a first-line medication for the treatment of neuropathic pain. Now, these medications are considered to be a second-line treatment (Attal et al., 2010; Dworkin et al., 2010). This change is the result of concern surrounding the presence of adverse side effects, a lack of evidence demonstrating long-term safety of use, evidence showing continuous use is often associated with the development of dependence, tolerance, opioid-induced hyperalgesia,

and / or addiction, and a lack of placebo-controlled trials assessing the efficacy of opioid treatment following SCI (Dworkin et al., 2007; Ballantyne & LaForge, 2007; Ballantyne & Mao, 2003; O'Connor & Dworkin, 2009; Trescot et al., 2008; Attal et al., 2009). In fact, it is known that, in the long term, approximately 20% of people will discontinue opioid treatment (Moore & McQuay, 2005), because of significant side effects such as respiratory depression, constipation, nausea, vomiting, sedation, dizziness, and headache (reviewed in Dellemijn, 1999; Cruccu, 2007; Dworkin et al., 2007). Notably 80% of patients will continue to use opioids, however, leaving them vulnerable to the potential development of addiction. In addition to these side effects, it has been demonstrated in both animal models and human studies that administration of analgesics such as morphine can potentiate the development of neuropathic pain and allodynia (Chang et al., 2007; Hook et al., 2007; Liang et al., 2008; Yu et al., 1997a, 1997b).

The lack of evidence regarding safety is particularly concerning when opioid analgesics are administered following SCI. Emerging evidence suggests that opioid administration can interact with spinal cord injury pathology to negatively impact locomotor recovery, the development of pain, and general health. These are concerns that are not always present in an uninjured population, but warrant investigation in the injured population. In a similar manner, the processes occurring after spinal cord injury are likely to influence the development of addiction. The following sections will discuss the consequences of opioid administration following spinal cord injury, focusing on the development of addiction, effects on recovery of locomotor function, and the development of pathological, paradoxical pain.

Consequences of Opioid Administration After SCI: Addiction

Traditionally, opioid receptor agonists are known for their analgesic (antinociceptive) and rewarding, hedonic properties. This is evidenced by their frequent use in the treatment of pain, and the common occurrence of abuse following recreational or therapeutic use. In fact, it is estimated that 18-45% of individuals using opioids for the management of chronic pain will abuse the drug (Ballantyne & LaForge, 2007; Compton & Volkow, 2006; Contet et al., 2008; Heinemann et al., 1992; Morasco & Dobscha, 2008; Trescot et al., 2008). Inconsistencies between reports of the incidence of addiction in the clinic, resulting in this wide range, stem from differences in definitions of abuse, methods of reporting, and populations being surveyed, along with a general lack of empirical research examining the efficacy of long-term opioid use and addictive potential (Bell & Salmon, 2009; Dersh et al., 2008; Hojsted & Sjogren, 2007; Morasco & Dobsha, 2008; Radnitz & Tirch, 1995). Some reports indicate that these estimates of opioid abuse are low, whereas others suggest they are high. As noted, the fluctuation in incidence may depend on the characteristics of the population being sampled. For example, including depressed individuals in these surveys of drug misuse paints a very different picture. Depressed individuals are not only prescribed opioids more frequently, but are also given higher doses of opioids (Koyyalagunta et al., 2013; Blanco et al., 2013; Dobscha et al., 2013). This is concerning as there is a reported increased incidence of depression in individuals with spinal cord injury (Bonanno et al., 2012; Al-Owesie et al., 2012; Migliorini et al., 2009; Bombardier et al., 2012). Given

the estimates of abuse and various factors that may contribute to abuse liability, the use of opioids for the management of pain must be further investigated.

Opioid analgesics are commonly trialed for efficacy in the period of time immediately following the injury (the acute phase) and as a long-term treatment for pathological pain (the chronic phase). In the acute phase of injury, opioid analgesics are used to treat nociceptive pain, which will resolve with healing, making the period of opioid use relatively short. Addiction has not typically been studied in this phase of injury. Previous experiments from our lab have, however, assessed the addictive potential of morphine in the acute phase of SCI using a conditioned place preference paradigm (Woller et al., 2012). In these studies, animals were given a contusion or sham injury or remained intact. Two days following injury, animals began conditioning: they were placed in a distinct context paired with an injection of morphine (2.5 mg/kg, i.p.) or in a separate context paired with saline. Animals received two context drug pairings (for both morphine and saline) over two days. When the preference was tested, interestingly, contused animals developed a stronger preference for the morphine-paired chamber than sham or intact controls, suggesting the contusion injury is increasing the addictive potential for morphine in this phase of injury. As this preference was based on just two drug-context pairings, the increased preference underscores the potential for addiction even with short-term use in the acute phase of SCI.

In the chronic phase of injury, opioids are used to manage neuropathic pain. This pain is often described as severe or excruciating and does not resolve with time. In this case, long-term use of opioids is expected. A review of cases of human opioid treatment

of chronic pain, however, revealed a low risk of dependence (Minozzi et al., 2013). Similarly, reports from extant literature suggest that a state of neuropathic pain decreases the addictive potential of opioids. Niikura and colleagues (2008) used a sciatic nerve ligation to induce neuropathic pain in rats. They found that the rats experiencing pain did not develop a conditioned place preference for morphine. Martin et al. (2007) also found rats experiencing neuropathic pain will self-administer less morphine than their pain free counterparts, at low doses. Additionally, they examined the rewarding effect of opioids using an intracranial self-stimulation paradigm and found the hedonic properties of morphine are reduced in rats experiencing neuropathic pain (Ewan & Martin, 2011). These studies would suggest the development of addiction is not a concern when opioids are used to treat neuropathic pain, but this is in contrast to reports of opioid addiction and abuse of opioid analgesics. Even though the addictive nature of opioids is well documented, little work has been done to examine the addictive potential of morphine following SCI.

Following SCI, molecular processes underlying the pathology of the injury may influence addiction. Specifically, the κ-opioid receptor (KOR) system is thought to play a role. Typically, this system is considered to be anti-reward. Activation of KOR, through increased dynorphin levels, has been shown to counter opioid-induced analgesia, and is known to counter the rewarding properties of drugs of abuse (Lutz & Kieffer, 2013; Wang et al., 2010). Following exposure to social or physical stressors, as spinal cord injury can be, there is an increase in activation of the KOR system (Lutz & Kieffer, 2013; Bruchas et al., 2010). This same activation has been demonstrated with

extended exposure to drugs of abuse, such as morphine (Lutz & Kieffer, 2013; Butelman et al., 2012; Wang et al., 1999). This would suggest morphine administration in the acute phase of spinal cord injury is likely to be less rewarding because the rewarding properties are being countered by activation of the KOR system. This may decrease the addictive potential of morphine following spinal cord injury.

However, this same system has been implicated in drug relapse, and may contribute to addiction (Chavkin, 2012; Wang et al., 2010). Clinically, not all individuals with a spinal cord injury are drug-free. In fact, Heinemann et al. (1988) found that up to 62% of SCI patients had misused drugs or alcohol at the time of their injury. This suggests that the injury, along with stress-induced increases in dynorphin, could increase the addictive potential of morphine in patients with previous history of drug abuse. Indeed, stress-related increases in dynorphin have been linked to increased conditioned place preference for cocaine and nicotine (McLaughlin et al., 2003, 2006; Schindler et al., 2010, 2012; Smith et al., 2012), and have been associated with increased risk of drug abuse in people (de Kloet et al., 2005). Thus, activation of the KOR may induce drug-seeking behavior.

Numerous complex processes are known to underlie the development of addiction. Some of these may be changed following injury, and in the transition between acute and chronic pain. Understanding how these variables will affect addiction after SCI is critical.

Consequences of Opioid Administration After SCI: Attenuation of Locomotor

Recovery

Addiction is not the only concern surrounding the use of opioid analgesics for the treatment of pain resulting from a spinal cord injury. Recent experimental evidence from our laboratory has demonstrated that a single, intrathecal (i.t.) administration of morphine 24-hours after spinal cord injury significantly attenuates recovery of locomotor function (Hook et al., 2009; 2011). In these experiments, animals received a moderate contusion injury and an intrathecal catheter was implanted. The next day, the extent of spared locomotor function was evaluated using the Basso, Beattie, Bresnahan (BBB) scale (Basso et al., 1995). All animals had equivalent scores and were divided into one of three groups: a vehicle, 30 μg, or 90 μg morphine group. Morphine was administered through the catheter, and recovery was monitored for 21-days. At the end of the recovery period, the animals that had received 90 μg morphine showed significantly lower locomotor recovery than those receiving saline, indicating that morphine administration is undermining recovery of locomotor function. With the exception of these recent studies by Hook (2009; 2011), the effects of exogenous opioid administration on recovery of locomotor function after SCI have received little attention.

The lack of experimental research is concerning because there is significant evidence that opioids may act synergistically with SCI to exacerbate cell death and decrease recovery of locomotor function. In the acutely injured spinal cord, glutamate levels increase in minutes, and return to normal within 1.5 hours (McAdoo et al., 2005). It is thought that, while dying cells can continue to release glutamate over days, the

levels are not high enough to contribute significantly to excitotoxicity (McAdoo et al., 2005). However, compounding this increase in glutamate levels through the administration of morphine (Lim et al., 2005), it is possible that SCI and opioid administration in the early phase of SCI can exacerbate and sustain neuronal (Lepore et al., 2011; Nottingham & Springer, 2003) and/or oligodendrocytic (Xu et al., 2004; McTigue, 2008; Almad et al., 2011) cell death, which could undermine recovery of locomotor function following injury.

Indeed, experimental evidence suggests agonists for the KOR and μ-opioid receptor (MOR) can have detrimental effects on locomotor function, by inducing central sensitization. For instance, it is known that intrathecal administration of dynorphin, an endogenous ligand for the KOR (Zhang et al., 1998), or intrathecal dynorphin A (2-17), a stable metabolite of dynorphin, induce long-lasting paralysis in intact rats (Headrick et al., 1995; Caudle & Isaac, 1987; Faden, 1990; Faden & Jacobs, 1983; Hemstapat et al., 2009). For example, Faden & Jacobs (1984) conducted a series of experiments in which they administered dynorphin peptides intrathecally and quantified the effects on motor function. They found dose-dependent decrements in motor function produced by dynorphin (1-17), dynorphin (1-13), dynorphin (1-8), and α-neo-endorphin. This hindlimb paralysis was evident within 10 minutes of administration, and was not blocked or reversed by naloxone administration (Faden & Jacobs, 1984). Further studies demonstrated that spinal levels of dynorphin increase with the severity of SCI (Faden et al., 1985), and following prolonged morphine administration (Mao et al., 2002). Moreover, inflammatory pain, such as that resulting from SCI, results in upregulation of

spinal dynorphin (Nahin et al., 1989; Ruda et al., 1988; Przewlocki et al., 1992). The paralysis and loss of tail flick reflex resulting from dynorphin activity are NMDAR-dependent (Caudle & Isaac, 1987; Long et al., 1987; Faden & Simon, 1988; Faden 1992; Woods & Zagen, 2001; Hemstapat et al., 2009), and treatment with a selective, noncompetitive NMDAR antagonist prior to dynorphin administration lessens resultant paralysis (Bakshi et al., 1992). Similarly, dynorphin potentiates NMDA currents (Lai et al., 1998) and increases internal calcium ion concentrations leading to enhanced neuronal loss. These effects are blocked by the NMDAR antagonist MK-801 (Hauser et al., 1999). This result implicates NMDAR in producing negative effects on locomotor function. Overall, whether increased by morphine administration or SCI, dynorphin is leading to paralysis via excitotoxicity.

Spinal cord injury also activates an immune response reminiscent of that produced with opioid administration. Interleukin (IL)-1β, a pro-inflammatory cytokine released from microglia has been identified as a key player affecting locomotor function following SCI (Nesic et al., 2001). IL-1β is expressed within 15-min following injury in a rodent model of SCI, and is one of the first cytokines released by activated microglia (Kim et al., 2006; Pineau & Lacroix, 2007). While it has been demonstrated that IL-1β levels increase as a result of injury, exacerbating these levels is likely to produce further trauma. In fact, intrathecal administration of IL-1 β for three days in intact rats causes significant and lasting motor deficits that are blocked by the concurrent administration of IL-1ra (Liu et al., 2008). Similarly, morphine administration has been shown to increase spinal levels of IL-1β in contused rats, relative to vehicle controls (Hook et al., 2011).

As with the application of IL-1β, increased IL-1β levels resulting from morphine administration yielded an attenuation of locomotor recovery (Hook et al., 2011). Antagonizing the IL-1 receptor before morphine administration blocked morphine-induced locomotor deficits in SCI rats (Hook et al., 2011). Experimental evidence indicates IL-1β can induce apoptosis by phosphorylation of p38 MAPK, activating the pro-apoptotic caspase-3 cascade (Mika, 2008; Springer et al., 1999). Furthermore, cytokines, such as IL-1β, can activate both cytochrome c and caspase 9, which lead to activation of the pro-apoptotic caspase-3 pathway. Cell death initiates the release of pro-inflammatory cytokines, NO, and reactive oxygen species (Block & Hong, 2005; Min et al., 2003, 2004; Cho et al., 2011), causing further, sustained activation of microglia, enhancing the pro-inflammatory environment of the injured spinal cord, and affecting locomotor recovery after SCI.

There are many routes by which opioid administration can exacerbate the pathology of spinal cord injury to contribute to increased loss of locomotor function. Morphine administration may increase glutamate availability, leading to excitotoxic cell death, Similarly, morphine will lead to activation of MOR and KORs and NMDARs, which can also induce processes initiating excitotoxicity. In addition, there is an increase in the levels of pro-inflammatory cytokines occurring as a result of injury and opioid administration, which lead to activation of apoptotic pathways, and sustained glial activation. Many of these same pathways can contribute to paradoxical pain.

Consequences of Opioid Administration After SCI: Paradoxical Pain

There is emerging evidence that opioid administration not only affects locomotor recovery, but also affects neuronal and glial systems to produce pathological pain. Indeed, the continuous administration of morphine can lead to the development of pain sensitization known as opioid-induced hyperalgesia (OIH; Mao, 2002; Lee et al., 2011). Opioid-induced hyperalgesia, has been observed following administration of intrathecal (i.t.) morphine (Mao et al., 1995), a fentanyl bolus (Célèrier et al., 2000), and with repeated heroin administration (Célèrier et al., 2001). Increasing the dose of the opioid does not relieve this pain; instead, relief requires a seemingly contradictory decrease in the dose (Lee et al., 2011). While the causal mechanisms for OIH are still largely unknown, the glutamatergic system and increased spinal dynorphin have emerged as important mediators (Mao et al., 2002; Silverman, 2009; Lee et al., 2011; Mayer et al., 1999). For instance, it has been suggested that central sensitization, resulting from excessive NMDAR activation can lead to the development of hyperalgesia (Costigan et al., 2009).

The development of OIH is related to increased NMDAR activation stemming from 1) increased NMDAR excitability and 2) increased glutamate availability. It is known that glutamate can cause persistent activation of NMDA receptors, leading to pathological pain (Jacquet, 1988b; Raigorodsky & Urca, 1987; Mao et al., 1994; Laulin et al., 2002). Similarly, repeated exposure to morphine increases NMDAR channel activation (Harris et al., 1996; Croul et al. 1998; Mao & Mayer, 2001). In support of this, treatment with an NMDA antagonist enhanced morphine analgesia (Mehta et al.,

2011). In addition, meeting the second criteria, chronic morphine administration can lead to the downregulation of spinal glutamate transporters, which increases glutamate availability (Lim et al. 2005). This additional glutamate availability, along with NMDAR excitation, increases NMDAR activation (Mao et al., 2002; Harris et al., 1996; Croul et al. 1998; Mao & Mayer, 2001). Activation of the NMDAR through hyperexcitation has been shown to produce pain and neuronal cell death by apoptosis (Mao et al., 1992; Mao et al., 1997).

As with recovery of locomotor function, a role of spinal dynorphin has also been established in OIH (Mao, 2002; Lee et al., 2011). Sustained morphine administration results in increased spinal levels of dynorphin (Vanderah et al., 2001), which are already increased as a result of SCI. Dynorphin is often pronociceptive (Cho & Basbaum, 1989; Caudle & Isaac, 1988; Kjander et al., 1990; Wang et al., 2001; Dubner & Ruda, 1992), and the release of this endogenous opioid can lead to the development of pain via the induction of central sensitization (Vanderah et al., 2000).

As discussed previously, glial cells also play a significant role in the development and maintenance of persistent pain following central nervous system (CNS) injury (Graeber & Christie, 2012; Graeber, 2010; Hulsebosch et al., 2009; Watkins et al., 2007; Re & Dubner, 2010; Gosselin et al., 2010; Graeber & Streit, 2010; Mika et al., 2013). Suter et al. (2007), for example, outlined potential glial-induced mechanisms involved in pain following injury (nerve, SCI, inflammation, etc.). It is important to note that various types of pain are manifest following injury, each with unique and shared mechanisms (for review, see Hulsebosch et al., 2009). Generally

speaking, however, microglia become activated in response to factors produced by damaged cells and contribute to pain states through their recruitment to the spinal cord dorsal horn (Gwak et al., 2012). Activation of microglia is positively associated with severity of allodynia following a moderate SCI or spinal nerve ligation (Detloff et al., 2008). Similarly, opioid-dependent activation of astrocytes and microglia affects the release of excitatory amino acids, nitric oxide (NO), reactive oxygen species (ROS), pro-inflammatory cytokines (e.g. IL-1β, TNFα), chemokines, and prostaglandins (Raghavendra et al., 2002; Aloisi, 2001; Dong & Beveniste, 2001; DeLeo et al., 2004). Of the pro-inflammatory cytokines, IL-1β, specifically, is known to be increased following morphine administration (Hook et al., 2011), and has been shown to induce allodynia and hyperalgesia when delivered intrathecally (i.t.) (Mika et al., 2008; Obreja et al.,2002; Opree & Kress, 2000). Glial activation alone can lead to neuronal hyperexcitability, neurotoxicity, and chronic inflammation, however, these effects, as they are initiated by SCI and interact with opioid administration, have not been examined. Evidence suggests that SCI and opioid administration, in combination, can lead to the development of pain through shared pathways (Woller & Hook, 2013).

Specific Aims

Experimentally, a rodent model of contusion injury is used to simulate the traumatic injury typically seen in humans (Sekhon & Fehlings, 2001). This rodent model of SCI relies on numerous behavioral tests to assess not only the development of pain, but also the recovery of locomotor function. Using these behavioral tests, it has been shown that a contusion injury produces symptoms of neuropathic pain in up to 80%

19

of rodents (Hulsebosch, 2002), compared to the estimated 66% of people that will experience similar pain characteristics. This makes the rodent contusion model clinically relevant in the study of pain. In addition, the rodent model of SCI can be used to assess the analgesic and addictive properties of drugs. The self-administration paradigm is commonly used to assess addiction in rodents, and in the case of SCI, simulates patient controlled analgesia. However, despite the clinical relevance, there have been no studies of addiction in this model of injury. It is important that an animal model is developed and used to delineate the potential for addiction after SCI, as well as the contribution of confounding factors such as pain or depression. With this in mind, the experiments in this dissertation are designed to characterize morphine self-administration following a spinal cord injury.

An animal model of SCI provides a unique opportunity to examine the addictive potential of morphine in a state of nociceptive pain and over the course of the development of neuropathic pain. In the acute phase of injury, defined here as days 1-7 post injury, animals are experiencing nociceptive pain as a result of the surgery itself. It is known that this pain resolves with healing and is well managed with opioid analgesics. Despite the frequent use for the treatment of pain, however, the addictive potential of morphine is not typically studied in this phase, or when opioids are being used to treat nociceptive pain. Thus, the first experiments (Aim I, Chapter III) will examine morphine self-administration in the acute phase of injury. In the early chronic phase of spinal cord injury, neuropathic pain is beginning to develop, and is well established in the late chronic phase. This pain does not resolve, and often gets worse, with time. The

20

abuse liability of morphine has been studied in cases of neuropathic pain, but not

neuropathic pain resulting from a spinal cord injury. Aim II (Chapter IV), therefore, will

examine the addictive potential of morphine in the early (days 14-20) and late (days 35-

42) chronic phases of spinal cord injury. In addition to characterizing the addictive

potential of morphine in the acute and chronic phase of injury, these studies will

examine secondary effects of morphine administration. In both the acute and chronic

phases of injury, recovery of locomotor function and weight loss will be examined.

We hypothesize that morphine acts synergistically with molecular process

occurring after spinal cord injury to contribute to attenuated recovery of locomotor

function and the development of pain (Woller and Hook, 2013). This possibility will be

addressed in the final aim (Aim III, Chapter V).

The discussion from the sections above can be tied together in one concept

(Table 1) demonstrating the interactions of opioid administration with the environment

of the spinal cord following injury, which can produce alterations in the addictive

potential of morphine, decrease recovery of locomotor function, and lead to the

development of pain. It is unlikely that any one factor is solely responsible for these

detrimental effects. Rather, imposing opioid administration onto the vulnerable, injured

spinal cord is likely to exacerbate and compound effects seen in either case individually,

to produce pathology. Thus, this idea hinges on a few key components: increased

glutamate, increased dynorphin levels, and increased release of pro-inflammatory

cytokines. Each of these components has been shown to have various negative effects

following opioid administration and spinal cord injury individually, but have not been

examined in combination. To begin to test this hypothesis, Aim III will examine the

development of opioid-induced hyperalgesia, the development of tolerance, and changes

in protein levels of TNFα, MOR, KOR, caspase 3, TLR2, and TLR4 occurring after

chronic morphine administration.

Concequence of SCI or opioid administration		Mechanisms common to SCI and opioid administration
Decreased locomotor function	Excitotoxic cell death	Increased extracellular glutamate
		Increased NMDAR activation
		Increased dynorphin levels
	Increased glial activation	Release glutamate, ATP, CGRP, pro-inflammatory cytokines, ROS, NO
	Apoptosis	Initiated via activation of caspase-3, increased Bax and decreased Bcl-2
		Causes release of proinflammatory cytokines
Pain development	Central sensitization	Increased NMDAR activaiton
		Increased extracellular glutamate
	Opioid-induced hyperalgesia	Increased NMDAR activaiton
		Increased glutamate availability
		Increased spinal dynorphin
	Increased glial activation	Release glutamate, ATP, CGRP, pro-inflammatory cytokines, ROS, NO, activation of MAPK pathwyas, TLR's
Addictive potential		Increased dynorphin levels
		Activation of TLRs

Table 1: Commonalities of opioid administration and SCI. The consequences of spinal cord injury and opioids administration are listed along with the mechanisms common to both.

23

CHAPTER II

GENERAL METHOD

Subjects

Male, Sprague-Dawley rats obtained from Harlan (Houston, TX) were used as subjects. Animals were 90-110 days old (300-350 g), and were individually housed in Plexiglas bins [45.7 (length) x 23.5 (width) x 20.3 (height) cm] with food and water available *ad libitum*. Subjects' bladders were expressed manually in the morning (8-9:30 AM) and evening (6-7:30PM) until they regained bladder control, which was defined as three consecutive days with an empty bladder at the time of expression. Animals were maintained on a 12-hour light-dark cycle. Except where noted, all behavioral testing occurred during the light portion of the cycle.

All of the experiments were reviewed and approved by the institutional care committee at Texas A&M and all NIH guidelines for the care and use of animal subjects were followed.

Surgery

Jugular Catheterization

To allow for the self-administration of morphine, animals were implanted with a jugular catheter. Rats were anesthetized using a combination of 80 mg/kg ketamine and 10 mg/kg xylazine, intraperitoneally (i.p.). While under anesthesia, a catheter consisting of a length of polyethylene (PE) 50 tubing was inserted into the jugular vein and tied to the vein. Using an 11-gauge stainless steel tube as a guide, the catheter was passed subcutaneously through the body of the animal so that it exited in the back. A back

mount cannula pedestal (model 313-00BM-10-SPC; Plastics One Inc., Roanoke, VA) was implanted subcutaneously and connected to the catheter. The back mount exited the skin between the scapulae and was covered with a dust cap. All incisions were closed using VetBond.

For the first 24-hours after surgery, the rats were housed in a recovery room maintained at 26.6°C. All subjects were treated (IP) with 100,000 U/kg penicillin G potassium immediately after surgery and again 2 days later. To help maintain hydration, the subjects were also given 3.0 ml of saline (0.9%, IP) following surgery. During the 5-day recovery period following surgery, the catheters were flushed with heparinized saline (0.25ml).

Contusion Injury

Five days after implantation of the jugular catheter, subjects were anesthetized with inhaled isoflurane (5% to induce anesthesia and 2-3% for maintenance), and an area approximately 4.5 cm above and below the injury site was shaved and disinfected with iodine. A 7 cm incision was made over the spinal cord, and two incisions extending 3 cm rostral and caudal to T12 were made on either side of the vertebral column. The lamina of the T12 vertebra was removed, exposing the spinal tissue. The vertebral column was then fixed within the MASCIS device (Constantini & Young, 1994; Gruner, 1992). Animals were given a moderate contusion injury by allowing the 10 g impactor (outfitted with a 2.5 mm tip) to drop 12.5 mm. The wound was closed with Michel clips. Sham subjects received a laminectomy only, and intact subjects received anesthesia only.

For the first 24-hours after surgery, the rats were housed in a recovery room as described previously. All subjects were treated (IP) with 100,000 U/kg penicillin G potassium immediately after surgery and again 2 days later. To help maintain hydration, the subjects were also given 3.0 ml of saline (0.9%, IP) following surgery. The Michel clips were removed 14 days following surgery.

Self-Administration Procedure

Apparatus

Self-administration took place in operant chambers (model E10-10; Coulbourn, Allentown, PA) enclosed in sound-attenuating cubicles. Each chamber was equipped with two levers with a stimulus light positioned over each. Infusion pumps (Razel Scientific Instruments, Stanford, CT) controlled drug delivery to each of the boxes, and a 20 ml syringe delivered an IV infusion (160 μl) over a 6-second timeframe. The chambers were interfaced with two IBM computers, which controlled drug delivery and recorded lever depressions.

Self-Administration Procedure

All animals were implanted with a jugular catheter, and 5 days later received a contusion or a sham injury or remained intact. Twenty-four hours following the contusion or sham surgery, locomotor function was assessed (see Assessment of Motor and Sensory Recovery) and the animals were placed in the self-administration chambers for 12-hours. In the chambers, depression of the right lever (FR1) resulted in an IV infusion of 0.0, 0.75, 1.5, or 3.0 mg morphine (equivalent to 0.56, 1.13, or 2.26 mg morphine base), with a maximum total dose of 30 mg morphine / day. This dose is

similar to what is used clinically in humans. Patients receive up to 120 mg/kg morphine

a day at the beginning of treatment (30 mg is between 75-120 mg / kg / day; Chu et al.,

2006), with doses increasing as needed for pain relief, often up to 180 mg morphine / kg

/ day (Clark, 2002; Schneider & Kirsh, 2010). We recorded the amount of morphine

administered for the first 7 days following injury.

In addition to the amount of morphine administered, we examined the rate of

administration. The computers recorded the time (min) between each lever depression.

These times, accounting for just the reinforced lever depressions, were summed, and

separated into 1-hour time bins. The cumulative number of reinforced lever depressions

made each hour was then multiplied by the dose administered to yield the cumulative

amount of morphine administered by hour.

Assessment of Motor and Sensory Recovery

Locomotor Recovery

Locomotor behavior was assessed using the Basso-Beattie-Bresnahan (BBB)

rating scale (Basso et al., 1995) in an open enclosure (99 cm diameter and 23 cm deep)

on the day following the contusion injury. The subjects were acclimated to the apparatus

for 5 min per day for 3 days prior to surgery. Twenty-four hours after surgery, each

subject was placed in the open field and observed for 4 min to assess locomotor

function. All observers had high intra- and inter-observer reliability (all r's >0.89), and

were blind to the subjects' experimental treatment.

Locomotor scores were transformed to help assure that the data were amendable

to parametric analyses (Ferguson et al., 2004). This transformation pools BBB scores 2-

4, removing a discontinuity in the scale. The transformation also pools scores from a region of the scale (14-21) that is seldom used for a moderate contusion injury. By pooling these scores, we obtain an ordered scale that is relatively continuous with units that have approximately equivalent interval spacing. Meeting these criteria allows us to apply metric operations (computation of mean performance across legs), improves the justification for parametric statistical analyses, and increases statistical power.

Girdle Reactivity

A grid map of the girdle zone for allodynic responding was made on the rats using an indelible marker (44 squares). A single von Frey filament with bending force of 204.14 mN (26 g force) was applied to each point on the grid, and vocalization responses were recorded on a grip map of that animal. For each subject, the total number of vocalizations were recorded (Nv) and normalized by the following formula: percent vocalizations=(Nv x 100)/total number applications (44).

Tactile Reactivity

Reactivity was assessed using von Frey stimuli formed from nylon monofilaments (Semmes-Weinstein Anesthesiometer; Stoelting Co., Chicago, IL) and applied to the plantar surface of the hindpaws. Subjects were placed into Plexiglas tubes [7.0 cm (internal diameter) x 20 cm (length)] that had 6 cm (length) x 1.7 (width) cm notches removed from the sides, to allow the hindlimbs to hang freely. After a 15-min acclimation period, the von Frey stimuli were applied sequentially at approximately 2 sec intervals until subjects withdrew the paw and vocalized. If no response was observed, testing was terminated at a force of 300 g. Each subject was

28

tested twice on each foot in a counterbalanced ABBA order. Test sequences were spaced 2 min apart. Stimulus intensity was reported using the formula provided by Semmes-Weinstein: Intensity= log10 (10,000*g force).

Thermal Reactivity

Thermal reactivity was assessed using radiant heat in the tail flick test. Subjects were placed in clear Plexiglas tubes with their tail positioned in a 0.5 cm deep groove, cut into an aluminum block, and allowed to acclimate to the apparatus (IITC Inc., Life Science, CA) and testing room for 15 min. The testing room was maintained at 26.5°C. Thermal thresholds were then assessed. Thermal reactivity was tested using a halogen light that was focused onto the rat's tail. Prior to testing the temperature of the light, focused on the tail, was set to elicit a baseline tail flick response in 3-4 sec (average). This pre-set temperature was maintained across all subjects. In testing, the latency to flick the tail away from the radiant heat source (light) was recorded. If a subject failed to respond, the test trial was automatically terminated after 8 s of heat exposure, to avoid tissue damage. Two tests occurred at 2-minute intervals, and the second test tail flick latencies were recorded as indices of nociceptive reactivity. To confirm that subjects did not respond in the absence of the stimuli, blank trials were also performed. A 'false alarm' was recorded if subjects made a motor or vocalization response during the blank tests. The blank trials were performed 1 min before or after each sensory test (in a counterbalanced fashion). No false alarms were recorded.

Western Blots

Animals (n=12) were deeply anesthetized with pentobarbital (50 mg/kg) and a 1 cm section of spinal cord around the lesion epicenter was rapidly removed. To determine the spatial (dorso-ventral) changes, the spinal cord tissue was further sectioned to yield dorsal and ventral portions. Total protein was extracted from the organic layer of the homogenized tissue containing protein and DNA using the QIAzol™ lysis reagent protocol for isolation of genomic DNA and/or proteins from fatty tissue (Qiagen, Valencia, CA). Following determination of the protein concentration, using the Bradford Assay (BioRad, Hercules, CA), protein samples were diluted in Laemmli sample buffer and stored at -80°C at known concentrations (usually 2-5ug/ul). Western blotting was used for the protein quantification of μ-opioid receptor (MOR), κ-opioid receptor (KOR), toll-like receptor (TLR) 2, TLR4, caspase 3, and TNFα. Equal amounts (30ug) of total protein were subjected to SDS-PAGE using 15% Tris-HCl precast gels (BioRad, Hercules, CA) for TNFa (estimated molecular weight of ~17kDA), caspase-3 (multiple bands between ~35kDA and 10kDA), and KOR (estimated molecular weight of 50kDA) or 12% Tris-HEPES precast gels (Pierce, Rockford, IL) for TLR4 (estimated molecular weight of 102kDA), TLR2 (estimated molecular weight of 100kDA), and MOR (estimated molecular weight of 50kDA). Following transfer onto PVDV membranes (Millipore, Bedford, MA), the blots were blocked for one hour in 5% blotting grade milk (BioRad, Hercules, CA) in Tris-Buffered Saline Tween-20 (TBST). After blocking, the blots were incubated overnight at 4°C in one of the following primary antibodies generated in rabbit: TNFα (1:500; #ARC3012 - Invitrogen,

Camarillo, CA), TLR2 (1:1000; #NBP1-95457- Novus Biologicals, Littleton, CO),

TLR4 (1:500; sc-30002- Santa Cruz Biotechnology, Inc, Santa Cruz, CA), Caspase-3

(1:1500; #NB100-56113- Novus Biologicals, Littleton, CO), MOR (1:400; sc-15310-

Santa Cruz Biotechnology, Inc, Santa Cruz, CA), KOR (1:750; #NB100-91902, Novus

Biologicals, Littleton, CO), or Actin (~43 kDA, 1:5000; ab8227- Abcam Inc.,

Cambridge, MA). With the exception of Actin, all primary antibodies were diluted in

blocking solution.

The following day, blots were washed in TBST (3 x 10 min) at room temperature

then incubated in HRP-conjugated goat anti-rabbit secondary antibodies (1:5,000;

#31460; Pierce, Rockford, IL) for 1 hour at room temperature. Following another 3 x 10

min series of washes, the blots were developed with ECL (Pierce, Rockford, IL) and

imaged with Fluorchem HD2 (ProteinSimple, Santa Clara, CA). Ratios of the integrated

densitometry of each protein of interest to the loading control (actin) were calculated,

normalized to sham vehicle controls and averaged for animals within each group.

Histology

In this experiment, animals (n=24) were deeply anesthetized with pentobarbital

(50 mg/kg) and perfused intracardially with phosphate buffered saline (PBS) followed

by 4% paraformaldehyde in PBS. A 1 centimeter section of spinal cord surrounding the

lesion center was removed and post-fixed in 4% PFA for 2 hours and then transferred to

30% sucrose for cryoprotection. Following a minimum of 72 hours in sucrose, the

section of cord was frozen in a cryomold with optimal cutting temperature (OCT)

compound. Twenty microns (20μm) horizontal sections were cut with a cryostat (Leica

Microsystems, Buffalo Grove, IL). Sections were then used for staining with cresyl

violet for Nissl substance and luxol fast blue for myelin (Beattie, 1992; Behrmann et al.,

1992).

Slides containing sections of spinal cord were placed in a 1:1 chloroform/ethanol

solution for 3 hours, and were then washed in 95% ethanol for 5 minutes. Slides were

put in Luxol Fast Blue overnight in a 50° C oven. The following day, slides were rinsed

in 95% ethanol, and were then quickly rinsed in distilled water (dH$_2$O). Slides then went

through a series of washes: 1 minute in lithium carbonate, 1 min in 70% ethanol, quick

rinse in dH$_2$0. These steps were repeated until the sections were light blue. Next, slides

were placed into cresyl violet for 1 min. Slides were then rinsed in dH$_2$O and

dehydrated for coverslipping.

In these sections, the total cross-sectional area of the cord and spared tissue was

assessed at the lesion center using MicrobrightField software as described by Hook et al.

(2011). Sections ±600, 1200, 1800, and 2400 μm from the lesion center (rostral and

caudal) were also traced and analyzed. Assessments were made by an experimenter who

was blind to the subject's treatment condition. Three indices of lesion magnitude were

derived: lesion, residual gray matter, and residual white matter. To determine the area of

lesion, an observer who was blind to the experimental treatments traced around the

boundaries of cystic formations and areas of dense gliosis (Hook et al., 2011). Nissl-

stained areas that contained neurons and glia of approximately normal densities denoted

residual gray matter. White matter was judged spared in myelin-stained areas lacking

dense gliosis and swollen fibers. These analyses yielded three parameters for each section: white matter area, gray matter area, and lesion area.

To control for variability in section area across subjects, we applied a correction factor derived from our sham vehicle animals. This correction factor is based on section widths and is multiplied by all area measurements to standardize area across analyses (see Grau et al., 2004). By standardizing areas across sections we were able to estimate the degree to which tissue is "missing" (i.e. tissue loss from atrophy, necrosis, or apoptosis). An accurate assessment of the degree to which a treatment has impacted, or lesioned, the cord includes both the remaining "damaged" tissue as well as resolved lesioned areas. When we sum the amount of "missing" tissue and the measure "damaged" area we derive an index of the relative lesion (% relative lesion) in each section that is comparable across sections. We can also compute the relative percent of gray and white matter remaining in each section, relative to sham controls.

Statistical Analyses

Comparisons between the amounts of morphine administered across surgery/dose/phase of injury, as well as the effects of morphine on recovery of function and molecular endpoints were analyzed using analysis of variance (ANOVA) and trend analyses. In experiments with a continuous independent variable (e.g. recovery period, rostral-caudal histological sections), mixed-design ANOVAs were used. In cases where significant between-subject differences were obtained (main effect of a single variable), group means were compared using the Duncan's New Multiple Range Test ($p < .05$). A non-parametric Kruskall-Wallace test was used to compare levels of the cleaved

caspase-3 band across groups. There does not appear to be a linear relation between levels of caspase-3 and the degree of apoptosis. Instead, numerous studies suggest that cell death will occur only when caspase activity reaches a critical threshold (Florentin and Arama, 2012). By ranking the levels of caspase-3 expressed across subjects and groups, the Kruskall-Wallace test allows for allows for a statistical comparison based on ranks of expression levels; a group expressing a higher level (mean rank) of expression would be more likely to surpass the critical threshold, and initiate cellular apoptosis.

CHAPTER III

SELF-ADMINISTRATION OF MORPHINE IN THE ACUTE PHASE OF

INJURY

As mentioned in the introduction, we have examined the addictive potential of morphine in the acute phase of SCI using a conditioned place preference (CPP) paradigm. In this previous study, contused animals developed a greater preference for a morphine-paired chamber at both a 1.25 and 2.5 mg/kg (i.p.) dose of morphine compared to sham and intact controls (Woller et al., 2012). This result suggests contused animals find morphine to be more rewarding. Why reward is increased in injured animals, however, is less clear. In the acute phase of injury, contused animals are experiencing pain as a result of injury. Thus, contused animals could be developing a preference for the analgesic properties associated with morphine administration. Indeed, the CPP paradigm has been used to assess spontaneous pain, and rats will develop a preference for a chamber paired with a pain-relieving drug (Navratilova et al., 2013). Critically, these pain-relieving drugs (e.g. clonidine) do not support a place preference in pain-free animals (Navratilova et al., 2013; King et al., 2009). The traditional view, using this paradigm, however, is that animals develop a conditioned place preference for the hedonic properties of morphine. This view suggests that contused animals may show an increased preference because spinal cord injury potentiates addiction. It is important to note, though, that after SCI both the analgesic and hedonic properties could be considered to be rewarding. This is an issue that must be further addressed.

35

In the current experiment, therefore, we used a self-administration paradigm to assess the rewarding properties of morphine following spinal cord injury. This paradigm is commonly used to assess drug reward, and is thought to provide a better model of addictive behavior (Koob et al., 2009). However, it has yet to be employed in the investigation of the addictive properties of drugs following spinal cord injury. The aim of this experiment was to examine the amount of morphine self-administered following a moderate contusion injury. The self-administration paradigm is also of clinical interest because it models patient-controlled delivery of analgesic agents (Martin et al., 2007; Sanchis-Segura & Spanagel, 2006). Based on the results of previous experiments using the conditioned place preference paradigm, we hypothesized that contused animals would administer more morphine than sham and intact controls, suggesting that injury is potentiating addiction.

Method

This experiment used a total of 12 rats (n=4). In this experiment, the surgeries and self-administration procedures are as described in Chapter II (General Method). Specifically for this study, sham, contused, and intact rats were placed into self-administration chambers for 12-hours, during their dark cycle (9pm-9am) for the first seven nights following injury. In the self-administration chambers, each lever depression resulted in the infusion of 1.5 mg morphine, and a total of up to 30 mg each session.

Results

Morphine Administered

We calculated the amount of morphine each subject self-administered based on the number of lever depressions, including only depressions when morphine was available (up to 20 presses in this dose). Although there was a tendency for sham controls to display higher levels of pressing than contused, which is consistent with the amount of morphine administered, there was not a significant main effect of surgery condition (both F's <2.44, $p > .05$). As shown in Figure 1 A & B, all subjects generally exhibited an increase in morphine administration over days (F $(6, 54) = 3.52, p < .05$). Contrary to our hypothesis, however, contused rats exhibited the lowest levels of morphine self-administration, yielding a main effect of surgery condition (F $(2, 59) = 5.29, p < .05$). No other differences were significant ($p > .05$).

Effects of Injury on Morphine Self-Administration are Dose-Dependent

In the first experiment, we found contused rats self-administered significantly less morphine than sham injured animals. However, there is considerable data to suggest that the effects of opioids depend on the dose, time, and route of administration (Yu et al., 1997a, 1997b). Therefore, this experiment examined whether the propensity for morphine administration after SCI is dose-dependent. We hypothesized that contused animals would continue to self-administer less morphine than sham controls, but this may depend on the dose.

Method

In this experiment, 32 animals (n=4) were implanted with a jugular catheter, and, five days later, were given a moderate contusion or sham injury (See General Method and Figure 2). Twenty-four hours later, baseline tests of locomotor function were conducted. The same day, animals were placed into self-administration chambers. Subjects were assigned to one of four groups based on their baseline locomotor score: 0.0 mg, 0.75 mg, 1.5 mg, or 3.0 mg/infusion. Baseline BBB scores were balanced across conditions. Irrespective of the morphine dose administered with each lever press, all subjects had access to a total of 30 mg morphine in each of the seven sessions.

Results

Morphine Administered

Examining the number of reinforced responses made overall, it was shown that, as expected, morphine treatment led to an increase in responses on the reinforced lever ($F_{(3, 24)} = 3.55$, $p < .05$). Again, net responses on the reinforced lever were not significantly affected by surgery condition (both F's < 2.49, $p < .05$).

In this experiment, the amount of morphine administered was once again calculated by examining the number of reinforced lever presses. Because the saline-treated subjects received no morphine, they were omitted from this analysis (Figure 3). As observed in the previous experiment, a contusion injury reduced self-administration at the intermediate dose (1.5 mg; Figure 3C). A lower dose of morphine led to a moderate level of self-administration in both groups (Figure 3B). At a high morphine
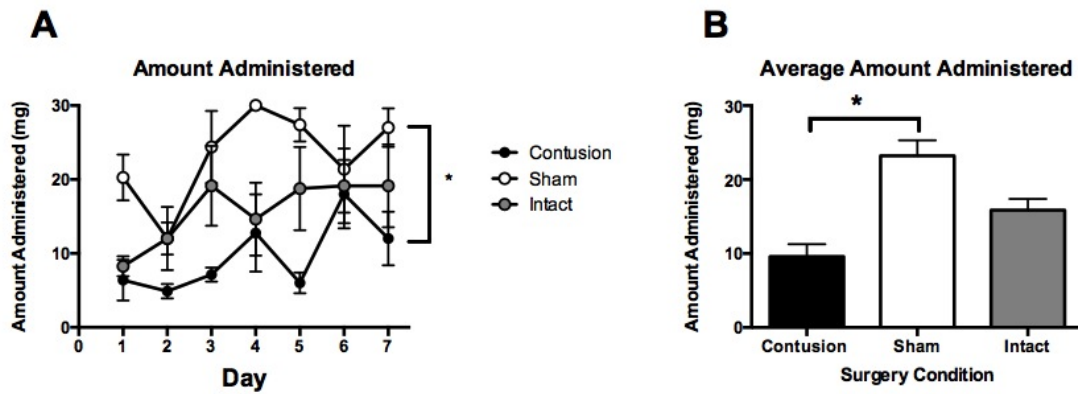
Figure 1: Morphine self-administered in the acute phase of SCI. The amount of morphine administered is shown across the seven sessions (A). Animals with a contusion injury self-administered significantly less morphine than sham animals (B). Neither sham nor contused animals were different from intact controls. $* p < .05$ Figure from Woller et al., 2012.

| Jugular Catheterization | 5-days → | Contusion or Sham Injury | 24-hours → | Self-Administration Chambers 0.0, 0.75, 1.5, or 3.0 mg |

Figure 2: Experimental Timeline: Dose Response. Five days following the implantation of a jugular catheter, animals received a contusion or sham injury. 24-hours later, they were placed into the self-administration chambers. They were placed into the chambers for 7 consecutive nights and had access to either 0.0, 0.75, 1.5, or 3.0 mg morphine, up to 30 mg each session.
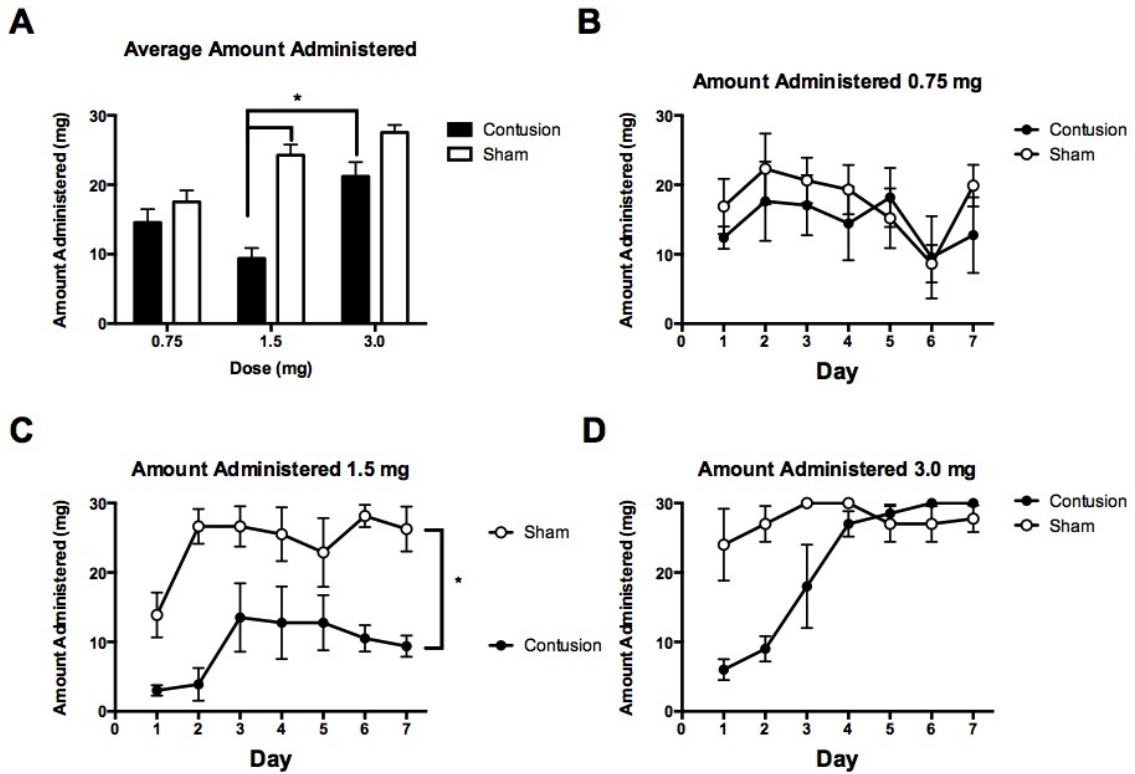
Figure 3: The amount of morphine self-administered in the acute phase of injury is dependent on dose. Panel A shows the average amount of morphine administered by each group. Contused and sham anima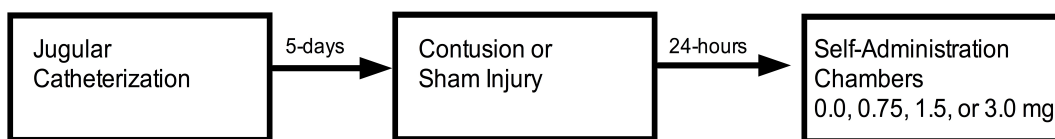ls self-administered the same amount of morphine in the 0.75 mg group (B). As in the first experiment, when given access to the 1.5 mg concentration (C), contused animals, administered significantly less morphine than sham animals. This same difference was seen for the first three days of administration in the 3.0 mg concentration (D). After the third day of administration, however, contused animals began to self-administer an amount of morphine equal to the sham animals. $* p < .05$ Figure from Woller et al., 2012.

concentration (Figure 3D), contused rats self-administered less morphine on days 1 and 2, but not days 4-7. An ANOVA yielded a significant main effect of drug dose and surgery (both F's > 5.59, $p < .05$). In addition, the amount of morphine administered varied across days and the change observed depended upon both drug and surgery condition (all F's > 2.73, $p < .05$). *Post-hoc* comparisons showed that contused rats given 1.5 mg of morphine administered less drug than sham-operated rats given the 1.5 mg dose and both of the 3.0 mg treated groups ($p < .05$). Contused and sham rats given 0.75 mg of morphine administered less than sham-operated animals given 3 mg ($p < .05$). No other differences were significant ($p > .05$).

Rate of Administration

In addition to the total amount of morphine administered, we examined the time it took animals to administer the morphine. For each session, animals have access to morphine for 12 hours. By examining the cumulative amount administered over each hour of the 12-hour session, we can infer the underlying cause of administration. Rapidly self-administering the full amount in a short period of time is indicative of addictive behavior. In contrast, a low, steady amount of administration over the 12-hour session would suggest the animal is administering the morphine as needed for pain relief.

Animals in the 0.0 mg group do not administer morphine, so the cumulative number of responses per hour, up to a maximum of 15 presses is indicated (Figure 4A). Over days, these animals showed a decrease in responding, but still make a few presses over several hours. Animals in the 0.75 mg dose are not depicted. At this low dose,

there was not a difference in morphine self-administration between contused and sham subjects, and we did not look at the rate data. In the moderate, 1.5 mg dose, (Figure 4B) sham animals reached a plateau in responding on Day 1 by 6 hours, having self-administered approximately 18 mg of morphine. In contrast, contused animals ceased to respond around 3 hours, and 5 mg of morphine. Neither group rapidly administered the entire amount available, and neither group shows signs of addictive behaviors at this time. In fact, the curve for the saline sham animals and the morphine sham animals looks very similar. This, however, changes over the course of the seven sessions. By Day 7, sham rats are administering nearly the full amount available in approximately 8 hours, whereas the saline animals have greatly decreased responding at this time. The contused animals administering morphine at a level only slightly increased from Day 1. On day 7, they administer around 10 mg in 5 hours.

In the high, 3.0 mg dose, Day 1 is very similar to that of the 1.5 mg dose. Sham animals are administering more morphine in a shorter period of time, but contused animals administered < 10 mg over the course of 3 hours. By day 7, there is a vast change in behavior. Now, both the sham and injured animals are administering nearly the entire amount of morphine available in 3-4 hours. This pattern begins to emerge around Day 5. The full 30 mg of morphine administered is an amount of morphine in excess of that needed to sustain analgesia. For example, to a 300g rat, 30 mg is 100 mg/kg; typically 10 mg/kg (systemic) produces a robust analgesia for up to 6 hours in these animals. This rapid administration seen in the 3.0 mg animals is indicative of addiction (Figure 4C).
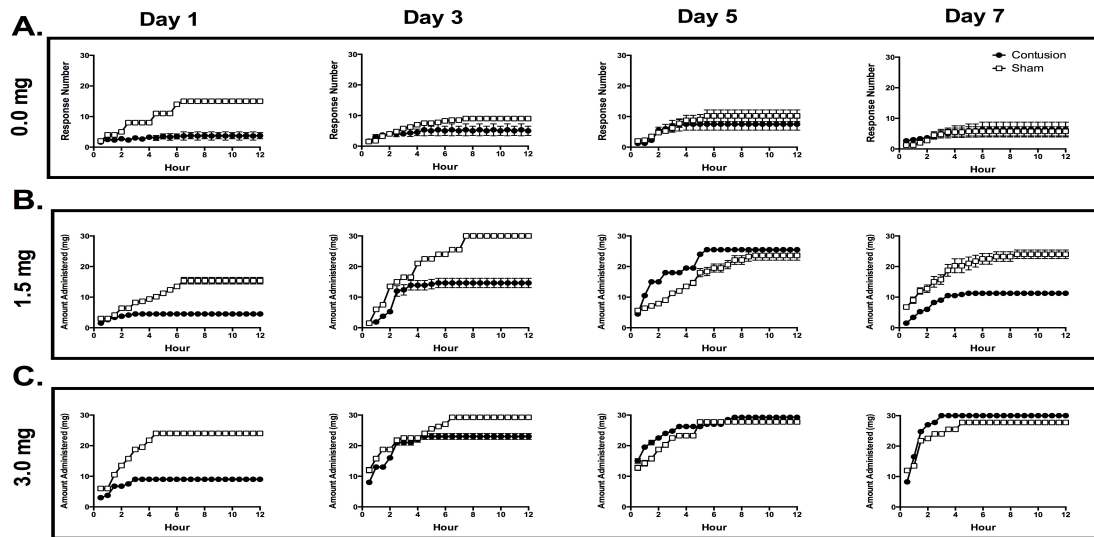
Figure 4: The cumulative amount of morphine administered by hour. The cumulative amount of morphine administered over the 12-hour sessions on days 1, 3, 5, and 7 are shown for the 0.0 mg (A), 1.5 mg (B), and 3.0 mg (C) groups. In contrast to contused animals in the 1.5 mg dose, contused subjects administering 3.0 mg/infusion show signs of addictive behavior by day 7 of administration.

Locomotor Recovery

Locomotor recovery was monitored for a 42-day period in both sham and contused animals. Sham control animals received a maximum converted BBB score of 12 on the day following surgery, and this remained unchanged throughout the period of recovery in both the vehicle and morphine groups. They were, therefore, omitted from further analyses. To assess the impact of morphine on recovery following a contusion injury, we divided the data into two phases: during the period of self-administration (days 1-7), and during the period of recovery (days 8-42). BBB scores did not differ significantly on day 1 post-injury ($F_{(3, 12)} < 1.0$, $p > .05$). During the period of self-administration, morphine treatment slowed recovery (Figure 5). While an ANOVA showed the main effect of drug treatment was not significant ($F_{(3, 12)} = 2.40$, $p > .05$), a trend analysis revealed a significant linear component ($F = 6.66$, $p < .05$). Neither the quadratic nor cubic trends were significant (both F's < 0.54, $p > .05$).

During the recovery period, morphine treated rats continued to perform below the saline-treated controls. Again, while the main effect of drug treatment was not significant ($F_{(3, 12)} = 3.05$, $p < .05$), a trend analysis yielded a significant linear component ($F = 7.72$, $p < .05$). Neither the quadratic nor cubic components were significant (both F's < 0.43, $p > .05$). *Post-hoc* comparisons of the group means showed that subjects who had received the highest concentration of morphine (3.0 mg) differed from the saline-treated controls (Figure 5B; $p < .05$). No other differences were significant ($p > .05$).

A.

Locomotor Recovery

Legend: -O- 0.0 mg   -□- 0.75 mg   -●- 1.5 mg   -■- 3.0 mg

B.

Average Day 42 Score
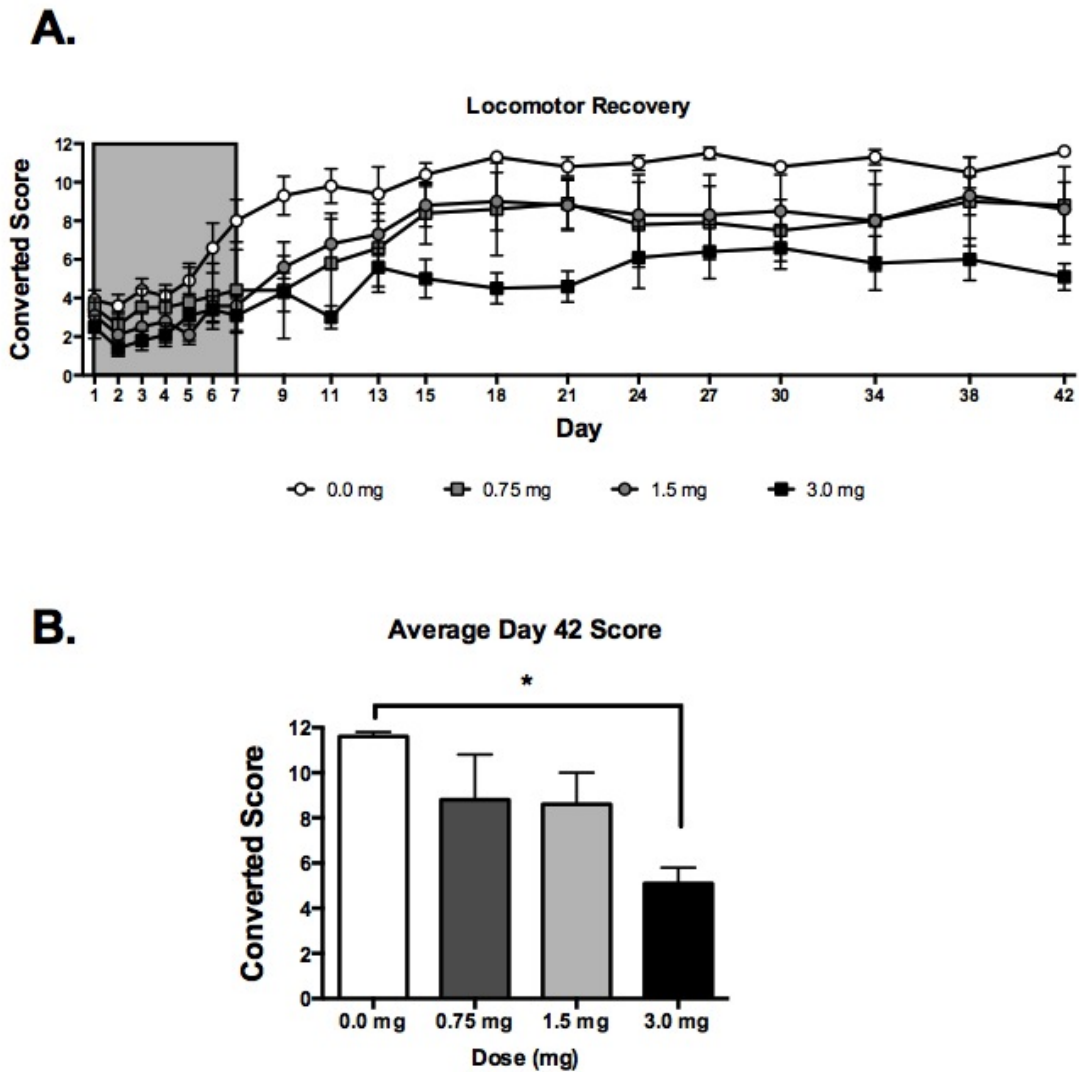
Figure 5: Effects of morphine administration in the acute phase of SCI.
Locomotor recovery scores are shown during the period of administration (gray box days 1-7) and during the recovery period (days 8-42). Despite similar scores prior to morphine treatment, animals administering the 3.0 mg concentration of morphine never recovered to the same level as those administering saline. Panel B shows the average converted BBB scores on Day 42. * $p < .05$ Figure from Woller et al., 2012.

It is important to note that because these animals were self-administering morphine, the amount administered in each group and dose varied. Morphine administration is likely to have an impact on locomotor recovery based on the total amount of morphine (mg/kg) administered rather than the dose administered (mg). To control for these differences, locomotor recovery was examined based on the amount each animal administered compared to their body weight (mg/kg). In doing this, it was found that animals administering on average above 50 mg/kg/day showed significantly decreased recovery relative to those administering less than 50 mg/kg/day and those administering saline (Figure 6). Supporting these results, an ANCOVA revealed a significant Day x Amount of morphine interaction ($F_{(11, 440)} = 2.04$, $p < .05$). *Post hoc* analyses indicated animals administering the high amount of morphine recovered significantly less function than those administering the low amount, or no morphine ($p < .05$).

Weight

As with locomotor recovery, the impact of morphine on weight change was assessed in two phases. Examining weight change during the period of self-administration (days 1-7), we found that morphine treatment led to weight loss over days independent of surgery condition. Supporting this, an ANOVA revealed main effects of drug treatment and day, as well as a Drug x Day interaction (both F's > 6.51, $p < .05$). No other terms were significant (all F's < 3.16, $p > .05$).
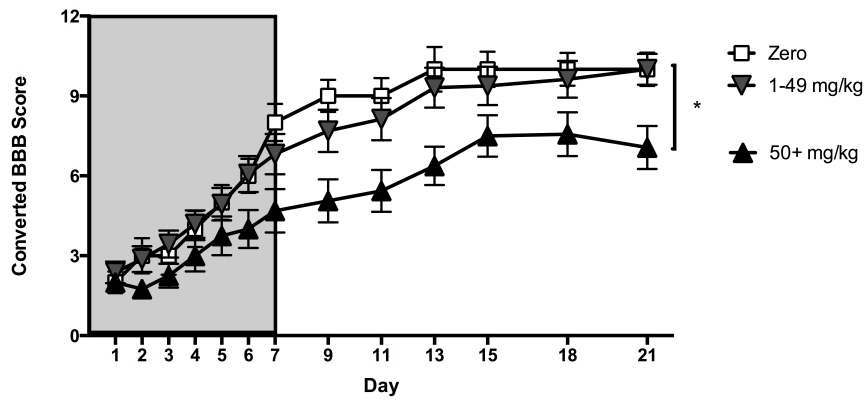
47

Figure 6: Recovery of locomotor function is dependent on the amount of morphine self-administered. Animals administering above 50 mg/kg/day on average showed significantly attenuated recovery of locomotor function relative to saline controls and those administering less morphine. * *p* < .05 Figure from Woller et al., 2012.
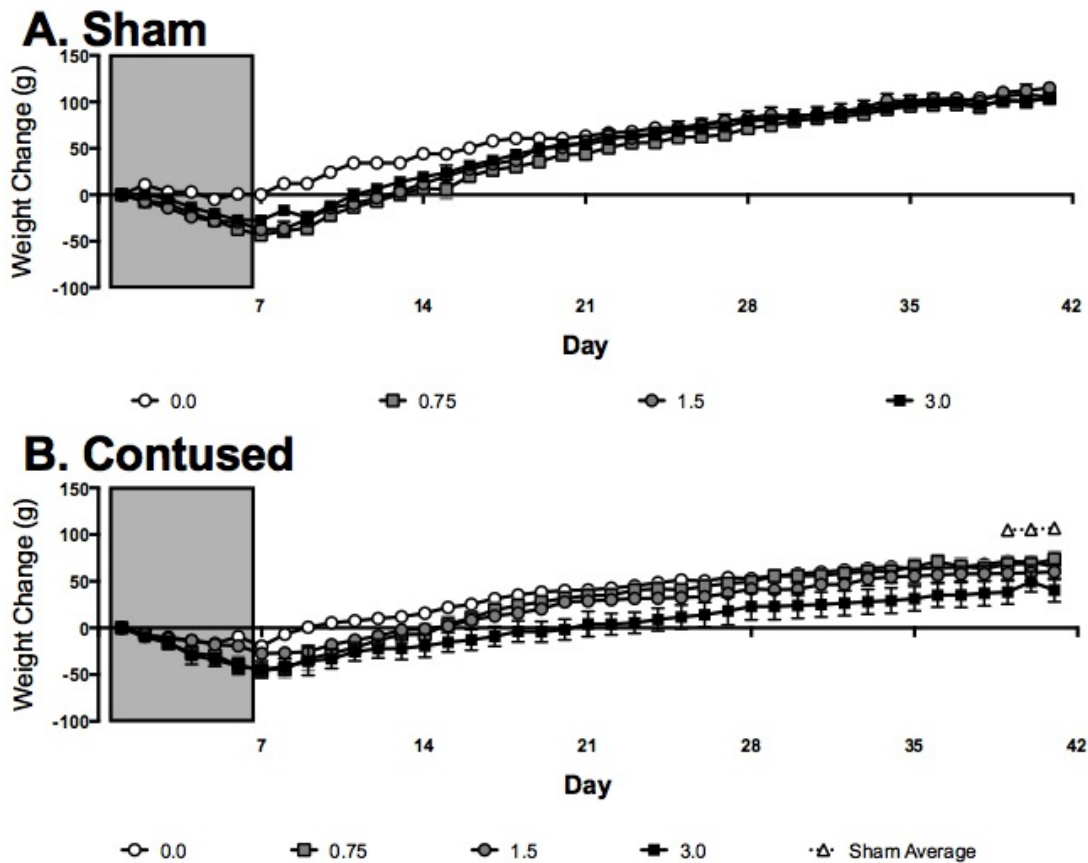
Figure 7: Effects of morphine administration on weight following SCI. Weights of sham (A) and contused (B) animals were monitored during the period of morphine administration (gray shaded areas) and during the period of recovery. During the period of administration, all animals lost weight. During the recovery period, a contusion injury increased weight loss. This weight difference is emphasized in B with the addition of the final average weight of sham animals superimposed on the figure of contused weights (open triangles). During the period of recovery, sham animals regained weight and there was no effect of morphine on the amount of weight gain. Contused animals regained weight dependent on the concentration of morphine administered. Subjects administering the 3.0 mg/morphine/lever press regained significantly less weight than as those administering saline, or low and moderate doses of morphine.

During the recovery period, the effect of morphine treatment on weight in the sham-operated rats waned across days (Figure 7A). As observed in prior studies (Hook et al., 2007, 2009, 2011; Woller et al., 2012), contused rats gained less weight (Figure 7B) than sham controls. While contused rats that received a low-to-moderate dose of morphine per lever press (0.75-1.5 mg) recovered weight to the same level as the saline-treated controls, subjects that had received the highest concentration (3.0 mg) exhibited poorer recovery of weight, and maintained a significantly lower weight than sham controls across recovery. An ANOVA confirmed that the main effects of drug, surgery, and day were significant (all F's > 3.48, $p < .05$). In addition, the change in weight observed across days depended upon both drug and surgery treatment (all F's > 1.90, $p < .0001$). The Drug x Surgery interaction was not significant ($F_{(3, 24)} < 1.89$, $p > .05$).

As with recovery of locomotor function, it is expected that, rather than the dose affecting weight loss most significantly, that the amount each animal was administering would be most important. Here, we also grouped animals into a zero, low (< 50 mg/kg/day morphine), and high (> 50 mg/kg/day) amount of morphine administered. We found that animals administering above 50 mg/kg/day showed greater weight loss than those administering less than 50 mg/kg/day (Figure 8). An ANOVA supported these results showing a main effect of amount of morphine ($F_{(2, 43)} = 10.29$, $p < .05$). In fact, *post hoc* analyses indicate animals administering a low amount of morphine were not significantly different from those in the saline group ($p < .05$).
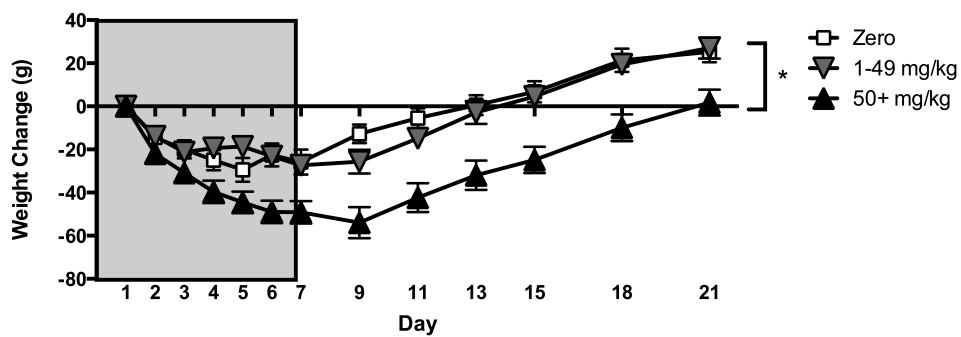
Figure 8: Weight loss is associated with administration of high amounts of morphine. As with locomotor recovery, animals administering the most morphine showed the highest amount of weight loss, relative to animals administering saline and lower amounts of morphine. * $p < .05$

Discussion

In the acute phase of a spinal cord injury, contused subjects self-administered significantly less morphine than their sham counterparts. This decreased self-administration was time- and dose-dependent. Contused subjects given access to the 1.5 mg dose of morphine self-administered significantly less morphine than their sham counterparts on days 3-7 of recovery. This was not the case for the higher (3.0 mg) dose. Contused animals rapidly administered the total amount of morphine available at a rate commensurate with sham controls. This rapid administration in the 3.0 mg dose group provides evidence that the decreased self-administration in the 1.5 mg group is not related to locomotor deficits or an inability to perform the response. In fact, administration of the high dose (3.0 mg) significantly undermined locomotor recovery, yet these animals were able to administer the entire amount available each session. Together, this suggests that the addictive potential of morphine is shifted in the acute phase of injury, but it is not negated. These results suggest that molecular processes occurring after a contusion injury are reducing the addictive potential of morphine in this phase of injury.

While our CPP results showed an increase in addictive potential in the acute phase of injury, we did not see the same effect using a self-administration paradigm. Morphine administration would be expected to engage neural systems associated with pleasure, and mechanisms that function to inhibit pain. We have shown that the dose of morphine used to induce a conditioned preference also produces robust antinociception. For this reason, injured rats may prefer the morphine-paired context because nociceptive

input (pain signals) is inhibited. In the absence of pain (sham and intact rats), the antinociceptive effect of morphine may have little reward value (with the low doses used in the present study), and as a result, yield only a weak preference for the morphine-paired context. However, just as humans will self-administer an opiate to bring pain relief, we would expect injured rats to maintain a level of opiate infusion sufficient to diminish tonic pain. Indeed, injured rats given access to the low (0.75 mg) and moderate (1.5 mg) concentrations of morphine administered approximately 10 mg/kg of morphine over the 12-hour training period, a dosage that would be sufficient to maintain antinociception for approximately 6 hours (the half life of morphine is 2-3 hours) if it were given in one administration. However, individual administrations (with each lever press) of the low (0.75 mg) and moderate (1.5 mg) doses may not provide sufficient analgesia with each administration for the animals to continue responding (thus allowing larger amounts of morphine to accumulate). This may explain the reduced administration seen in the contused groups administering 0.75 mg and 1.5 mg relative to sham controls treated with 1.5 mg of morphine, and both groups treated with the 3.0 mg dose.

Although motivational variables remain to be determined, the behavior of the sham rats treated with the higher doses of morphine is suggestive of addiction. In a self-administration task, addictive behavior is evident from the near-maximal responding to obtain the full amount of opiate available. Inspection of the amount of morphine taken by sham-operated rats indicated that an addictive tendency emerged at a moderate concentration (1.5 mg), and was fully evident at the high concentration (3.0 mg).

Injured rats did not engage in the same level of addictive behavior, and even at the high concentration of morphine, this effect did not emerge until late in training. Following injury there are several underlying molecular changes occurring that may cause the shift in addictive potential seen in contused animals. Activation of the KOR has been thought to oppose morphine-induced analgesia, and has been associated with a decreased preference for a morphine-paired context (Suzuki et al., 1999). Following injury, there is an increase in the amount of dynorphin, the endogenous ligand for the KOR, commensurate with injury severity. Activation of the KOR might mask the rewarding effects of lower doses of morphine although it may not be sufficient to counter the rewarding effects associated with higher doses.

Another potential explanation for the decreased self-administration seen in contused animals involves the inflammatory response to injury. Immediately following SCI, there is an increase in the number of lymphocytes, microglia, and astrocytes at the injury site (Ankeny & Popovich, 2009; Ankeny et al., 2006; Alexander & Popovich, 2009). It is now generally accepted that microglia and astrocytes play a role in the development of chronic and neuropathic pain (See Mika et al., 2013 for review). Interactions of these cell types, along with the release of pro-inflammatory cytokines are thought to contribute to a loss of opioid analgesic efficacy (DeLeo & Yezierski, 2001; Mika, 2008; Watkins et al., 2003). In addition, microglial expressing the non-classic opioid receptor, TLR4, are activated within 72 hours of SCI (Kigerl et al., 2007), and this activation is further increased by chronic morphine administration (Cao et al., 2010). Spinal cord injury, TLR4 activation, and morphine administration all increase mRNA

and protein levels of the pro-inflammatory cytokine IL-1β in spinal cord tissue (Hutchinson et al., 2008b; Liu et al., 2008; Lewis et al., 2010; Hook et al., 2011), which could modulate opioid effectiveness (Hutchinson et al., 2008; 2011) by increasing pain and reducing analgesic efficacy, and this process could contribute to injury-induced changes in opiate self-administration.

Increased levels of pro-inflammatory cytokines could also undermine locomotor recovery. In this study, administration of the high dose of morphine (3.0 mg) significantly undermined recovery of locomotor function. The low, 0.75 mg, and moderate, 1.5 mg, doses, however, had no effect. These data concur with our previous studies showing that the administration of a high amount of i.t. morphine (90 μg) significantly attenuated recovery of locomotor function, but a moderate (30 μg) amount had no effect (Hook et al., 2009; 2011). These data also extend our previous studies by showing that morphine is not just having an effect when applied directly to the spinal cord, but can also impact recovery of locomotor function when applied via an intravenous route, which is typically utilized clinically. Importantly, the amount these animals administer, even in the high condition, is low relative to the amount used clinically for the treatment of pain. SCI patients will receive up to 180 mg /kg a day, with doses increasing as necessary for the management of pain (Magrinelli et al., 2013).

As noted above, increased pro-inflammatory cytokine levels and other molecular changes inherent to SCI and opioid administration may result in decreased locomotor recovery. As discussed in the Introduction (Chapter I), increases in the levels of IL-1β following injury have been linked to a decrease in recovery of locomotor function. Both

SCI and morphine administration have been shown to increase levels of pro-inflammatory cytokines (Hook et al., 2011), and increased expression of IL-1β appears to be necessary for the morphine-induced attenuation of recovery after SCI. It has been suggested that these motor deficits related to increased IL-1β are the result of glutamate toxicity and increased neuronal loss (Liu et al., 2008). Through activation of the KOR or increased glial activation, and increased glutamate levels, it is possible that SCI and opioid administration in the early phase of injury can exacerbate and sustain neuronal (Lepore et al., 2011; Nottingham and Springer, 2003) and/or oligodendrocytic (Almad et al., 2011b; McTigue, 2008; Xu et al., 2004) cell death, which could undermine recovery of locomotor function following injury.

These changes occurring at a neuronal and glial level, and occurring in the acute phase of injury, can be synergistically exacerbated by the administration of morphine, which could lead to an attenuation of locomotor recovery (for review, see Woller & Hook, 2013). Alternatively, there is an increase in dynorphin levels in the acute phase of SCI. This endogenous KOR agonist has been shown to play a role in the pathology of SCI (Krumins & Faden, 1986) and administration produces paralysis in intact rats through activation of the NMDAR (Bakshi et al., 1992). Morphine binding to the kappa receptor, on top of the increased levels of dynorphin resulting from the injury, may exacerbate neuronal loss, and lead to decreased recovery of locomotor function.

Morphine administration also resulted in weight loss during the period of administration in both contused and sham subjects. In injured animals, the high dose of morphine caused an increase in weight loss relative to the other doses. Furthermore, as

with recovery of locomotor function, this effect was dependent on the amount of morphine being administered. Chronic morphine administration is often associated with weight loss occurring as a result of decreased food intake (Nogueiras et al., 2012; Ferenczi et al., 2010). In these animals, however, we did not see a difference in food intake during the period of self-administration (data not shown), when the most robust weight loss occurred. Alternatively, weight loss can occur as a part of a withdrawal syndrome (Gu et al., 2005) or dehydration, which were not directly assessed in this study, but can be assessed further in future studies.

From a clinical perspective these data indicate that, it is important that patients are not given a high dose of morphine in the acute phase of SCI. Administration of a high dose of morphine leads to an increase in addictive behavior, a decrease in locomotor recovery, and an increase in weight loss. Low to moderate amounts of morphine, however, do not seem to produce to these same effects.

CHAPTER IV

SELF-ADMINISTRATION OF MORPHINE IN THE CHRONIC PHASE OF INJURY

The addictive potential of morphine varies depending on the experimental design (conditioned place preference or self-administration paradigm) and dose administered. Indeed, the previous study investigated morphine self-administration in the acute phase of injury and revealed dose-dependent effects. Contused animals, when given access to a moderate dose of morphine (1.5 mg/ lever depression), self-administered significantly less morphine than their sham counterparts. When given access to a higher concentration, 3.0 mg / lever depression, contused animals increased the amount of morphine administered over the first three days to self-administer nearly the full 30 mg available to them for each session in the final four days. These results suggest there is a shift in the addictive potential of morphine in the acute phase of injury.

As mentioned in the introduction, opiates are used for the treatment of both nociceptive pain in the acute phase of injury, and neuropathic pain, which develops in the chronic phase of SCI. Anecdotally, abuse liability is said to be reduced when opioids are used for the treatment of neuropathic pain. Indeed, there is evidence in both human and animal literature to support this claim (Minozzi et al., 2013; Ewan & Martin, 2011). However, individuals with a SCI are likely to develop conditions, such as depression, which further increase the prescription of opioids. In addition, abuse liability has not been studied using a model of central neuropathic pain. While this model of neuropathic

pain should be comparable to others, and show a reduction in addictive potential of morphine, this remains to be tested.

In addition to examining the abuse liability of morphine in the chronic phase of injury, we are able to use the self-administration paradigm to examine the effects of repeated morphine administration on long-term recovery of function. Indeed, the previous study demonstrated that self-administration of morphine at amounts greater than 50 mg/kg/day significantly attenuated recovery of locomotor function when administration occurred from days 1-7 post SCI (Chapter III). In the rodent model of contusion injury, we typically see a plateau in recovery of locomotor function at two weeks post injury (Hook et al., 2004). Whether morphine administration would have a similar effect in this later phase of SCI has not been examined.

To address these issues, the current study extends the original finding on the addictive potential of morphine, and the morphine-induced attenuation of locomotor recovery. Here, the propensity for self-administration is examined in the chronic phase of SCI. This is important because the contusion model of spinal cord injury typically leads to the development of neuropathic pain in approximately 80% of subjects (Hulsebosch, 2002). However, signs of this pain do not begin to appear until around 14 days following injury, with established neuropathic pain being present around 35 days post injury (Crown et al., 2006). This study tests the hypothesis that a state of neuropathic pain lowers the addictive potential of morphine in the chronic phase of SCI.

Method

In this experiment, 60 animals (n=6) were given a sham or contusion injury 5 days after the implantation of a jugular catheter. Twenty-four hours after the contusion injury, baseline tests of locomotor and sensory reactivity were conducted. After balancing day 1 BBB scores, animals were divided into groups (Figure 9). In one group, animals were placed into the self-administration chambers for 7 nights beginning 24-hours after injury. In the chambers, animals were given access to 0.0 or 1.5 mg morphine/ lever depression, up to 30 mg morphine/ session. A second group of animals were placed into the self-administration chambers in the early chronic (Days 14-21) phase of injury. As with the acute group, these animals were placed into the self-administration for 12 hours during their dark cycle for 7 nights. In the chambers, they were given access to 0.0 or 1.5 mg morphine/ lever depression, up to 30 mg morphine/ session. Animals in the final group were placed in the chambers in the late chronic phase of injury (Days 35-41). These animals had access to 1.5 mg morphine/ lever depression, up to 30 mg/session, for 7 sessions.

Results

Morphine Self-Administration is Decreased in the Acute Phase of Spinal Cord Injury

Baseline Sensory Reactivity. Twenty-fours following a contusion or sham injury, baseline sensory reactivity tests were conducted. These tests measured mechanical allodynia, thermal hyperalgesia, and the development of at-level neuropathic pain (girdle test). Using von Frey filaments to measure mechanical allodynia, we found

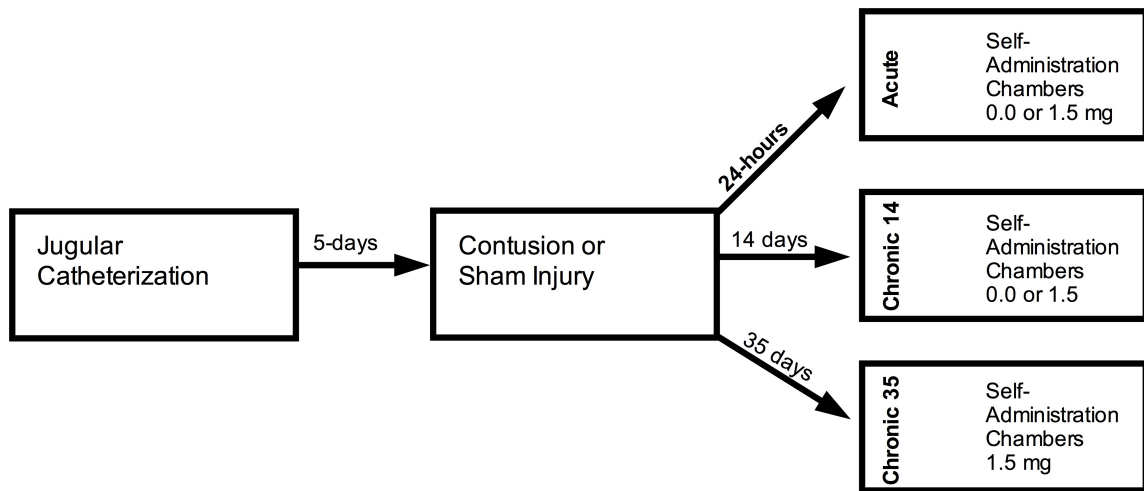*Figure 9: Experimental timeline: phase of injury.* In this experiment, animals were implanted with a jugular catheter, and received a contusion or sham injury 5 days later. One group of rats was placed into the self-administration chambers 24-hours following SCI, another group 14-days following SCI, and a third 35-days following SCI. Each group had access to 1.5 mg morphine/ lever depression, up to 30 mg each session.

that sham animals withdrew their hindlimb at a lower threshold than contused animals (F (1, 22) = 17.87, $p < .05$, Figure 10A). Vocalizations to the mechanical stimuli were also recorded. Here, sham animals vocalized at a lower threshold than contused animals, but this result only approached significance (F (1, 22) = 3.98, $p = .059$, Figure 10A). The reduced spinal-supraspinal communication in the acute phase of a contusion injury likely reduced reactivity to stimuli applied below the level of the lesion. While contused animals were less reactive for the measure of below-level mechanical allodynia, however, they vocalized more than sham animals on the test of at-level pain, girdle reactivity (F (1, 21) = 6.93, $p < .05$, Figure 10B). No other results approached significance (F's < 1.2, $p > .05$).

Morphine Administered. As found in the previous study (Chapter III), the contused subjects administered significantly less morphine (1.5 mg/lever press) than sham subjects in the acute phase of injury. Contused animals self-administered only 8.4 mg on average versus 21.1 mg in the sham group (Figure 11). An ANOVA revealed a main effect of surgery (F (1, 10) = 15.43, $p < .05$).

Rate of Administration. In these experiments, we again examined how rapidly animals administered morphine (Figure 12). The patterns seen here are nearly identical to those in the first experiment. On Day 1, contused and sham rats diverge in the amount of morphine administered, and the amount of time spent pressing the lever. Across days, contused rats maintain a moderate level of morphine administration, and administer morphine for eight hours (on Day 7). The amount administered, and the rate at which it

A. Tactile Response
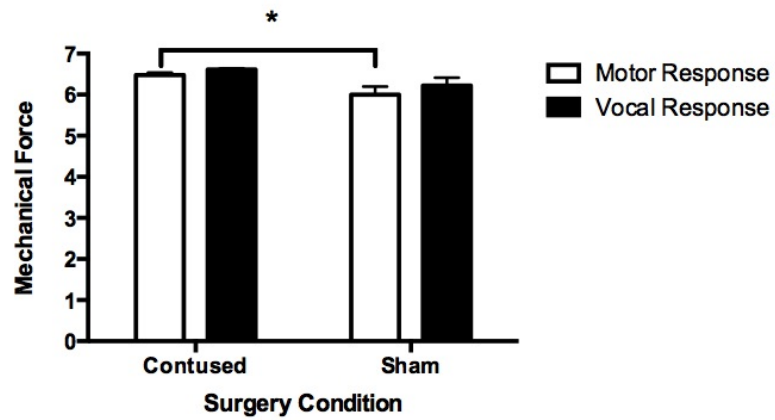
B. Girdle Response
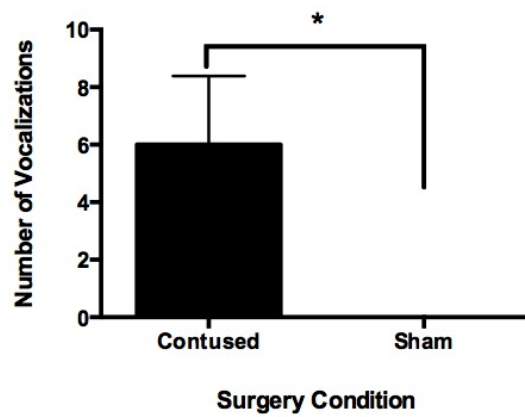
Figure 10: Assessment of sensory reactivity prior to morphine administration. Baseline tests of mechanical reactivity (A) and girdle reactivity (B) were conducted prior to morphine administration. Contused animals were less reactive on motor tests of mechanical reactivity. However, contused rats vocalized more in response to mechanical stimuli applied at and above the level of injury. * $p < .05$
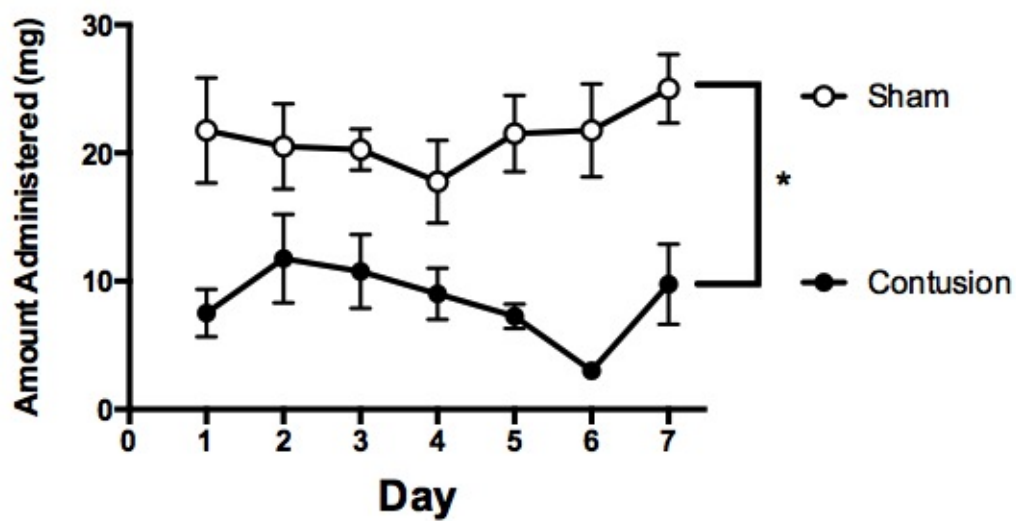
Figure 11: The amount of morphine self-administered in the acute phase of SCI. Contused animals administered significantly less morphine than sham animals when given access to a 1.5 mg per lever depression. * = $p < .05$
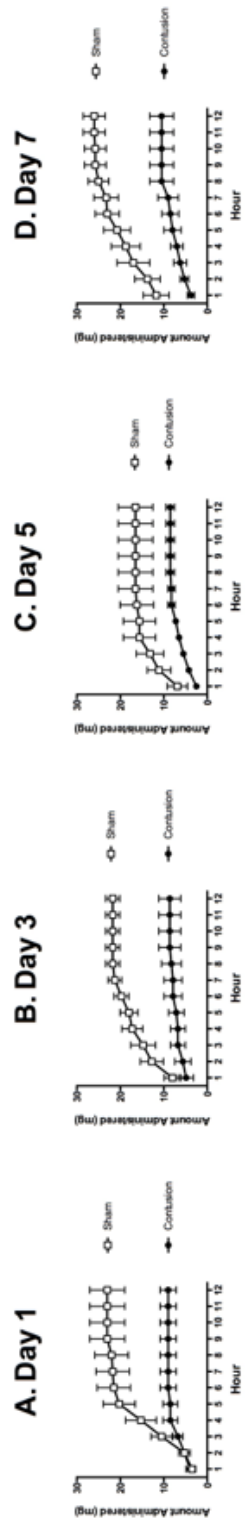
Figure 12: Rate of administration in the acute phase. The cumulative amount of morphine administered by hour on Day 1 (A), Day 3 (B), Day 5 (C), and Day 7 (D) is shown. As with the previous experiment, the 1.5 mg dose leads to an increase in responding in the sham animals, but the administration occurs over a longer period of time. The contused animals maintain a moderate amount of administration over a 6-8 hour period.

is administered suggests these rats are not administering for the hedonic properties of morphine.

Locomotor Recovery. As in the previous experiment, locomotor recovery was monitored for a 42-day period in both sham and contused subjects. Again, sham controls received a maximum converted BBB score of 12 on the day following surgery, and this remained unchanged throughout the period of recovery in both vehicle and morphine groups. Subsequent analyses, therefore, focused on the recovery of the contused groups only. To investigate the effect of morphine administration on recovery of locomotor function, we separated the data into a period of administration (Days 1-7) and a period of recovery (Days 8-42). As in the previous study (Chapter III), there was no significant effect of morphine administration on recovery of locomotor function at the dose administered (F's < 1.0, $p > .05$). Administering 10 mg/morphine per session or less, contused animals recovered to the same level of locomotor function as those administering saline (Figure 13).

Weight. As with locomotor recovery, weight data was separated into a period of administration (Days 1-7) and a period of recovery (Days 8-42). During administration, contused and sham animals lost weight in both the 0.0 mg and 1.5 mg groups (Figure 14 A & B). Contused animals in the saline group lost more weight during this phase of injury. These results are supported by a Day x Drug Condition interaction (F (6, 120) = 2.81, $p < .05$).
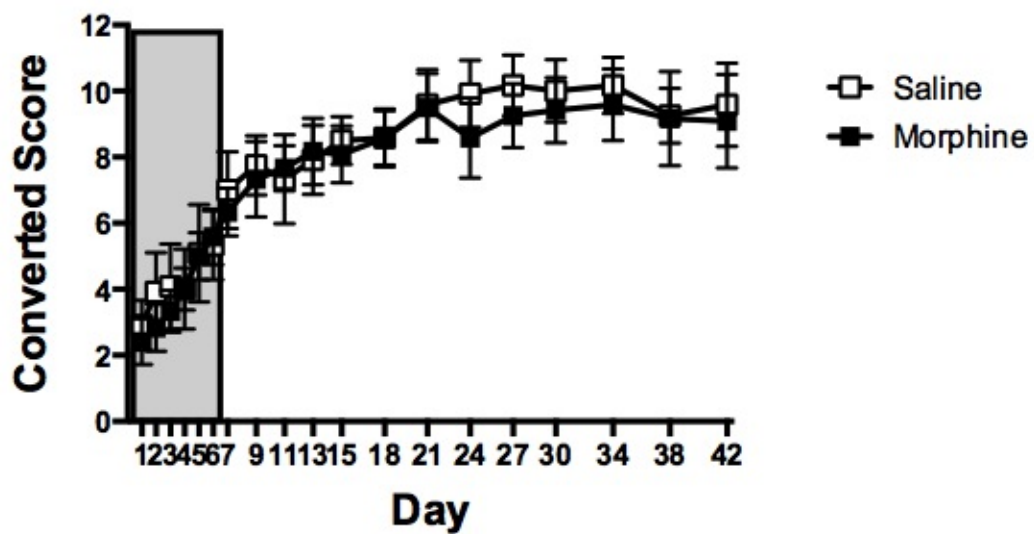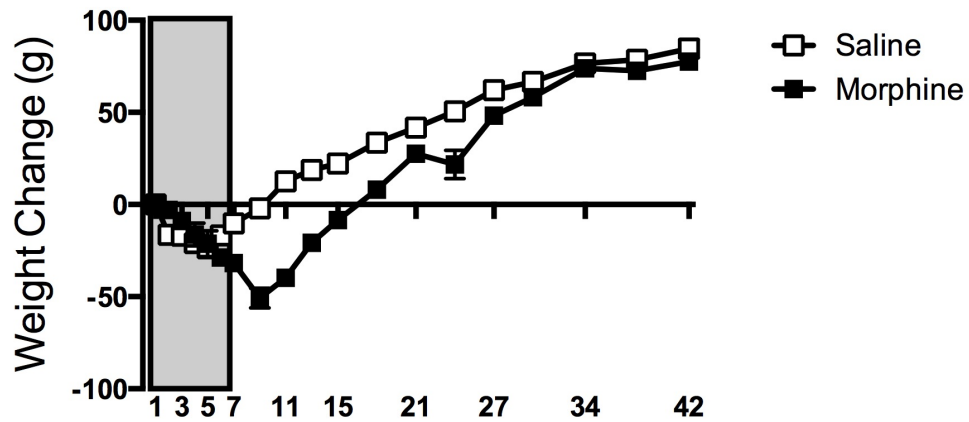
Figure 13: Effects of morphine administration on recovery of locomotor function. As in the previous experiment, a 1.5 mg concentration of morphine did not affect recovery of locomotor function when administration began in the acute phase of SCI (shaded area).

During the period of recovery (days 8-42), contused animals maintained a lower weight than sham animals in both the 0.0 mg and 1.5 mg groups (Figure 14 A & B). Morphine administration did not significantly increase this weight loss in the contused groups. Weight loss in the sham animals was increased by morphine administration, but this effect did not persist throughout the recovery period. There were significant main effects of drug condition (F (1, 20) = 6.66, $p < .05$) and surgery (F (1, 20) = 11.48, $p < .05$) on weight loss. As can be seen in Figure 14, morphine treated animals lost more weight than saline controls, and the contusion produced greater weight loss than the sham injury. In addition, there were significant Day x Drug Condition (F (11, 220) = 7.66, $p < .05$), Day x Surgery (F (11, 220) = 7.94, $p < .05$), and Day x Drug Condition x Surgery (F (11, 220) = 6.99, $p < .05$) interactions. *Post hoc* tests indicated a significant difference between sham animals administering morphine and sham animals administering saline ($p < .05$).

Sensory Reactivity. The tests of girdle, tail flick and hindpaw reactivity were repeated 42-days after the contusion or sham injury. At this time point, there was no effect of morphine administration on mechanical reactivity, thermal hyperalgesia, or girdle reactivity (All F's < 3.84, $p > .05$). However, a contusion injury significantly decreased tail flick latency, regardless of drug treatment (F (1, 1) = 8.65, $p < .05$, Figure 15). No other results approached significance (F's < 4.1, $p > .05$).

A. Sham

B. Contused
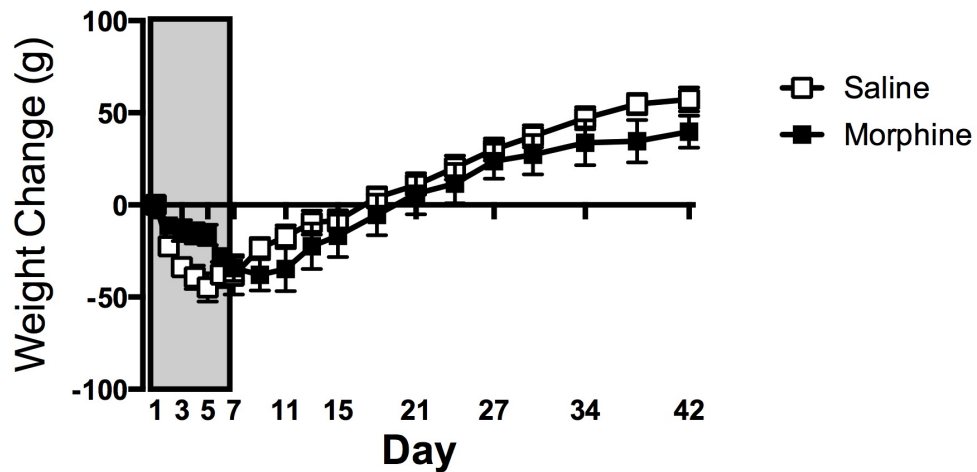
Figure 14: Morphine administration induces weight loss. Both contused and sham animals lost weight during the period of self-administration (shaded area). Contused animals (B) lost more weight than sham animals (A). While morphine administration did not increase weight loss during the period of administration, it significantly slowed regain of weight.
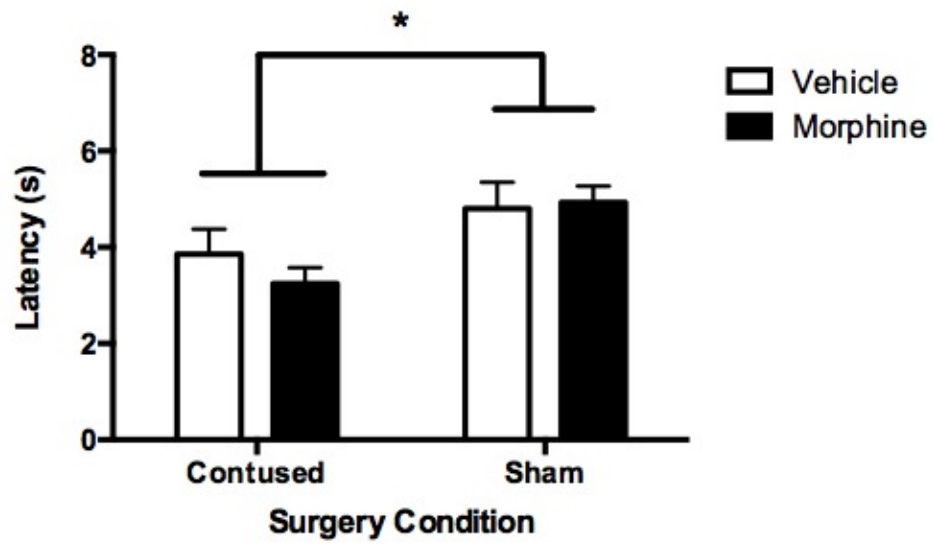
Figure 15: Tests of sensory reactivity after morphine administration. 42-days following injury, contused animals showed a significantly decreased tail flick latency. There was not a lasting effect of morphine administration. *p < .05

Morphine Self-Administration is Not Decreased in the Chronic Phase of Injury

In the acute phase of injury, we observed a decrease in morphine self-administration in injured animals. These animals, however, are not yet experiencing neuropathic pain. The idea that neuropathic pain reduces the abuse liability of opioids has not been examined following a spinal cord injury. Thus, we assessed the addictive potential of morphine in the chronic (after symptoms of neuropathic pain have developed) phase of SCI using a self-administration paradigm.

Sensory Reactivity. In this group, baseline tests of sensory reactivity were conducted 14 days after injury, before morphine self-administration sessions began. At this time, contused animals withdrew their hindlimbs to a lower force von Frey filament than sham animals, indicating these animals are showing signs of mechanical allodynia. There was a main effect of surgery on mechanical reactivity thresholds ($F_{(1, 22)} = 13.84$, $p < .05$, Figure 16). There were no differences in vocalization to a mechanical stimulus, in thermal hyperalgesia, or in response to girdle stimulation (All F's < 1.0, $p > .05$).

Morphine Administered. In contrast to the acute phase of injury, we found contused and sham animals took similar amounts of morphine when administration began 14-days following injury ($F < 1.0$, $p > .05$). On average, injured animals self-administered 25.61 mg / day while sham rats self-administered 19.86 mg / day (Figure 17).
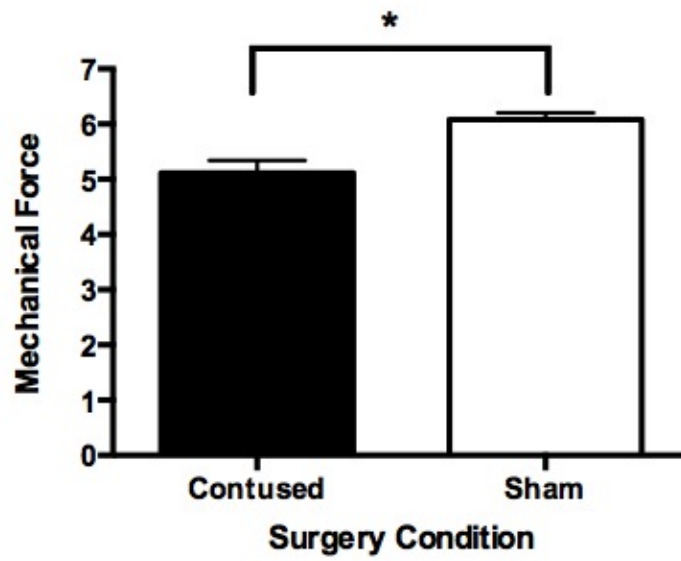
Figure 16: Tests of tactile reactivity prior to morphine administration. 14-days post surgery, contused animals were showing signs of mechanical allodynia when compared to sham controls. * *p* < .05
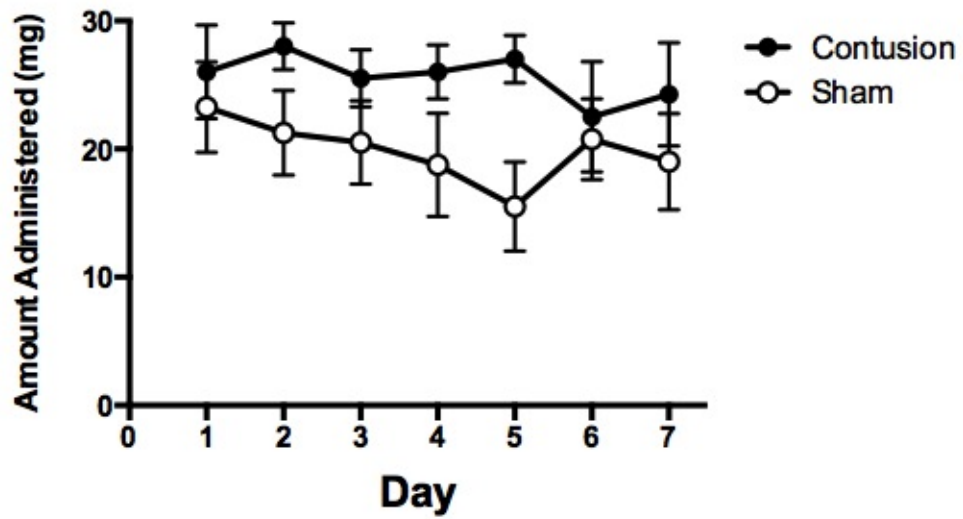
Figure 17: Morphine self-administration in the early chronic phase of SCI. In this early chronic phase of injury, contused animals administered as much morphine as sham animals.

Rate of Administration. In the early chronic phase of injury, the rate of administration in the contused rats is different than the acute phase. On day 1 of administration, contused animals administered an amount of morphine commensurate with sham controls. They administered around 20 mg of morphine in the first five hours of the self-administration session. The amount they administered increased over days, and, by day 7, they were administering nearly the entire 30 mg. This administration occurred over ten hours of the session. While the amount they are administering is greatly increased, it is administered over a longer period of time (Figure 18).

Locomotor Recovery. In this experiment, the 42 days were separated into three periods: prior to morphine administration (Days 1-13), during morphine administration (Days 14-20), and during recovery (Days 21-42). There was no effect of morphine on recovery of locomotor function in any of these periods (all F's < 1.0, $p > .05$; Figure 19).

Weight. As with recovery of locomotor function, weight change was examined in three periods. Prior to morphine administration (days 1-13), contused animals lost weight relative to sham controls (Figure 20 A & B). This result is supported by a significant main effect of surgery (F $(1, 20) = 54.75$, $p < .0001$) and a Day x Surgery interaction (F $(9, 180) = 13.39$, $p < .0001$).

During the period of morphine administration, contused rats continued to weigh less than sham animals and weight loss was increased by the administration of morphine. There was a significant main effect of surgery (F $(1, 20) = 26.52$, $p < .0001$), and a Day x Drug Condition interaction (F $(1, 20) = 9.41$, $p < .01$) for weight loss in this period. As
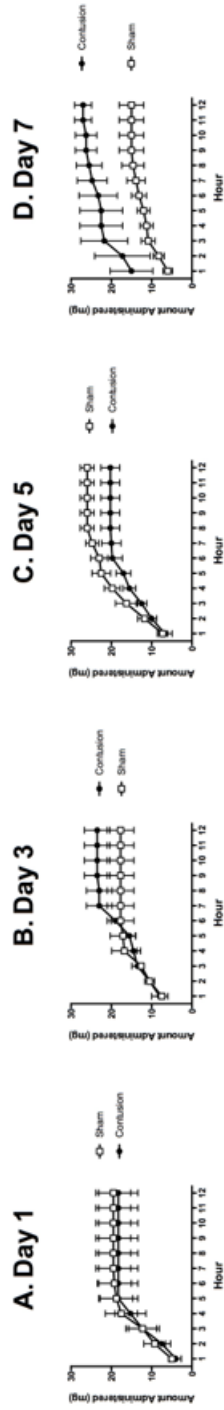
Figure 18: Rates of administration in the early chronic phase. In the early chronic phase of SCI, contused animals begin by administering more morphine than contused animals in the acute groups. However, rather than increasing the rate of administration over days, they gradually increase the amount of morphine administered.
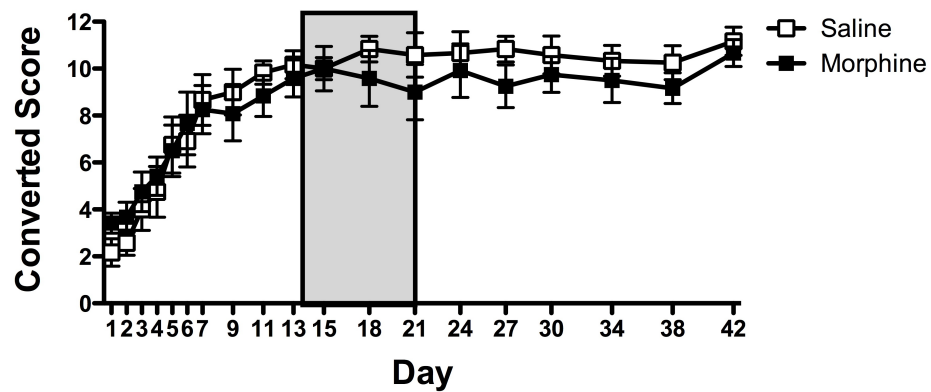
Figure 19: Locomotor recovery is not affected by morphine administration in the chronic phase of SCI. In this early chronic phase of injury, morphine administration, even at the high amount administered here, did not affect recovery of locomotor function.

A. Sham
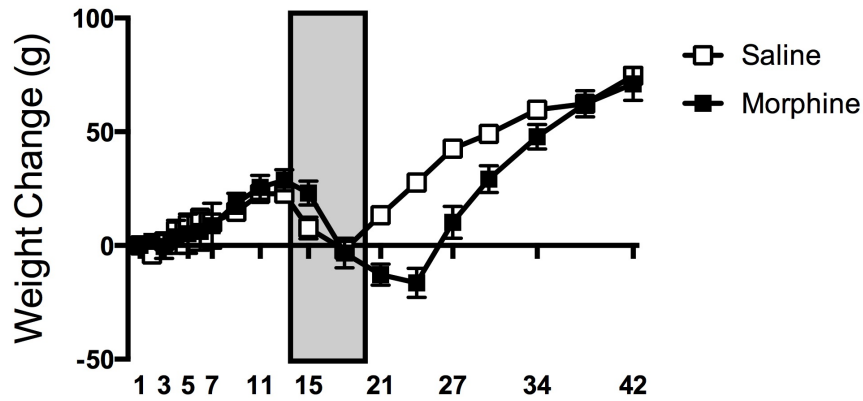
B. Contused
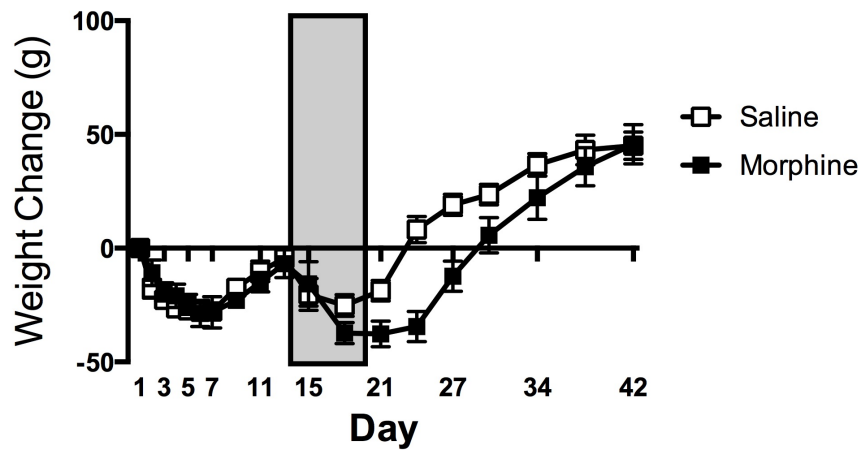
Figure 20: Morphine administration leads to weight loss in the chronic phase of SCI. Prior to morphine administration, contused animals (B) lost more weight than sham animals (A). During the period of administration, contused animals continued to lose weight, and contused animals administering morphine weighed less than all other groups. During the recovery period, all rats regained weight, but contused animals remained smaller. In addition, contused rats administering morphine weighed less than those administering saline.

can be seen in Figure 20 A & B, contused rats administering morphine weighed significantly less than injured subjects treated with saline or sham subjects.

During recovery (days 21-42), all rats began to regain weight, but contused rats remained smaller than their sham counterparts. In addition, both contused and sham animals administering morphine weighed less than injured and sham animals administering saline. Supporting these results, an ANOVA yielded a significant main effect of drug condition (F (1, 20) = 10.64, $p < .01$), and surgery (F (1, 20) = 16.99, $p < .001$), and a significant Day x Drug Condition interaction (F (6, 120) = 20.918, $p < .0001$). As found previously, morphine administration increased weight loss as did the contusion, relative to sham, injury. *Post hoc* analyses indicated that contused animals administering morphine lost significantly more weight than the contused animals administering saline ($p > .05$), weighing only 4.86 grams more on Day 42 than on Day 1, versus 22.41 grams more in the saline group.

Sensory Reactivity. Forty-two days after injury, there was no lasting effect of morphine administration on measures of sensory reactivity (all F's < 1, $p > .05$). A contusion injury significantly increased vocalizations on the girdle test of at-level neuropathic pain (F (1, 1) = 5.75, $p < .05$, Figure 21). No other results approached significance (all F's < 1.1, $p > .05$).
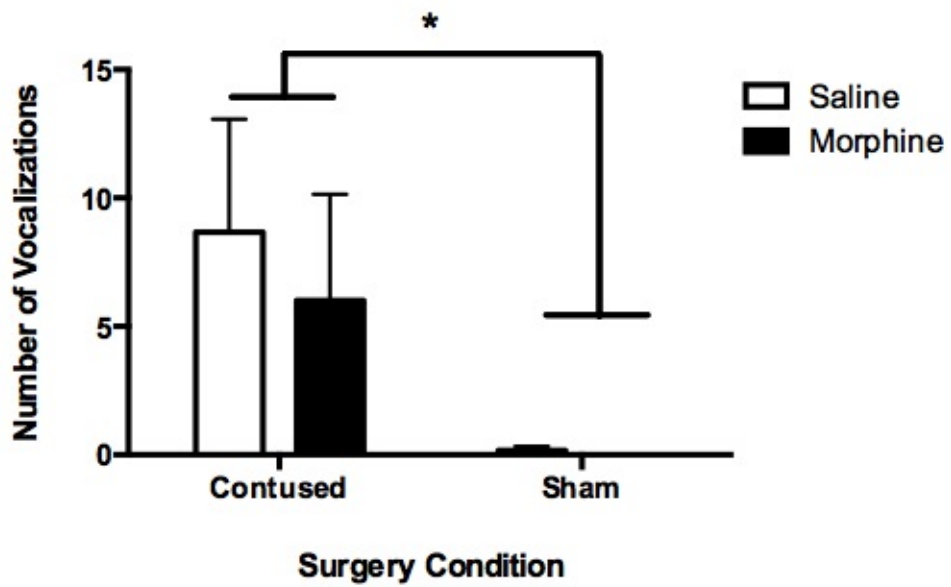
Figure 21: Tests of neuropathic pain conducted after morphine administration. Forty-two days following injury, contused animals vocalized significantly more on a test of at-level girdle reactivity. There was no lasting effect of morphine treatment. * $p < .05$

Contused Rats Showing Established Neuropathic Pain do Not Self-Administer Less

Morphine

The results for the early chronic phase are in contrast to those in the literature reporting a reduced addictive potential of morphine in peripheral injury models with neuropathic pain. In the contusion model of SCI, symptoms of neuropathic pain typically begin to develop around 14 days post injury. However, in our study, many of the animals were not showing signs of neuropathic pain at this early time point. Thus, we extended the period of administration to begin 35 days after injury, when neuropathic pain is more established. Here, we examined only the amount of morphine administered in contused and sham animals given access to 1.5 mg of morphine and their sensory reactivity. We did not monitor recovery in these animals.

Sensory Reactivity. Baseline measures of sensory reactivity were recorded on day 35 following injury, before initiation of self-administration sessions. In a test of mechanical allodynia, contused subjects were less reactive than sham animals ($F_{(1, 10)}$ = 7.65, $p < .05$), but were not different in vocalizations ($F < 1.0$, $p > .05$). At this time, however, we found contused animals showed a shorter tail flick latency than sham animals, indicating the development of thermal hyperalgesia. This result approached significance ($F_{(1, 10)} = 3.84$, $p = .07$). In addition, we found contused animals vocalized more in the girdle test than sham animals (an average of 10.2 vocalizations vs 1.0, respectively) indicating the development of neuropathic pain. This result, however, was not significant ($F < 1.0$, $p > .05$).

As we were testing the effect of neuropathic pain on morphine self-administration, the initial lack of statistical significance for measures of neuropathic pain was concerning. In this model of injury, however, not all contused animals will develop neuropathic pain, and this can make groups too small to have power to yield statistical significance. Thus, we separated contused animals into groups based on the girdle test, an indicator of the development of at-level neuropathic pain. We found four of six animals were showing signs of neuropathic pain (Figure 22).

Morphine Administered. As in the early chronic phase, contused animals administered as much morphine (21.5 mg / day average) as sham controls (22.9 mg / day average), shown in Figure 23A. An ANOVA showed there was not a difference between these two groups (F (1, 10) = 40.74, $p < .05$).

The animals experiencing neuropathic pain did not self-administer a different amount of morphine than pain-free contused or sham animals, though the result did approach significance (F (1, 9) = 3.80, $p = .08$). Sham animals administered an average of 22.89 mg morphine, pain-free contused animals self-administered an average of 25.29 mg, and contused animals showing signs of neuropathic pain self-administered an average of 19.61 mg (Figure 23B). Further supporting similar levels of morphine self-administration in animals experiencing neuropathic pain, use of girdle reactivity as a covariate in examining the amount of morphine administered did not yield a significant result (F $<1.0, p > .05$).

Rate of Administration. As with the early chronic phase, animals in this phase of injury begin by administering approximately 25 mg of morphine in 6 hours on day 1
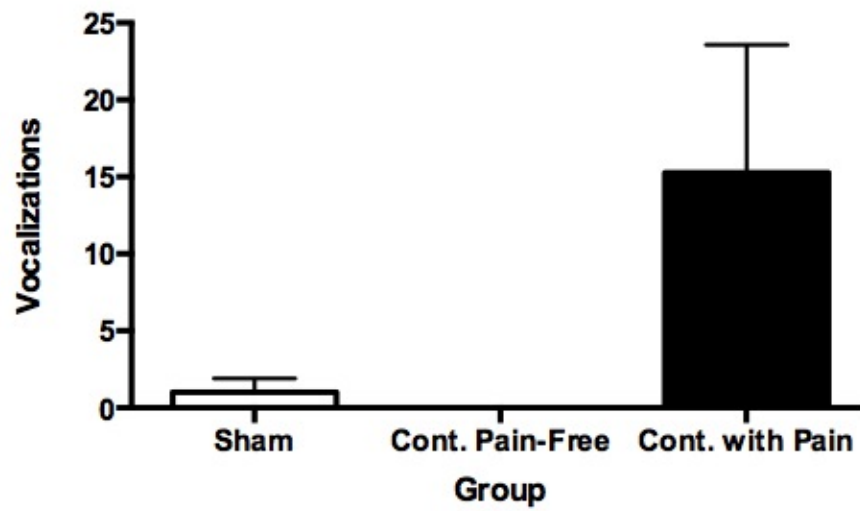
Figure 22: Most contused animals are experiencing pain prior to morphine administration. Four of six contused animals were showing signs of neuropathic pain. No sham animals showed similar signs of pain.

A.

B.
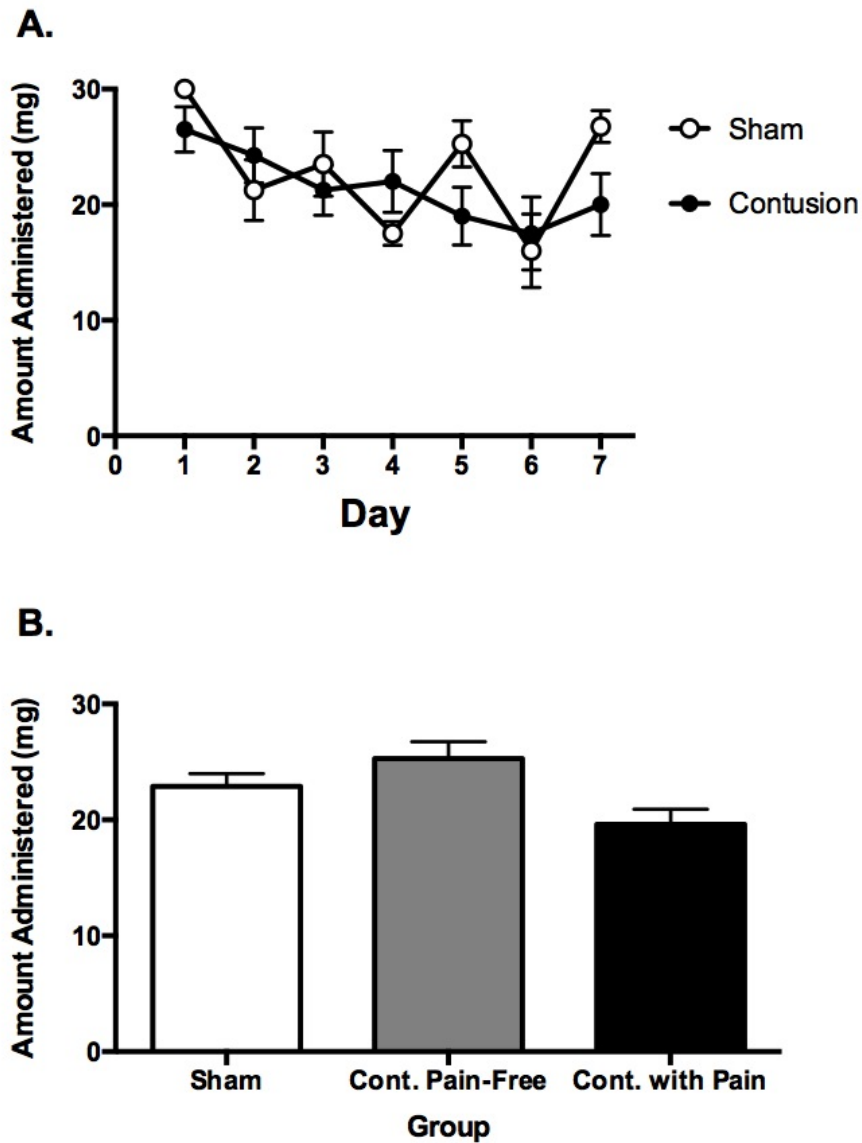
Figure 23: Amounts of morphine administered based on pain level. In the late chronic phase of injury, contused animals administered as much morphine as sham animals (A). When contused animals are separated into a pain-free group and a group experiencing pain, there was not a significant difference in the amount of morphine administered (B).
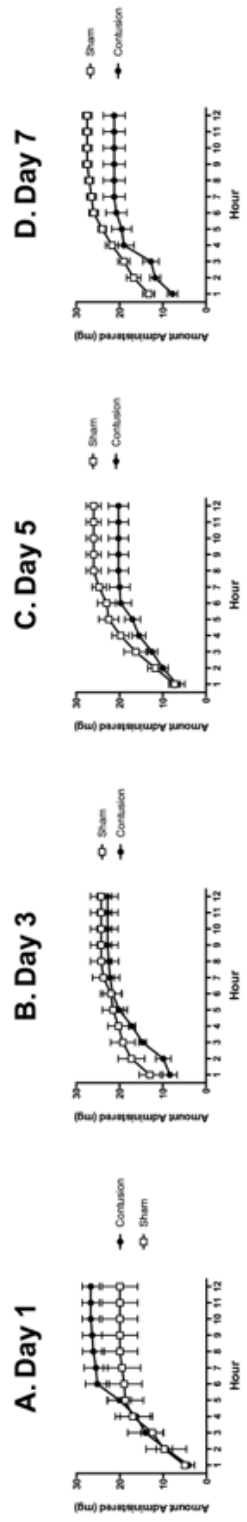
Figure 24: Rates of administration in the chronic phase of SCI. Contused animals decreased the amount of morphine self-administered over days, and remained consistent in the amount of time they administered.

(Figure 24). Rather than increasing the rate and amount administered over days, however, they remained consistent in the time they administered (6-7 hours).

Discussion

These data do not support the hypothesis that neuropathic pain lowers the addictive potential of morphine after SCI. In contrast, the data reported here indicate that the decreased abuse liability observed in the acute phase of a contusion injury has receded by the chronic phase. In the current studies, contused animals in the acute phase of injury self-administered significantly less morphine than their sham counterparts. By the early chronic phase, however, when symptoms of neuropathic pain are just beginning to develop we found that contused animals self-administered as much morphine as sham animals. In addition, contused animals in the chronic phase of injury, experiencing neuropathic pain, self-administered an amount of morphine commensurate with their pain-free counterparts and sham animals. Our results demonstrate that the addictive potential of morphine varies depending on the phase of injury in which it is administered and neuropathic pain symptoms do not negate abuse liability in this model.

In peripheral models of neuropathic pain, reports from the literature indicate that the presence of pain reduces the abuse liability of morphine. The variation in results between our studies, using a central model of neuropathic pain, and peripheral models may stem from differences in experimental design (e.g. amount of morphine available, length of self-administration sessions), the amount of time from injury onset to initiation of self-administration sessions, and prior experience in self-administration chambers. For example, Martin et al. (2007) found dose-dependent decreases in morphine self-

administered following a spinal nerve ligation. Animals experiencing pain self-administered less morphine at doses up to 180 μg/kg, but not at higher (up to 600 μg/kg) doses. The doses used in their study, compared to ours (and those used clinically), are low: 0.18 mg/kg versus approximately 5 mg/kg in our 1.5 mg group. Accordingly, higher doses of morphine would not lead to differential self-administration in contused versus sham animals. While lower doses were not investigated in the current study, it is possible that contused animals would self-administer significantly less morphine than sham controls at lower doses. Indeed, in the acute phase of injury, we found contused animals self-administered significantly less of the 1.5 mg dose, compared with a 3.0 mg dose, but this difference did not persist into the chronic phase of injury.

Our experimental design also differed in the length of the self-administration sessions. In studies of cocaine self-administration, session length has been shown to be an important factor in determining both the rate (infusions/ hour) and amount of cocaine self-administered (Ahmed & Koob, 1998; Wee et al., 2007). In a 1-hour session, rats maintained a low, stable rate of administration (Ahmed & Koob, 1998). In a 12-hour session, however, more cocaine was self-administered, and was administered in a shorter amount of time (Wee et al., 2007). The Martin et al. (2007) study used four, 1-hour sessions in which different doses of drug were available each hour. In contrast, our studies have all used 12-hour sessions. With a 12-hour session, it is anticipated that rats will self-administer more morphine more rapidly than would be seen in a 1-hour session. The 12-hour session, therefore, provides a good index of addictive behavior. It is important to note that the 12-hour session also more closely resembles patient controlled

analgesia, making use of longer sessions clinically relevant in the study of addictive behavior.

With the 12-hour session allowing for the assessment of addictive behavior, we found contused animals administering the 1.5 mg dose in the acute phase of injury did not develop addictive behaviors. These contused animals maintained a low rate of morphine administration across the seven sessions. This finding suggests that the acute, inflammatory pain these animals are experiencing is altering the rewarding properties of the drug. This change in abuse liability could result from several potential mechanisms. For example, a decrease in analgesic efficacy could reduce morphine self-administration. In the absence of rewarding analgesic effects, contused subjects given access to the 1.5mg dose may not be motivated to continue administering the drug. Supporting this, Martin et al., (2007) found that low doses of morphine (below 180 μg/kg) that did not reverse mechanical allodynia also did not support self-administration. Only doses that reversed mechanical allodynia maintained self-administration (Martin et al., 2007). The analgesic efficacy of the 1.5 mg dose of morphine was not tested in the current study. Theoretically, however, a decreased analgesic efficacy, in our model, could result from increased glial activation. Indeed, glial activation, characteristic of the acute phase of SCI, has been shown to counter morphine-induced analgesia (Mika, 2008). Also characteristic of the acute phase of SCI are increases in spinal dynorphin. As with glial activation, antinociceptive effects of morphine are decreased by elevations in spinal dynorphin (Malan et al., 2000). Together, in the acute phase of SCI, increased glial activation and increased spinal dynorphin may act to decrease the rewarding, analgesic

properties of morphine. Thus, low amounts of morphine are less rewarding and lead to decreased morphine self-administration in the acute phase of SCI. Higher doses, however, appear to overcome these anti-analgesic effects. This idea should be examined further in future studies.

In the chronic phase of injury, when neuropathic pain symptoms are displayed, many of the acute changes in glial activation and endogenous opioid activity have subsided. For example, a study by Adjan et al. (2007) found that dynorphin increases, following a T10 contusion injury in mice, is associated with the expression of caspase-3 in greater than 90% of all neurons, oligodendrocytes, and astrocytes. These increases were seen at 4 hours, but not 24-hours following SCI (Adjan et al., 2007). Importantly, our morphine administration began approximately 24 hours after SCI. Morphine administration, at this time, may prolong the elevation in dynorphin levels in this vulnerable phase of injury. Similarly, following SCI astrocytes in the thoracic spinal dorsal horn are most activated from 24-hours to 7-days post injury, and microglia are most activated at 24-hours (Gwak et al., 2012). While glial activation continues for up to 180 days following SCI (Gwak et al., 2012), and increases in pro-inflammatory cytokines are seen up to 28 days, the levels are not as high as in the acute phase of SCI. The reduced levels of glial activation and dynorphin may unmask the addictive potential of morphine in the chronic phase of SCI.

Changes in the molecular milieu inherent to the acute and chronic phase of SCI may also explain the differential effects of morphine on locomotor recovery. In the previous experiment, self-administration of morphine at amounts greater than 50

mg/kg/day day significantly attenuated recovery of locomotor function when administration occurred from days 1-7 post SCI (Chapter III). In the current experiments, animals in the acute phase of injury did not self-administer an amount over 50 mg/kg/day, and did not show attenuated recovery of locomotor function. This replicates our previous result at this dose (Chapter III). However, contradicting these effects, we found contused animals administering over 50 mg/kg/day in the chronic phase of injury did not show attenuated recovery of locomotor function, compared to those administering saline. These data suggest that there are "windows of vulnerability" after SCI, in which morphine produces adverse effects on the recovery of locomotor function. It appears that morphine does not affect locomotor function after 'spontaneous recovery' has plateaued, as is typically seen in the rodent contusion model around 14 days (Hook et al. 2004).

In the previous chapter, we hypothesized that opioid administration may be compounding molecular changes characteristic of acute SCI to produce detrimental effects. As discussed previously, by the chronic phase of injury, many of these molecular processes, including changes in endogenous opioid activity and glial activation, have stopped or are muted from the time immediately post injury. Therefore, it is not expected that opioid administration, in the chronic phase of SCI, will have the same effects as in the acute phase of SCI. For example, following SCI, there is an immediate increase in the levels of extracellular glutamate (McAdoo et al., 2005). Typically, these levels return to normal over the course of a couple hours (McAdoo et al., 2005), thus producing a short-lived effect on excitotoxicity and apoptosis. Upon

further investigation, it was found that application of glutamate to the intact spinal cord, at levels reached following SCI, results in maximal apoptosis (of neurons and oligodendrocytes) 6 hours to 24 hours following application, with no additional apoptosis being seen from 3-28 days (Xu et al., 2008). At an early time following injury, it is hypothesized that opioid administration contributes to increased levels of extracellular glutamate to prolong the period of excitotoxicity, and increase cell loss. By the chronic phase of injury, however, there is unlikely to be a compounding effect of opioid administration because the endogenous levels of glutamate have returned to normal and apoptosis is no longer occurring. Therefore, opioid administration would produce detrimental effects on recovery of locomotor function in the acute, but not the chronic phase of SCI.

In these experiments, we also saw weight loss occurring as a result of morphine administration. Weight loss can occur as a result of withdrawal syndrome (Gu et al., 2005), which may contribute the weight loss we observed in the early chronic phase of injury. Indeed, the high amounts of morphine administered each day in the early chronic phase of injury led to signs of withdrawal (e.g. wet-dog shakes, teeth chewing) throughout the period of administration and into the recovery period, though these were not quantified. Additionally, morphine administration has been shown to affect food intake (Nogueiras et al., 2012; Ferenczi et al., 2010). As there were no effects on food consumption in the first experiments, we did not monitor food intake in this study. However, it is worth noting that an altered feeding pattern contributing to weight loss remains a possibility.

Despite these plausible explanations, we propose that the weight loss is not due solely to withdrawal or alterations in food/fluid consumption. Hook et al. (2009; 2011) have shown that a single intrathecal administration of morphine produces lasting weight loss in rats. This weight loss cannot result from withdrawal, and acute administration of morphine does not significantly affect food intake (Ferenczi et al., 2010). This result suggests that opioid administration is causing a dysregulation in the acute phase of injury that contributes to these effects. Supporting this notion further, the administration of high doses of morphine in the early chronic phase did not have as significant an impact on weight loss, nor as lasting an effect, as high doses of morphine administered in the acute phase of injury. In the chronic phase, animals lost weight during morphine administration, but recovered to the same level as saline-treated rats, and gained more weight than sham controls. In contrast, animals administering the high dose in the acute phase of injury lost significantly more weight, never recovered weight to the same level as saline-treated rats, and were far below sham animals. These results suggest that there are complex interactions occurring with SCI and opioid administration that affect weight in a dose- and phase-dependent manner. The root cause of this weight loss remains to be determined.

Overall, the data presented here suggest that the phase of injury critically affects the adverse outcomes associated with morphine administration. Nonetheless, the use of morphine may be fraught with adverse consequences, irrespective of the time of use. We have shown that morphine administered in the acute, but not the chronic phase of injury attenuates recovery of locomotor function. Based on these findings, use of opioid

analgesics, at high doses, should be avoided in the acute phase of injury. In the chronic phase, however, even a moderate dose of morphine leads to addictive behavior in injured animals, suggesting that a state of neuropathic pain does not reduce the addictive potential of morphine. Thus, the use of opioids analgesics should be closely monitored in this phase of injury. As one of few effective analgesics available for the treatment of pain after SCI, it is difficult to suggest that morphine be removed as a potential treatment for the injured population. Instead, future studies must focus on the molecular mechanisms underlying morphine administration, as well as the potential synergistic actions of opioids and SCI pathology (Woller & Hook, 2013), to negatively affect addiction, locomotor recovery, pain, and general health.

CHAPTER V

RECEPTOR SYSTEMS CONTRIBUTING TO DECREASED RECOVERY OF

LOCOMOTOR FUNCTION

The experimental results described in the previous chapters suggest that changes occurring in the acute phase of SCI leave the spinal cord vulnerable to the adverse secondary consequences of morphine administration. Specifically, morphine administration in the acute phase of injury leads to deficits in locomotor recovery that are not seen with administration in the chronic phase of SCI. It has been suggested that changes in the dynorphin/KOR (kappa opioid receptor) system and increased glial activation contribute to hyperexcitability, and a loss of cells, which could underlie these deficits (Chapter I). However, whether locomotor deficits associated with morphine administration result from activation of one (or both) of these systems has not been investigated. In addition to these two systems, morphine is known to bind in a classic fashion to the MOR (mu opioid receptor), and in a non-classic manner to both TLR2 (Toll-like receptor 2) and TLR4 (Toll-like receptor 4; for review see Hutchinson et al., 2011). The involvement of each receptor system in contributing to morphine-induced locomotor deficits following SCI has not been addressed.

As with glial activation and the KOR system, there is evidence indicating that activation of TLRs may contribute to the detrimental effects of morphine administration. Morphine binds to both TLR2, found on neurons, and TLR4 located on microglia and activated astrocytes. Upon activation, these receptors initiate pathways resulting in the release of the pro-inflammatory cytokine TNFα (Tumor necrosis factor α; Hutchinson,

2011; Papadopoulos et al., 2013; Ozinsky et al., 2000). Increased release of TNFα has

been associated with a variety of pathologies including the development of allodynia in

inflammatory and peripheral-injury induced pain (Garraway et al., (submitted); Schäfers

et al., 2003b; Zhang et al., 2011; Leung & Cahill, 2010). In addition, TNFα enhances

neuronal excitability (Chen et al., 2011) and increases trafficking of calcium-permeable

AMPARs, an effect that can ultimately lead to cell death (Ferguson et al., 2008).

Despite evidence indicating that engaging TNF receptors can lead to apoptosis

via the caspase-3 pathway (Guadagno et al., 2013), there has been little work done

linking activation of TLR2 or TLR4 to altered recovery of locomotor function after SCI.

TLRs (except TLR3) signal through an adaptor protein, myeloid differentiation primary

response gene 88 (MyD88; Kawai & Akira, 2007; Okun et al., 2009). The MyD88-

dependent pathway leads to the activation of NF-κB and results in the production of pro-

inflammatory cytokines, such as TNFα (for review see Hutchinson et al., 2011). A

recent study by Yao et al. (2012) examined the effect of inhibiting the MyD88 pathway

following compression SCI. They found early (immediately after injury) treatment with

a peptide inhibiting MyD88 signaling decreased levels of the pro-inflammatory

cytokines TNFα and IL1-β. This single treatment also reduced activation of caspase-3,

resulting in fewer apoptotic cells, greater numbers of spared neurons in the proximity of

the injured area, decreased lesion size, and improved recovery of locomotor function

(Yao et al., 2012). This study suggests that TLR signaling following SCI is contributing

to secondary pathology. Given these effects, it is possible that morphine-induced

activation of TLRs after SCI contributes further to secondary pathology, and significantly impairs recovery of locomotor function.

The aim of this study, therefore, was to examine changes in these receptor systems occurring after injury and prolonged morphine administration. Specifically, we used western blots to examine alterations in protein expression of KOR, MOR, TLR2, TLR4, TNFα, and caspase-3. In addition, fast blue and cresyl violet analyses allowed us to examine the effects of morphine administration on spared gray matter, white matter, and overall lesion size.

Method

Rats were implanted with a jugular catheter, and five days later were given a moderate contusion or sham injury, as outlined in Chapter II. In this study, the experimenter administered morphine intravenously beginning 24 hours after the contusion or sham injury. Animals were given 5 mg morphine / infusion through the back mount attached to the jugular catheter (i.v.) for 7 days on the following schedule: 10 mg (days 1 and 2), 20 mg (days 3 and 4), and 30 mg (days 5, 6, and 7). Control animals received an equivalent number and volume of infusions of 0.9% saline. Sensory reactivity tests were conducted on days 1, 3, 5, and 7 prior to administration of drugs in the morning, and in the evening, approximately 30 minutes after the last infusion. Twenty-four hours after the last morphine infusion, animals were sacrificed and spinal tissue was collected for western blot and histology as outlined in the General Method section.

Results

Development of Tolerance

In the previous studies, we were unable to monitor the development of tolerance across the seven self-administration sessions because the time since the last infusion of morphine varied, as did the amount of morphine self-administered each day. Here, rats were given experimenter-controlled escalating doses of morphine over the course of seven days, and we were able to monitor the development of tolerance by conducting tests of sensory reactivity in the evening, after the last dose of morphine had been administered.

Morphine administration produced a robust analgesia when administered 24-hours after a sham or contusion injury; all animals reached the maximum 8 second tail flick latency. Animals given saline do not show a change from their morning tail flick latency (Figure 25). An ANOVA revealed a significant main effect of drug condition ($F (1, 32) = 28.45, p < .05$).

Examining motor responses, to a mechanical stimulus, made over the seven days of administration revealed significant main effects of surgery ($F (1, 32) = 47.97, p < .0001$), drug treatment ($F (1, 32) = 82.81, p < .0001$), and a Surgery x Drug interaction ($F (1, 32) = 37.97, p < .0001$). In general, contused subjects only respond to higher force von Frey filaments. As shown in Figure 26A, morphine treatment led to a significant decrease in tactile reactivity; contused animals reached the maximum filament force without withdrawing the hindlimb. By days 5 and 7, however, sham animals no longer

96
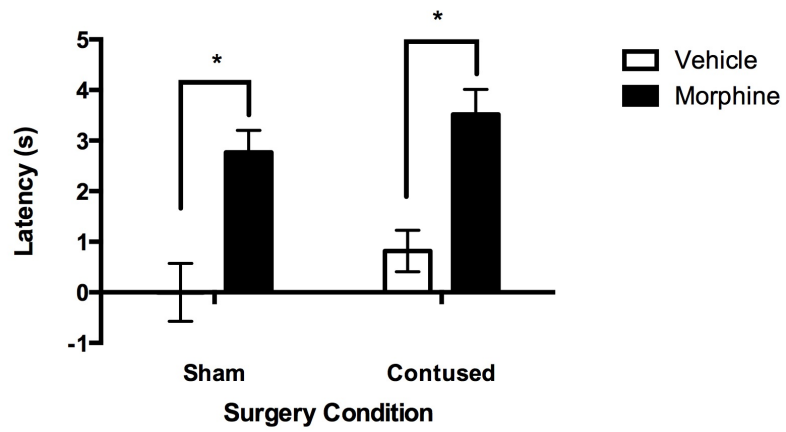
Figure 25: Analgesic response to the first administration of morphine. A change from baseline response to the first day of morphine administration is shown. All animals treated with morphine showed an increase in tail flick latency, while those given saline showed no change. * *p* < .05
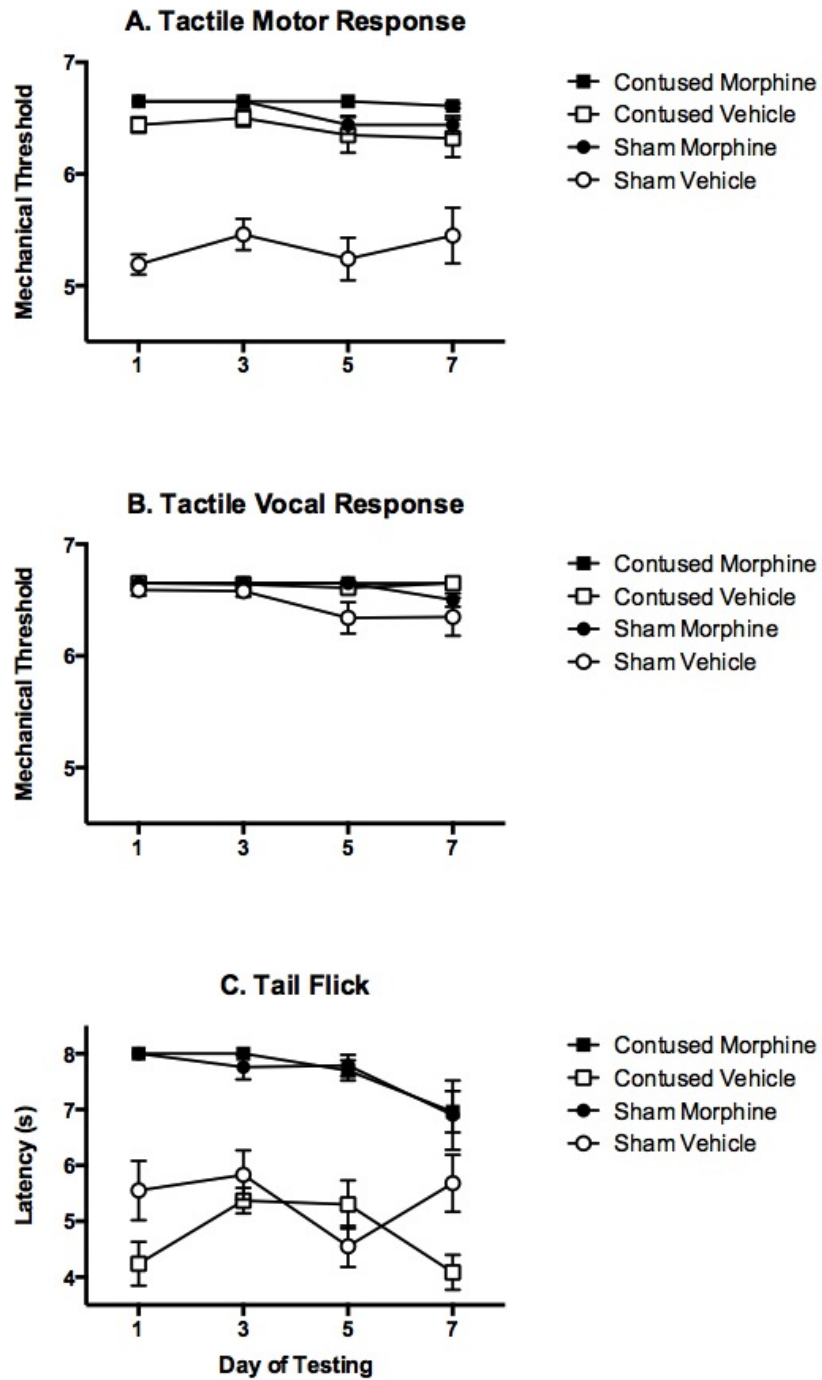
Figure 26: The development of tolerance to morphine. In measures of mechanical allodynia (A & B) and thermal hyperalgesia (C), there were signs of the development of tolerance. On day 5, animals treated with morphine were no longer reaching the maximum response to a mechanical or thermal stimulus.

reached the maximum threshold, indicating the development of tolerance. These changes across testing sessions, however, were not significant (F's < 2, p > .05). Similar changes were seen in vocal responses across testing sessions (Figure 26 B). In general, contused animals were less reactive than sham controls (F (1, 32) = 8.7, p < .05). Again, morphine treatment significantly increased response threshold (F (1, 32) = 4.90, p < .05). By day 7, sham animals began vocalizing in response to mechanical stimulation, but this was not seen in contused animals. These results, across testing sessions, were not significant (F's < 1, p > .05).

Measuring thermal hyperalgesia, there was a main effect of morphine treatment (F (1, 32) = 156.53, p < .0001). Animals treated with morphine showed increased tail flick latencies. However, this effect waned across days, indicating the development of morphine-induced tolerance. By day 7, sham and contused rats, while still showing increased tail flick latencies relative to vehicle-treated controls, no longer reached the maximum tail flick latency of 8 seconds. This is despite treatment with 30 mg (an amount approximately equal to100 mg/kg) of morphine. An ANOVA showed an effect of day of treatment (F (3, 32) = 3.54, p < .05), but no interactions were significant (F's < 3.0, p > .05).

Development of Opioid-Induced Hyperalgesia

In previous experiments, we had not been able to assess the development of opioid-induced hyperalgesia because of the varying amounts of morphine administered across self-administration sessions. Opioid-induced hyperalgesia (OIH) is a phenomenon in which the repeated administration of morphine leads to the development

of pain, either mechanical or thermal. By assessing sensory reactivity in the morning, before any morphine administration began, we were able to monitor the development of OIH in these animals.

The development of OIH was first assessed by examining withdrawal of the hindlimb to a von Frey filament. Again, contused animals tend to be less reactive on this test, and this was confirmed by a significant main effect of surgery ($F(1, 32) = 38.54$, $p < .05$). Over days of testing, sham and contused animals treated with morphine became much more reactive and began withdrawing their hindlimb at lower forces. Sham animals treated with morphine, by day 7, were more reactive than sham animals treated with saline. These results indicate morphine treatment is leading to the development of pain (OIH). An ANOVA confirmed these results showing a significant effect of day of testing ($F(3, 96) = 23.73$, $p < .05$), and Day x Drug ($F(3, 96) = 5.93$, $p < .05$) and Day x Surgery x Drug ($F(3, 96) = 5.48$, $p < .05$) interactions.

Vocalizations in response to tactile stimulation showed a similar, though more dramatic, effect. Contused animals are less reactive than sham animals, in general ($F(1, 32) = 12.61$, $p < .05$). Morphine treatment led to a significant decrease in threshold required to elicit a response ($F(1, 32) = 9.68$, $p < .05$), and this effect was greatest in the sham animals ($F(1, 32) = 4.40$, $p < .05$). Over days, animals treated with morphine showed the development of OIH. This was supported by a significant Day x Drug interaction ($F(3, 96) = 6.89$, $p < .05$).

A. Tactile Motor Response

B. Tactile Vocal Response

C. Tail Flick
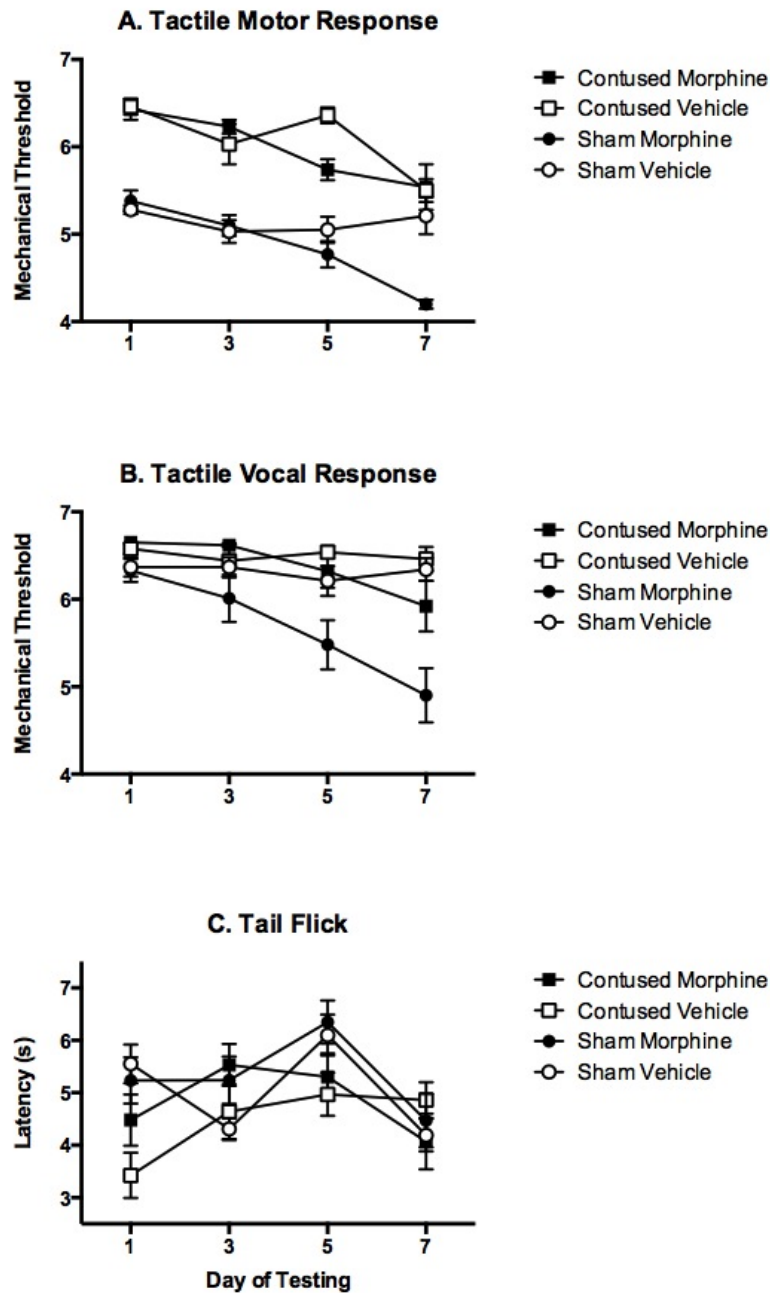
Figure 27: The development of opioid-induced hyperalgesia. Animals were tested in the morning, prior to any morphine treatment. Over days of testing, animals being treated with morphine showed increased reactivity (withdrawal of the hindlimb and vocalization) to mechanical stimuli. The effect was most pronounced in sham animals.

Using the tail flick test to measure thermal hyperalgesia (Figure 27C), contused animals exhibited a faster tail flick than sham animals (F (1, 32) = 5.05, $p < .05$). All animals exhibited similar tail flick latencies regardless of prior treatment with morphine. There was no evidence for the development of opioid-induced hyperalgesia on this measure.

Locomotor Recovery

As in previous experiments, we assessed recovery of locomotor function using the BBB scale. Previously, in the acute phase of injury, we found a high amount of morphine administration significantly attenuated recovery of locomotor function, but a low dose did not. In this experiment, all animals were given an average of 21.4 mg each day. This corresponds to approximately 70-74 mg/kg/day in these rats, an amount that, based on previous results, should attenuate functional recovery. As expected, contused rats treated with morphine recovered significantly less function over the first week than contused animals treated with saline, despite having equal converted BBB scores on day 1 (1.6 in the morphine group and 1.8 in the vehicle). This result is supported by an ANCOVA showing Day x Drug Condition interaction (F (5, 75) = 5.27, $p < .05$). Contused animals treated with morphine recovered to an average converted BBB score of 4.8, while those treated with saline recovered to a 7.1, in the first seven days (Figure 28).
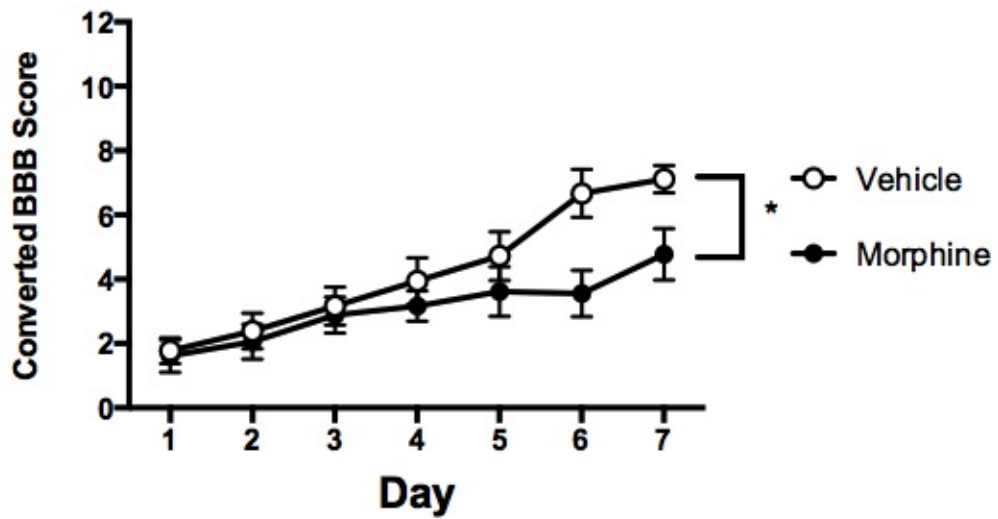
Figure 28: Locomotor recovery following morphine administration. Contused subjects treated with high amounts of morphine recovered significantly less locomotor function over the first week than did animals treated with saline. * $p < .05$
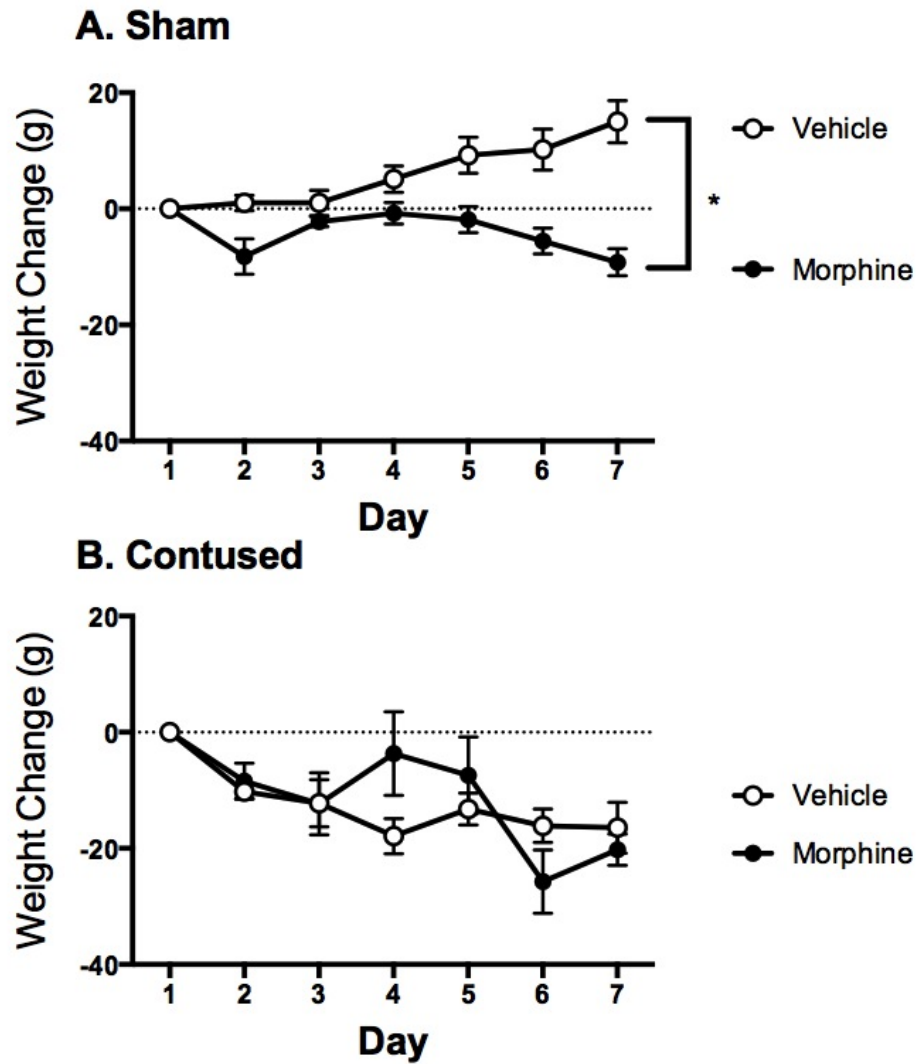
Figure 29: Weight loss occurring during morphine administration. Sham animals (A) treated with morphine lost significantly more weight during the seven days of administration than sham animals treated with vehicle. In contrast, all contused (B) animals lost weight throughout the seven days, and this was not affected further by morphine administration. * $p < .05$

Weight

In previous experiments, we have shown morphine administration leads to a significant weight loss. Here, we found sham rats given morphine lost weight over the first week (Figure 29A), while those given saline did not. As found previously (Chapter IV), contused rats lost weight in the first week of injury irrespective of drug treatment (Figure 29B). This weight loss, characteristic of a contusion injury, likely masked additional effects of morphine in the truncated assessment period. Nonetheless, there was a main effect of surgery (F (1, 32) = 24.18, $p < .05$), a Day x Surgery (F (6, 192) = 9.88, $p < .05$), Day x Drug Condition (F (6, 192) = 6.59, $p < .05$), and a Day x Surgery x Drug Condition (F (6, 192) = 2.57, $p < .05$) interaction. *Post hoc* analyses indicated that sham animals treated with morphine weighed significantly less than sham animals treated with vehicle.

Histology

Following morphine administration, we examined the effect of contusion injury and morphine administration on spinal tissue. Tissue was analyzed as three regions within the same 1-cm section. Rostral tissue was defined as -2400, -1800, and -1200 μm from the impacted region. Center tissue was defined as -600, 0, and 600 μm, and caudal was 1200, 1800, and 2400 μm from the impacted region (Figure 30).

We found that a contusion injury significantly decreased the amount of gray matter at the site of injury (center), as well as rostral and caudal to the injury site (Figure 31A) relative to sham subjects. These results are supported by an ANOVA showing a significant main effect of surgery condition (center: F (1, 8) = 17.05, $p < .05$; rostral: F
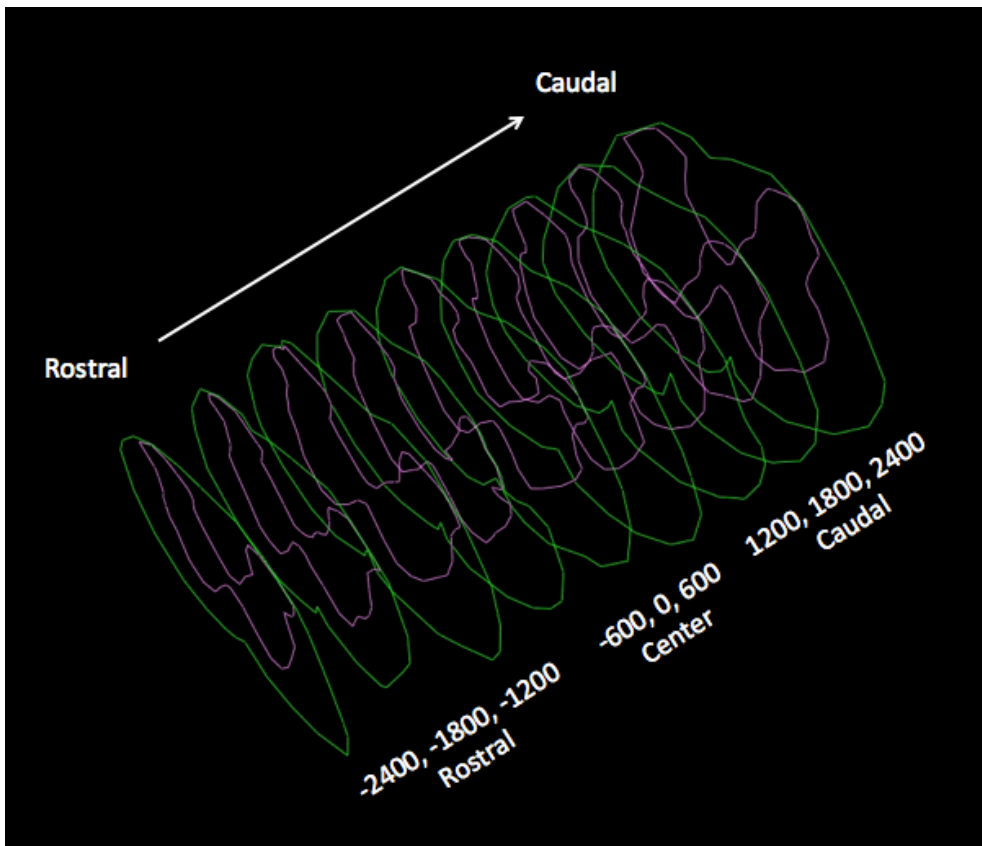
Figure 30: Spinal sections for histology. The nine sections used for analyses are depicted. The first three sections were considered the area rostral to injury, the next three contained the lesion center, and the last three were caudal to the injury. In this sham vehicle subject, gray matter is outlined in pink and white matter in green.
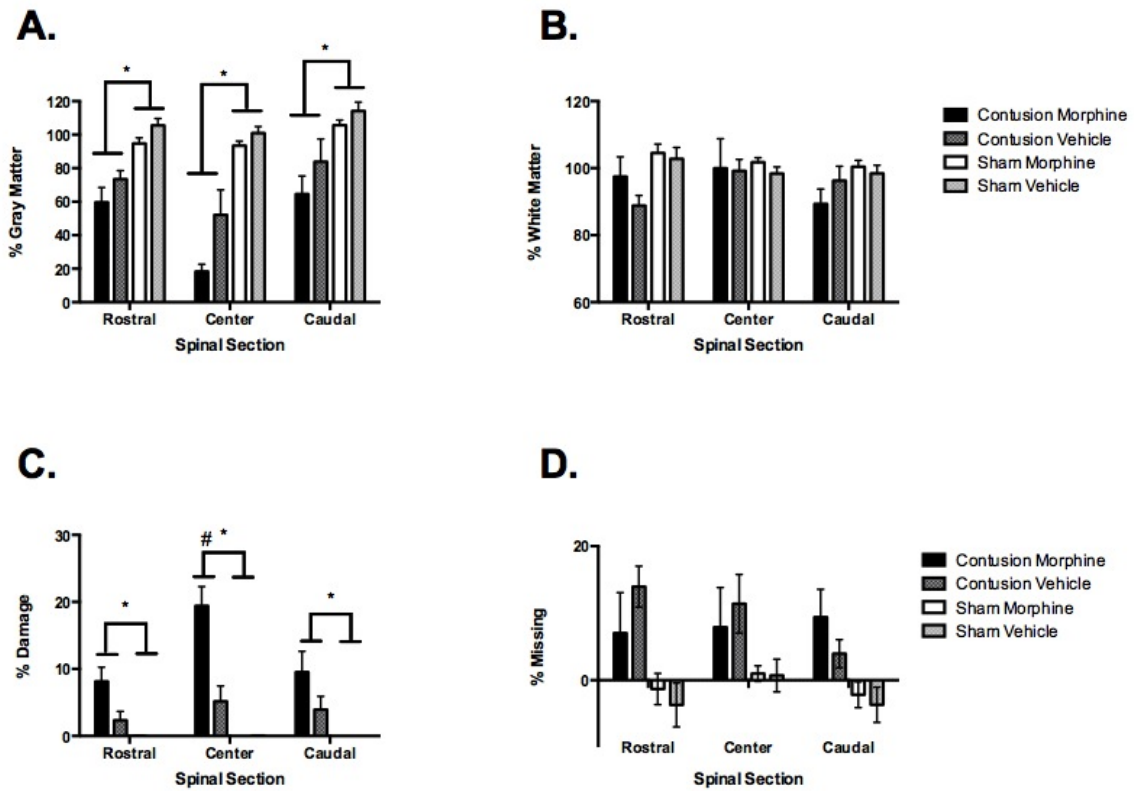
Figure 31: Analysis of tissue. The percentage of gray matter (A) was significantly reduced by contusion injury at all regions of the spinal cord, but white matter was not changed (B). Overall, a contusion injury increased the amount of damage to the spinal cord (C). The amount of missing tissue (D) was not different across surgery conditions. Damage was increased by morphine administration at the center of the spinal cord in contused animals. $* p < .05$, #, significant group difference.

(1, 8) = 28.50, $p < .05$; caudal: F (1, 8) = 7.00, $p < .05$). There was not a main effect of

drug treatment (F's < 3.81, $p > .05$). In the area rostral to the injury center, there were

significant Distance x Surgery (F (2, 16) = 5.64, $p < .05$) and Distance x Drug

interactions (F (2, 16) = 6.89, $p < .05$) indicating that the amount of gray tissue

remaining varied depending on the spatial location in the cord. The area closest to the

lesion center had the least gray tissue remaining, and there was significantly less gray

matter in morphine-treated animals. At the center of the lesion and the area caudal, no

other results approached significance (F's < 3.37, $p > .05$).

As shown in Figure 31B, at seven days post injury, there was no effect of

contusion injury or morphine administration on the percentage of white matter spared

(all F's (rostral, center, and caudal) < 2.90, p's > .05). This is expected as cell loss and

cavitation progress from the gray matter outward toward the white matter with time,

eventually leaving just a rim of spared white matter (James et al., 2011).

When examining the percentage of lesioned tissue, we found a contusion injury

significantly increased lesion area in the rostral, center, and caudal regions of the spinal

cord (Figure 31C; rostral: (F (1, 8) = 6.66, $p < .05$; center: F (1, 8) = 22.85, $p < .05$;

caudal: F (1, 8) = 13.18, $p < .05$). Morphine administration further increased the

percentage of damage in the center area of the spinal cord in contused animals. An

ANOVA revealed a significant main effect of drug condition (F (1, 8) = 7.66, $p < .05$).

Also at the center of the lesion, there were significant Surgery x Drug (F (1, 8) = 7.80, p

< .05) and Distance x Surgery interactions (F (2, 16) = 3.84, $p < .05$). Morphine
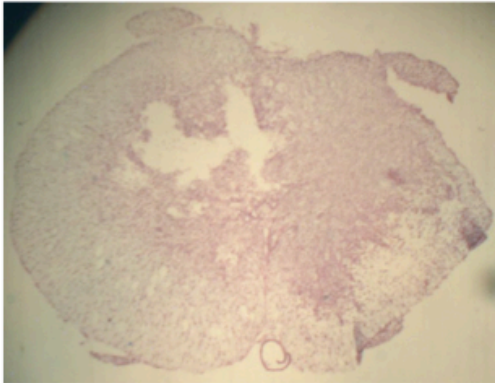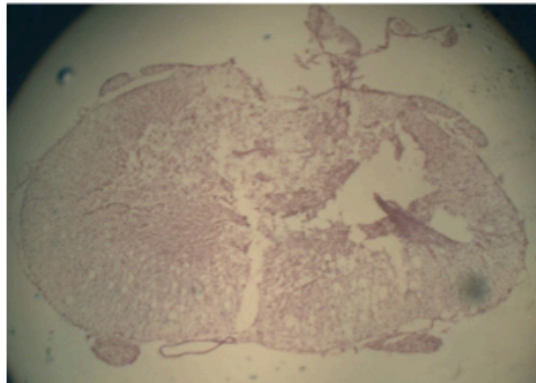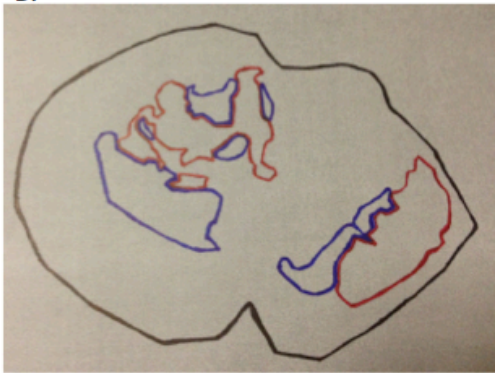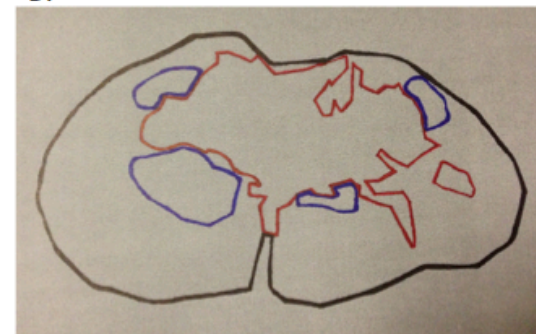
Figure 32: Morphine treatment increases lesion size at the center of the cord.
Contused vehicle animals (A) show lesion area (the red outline; B), but this is
increased in contused animals treated with morphine (C & D). Black = outline of
white matter. Blue = outline of normal gray matter. Red= outline of lesion area.

treatment increased the percentage of lesion area in contused animals only, and had the most impact in the area closest to the center of the injury (Figure 32).

In previous experiments (Hook et al., 2011; Grau et al., 2004), the amount of missing tissue was also assessed. Missing tissue accounts for tissue lost as a result of factors such as atrophy, necrosis, or apoptosis that are not accounted for in analyses of lesion area alone (see Hook et al., 2011). As shown in Figure 31D at this early time point, we did not see a significant amount of missing tissue in any of the groups (all F's < 4.86, p's > .05).

Western Blot

At the time of tissue collection, cords were hemisected to allow for the examination of dorsal-ventral changes in protein expression. With these sections, we compared changes in each half individually, the changes in dorsal versus ventral, and changes in an average "overall" cord. Analyses of changes in the dorsal versus ventral portion of the spinal cord were conducted to examine whether protein levels were differentially expressed in the two halves (a within subjects variable). Specifically, changes in protein expression for the MOR, KOR, TLR2, TLR4, TNFα, and caspase-3 were assessed.

We used β-Actin as a control protein, and analyzed protein expression levels for differences. In these samples, there were no significant differences in β-actin expression in the ventral portions (Mini: F's < 1.0, p > .05; Criterion: F's < 1.5, p > .05). In the dorsal portions, there was a significant effect of surgery on the Mini gels (F (1, 19) = 7.968, p < .05) and a significant Surgery x Drug interaction (F (1, 19) = 8.51, p < .05).

Also in the dorsal portions on the Criterion gels, there was a significant Surgery x Drug interaction (F (1, 19) = 4.60, $p < .05$). Future analyses will include tubulin as an endogenous control. However, for these analyses, we used β-actin despite the differences.

Effects of Repeated Morphine Administration and Contusion Injury on MOR and KOR Protein Expression

In examining protein expression levels in the dorsal portion of the spinal cord (Figure 33 A & B), we found increased MOR protein in the sham animals treated with morphine, but decreased MOR protein in contused animals treated with morphine. The contused animals treated with saline showed no change. There were, however, no significant differences (F's < 2.97, $p > .05$). The change in MOR protein levels on the ventral half were similar to the dorsal, but sham animals treated with morphine did not show as much of an increase. No results approached significance (F's < 1.20, $p > .05$).

Comparing the dorsal and ventral halves, there was a divergent effect of morphine treatment on sham and contused animals: contused animals treated with morphine showed decreased MOR protein levels while sham animals treated with morphine showed increased MOR protein levels. Contused animals treated with saline showed no difference from sham animals treated with saline. These results were, however, not significant (F's < 3.23, p's $> .05$). Averaging the two halves into an "overall" cord also yielded no significant results (F's < 2.63, $p > .05$).
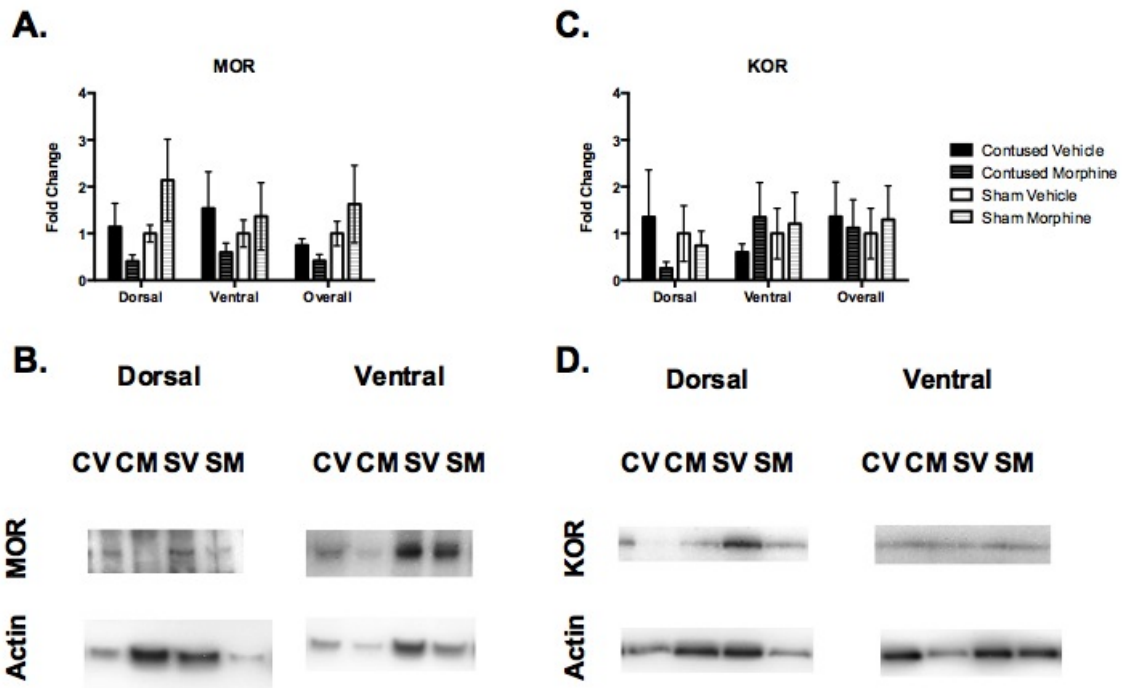
Figure 33: MOR and KOR protein levels. There are no significant differences in MOR (A & B) or KOR (C & D) protein levels in the dorsal, ventral, or overall cord. CV = contused vehicle, CM = contused morphine, SV = sham vehicle, SM = sham morphine.

Levels of KOR protein, Figure 33 C & D, were not changed by either surgery condition or drug treatment in the dorsal portion of the spinal cord (F's < 1.2, p > .05). Contused subjects treated with morphine, however, appeared to have lower levels of KOR protein than the other three groups. In the ventral portion of the spinal cord, there were, again, no differences in KOR protein levels (F's < 1.0, p > .05). Comparing the dorsal and ventral halves of the cord, there were no changes in KOR protein levels (F's < 2.0, p > .05). Nor were there changes when looking at the "overall" cord. Here, KOR protein levels seemed equal between groups (F's < 1.0, p > .05).

Effects of Repeated Morphine Administration and Contusion Injury on TLR2 and TLR4 Protein Expression

In examining changes in TLR2 protein levels in the dorsal portion of the spinal cord (Figure 34 A & B), we saw a decrease in contused animals treated with morphine. All other groups were at the same level as sham animals treated with saline. These differences were not significant (F's < 1.50, p > .05). On the ventral portion of the spinal cord, there were no changes in TLR2 protein levels (F's < 1, p > .05).

Comparing the dorsal half to the ventral half of the cord, there seemed to be a slight decrease in TLR2 protein in contused subjects treated with morphine, but this was not significant nor were there any other significant differences (F's < 1.5, p > .05). These results are also reflected in the analysis of the "overall" cord (F's <1, p > .05).

In the dorsal portion of the spinal cord, there were no significant differences in TLR4 protein as a result of surgery condition or morphine treatment (F's < 2.00, p >
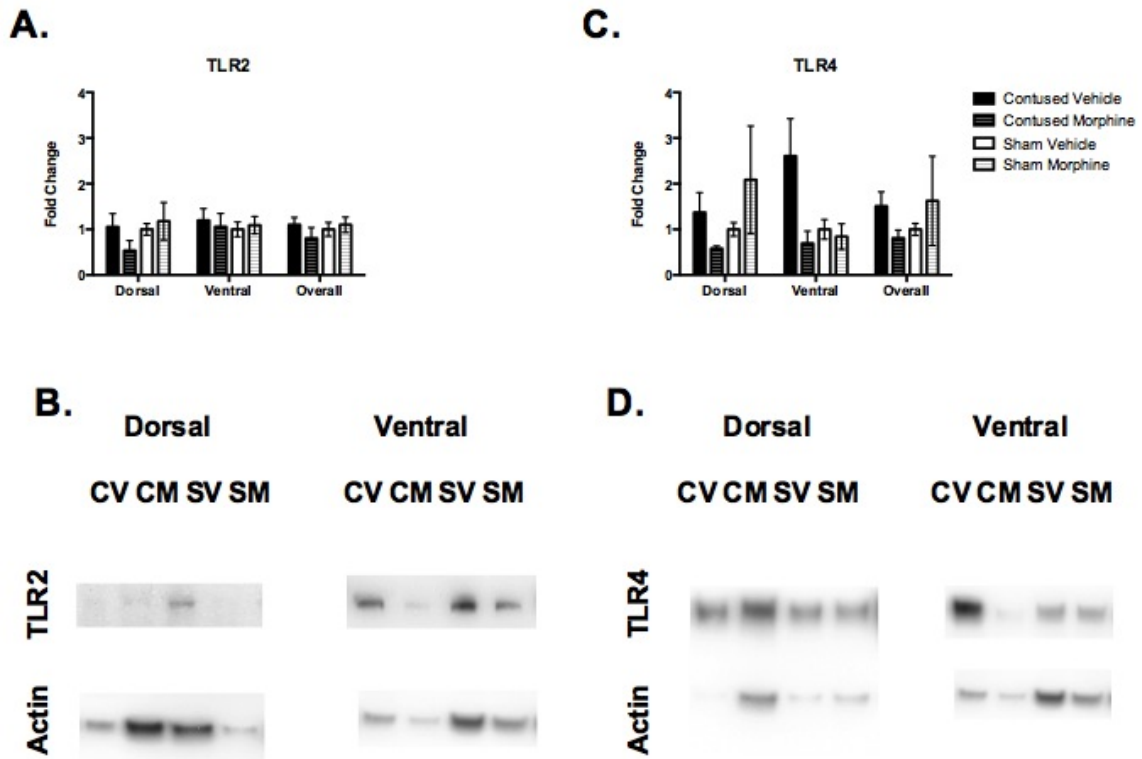
Figure 34: TLR2 and TLR4 protein levels in the spinal cord. There were no changes in TLR2 (A & B) and TLR4 (C & D) protein expression in the dorsal and ventral portions of the spinal cord. CV = contused vehicle, CM = contused morphine, SV= sham vehicle, SM = sham morphine.

.05). Again, though, there appeared to be divergent effects of morphine treatment on contused versus sham subjects (Figure 34 C & D), and no change in the subjects treated with saline. TLR4 protein levels were quite different in the ventral portion of the spinal cord. In this portion, morphine treatment had no effect relative to saline animals treated with saline. Contused animals treated with saline, however, had increased TLR4 protein levels. These results were not significant (F's < 4.00, p > .05).

Comparing the dorsal portion of the cord to the ventral, there was a significant Surgery x Drug interaction stemming from the results described above (F (1, 17) = 4.56, p < .05). No other differences approached significance (F's < 3.00, p > .05). Similarly, there were no differences in TLR4 protein expression when looking at the "overall" cord (F's < 1.50, p > .05).

Effects of Repeated Morphine Administration and Contusion Injury on TNFα Protein Expression

In the dorsal portion of the spinal cord, protein levels of TNFα (Figure 35 A & B) in contused rats treated with morphine appeared to drop to almost nothing. There were no changes in TNFα in the other three groups. No differences were significant (F's < 1.5, p > .05). In the ventral potion of the cord, all groups seemed to have decreased TNFα protein levels relative to sham animals treated with saline, but these differences were not significant (F's < 2.0, p > .05). There was a similar lack of significance when comparing the dorsal and ventral sections (F's < 2.0, p > .05), and when looking at the "overall" cord (F's < 1.5, p > .05).

A.
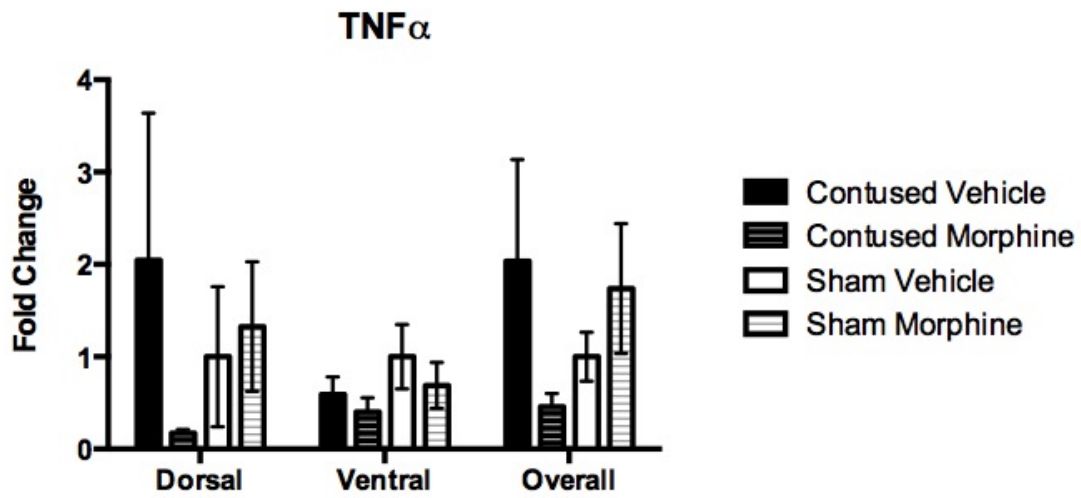
B.
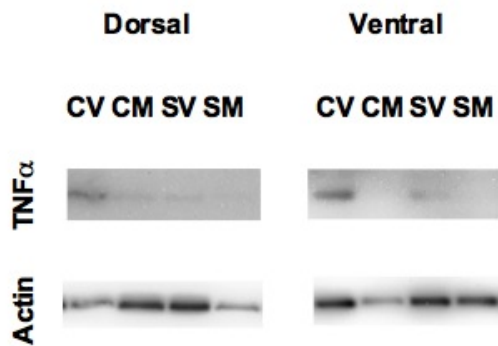
Figure 35: Protein levels of TNFα in the spinal cord. Following prolonged treatment with morphine, there are no changes in TNFα protein in either portion of the cord, or overall. Protein levels were also unchanged by surgery (A & B). CV = contused vehicle, CM = contused morphine, SV = sham vehicle, SM = sham morphine

Effects of Repeated Morphine Administration and Contusion Injury on Caspase-3

Protein Expression

Caspase-3 presents as several bands between ~17 kDa and ~35 kDa, and we analyzed the band at approximately 35kDA, which represents the cleaved caspase product. There were no significant differences in caspase-3 protein in the dorsal portion of the spinal cord (Figure 36 A & B; $p > .05$) or in the ventral portion ($p > .05$).

Changes in Protein Expression 24-hours After i.t. Morphine Administration

Although there were no significant differences in protein expression for the morphine and saline-treated animals, there were trends for divergent expression across groups. Moreover, functionally we found significant effects on locomotor recovery, weight loss, and behavioral changes indicating the development of tolerance and opioid-induced hyperalgesia. We hypothesized that, in only examining protein levels 24-hours after cessation of morphine administration, and 8-days after contusion injury, we missed the window of significant changes occurring at an earlier time (i.e. on Day 1 of morphine administration). To test this, we examined changes in protein expression 24-hours following the administration of i.t. morphine (48-hours after contusion injury), which has also been shown to result in attenuated recovery of locomotor function and weight loss (Hook et al., 2009; 2011).
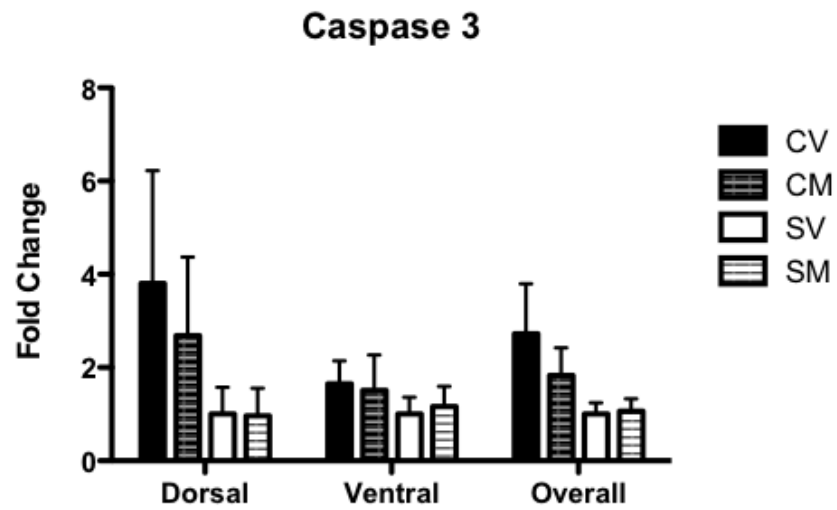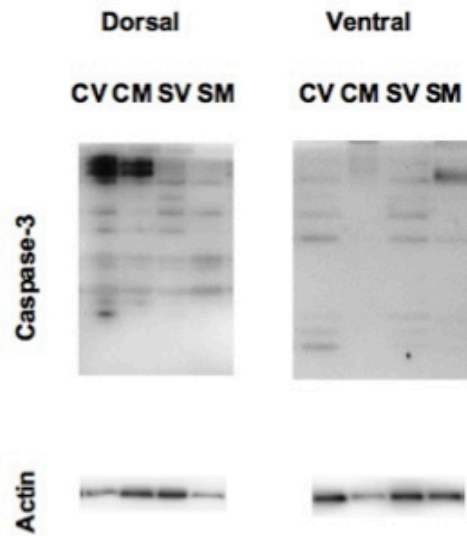
Figure 36: Protein levels of caspase-3 in the spinal cord. Caspase-3 protein was neither changed by spinal cord injury nor morphine administration (A & B). CV = contused vehicle, CM = contused morphine, SV = sham vehicle, SM = sham morphine

Method

An additional 21 animals (n=7) were used to examine the effects of acute i.t. morphine administration on changes in protein expression. These animals were given a contusion (n=14) or sham injury (n=7) injury and a PE 10 catheter was implanted with the tip positioned 2 cm caudal to the lesion center. Twenty-four hours after injury, subjects were given an intrathecal injection of 90 µg morphine or vehicle. The next day animals were deeply anesthetized and a 1 cm section of the spinal cord was collected and immediately frozen in liquid nitrogen, as described in the General Methods (Chapter II). Spinal cords in this experiment were not cut into dorsal and ventral portions because there were no significant differences in the first experiment. In addition, we wanted to focus on the changes induced by spinal cord injury and whether morphine administration caused further changes in contused animals treated with morphine. Thus, we did not include a sham group treated with morphine.

Results

Here, we again examined β-Actin levels for differences between groups. In these experiments, there were no significant differences in the levels of β-Actin on the mini or criterion gels (F's < 2.5, $p > .05$).

Effects of Acute Morphine Administration and Contusion Injury on MOR and KOR Protein Expression

Similar to the changes seen with the repeated i.v. administration of morphine, we found protein levels of the MOR were significantly decreased by a contusion injury (F

(1, 18) = 11.12, $p < .05$; Figure 37 A & B). This result is consistent with those seen after a peripheral nerve injury (Lee et al., 2011). Protein levels were not further affected by morphine administration (F < 1.0, $p > .05$).

As shown in Figure 37 C & D, there were no changes in protein expression levels of the KOR 24-hours following intrathecal morphine administration (48-hours following a contusion injury). All groups exhibited similar levels of KOR protein (F's < 1.0, p's > .05).

Effects of Acute Morphine Administration and Contusion Injury on TLR2 and TLR4

Protein Expression

Examining the protein levels of TLR2 failed to reveal a significant effect of contusion injury or morphine treatment (F's < 3.26, p's > .05, Figure 38 A & B). However, morphine treatment appears to decrease TLR2 protein levels, and this result trended toward significance ($p = 0.09$). The changes seen following a single administration of morphine are comparable to those seen with repeated i.v. administration of morphine where treatment, in the contused subjects, appeared to slightly decrease TLR2 protein levels.

As seen in Figure 36 C & D, acute, i.t. treatment with morphine did not have an effect on TLR4 protein levels (F < 1.0, $p > .05$), but TLR4 protein levels were significantly increased in subjects with a contusion injury (F (1, 18) = 7.54, $p < .05$).
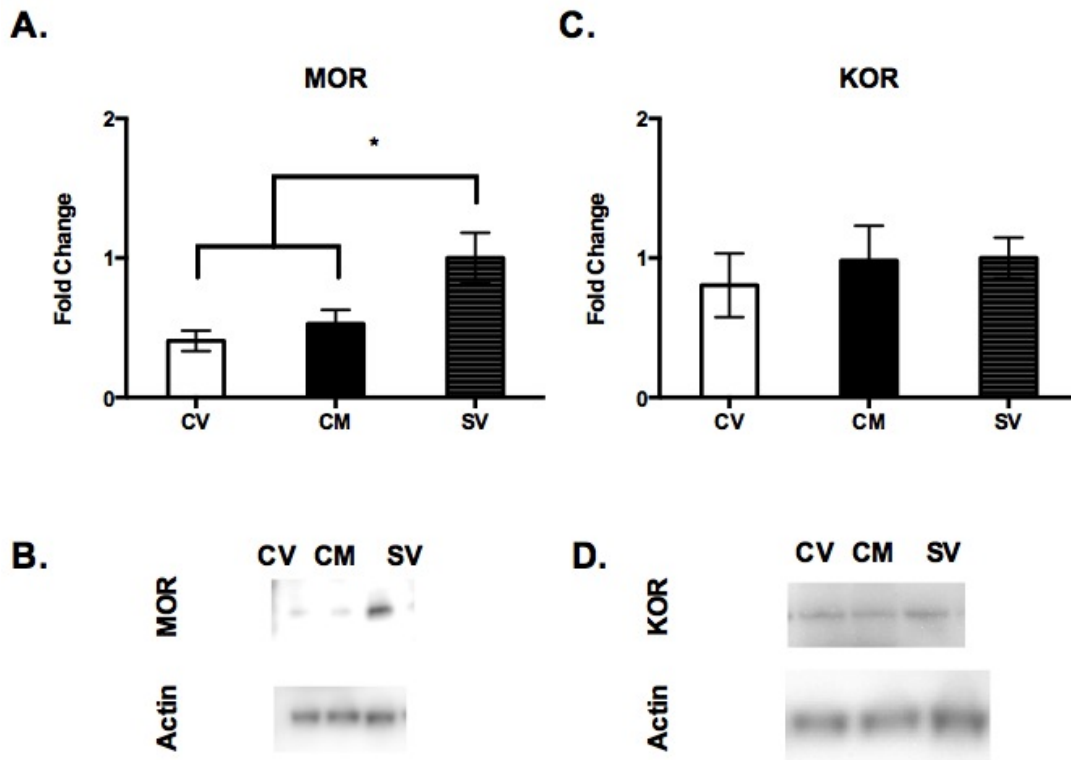
Figure 37: Protein levels of MOR and KOR in the spinal cord following i.t. morphine administration. A contusion injury significantly decreased KOR protein in the spinal cord (A & B). There were no differences in spinal KOR protein. * p < .05, CV = contused vehicle, CM = contused morphine, SV = sham vehicle.

Effects of Acute Morphine Administration and Contusion Injury on TNFα Protein Expression

Twenty-four hour following the intrathecal administration of morphine, there were no changes in TNFα protein levels as a result of morphine administration ($F < 1.0$, $p > .05$) or contusion injury ($F < 2.0$, $p > .05$; Figure 39).

Effects of Acute Morphine Administration and Contusion Injury on Caspase-3 Protein Expression

Again, we analyzed the cleaved caspase-3 product. Here, we found caspase-3 is increased in contused animals relative to sham controls (Kruskall Wallis test, $p < .05$), but this is not further affected by morphine administration (Mann-Whitney U, $p > .05$; Figure 40).

Discussion

In this experiment, we used behavioral, histological, and molecular methods to examine the effects of morphine administration following a contusion injury. Animals were treated with high quantities of intravenous morphine for seven days, mimicking the pattern of the self-administration seen in Chapter III. We found that prolonged treatment with morphine led to the development of tolerance beginning on day 5 of treatment. On the test of mechanical allodynia, contused rats only showed slight signs of tolerance developing on day 7 of administration. On the tail flick test of thermal reactivity, however, a reduction in the analgesic efficacy of morphine was evident in both sham and contused rats on Day 5 of administration. Together, these behavioral data indicate that animals are becoming tolerant to morphine. The data also suggest that hyperalgesia

develops with chronic administration of morphine. Opioid induced hyperalgesia was particularly evident in the sham rats, but also clear following a contusion, on the measure of tactile reactivity. Overall, these data suggest that the increases in morphine administration, seen in the acute phase of a contusion injury may be due, in part, to the development of tolerance and opioid induced hyperalgesia.

While the behavioral effects of repeated opioid administration on hyperalgesia and tolerance are clear, the molecular changes that might mediate these effects are not. In the literature, tolerance has been linked to activation of TLR4 (Hutchinson et al., 2011). Through activation of TLR4, IL-1β is released and has been shown to oppose the analgesic properties of morphine (Hutchinson et al., 2011). In these experiments, however, we did not see a change in spinal expression of TLR4 protein after 7 days of tolerance-inducing morphine treatment. On the basis of these data, we could suggest that TLR4 is not mediating tolerance in the SCI model. Indeed, others have shown that morphine-induced tolerance is not mediated solely by TLR4 activation (Fukagawa, 2013). Alternatively, activation of TLR4 may lead to increased pro-inflammatory cytokine expression after SCI, without changes in receptor expression level, and thereby mediate tolerance. Indeed, Hook et al. (2011) found that IL-1 β levels are increased within 30 minutes of intrathecal morphine administration, relative to vehicle-treated SCI controls. Further, Hook et al. (2011) demonstrated that treatment with an IL-1 receptor
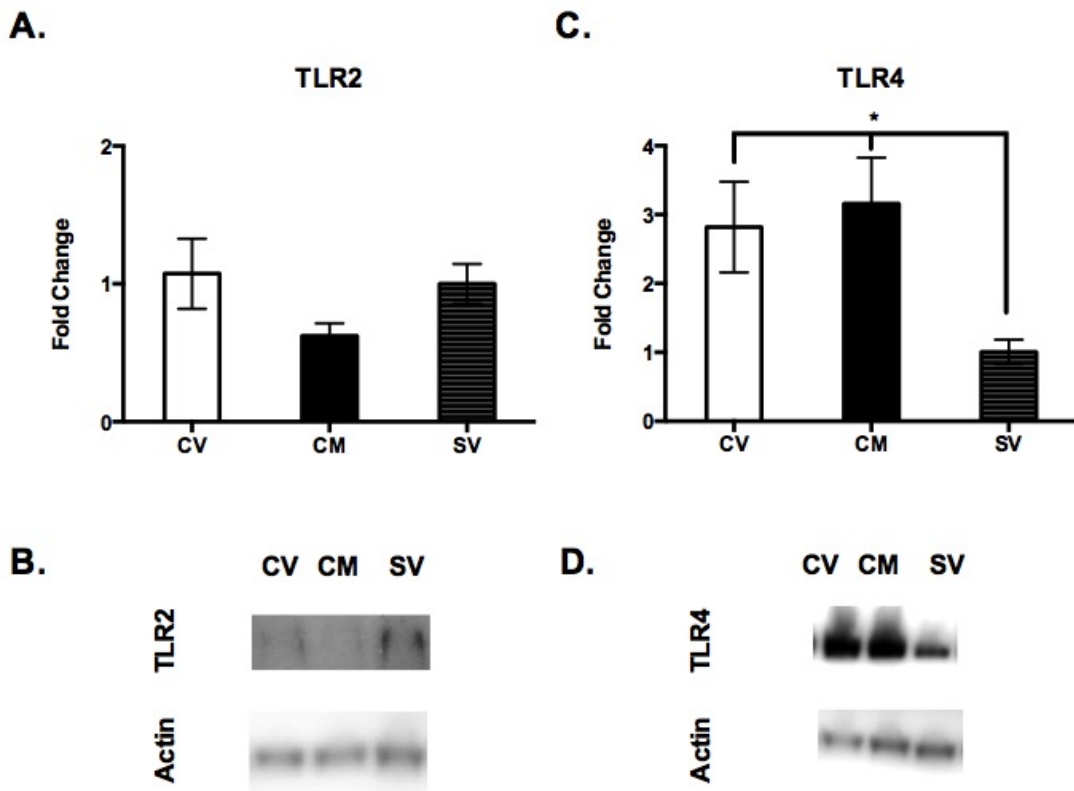
Figure 38: Spinal TLR2 and TLR4 protein levels following i.t. morphine administration. There were no changes in spinal TLR 2 protein expression (A & B). Spinal levels of TLR4 were increased by a contusion injury (C & D). * $p < .05$, CV = contused vehicle, CM = contused morphine, SV = sham vehicle.

A.
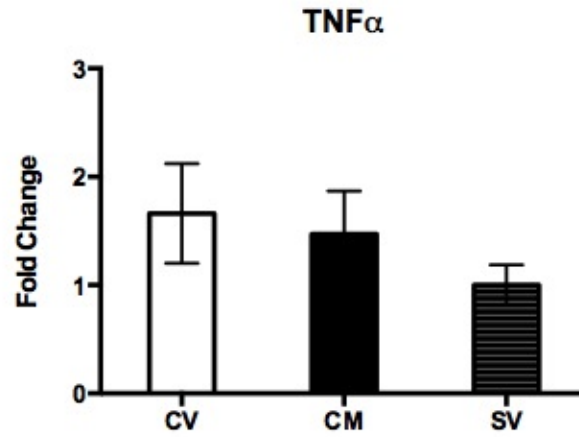
B.
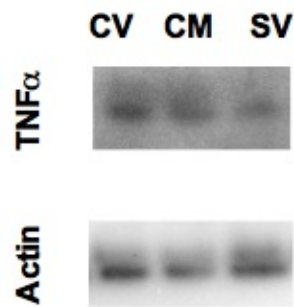
Figure 39: Protein expression of TNFα in the spinal cord. There were no changes in spinal levels of TNFα following a contusion injury or morphine administration. CV = contused vehicle, CM = contused morphine, SV = sham vehicle

A.
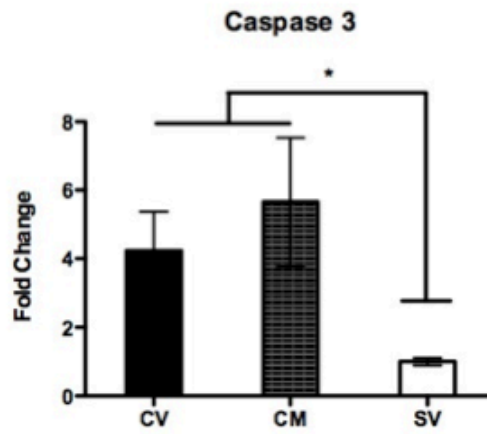
Caspase 3

B.

Figure 40: Protein levels of caspase-3 in the spinal cord. There was a significant increase in spinal levels of caspase-3 following a contusion injury. CV = contused vehicle, CM = contused morphine, SV = sham vehicle. *$p < .05$

antagonist not only blocked the morphine-induced attenuation of locomotor recovery, but also prevented increases in at-level neuropathic pain symptoms that were potentiated by morphine administration. Future investigation of the temporal expression of pro-inflammatory cytokines is warranted with respect to tolerance, as well as hyperalgesia. Moreover while the data presented here do not explain the molecular mechanisms underlying tolerance/hyperalgesia, it does provide insight into the reduced analgesic efficacy of morphine that was noted in these earlier studies (Hook et al. 2007; Hook et al., 2009). For example, following acute SCI 30 μg of intrathecal morphine, a dose providing robust analgesia in sham-operated rats, is ineffective in providing complete analgesia (Hook et al., 2009). This could result from a decrease in spinal MOR. Indeed, we found that a contusion injury significantly decreased MOR protein 48-hours following SCI. There were no changes 8-days following SCI. Similarly, Lee et al. (2011) examined MOR expression following spinal nerve injury and found injury down-regulated MOR mRNA and protein in the DRG, and decreased protein expression in the spinal cord. Interestingly, they also found decreased MOR protein at L4 (an uninjured area) 7 days after injury, but not 14-days following injury. Decreases in receptor number can contribute to reduced analgesic efficacy. Together, these results suggest a spatial localization of MOR protein expression following injury, but a highly variable temporal expression. This result demonstrates the need to examine MOR protein at multiple time points following injury to better understand analgesic efficacy.

Opioid-induced hyperalgesia is a phenomenon in which continuous administration of morphine leads, paradoxically, to the development of increased pain

sensitivity. We saw behavioral signs of OIH developing in our animals, but the mechanisms underlying the development of OIH are less clear. Mechanisms underlying OIH are thought to be similar to those underlying the development of neuropathic pain, with glial cells playing a critical role. As it has been implicated in the development of pain, excitotoxicity occurring via activation of the KOR system is thought to be involved in the development of OIH. In the current experiments, we failed to detect a significant change in KOR protein following SCI or morphine administration. This is not entirely unexpected as many reports suggest increased levels of dynorphin lead to increased activation of the KOR, but not necessarily increases in KOR expression (Faden, 1990; Faden et al., 1985; Abraham et al., 2001). Thus, the levels of KOR protein expression may remain stable, while activation in this system is increased. This needs to be further explored.

In addition to tolerance and hyperalgesia, we also found that chronic morphine exposure, as found for i.t. morphine (Hook et al., 2009; 2011), increased lesion size at the injury center. Morphine administration did not have effects rostral or caudal to the injury center, but the increased cell loss at the lesion center resulted, behaviorally, in decreased recovery of locomotor function. Morphine treatment can initiate loss of cells through binding at classic and non-classic receptors. Accordingly, neurons express TLR2, and, upon activation by ligands such as morphine, initiate pro-apoptotic signaling (Yao et al., 2012; Li et al., 2009). In the current studies, however, we failed to find significant changes in TLR2 but did find significantly increased caspase-3 protein 48-hours following a contusion injury. While this is in contrast to studies of spinal cord

injury, which show increased levels of TLR2 for 14-days following SCI (Kigerl and

Popovich, 2007), the result corresponds to increased caspase-3 activity seen after injury

(Lu et al., 2013; Yao et al., 2012). Additionally, our results contradict those examining

changes in TLR2 expression following chronic morphine administration. For example,

Li et al. (2010) showed chronic morphine treatment increased both TLR2 mRNA and

protein in neuronal cultures following 4 days of morphine treatment. They also found 6

days of morphine treatment increased caspase-3 levels, but TLR2 deficient neurons were

not susceptible to this apoptosis (Li et al., 2010). Importantly, many of these studies on

TLR2 are conducted *in vitro* and the interaction of opioid administration with SCI has

not been investigated. As TLR2 receptor are predominantly located on neurons, it is

possible that increased neuronal apoptosis is contributing to the lower levels of TLR2

protein. This issue can be examined further with immunohistochemistry.

As with TLR2, TLR4 binds morphine in a non-classic fashion. While TLR2 is

predominantly located on neurons, TLR4 is found on microglia and activated astrocytes

(see Hutchinson et al., 2011). Therefore, morphine binding to TLR4 would not cause

the direct apoptosis of neurons, but, via signaling cascades, could contribute to cell loss.

As mentioned previously, TLR4 signals through one of two adaptor proteins: MyD88 or

Toll/IL-1 receptor domain-containing adapter-inducing interferon-β (TRIF; see

Hutchinson et al., 2011). Through the MyD88 adaptor protein, TLR4 signaling activates

NF-κB and results in the production of pro-inflammatory cytokines (e.g. IL-1β and

TNFα), and can lead to the activation of caspase-3. Indeed, these pathways are activated

following SCI (Yao et al., 2012), and a spinal cord injury significantly increases TLR4

mRNA from 3-7 days (Kigerl & Popovich, 2007). Consistent with this data, 8 days following SCI, we did not see changes in TLR4 protein. However, there was a significant increase in TLR4 protein 48-hours following a contusion injury, suggesting TLR4 activation may contribute to cell loss, via increased release of pro-inflammatory cytokines in the early acute phase of SCI. This effect does not appear to be mediated through TNFα as we failed to find significant changes in protein 48-hours or 8-days following contusion injury, but may be mediated through other pro-inflammatory cytokines, such as IL-1B (Hook et al., 2011).

Together, these studies demonstrate the need to further investigate the effects of morphine following SCI. At the times examined, we found very few significant changes. However, the literature suggests that expression of various proteins fluctuates with time post injury and morphine administration. In addition, the dose of morphine administered seems to play a key role in initiating pro-inflammatory pathways. We chose our experimental treatment (7 days of administration of high quantities of morphine) because high amounts of intravenous morphine had been shown to produce detrimental effects, while lower doses did not. The data shown here, replicated these behavioral results, but failed to find significant changes in protein expression. We hypothesize that morphine may be having the greatest impact in a more acute phase of SCI (Days 1-2), and that changes in protein expression may be more apparent at time points closer to drug administration (i.e. 3 hours after administration). Future studies should examine earlier time points following SCI and morphine administration, and use multiple methods (e.g. immunohistochemistry, PCR) to assess changes.

CHAPTER VI[2]

GENERAL DISCUSSION AND CONCLUSIONS

The experiments in this dissertation examined the abuse liability of morphine in the acute (Chapter III) and chronic (Chapter IV) phases of SCI. We found a reduced addictive potential of morphine at a moderate dose in the acute, but not the chronic phase of injury. In addition, we examined the impact of morphine administration on recovery of locomotor function and the development of pain (Chapters III and IV). We found morphine administration significantly undermined recovery of locomotor function in the acute, but not the chronic phase of injury. After the cessation of morphine administration, we did not find an affect of administration on long-term pain reactivity. These results indicate that molecular processes occurring in the acute phase of injury as a result of the contusion injury seem to be "protecting" the animal from addiction, but contributing to decreased recovery of locomotor function. Finally, we examined the receptor systems that may underlie the detrimental effects of morphine on recovery of locomotor function (Chapter V). In the introduction, it was hypothesized that opioid administration following SCI could potentiate excitotoxic cell death (via increased dynorphin levels and glutamate dysregulation), and increase glial activation resulting in increased release of pro-inflammatory cytokines. Indeed, increased activation of the KOR and increased levels of pro-inflammatory cytokines have been associated with reduced analgesic efficacy of morphine and locomotor deficits. In our study, however,

we failed to find significant effects of SCI or morphine administration on spinal KOR protein or TNFα. A contusion injury significantly increased caspase-3 protein 48-hours, but not 8-days after SCI, and morphine administration had no additional effect. These experimental results will be discussed below in regards to addiction, recovery of locomotor function, and the development of pain.

Consequences of Opioid Administration After SCI: Addiction

In these experiments, we examined the abuse liability of morphine in the acute and chronic phases of SCI. In the acute phase of injury, we found dose-dependent effects on morphine self-administration. Animals given access to 1.5 mg morphine self-administered significantly less morphine than sham controls. In contrast, animals given access to a higher dose, 3.0 mg / infusion, increase intake over the first 3 sessions to administer nearly the full amount of morphine available on days 4-7, a behavior indicative of addiction. In the early chronic and chronic phases of injury, however, this difference did not persist. Contused animals given access to 1.5 mg morphine administered nearly the full amount available each session in the early chronic phase of SCI. In the chronic phase, a similar pattern emerged. Contused animals self-administered nearly the full amount of morphine available each session, suggesting that morphine reward is altered in the acute, but not the chronic, phase of SCI.

Acute Pain and Morphine Reward

Previously, we hypothesized that increases in spinal dynorphin levels, resulting from morphine administration and SCI, activate spinal KORs to oppose morphine-induced analgesia, and decrease reward (decrease morphine self-administration).

132

Indeed, as was discussed in Chapter IV, the difference in self-administration between the acute and chronic phases of SCI may be due to decreased analgesic efficacy of morphine, making the 1.5 mg dose less rewarding in the acute phase than the chronic. It would, therefore, not maintain morphine self-administration. In our analyses of protein expression of the kappa opioid receptor, however, we failed to see significant differences at a spinal level. This alone does not, however, rule out a role for a kappa-mediated reduction in the potential for addiction after SCI. While we did not see spinal changes in the KOR, increased dynorphin activation of the KOR does not necessarily lead to an alteration in the number of KORs (Faden, 1990; Faden et al., 1985, Abraham et al., 2001). In addition, while spinal changes in KOR activation could decrease reward from an analgesic standpoint, supraspinal sites are involved in addictive processes, and changes in these systems were not examined.

Indeed, in the current studies, we administered i.v. morphine, which will bind at spinal and supraspinal sites. Exposure to drugs of abuse, such as morphine, increases striatal dynorphin levels and KORs (see Butelman et al., 2012 and Niikura et al., 2010). As with spinal activation, increased activation of the KOR is also thought to oppose reward at a supraspinal level (see Niikura et al., 2010). For example, genetic differences in brain dynorphin levels have been shown to make various strains of mice less responsive to the rewarding effects of morphine (Gieryk et al., 2010). Mouse strains with high endogenous levels of dynorphin will not develop a preference for a morphine-paired context at a low dose of morphine. When the KOR is antagonized with nor-BNI, the same dose of morphine will produce a conditioned place preference (Gieryk et al.,

2010). Furthermore, it is known that dynorphin binds to KOR in the nucleus accumbens (NAcc) to decrease dopamine (DA) levels, supporting the idea that activation of the KOR antagonizes reward, even at supraspinal sites (Di Chiara & Imperato, 1988; Skoubis et al., 2001; Spanagel et al., 1990). This antagonistic effect, however, varies with the cycle of addiction.

There are differential effects of MOR receptor activation with and without concurrent KOR activation. When MOR's are activated along with KOR's (as in analgesic use following SCI), the activation of the KOR is though to "protect" from addiction, by opposing MOR-mediated reward. However, in the absence of MOR activation, KOR agonists are known to produce dysphoria (Aldrich, 2003) and are aversive to rodents (Shippenberg et al., 2001). In the withdrawal / abstinence stage of addiction (when there is no MOR activation), the dysphoria associated with increased KOR activation is thought to underlie drug seeking. In addition, conditions of stress can contribute to increases in dynorphin levels, and, therefore, KOR activation. Stress-induced anxiety and increases in dynorphin levels increase the risk of drug abuse in people (de Kloet et al., 2005) and reinstate drug seeking in animals (Shaham et al., 2000). These data indicate that, rather than protecting from addiction, increased levels of dynorphin can contribute to drug relapse. Following SCI, the stress related to sustaining the injury (i.e. the numerous consequences associated with injury affecting quality of life) could potentially lead to increased risk of drug abuse, in patients with a prior history of drug use.

Indeed, in our rodent model of SCI, we have studied the development of addictive behavior under ideal conditions. Our animals have no prior experience with drugs of abuse. How prior exposure to drugs of abuse affects addiction in the SCI model is an important question. In the general population, opioid abuse and addiction have become quite prevalent, especially among people aged 12-25 (NIDA, 2011). According to statistics published by the Christopher Reeve Foundation (2009), the majority of people will sustain a spinal cord injury between 25-40 years of age, making it likely that these individuals will have prior history with drugs of abuse. Indeed, Heinemann et al. (1988) found that up to 62% of SCI patients had misused drugs or alcohol at the time of their injury. This history with drugs of abuse makes finding adequate therapies to treat pain difficult, and makes these individuals susceptible to the development of addiction when using opioids for the treatment of pain following SCI. These are issues that need to be further addressed.

In addition to the role of the KORs in analgesia and addiction, MORs also play a role. Traditionally, agonists of MORs, are associated with the analgesic properties of morphine. In the last experiment, however, we found that a contusion injury significantly decreased spinal MOR protein levels. Thus, decreased analgesic efficacy of morphine in the acute phase of injury could result from a decreased number of MORs. Indeed, it has been shown contused rats require a higher dose of intrathecal morphine to produce antinociception (Hook et al., 2009). Whether similar changes occur at a supraspinal level as a result of injury have yet to be investigated, but are quite possible and would have implications for pain control and addiction.

Neuropathic Pain and Morphine Reward

Despite the highly addictive nature of opioids, there is evidence to suggest that a state of neuropathic pain will decrease the risk of addiction to opioids used for treatment (e.g. Minozzi et al., 2013; Niikura et al., 2008; Martin et al., 2007; Ewan & Martin, 2011; Niikura et al., 2010). Indeed, when separating animals by pain state, contused animals experiencing neuropathic pain trended toward a decrease in morphine self-administration relative to their pain-free counterparts. While this result was not significant, there was an unequal sample size, leaving only 2 rats in the pain-free condition. There is a need for additional work examining the effects of pain on morphine self-administration because it has been shown that pain alters supraspinal reward circuitry. For example, Ozaki et al. (2003) have shown that morphine-induced release of dopamine (DA) in the nucleus accumbens (NAcc) is reduced following sciatic nerve ligation (SNL), indicating a reduction in reward potential in animals experiencing neuropathic pain. In addition, chronic morphine administration has been shown to increase ERK activation in the ventral tegmental area (VTA), where it increases activation of tyrosine hydroxylase (TH; Berhow et al., 1996). Tyrosine hydroxylase is necessary for the synthesis of dopamine. Thus, ERK activation, occurring as a result of chronic morphine administration, in the VTA stimulates DA production. However, following SNL, levels of p-ERK following morphine administration are significantly decreased (Ozaki et al., 2004), leading to a decrease in opioid reward due to decreased dopamine availability. Whether similar changes occur as a result of SCI-induced

neuropathic pain, and with morphine administration have yet to be investigated, but, is important from a clinical standpoint.

Tolerance

Morphine reward can also be affected by the development of tolerance. In the final experiment (Chapter V), we examined the development of tolerance to morphine following the repeated administration of high doses. We found evidence of tolerance developing on days 5-7 of administration. In previous experiments (Chapter III), we were unable to say whether morphine administration was increasing in the acute phase of injury due to the development of addiction, or tolerance. In the 3.0 mg / infusion animals, we saw an escalation in the amount of morphine self-administered beginning on day 3, and these animals reached the same level of administration as sham controls by day 4. With experimenter-controlled administration of morphine, however, signs of tolerance did not begin to develop until the 5th day of administration, suggesting animals are increasing their intake for reasons other than the development of tolerance. However, the development of tolerance could maintain the high rates of administration seen on days 5-7. This is concerning because the clinical SCI population is faced with long-term, high-dose opioid treatment. The development of tolerance in these individuals will result in increased opioid intake, further increasing their risk of experiencing significant side effects such as respiratory suppression, sedation, and attenuated recovery of locomotor function when treatment is initiated in the acute phase of SCI.

Together, these studies suggest morphine administration and neuropathic pain can cause alterations at a spinal and supraspinal level to influence the rewarding properties of morphine. This can result from decreased analgesic efficacy, decreased release of dopamine, and the development of tolerance. These issues make the use of opioids for the treatment of chronic pain more likely to lead to drug-seeking behaviors. In addition to these concerns, however, opioid administration has also been shown to affect recovery of locomotor function in experimental models.

Consequences of Opioid Administration After SCI: Attenuation of Locomotor Recovery

Previous studies have shown morphine administration undermines recovery of locomotor function (Hook et al., 2009; 2011). In these studies, morphine was applied directly to the spinal cord, a route of administration that is not frequently used clinically. In the current studies, we used a clinically relevant, intravenous delivery of morphine. Intravenous infusions are similar to patient controlled analgesia used in hospital settings. Using this route of administration, we found that morphine administration significantly undermines recovery of locomotor function in the acute phase of SCI. This effect, however, was dependent on the amount of morphine being administered. Animals administering on average 50+ mg/kg/day showed significantly impaired recovery. Those administering less, recovered to the same level as saline controls. In the current studies, impaired recovery was associated with significantly increased lesion size. Surprisingly, however, in the chronic phase of injury all contused animals self-administered more than 50 mg/kg/day and did not show an attenuation of locomotor

recovery. This suggests the spinal cord is more vulnerable to the effects of morphine administration in the acute, rather than the chronic, phase.

As discussed previously, it was hypothesized that increased dynorphin levels, resulting from injury and morphine administration would increase activation of the KOR to increase excitotoxicity and paralysis. In intact rats, increases in spinal dynorphin are associated with locomotor deficits (paralysis) (Faden & Jacobs, 1984; Faden, 1990; Bakshi et al., 1992), and spinal cord injury is known to cause an increase in dynorphin levels (Faden et al., 1985; Przewlocki et al., 1988; Yakovlev & Faden, 1994), contributing to the secondary pathology of injury. We would expect to see the greatest synergistic effect of opioid administration and SCI in the acute phase of injury, when endogenous dynorphin levels are still increased as a result of sustaining the injury, leading to the pattern of results we observed. Indeed, a study by Adjan et al. (2007) found that dynorphin, following a T10 contusion injury in mice, is associated with the expression of caspase-3 in greater than 90% of all neurons, oligodendrocytes, and astrocytes. These differences were seen at 4 hours, but not 24-hours following SCI (Adjan et al., 2007). Thus opioid administration, which also increases dynorphin levels, is likely to have the greatest synergistic effect when applied soon (within the first 24 hours) after injury.

While we saw behavioral effects of morphine administration, we did not see changes in KOR or caspase-3 protein following 8 days of morphine administration. However, we were assessing changes in protein following 7 days of morphine administration, and the tissue was collected 8-days following injury. This is definitely

outside the window in which Adjan et al. (2007) reported significant increases in caspase-3. Similarly, we had a group of animals in which we assessed changes in protein levels 24-hours after morphine administration, and 48-hours after a contusion injury. In this group of animals, there was a significant increase in caspase-3 protein, especially in the contused animals treated with morphine. Together, these results suggest that morphine administration is having an immediate effect, and there is not an additional effect with increased morphine administration. In fact, it is possible that the morphine administered within the first 24-hours of injury is leading to these detrimental effects. It is imperative that this issue is examined and the effects of morphine administration following SCI are assessed at an earlier time following morphine administration.

While increased activation of the KOR may be producing an immediate effect to decrease recovery of locomotor function, glial activation, resulting in the release of pro-inflammatory cytokines (such as IL-1β) has also been shown to affect recovery of locomotor function (Hook et al., 2011). As with alterations in dynorphin levels, increased glial activation is also characteristic of the acute phase of SCI. In fact, following SCI, astrocytes in the thoracic spinal dorsal horn are most activated from 24-hours to 7-days post injury, and microglia are most activated at 24-hours (Gwak et al., 2012). Upon activation, glial (actrocytes and microglia) cells, rather than maintaining homeostasis, become dysfunctional and contribute to neuronal hyperexcitability (Gwak & Hulsebosch, 2009). Morphine administration can contribute to dysfunction through binding at TLR4. In fact, we found a significant increase in spinal TLR4 protein levels

following SCI, likely due to increases in glia in the injured spinal cord. Upon activation, glial cells release pro-inflammatory cytokines (IL-1β and TNFα) that contribute to inflammation and secondary damage (Pineau & Lacroix, 2007; Nesic et al., 2005; Donnelly & Popovich, 2008; Song et al., 2001; Choi et al., 2010). Increases in pro-inflammatory cytokine levels cause further activation of glial cells. In this cycle, exacerbating glial activation (and levels of pro-inflammatory cytokines) through the administration of morphine can produce detrimental effects. In these experiments, we did not find significant differences in the overall levels of TNFα following morphine administration or SCI, suggesting TNFα is not contributing to this cycle of glial activation and cell damage. However, SCI has been shown to increase the levels of IL-1β, and morphine administration further increases the levels of IL-1β (Hook et al., 2011). It is thought that the increased IL-1β is contributing to the deficits in locomotor function associated with morphine administration. This is supported by data showing antagonizing the IL-1 receptor prior to morphine administration blocks the attenuation of locomotor function (Hook et al., 2011).

Changes in glial activation and the release of pro-inflammatory cytokines are not characteristic of the chronic phase of SCI. While glial activation continues for up to 180 days following SCI (Gwak et al., 2012), and increases in pro-inflammatory cytokines are seen up to 28 days, the levels are not as high as in the acute phase of SCI. In addition, it is unlikely that any one of the processes affecting the acute phase of SCI is causing deficits in locomotor function alone. Rather, it is likely a combination of all factors that push the system to hyperexcitability, and dysfunction. Thus, morphine administration,

141

in the chronic phase of SCI, does not contribute as significantly to dysfunction as it does in the acute phase. This indicates that, aside from concerns of addiction, morphine use for the treatment of pain in this phase of injury will not affect recovery of locomotor function.

Consequences of Opioid Administration After SCI: Pain

Pain is a common consequence of SCI, and is often treated with opioid analgesics. Unfortunately, long-term treatment with opioids can, paradoxically, lead to the development of pain. In these experiments, we assessed the development of opioid-induced hyperalgesia, and the effects of opioid administration on pain reactivity long after cessation of administration. Following repeated administration of morphine, we saw the development of OIH, but there was no lasting effect of morphine administration in the chronic phase of injury. Contused animals show increased reactivity at the end of the 42-day period relative to sham animals, but there was not a difference between contused animals treated with morphine versus those treated with saline. This was true of morphine administration in the acute and chronic phases of SCI. These results suggest spinal cord injury is not affecting the development of OIH, as both contused and sham animals showed signs of increased reactivity following repeated morphine administration, and that morphine administration is not leading to long-lasting changes in pain reactivity after the cessation of use.

The development of OIH, limits the clinical utility of morphine (Tawfic et al., 2013). In addition, opioid-induced hyperalgesia and the development of tolerance are difficult to distinguish in a patient population, and treatment of each differs. While

tolerance requires escalating doses of morphine to relieve reports of increased pain, OIH requires a reduction (Mao et al., 2002). Animal models are needed to identify molecular biomarkers, and the causal mechanisms, associated with these two distinct phenomena. Research examining the development of tolerance and OIH has often focused on shared mechanisms, specifically spinal dynorphin, increased glutamate availability (leading to NMDA-mediated excitation), and, more recently, glial activation (Mao et al., 2002; Vanderah et al., 2001; Ferrini et al., 2013). This recent research indicates that the tolerance and OIH result from separate mechanisms. For example, Ferrini et al. (2013) have demonstrated a role of spinal microglia in the development of OIH, but not tolerance. They showed morphine administration activated microglia and stimulated the release of brain-derived neurotrophic factor (BDNF) (Ferrini et al., 2013). This led to impaired Cl^- homeostasis in the spinal dorsal horn, resulting in hyperalgesia (Ferrini et al., 2013). This pathway has also been implicated in the development of hyperalgesia independent of opioid administration (Trang et al., 2011). Further understanding of these phenomena is important for the continued use of morphine in the treatment of chronic pain.

General Health Concerns

Although spinal cord injury directly damages tissue at the level of the spinal cord only, its effects are seen throughout the CNS and periphery. While this dissertation focused on the neuronal and glial effects of opioid administration following SCI, it should be noted that SCI also induces a number of changes that can decrease the overall health of the individual. If fact, a leading cause of death in both the acute and chronic

phases of SCI is cardiovascular disease (Furlan & Fehlings, 2008; Popa et al., 2010). In addition, individuals can experience changes in blood pressure, heart rate, blood flow, hypotension, bradycardia, deep vein thrombosis, and are at long-term risk for coronary heart disease and athrosclerosis (see Furlan & Fehlings, 2008; Popa et al., 2010; Grigorean et al., 2009 for review). Several of these metrics, specifically, blood pressure, heart rate, and body temperature have been shown to be altered by morphine administration in an experimental setting (Froger-Colléaux et al., 2011). These changes can significantly impact the health of the individual. Additionally, patients with a cervical or high thoracic (above T6) injury will frequently experience autonomic dysreflexia, which is characterized by an increase in blood pressure in response to stimulation (noxious or not) below the level of injury, often bowel or bladder distension (Rabchevsky, 2006; Weaver et al., 2002; Weaver et al., 2006). This sudden increase in blood pressure, while often within normal limits for healthy individuals, can be life-threatening (Furlan & Fehlings, 2008) in individuals with SCI. This is also problematic in individuals not experiencing autonomic dysreflexia.

Similarly, in a population susceptible to bladder infections, pressure ulcers, pneumonia, gastrointestinal infections, and frequent hospitalizations, immune suppression is dangerous. If fact, infections are the leading cause of death in individuals with cervical or high thoracic injuries (DeVivo et al., 1989). In the SCI population, immune (non-microglial / astrocyte responses) suppression results from dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis. Morphine may further reduce the systemic immune response by decreasing lymphocyte levels (Weed et al., 2006).

144

Clinically, the effects of morphine on immunosuppression are also quite clear (Eisenstein et al., 2006; Mellon & Bayer, 1998; Odunayo et al. 2010) as an opioid-induced immune suppression is thought to play a role in the increased incidence of bacterial infections in heroin addicts (Zhang et al., 2008). Experimentally, increased bacterial infection is also evident in the liver, spleen, kidneys, heart, and lungs of mice receiving morphine (Zhang et al., 2008). Imposing opioid-induced immune suppression onto a population vulnerable to infections should be considered before beginning treatment with an opioid.

These are not the only concerns when using morphine for pain relief. In our experiments, morphine treatment led to weight loss in contused and sham animals (Chapters III – V). Administration of a high amount of morphine was associated with the greatest loss. Typically, chronic morphine administration is associated with weight loss occurring as a result of decreased food intake (Nogueiras et al., 2012). In these animals, however, we did not see a difference in food intake during the period of self-administration, when the most robust weight loss occurred. It is possible that we did not get an accurate measure of food intake because all animals were on a disrupted schedule. During the self-administration sessions, animals were removed from their home cages for 12-hours, during their active cycle. Thus, all animals could have decreased their food intake, even those administering saline, resulting in the lack of differences between groups. Indeed, even saline animals show weight loss during the 7-days associated with self-administration sessions (Figure 20). Altered food intake, however, is not the sole cause for weight loss in these animals. Hook et al. (2009, 2011) have shown a single

145

intrathecal administration of morphine also induced lasting weight loss. Though it has not been examined in the experiments mentioned, this treatment should not affect food intake. On the contrary, acute treatment with opioids has been shown to increase food intake (Levine et al., 1988), leaving other factors responsible for this weight loss.

Alternatively, weight loss can occur as a part of withdrawal syndrome (Gue et al., 2005), which may contribute to the weight loss we observed in the early chronic phase of injury. Indeed, the high amounts of morphine led to signs of withdrawal (e.g. wet dog shakes, teeth chewing) throughout the period of administration and into the recovery period. However, as mentioned above, a single intrathecal administration of morphine also results in a lasting weight loss, and this treatment does not lead to withdrawal. Thus, it is unlikely that withdrawal is the sole contributor to weight loss.

Weight loss associated with morphine administration is commonly seen, but the typical explanations for this loss do not hold in our studies. We propose, instead, morphine administration is affecting metabolic and physiological variables and increasing weight loss. For instance, chronic morphine treatment results in decreased caloric efficiency (Ferenczi et al., 2010), increased body temperature, increased heart rate, increased arterial blood pressure (Froger-Colléaux et al., 2011), and alterations in blood brain barrier permeability (Sharma & Ali, 2006; Lynch & Banks, 2008). How these factors affect weight following morphine administration in the SCI model need to be investigated.

146

Clinical Implications

Opioids have long been a standard in the treatment of pain following SCI. In the chapters above, however, we have shown that opioid administration can interact with SCI pathology to negatively impact locomotor recovery. In the absence of descending inhibition, the spinal cord may be particularly vulnerable to increased levels of glutamate, resulting in NMDA-mediated excitotoxicity initiated by classic and non-classic opioid receptor activation, and resulting in detrimental effects. Nonetheless, for patients faced with a lifetime of intractable pain, opioids as effective analgesics cannot simply be removed as a treatment. For this reason, continued research is needed to develop guidelines for the safe use of these agents following SCI.

Indeed dose-dependent effects on recovery of locomotor function have been seen with morphine administration via either an intrathecal or intravenous route. Hook et al., (2009) found that a single 90 μg, but not 30 μg, intrathecal administration of morphine attenuates recovery of locomotor function. Similarly, we have shown that administration of high amounts of intravenous morphine, a more clinically relevant route of administration, also results in the attenuation of locomotor function and decreased weight, while the administration of lower amounts only results in weight loss. Considering there is no maximum dosage for opioids, and patients are often started on 10-15 mg morphine every 4 hours, often up to 300+ mg a day (Magrinelli et al., 2013) for the treatment of pain, this result is concerning. Together, this data suggests that high doses of morphine administered either intrathecally or intravenously in the acute phase can have adverse consequences on recovery of locomotor function following

experimental SCI. Doses given clinically in this phase of injury should be monitored carefully.

Equally important is the time at which opioid administration begins. The acutely injured spinal cord environment differs from that of the chronic phase. As discussed, opioid administration in the acute phase can exacerbate the pathology (e.g. increase excitotoxicity, increase glial activation) of SCI. In the chronic phase of injury, however, many of the inflammatory and endogenous opioid responses have subsided. Indeed, administration of opioids in the chronic phase of SCI does not lead to an attenuation of recovery of locomotor function. While this data suggests opioid administration may not be detrimental to recovery of locomotor function, opioid administration led to addictive behavior in these experiments. Because of this risk, opioids have been downgraded to a second-line medication for the treatment of pain (Attal et al., 2010). It has also been suggested that opioids are not an effective-long term treatment in SCI patients, and that morphine should be removed as a treatment option (Smith & Meek, 2011).

Apart from monitoring dose, route, and phase of injury in which morphine is administered, research is needed to examine potential drug combinations that can improve the analgesic efficacy of morphine while preventing the negative (e.g. decreased locomotor function, development of tolerance) consequences. Studies have shown that administration of an NMDA antagonist is an effective treatment for attenuation of both neuropathic pain and recovery of locomotor function. This treatment, however, is associated with unwanted side effects such as hallucination, dysphoria, and locomotor deficits, making it unsuitable for clinical use. In addition,

while an NMDA antagonist has been successful in promoting recovery of function in animal models (Lonjon et al., 2010), the success in clinical trials is yet to be determined (Kwon et al., 2010). Thus, it is important to look at alternative therapies. For instance, Kim et al. (2012) found that treatment with an NR2B subunit specific antagonist produces fewer unwanted side effects and reverses mechanical allodynia when administered two weeks following a contusion injury. In addition, a number of studies have shown that treatment with an antagonist for the NR2B subunit blocks morphine-induced reward (Ma et al., 2006a, Ma et al., 2011; Narita et al., 2000). For instance, Ma et al. (2006a) showed that treatment with Ifenprodil, an antagonist specific for the NR2B subunit of the NMDA receptor, decreased a morphine-induced conditioned place preference. The same treatment did not decrease a place preference for natural reinforcers (e.g. food consumption or social interaction). Thus, coadministration of an NR2B subunit antagonist with morphine could be a potential treatment for pain following SCI and could lower the abuse potential of morphine.

In addition, activation of TLR4 is responsible for many of the negative effects of morphine administration. It has been demonstrated that blockade of TLR4 increases opioid efficacy (Hutchinson et al., 2007; Johnston et al., 2004), and blocks other negative effects of opioids such as respiratory depression (Hutchinson et al., 2008a; 2008b; 2008c). Yet, as discussed previously, while blocking TLR4 can decrease pain and increase efficacy, it can contribute negatively to recovery of locomotor function after SCI. Potentially, if the blockade of TLR4 activity was via selective inhibition of microglia (e.g. blockade with minocycline), it may prove an effective treatment strategy.

The timing of administration of a TLR4 antagonist may also be critical: administration of an antagonist may be beneficial in conjunction with morphine in the chronic, but not acute phase of injury. Whether antagonizing TLR4 with (+)-naloxone can be used repeatedly with morphine for the treatment of pain (and prevention of addiction) has yet to be determined. As combination drug therapies are being used for the treatment of chronic pain with some success (for review, see Mao et al., 2011), these possibilities need to be explored.

Given the numerous negative consequences associated with opioid administration in the spinally injured population, clinical opioid use must be further evaluated and refined. However, the use of opioids is not only relevant to SCI pain. Central pain resulting from stroke, or TBI, falls under the same treatment guidelines as those for pain resulting from SCI. While the pathology differs from that of SCI, there may be similar concerns related to opioid use in other central models of pain or disease that need to be considered before initiation of an opioid treatment regimen.

REFERENCES

Abraham, K.E., McGinty, J.F., & Brewer, K.L. (2001). Spinal and supraspinal changes in opioid mRNA expression are related to the onset of pain behaviors following excitotoxic spinal cord injury. Pain, 90: 181-190.

Adjan, V.V., Hauser, K.F., Bakalikin, G., Yakovleva, T., Gharibyan, A., Scheff, S.W., & Knapp, P.E. (2007). Caspase-3 activity is reduced after spinal cord injury in mice lacking dynorphin: differential effects on glia and neurons. Neuroscience, 724-736.

Ahmed, S.H. & Koob, G.F. (1998). Transition from moderate to excessive drug intake: change in hedonic set point. Science, 282: 298.

Al-Owesie, R.M., Moussa, N.M., & Robert, A.A. (2012). Anxiety and depression among traumatic spinal cord injured patients. Neurosciences (Riyadh), 17 (2): 145-150.

Alexander, J.K. & Popovich, P.G. (2009). Neuroinflammation in spinal cord injury: therapeutic targets for neuroprotection and regeneration. Prog Brain Res, 175: 125-137.

Almad, A., Lash, A.T., Wei, P., Lovett-Racke, A.E., & McTigue, D.M. (2011a). The PPAR alpha agonist gemfibrozil is an ineffective treatment for spinal cord injured mice. Exp Neurol, 232 (2): 309-317.

Almad, A., Sahinkaya, F.R., McTigue, D.M. (2011b). Oligodendrocyte fate after spinal cord injury. Neurotherapeutics, 8 (2): 262-273.

Aloisi, F. (2001). Immune function of microglia. Glia, 36 (2), 165-179.

Anderson, K.D. (2004). Targeting recovery: priorities of the spinal cord-injured

  population. J Neurotrauma, 21 (10): 1371-1383.

Ankeny, D.P. & Popovich, P.G. (2009). Mechanisms and implications of adaptive

  immune responses after traumatic spinal cord injury. Neuroscience 158, 1112-

  1123.

Ankeny, D.P., Lucin, K.M., Sanders, V.M., McGaugy, V.M., & Popovich, P.G. (2006).

  Spinal cord injury triggers systemic autoimmunity: evidence for chronic B

  lymphocyte activation and lupus-like autoantibody synthesis. J Neurochem, 99:

  1073-1087.

Attal, M., Cruccu, G., Baron, R., Hanpää, M., Hansson, P., Jensen, T.S., Nurmikko, T.,

  Eruopean Federation of Neurological Societies (2010). EFNS guidelines on the

  pharmacological treatment of neuropathic pain: 2010 revision. Eur J Neurol, 17

  (9): 1113-e88.

Attal, N., Mazaltarine, G., Perrouin-Verbe, B., & Albert, T. (2009). Chronic

  neuropathic pain management in spinal cord injury patients. What is the

  efficacy of pharmacological treatments with a general mode of administration?

  (oral, transdermal, intravenous). Ann Phys Rehab Med, 52: 124-141.

Avellino, A.M., Hart, D., Dailey, A.T., MacKinnon, M., Ellegala, D., & Kliot, M.

  (1995). Differential macrophage responses in the peripheral and central nervous

  system during wallerian degeneration of axons. Exp Neurol, 136 (2): 183-198.

Bakshi, R., Ni, R.X., Faden, A.I. (1992). N-methyl-D-aspartate (NMDA) and opioid

  receptors mediate dynorphin-induced spinal cord injury: behavioral and

histological studies. Brain Res, 580 (1-2): 255-264.

Ballantyne, J.C. & LaForge, K.S. (2007). Opioid dependence and addiction during opioid treatment of chronic pain. Pain, 129: 235-255.

Ballantyne, J.C. & Mao, J. (2003). Opioid therapy for chronic pain. N Engl J Med, 349: 1943-1953.

Bao, F. & Liu, D. (2003). Peroxynitirte generated in the rat spinal cord induces apoptoic cell death and activates caspase-3. Neuroscience, 116: 59-70.

Basso, D.M., Beattie, M.S., & Bresnahan, J.C. (1995). A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma, 12 (1): 1-21.

Bell, K. & Salmon, A. (2009). Pain, physical dependence, and pseudoaddiction: redefining addiction for "nice" people? Intern J Drug Policy, 20: 170-178.

Bennett, A.D., Chastain, K.M., & Hulsebosch, C.E. (2000a). Alleviation of mechanical and thermal allodynia by CGRP(8-37) in a rodent model of chronic central pain. Pain, 86: 163-175.

Bennett, A.D., Everhart, A.W., & Hulsebosch, C.E. (2000b). Intrathecal administration of an NMDA or a non-NMDA receptor antagonist reduces mechanical but not thermal allodynia in a rodent model of chronic central pain after spinal cord injury. Brain Res, 859: 72-82.

Berhow, M.T., Hiroi, N., Nestler, E.J. (1996). Regulation of ERK (extracellular signal regulated kinase), part of the neurotrophin signal transduction cascade, in the rat mesolimbic dopamine system by chronic exposure to morphine or cocaine. J Neurosci, 16 (15): 4707-4715.

153

Bettoni, I., Comelli, F., Rossini, C., Granucci, F., Giagnoni, G., Peri, F., & Costa, B. (2008). Glial TLR4 receptor as a new target to treat neuropathic pain: efficacy of a new receptor antagonist in a model of peripheral nerve injury in mice. Glia, 56: 1312-1319.

Blanco, C., Iza, M., Schwartz, R.P., Rafful, C., Wang, S., & Olfson, M. (2013). Probability and predictors of treatment-seeking for prescription opioid use disorders: a national study. Drug Alcohol Depend, [Epub ahead of print].

Block, M.L. & Hong, J.S. (2005). Miroglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. Prog Neurobiol, 76 (2): 77-98.

Bombardier, C.H., Fann, J.R., Tate, D.G., Richards, J.S., Wilson, C.S., Warren, A.M., Temkin, N.R., Heinemann, A.W., PRISMS Investigators (2012). An exploration of modifiable risk factors for depression after spinal cord injury: which factors should we target? Arch Phys Med Rehabil, 93 (5): 775-781.

Bonanno, G.A., Kennedy, P., Galatzer-Levy, I.R., Lude, P., & Elfström, M.L. (2012). Trajectories of resilience, depression, and anxiety following spinal cord injury. Rehabil Psychol, 57 (3): 236-247.

Bruchas, M.R. et al., (2010). The dynorphin/kappa opioid systems as a modulator of stress-induced and pro-addictive behaviors. Brain Res 1314, 44-55.

Butelman, E.R., Yuferov, V., & Kreek, M.J. (2012). K-opioid receptor/dynorphin system: genetic and pharmacotherapeutic implications for addiction. Trends in Neuroscience, 35 (10): 587-596.

154

Cairns, D.M., Adkins, R.H., & Scott, M.D. (1996). Pain and depression in acute traumatic spinal cord injury: origins of chronic problematic pain? Arch Phys Med Rehabil, 77 (4): 329-335.

Cao, F., Gao, F., Xu, A.J., Chen, Z.J., Chen, S.S., Yang, H., Yu, H.H., Mei, W., Liu, X.J., Xiao, X.P., Yang, S.B., Tian, X.B., Wang, X.R., & Tian, Y.K. (2010). Regulation of spinal neuroimmune responses by prolonged morphine treatment in a rat model of cancer induced bone pain. Brain Res, 1326: 162-173.

Cardenas, D.D., Bryce, T.N., Shem, K., Richards, J.S., Elhefni, H., (2004). Gender and minority differences in the pain experience of people with spinal cord injury. Arch Phys Med Rehabil, 85: 1774-1781.

Carlson, S.L., Parrish, M.E., Springer, J.E., Doty, K., & Dossett, L. (1998). Acute inflammatory response in spinal cord following impact injury. Exp Neurol, 151: 77-88.

Caudle, R.M. & Isaac, L. (1987). Intrathecal dynorphin (1-13) results in an irreversible loss of the tail-flick reflex in rats, Brain Res., 435: 1-6.

Caudle, R.M. & Isaac, L. (1988). A novel interaction between dynorphin (1-13) and an N-methyl-D-aspartate site. Brain Res, 443 (1-2): 329-332.

Célèrier, E., Laulin, J.P., Corcuff, J.B., Le Moal, M., & Simonnet, G. (2001). Progressive enhancement of delayed hyperalgesia induced by repeated heroin administration: a sensitization process. J Neurosci, 21 (11): 4074-4080.

Célèrier, E., Rivat, C., Jun, Y., Laulin, J.P., Larcher, A., Reynier, P., & Simonnet, G. (2000). Long-lasting hyperalgesia induced by fentanyl in rats: preventive effect

of ketamine. Anesthesiology, 92 (2): 465-472.

Celik, E.C., Erhan, B., & Lakse, E. (2012). The clinical characteristics of neuropathic

pain in patients with spinal cord injury. Spinal Cord, 50 (8): 585-589.

Chang, G., Chen, L., & Mao, J. (2007). Opioid tolerance and hyperalgesia in chronic

pain patients after one month of oral morphine therapy: a preliminary

prospective pain study. J Pain 7, 44-48.

Chavkin, C. (2013). Dynorphin- still and extraordinarily potent opioid peptide.

Molecular Pharmacology, 83, 729-736.

Cho, D.C., Cheong, J.H., Yang, M.S., Hwang, S.J., Kim, J.M., Kim, C.H. (2011). The

effect of minocycline on motor neuron recovery and neuropathic pain in a rat

model of spinal cord injury. J Korean Neurosurg Soc, 49: 83-91.

Cho, H.J. & Basbaum, A.I. (1989). Ultrastructural analysis of dynorphin B-

immunoreactive cells and terminals in the superficial dorsal horn of the

deafferented spinal cord of the rat. J Comp Neurol, 281 (2): 193-205.

Chu, L.F., Clark, D.J., & Angst, M.S., (2006). Opioid tolerance and hyperalgesia in

chronic pain patients after one month of oral morphine therapy: a preliminary

prospective pain study. J Pain, 7: 44-48.

Clark, J.D. (2002). Chronic pain prevalence and analgesic prescribing in a general

medical population. J Pain Symptom Manage, 23: 31-37.

Cohen, J.J. (1993). Apoptosis. Immunol Today, 14 (3): 126-130.

Compton, W.M., & Volkow, N.D. (2006). Major increases in opioid analgesic abuse in

the United States: concerns and strategies. Drug Alcohol Depend, 81: 103-107.

Constantini, S. & Young, W. (1994). The effects of methylprednisolone and the ganglioside GM1 on acute spinal cord injury in rats. J Neurosurg, 80: 97-111.

Contet, C., Filliol, D., Matifas, A., & Kieffer, B.L. (2008). Morphine-induced analgesic tolerance, locomotor sensitization, and physical dependence do not require modification of mu opioid receptor, cdk5 and adenylate cyclase activity. Neuropharmacology, 54: 475-486.

Costigan, M., Scholz, J., & Woolf, C.J. (2009). Neuropathic pain: a maladaptive response of the nervous system to damage. Annu Rev Neurosci, 32: 1-32.

Croul, S., Radzievsky, A., Sverstiuk, A., & Murray, M. (1998). NK1, NMDA, 5HT1a, and 5HT2 receptor binding sites in the rat lumbar spinal cord: modulation following sciatic nerve crush. Exp Neurol, 154 (1): 66-79.

Crown, E.D., Ye, Z., Johnson, K.M., Xu, G.Y., McAdoo, D.J., & Hulsebosch, C.E. (2006). Increases in the activated forms of ERK 1/2, p38 MAPK, and CREB are correlated with the expression of at-level mechanical allodynia following spinal cord injury. Exp Neurol, 199 (2): 397-407.

Cruccu, G. (2007). Treatment of painful neuropathy. Curr Opin Neurol, 20 (5): 531-555.

David, S., Bouchard, C., Tsatas, O., & Giftochristos, N. (1990). Macrophages can modify the nonpermissive nature of the adult mammalian central nervous system. Neuron, 5 (4): 463-469.

de Kloet, E.R., Joels, M., & Holsboer, F. (2005). Stress and the brain: from adaptation to disease. Nat Rev Neurosci, 6: 463-475.

DeLeo, J.A. & Yezierski, R.P. (2001). The role of neuroinflammation and neuroimmune activation in persistent pain. Pain, 90 (1-2): 1-6.

DeLeo, J.A., Tanga, F.Y., & Tawfik, V.L. (2004). Neuroimmune activation and neuroinflammation in chronic pain and opioid tolerance/hyperalgesia. Neuroscientist, 10 (1): 40–52.

Dellemijn, P. (1999). Are opioids effective in relieving neuropathic pain? Pain, 80 (3): 453-462.

Dersh, J., Mayer, T.G., Gatchel, R.J. Polatin, P.B., Theodore, B.R., & Mayer, E.A.K. (2008). Prescription opioid dependence is associated with poorer outcomes in disabling spinal disorders. Spine, 33: 2219-2227.

Desbarats, J., Birge, R.B., Mimouni-Rongy, M., Weinstein, D.E., Palermen, J.S., & Newell, M.K. (2003). Fas engagement induces neurite growth through ERK activation and p35 upregulation. Nat Cell Biol, 5 (2): 118-125.

Detloff, M.R., Fisher, L.C., McGaughy, V., Longbrake, E.E., Popovich, P.G., & Basso, D.M. (2008). Remote activation of microglia and pro-inflammatory cytokines predict the onset and severity of below-level neuropathic pain after spinal cord injury in rats. Exp Neurol, 212 (2): 337-347.

DeVivo, M.J., Kartus, P.L. Stover, S.L., Rutt, R.D., & Fine, P.R. (1989). Causes of death for patient with spinal cord injuries. Arch Intern Med, 149 (8): 1761-1766.

Di Chiara, G., & Imperato, A. (1988). Opposite effects of mu and kappa opiate agonists on dopamine release in the nucleus accumbens and in the dorsal caudate of

freely moving rats. J Pharmacol Exp Ther, 244: 1067-1080.

Dobscha, S.K., Morasco B.J. Duckart, J.P., Macey, T., & Deyo, R.A. (2013). Correlates of prescription opioid initiation and long-term opioid use in veterans with persistent pain. Clin J Pain, 29 (2): 102-108.

Dong, Y. & Benveniste, E.N. (2001). Immune function of astrocytes. Glia, 36: 180-190.

Donnelly, C. & Eng, J.J. (2005). Pain following spinal cord injury: the impact on community reintegration. Spinal Cord, 43 (5): 278-282.

Dubner, R. & Ruda, M.A. (1992). Activity-dependent neuronal plasticity following tissue injury and inflammation. Trends Neurosci, 15 (3): 96-103.

Dworkin, R.H., O'Connor, A.B., Audetter, J., Baron, R., Gourlay, G.K., Haanpää, M.L., Kent, J.L., Krane, E.J., Lebel, A.A., Levy, R.M., Mackey, S.C., Mayer, J., Miaskowski, C., Raja, S.N., Rics, A.S., Schmader, K.E., Stacey, B., Stanos, S., Treede, R.D., Turk, D.c., Walco, G.A., & Wells, C.D. (2010). Recommendations for the pharmacological management of neuropathic pain: an overview and literature update. Mayo Clin Proc, 85 (3 Suppl): S3-S14.

Dworkin, R.H., O'Connor, A.B., Backonja, M., Farrar, J.T., Finnerup, N.B., Jensen, T.S., Kalso, E.A., Loeser, J.D., Miaskowski, C., Nurmikko, T.J., Portenoy, R.K., Rice, A.S., Stacey, B.R., Treede, R.D., Turk, D.C., & Wallace, M.S. (2007). Pharmacologic management of neuropathic pain: evidence-based recommendations. Pain, 132 (3): 237-251.

Eisenstein, T.K., Rahim, R.T., Feng, P., Thingalaya, N.K., & Meissler, J.J. (2006).

Effects of opioid tolerance and withdrawal on the immune system. J Neuroimmune Pharmacol, 1: 237-249.

Ewan, E.E. & Martin, T.J. (2011). Rewarding electrical brain stimulation in rats after peripheral nerve injury: decreased facilitation by commonly abused prescription opioids. Anesthesiology, 115 (6): 1271-1280.

Faden, A.I. (1990). Opioid and non-opioid mechanisms may contribute to dynorphin's pathophysiologic actions in spinal cord injury. Ann Neurol, 27: 67-74.

Faden, A.I. (1992). Dynorphin increases extracellular levels of excitatory aminoacids in the brain through a non-opioid mechanism. J Neuroscience, 12 (2): 425-429.

Faden, A.I. & Jacobs, T.P. (1983). Dynorphin induces partially reversible paraplegia in the rat. Eur J Pharmacol, 91: 321-324.

Faden, A.I. & Jacobs, T.P. (1984). Dynorphin-related peptides cause motor dysfunction in the rat through a non-opiate action. Br J Pharmacol, 81: 271-276.

Faden, A.I., Knoblach, S., Mays, C., & Jacobs, T.P. (1985). Motor dysfunction after spinal cord injury is mediated by opiate receptors. Peptides 6 (Suppl 1): ll-13.

Faden, A.I., Molineaw, C.J., Rosenberger, J.G., Jacobs, T.P., & Cox, B.M. (1985). Endogenous opioid immunoreactivity in spinal cord following traumatic injury. Ann Neurol 17: 386-390.

Faden, A.I., Simon, R.P. (1988). A potential role for excitotoxins in the pathophysiology of spinal cord injury. Ann Neurol 23: 623-626.

Fenollosa, P., Pallares, J., Cervera, J., Pelegrin, F., Inigo, V., Giner, M., & Forner, V. (1993). Chronic pain in the spinal cord injured: statistical approach and

pharmacological treatment. Paraplegia, 31 (11): 722-729.

Ferenczi, S., Núñez, C., Pintér-Kübler, B., Földes, A., Martín, F., Ladnyánszky Márkus, V., Milanés, M.V., & Kovács, K.J. (2010). Changes in metabolic-related variables during chronic morphine treatment. Neurochemistry International, 57: 323-330.

Ferguson AR, Christensen RN, Gensel JC, Miller BA, Sun F, Beattie EC, Bresnahan JC, Beattie MS (2008) Cell death after spinal cord injury is exacerbated by rapid TNF alpha-induced trafficking of GluR2-lacking AMPARs to the plasma membrane. J Neurosci 28:11391-11400.

Ferguson, A.R., Hook, M.A., Garcia, G., Bresnahan, J.C., Beattie, M.S., and Grau, J.W. (2004) A simple transformation that improves the metric properties of the BBB scale. J Neurotrauma 21, 1601-1613.

Ferrini, F., Trang, T., Mattioli, T.A.M., Laffray, S., Del'Guidice, T., Lorenzo, L.-E., Castonguay, A., Doyon, N., Zhang, W., Godin, A.G., Mohr, D., Beggs, S., Vandal, K., Beaulieu, J.-M., Cahill, C.M., Salter, M.W., & De Koninck, Y. (2013). Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl- homeostasis. Nature Neuroscience, 16 (2): 183-194.

Finnerup, N.B. & Baastrup, C. (2012). Spinal cord injury pain: mechanisms and management. Curr Pain Headache Rep, Epub ahead of print.

Finnerup, N.B., Johannesen, I.L., Sindrup, S.H., Bach, F.W., & Jensen, T.S. (2001). Pain and dysesthesia in patients with spinal cord injury: a postal survey. Spinal Cord, 39: 256-262.

161

Finnerup, N.B., Sindrup, S.H., & Jensen, T.S. (2010). The evidence for pharmacological treatment of neuropathic pain. Pain, 150 (3): 573-581.

Fleming, J.C., Norenberg, M.D., Ramsay, D.A., Dekaban, G.A., Marcillo, A.E., Saenz, A.D., Pasquale-Styles, M., Dietrich, W.D., & Weaver, L.C. (2006). The cellular inflammatory response in human spinal cords after injury. Brain, 129 (pt 12): 3249-3269.

Froger-Colléaux, C., Rompion, S., Guillaume, P., Porsolt, R.D., Castagné, V., & Moser, P. (2011). Continuous evaluation of drug withdrawal in the rat using telemetry: effects of morphine and chlordiazepoxide. J Pharmacol Toxicol Methods, 64 (1): 81-88.

Fukagawa, H., Koyama, T., Kakuyama, M., & Fukuda, K. (2013). Microglial activation involved in morphine tolerance is not mediated by toll-like receptor 4. J Anesth, 27: 93-97.

Furlan, J.C. & Fehlings, M.G. (2008). Cardiovascular complications after acute spinal cord injury: pathophysiology, diagnosis, and management. Neurosurg Focus, 25 (5): E13.

Gieryk, A., Ziolkowska, B., Solecki, W., Kubik, J., & Przewlocki, R. (2010). Forebrain PENK and PDNY gene expression levels in three inbred strains of mice and their relationship to genotype-dependent morphine reward sensitivity. Psychopharmacology, 208: 291-300.

Gosselin, R.D., Suter, M.R., Ji, R.R., & Decosterd, I. (2010). Glial cells and chronic pain. Neuroscientist, 16 (5): 519-531.

Graeber, M.B. (2010). Changing face of microglia. Science, 330 (6005): 783-788.

Graeber, M.B. & Christie, M.J. (2012). Multiple mechanisms of microglia: a
gatekeeper's contribution to pain states. Exp Neurol, 234 (2): 255-261.

Graeber, M.B. & Streit, W.J. (2010). Microglia: biology and pathology. Acta
Neuropathol, 119 (1): 89-105.

Grau, J.W., Washburn, S.N., Hook, M.A., Ferguson, A.R., Crown, E.D., Garcia, G.,
Bolding, K.A., & Miranda, R.C. (2004). Uncontrollable stimulation undermines
recovery after spinal cord injury. J Neurotrauma, 21 (12): 1795-1817.

Grigorean, V.T., Sandu, A.M., Popescu, M., Iacobini, M.A., Stoian, R., Neasu, C., &
Popa, F. (2009). Cardiac dysfunctions following spinal cord injury. J Med and
Life, 2 (2): 133-145.

Gruner, J.A. (1992). A monitored contusion model of spinal cord injury in the rat. J
Neurotrauma, 0: 123-126.

Gu, J., Yao, M., Wang, J., Zhou, W., Yang, G., Liu, H., Zhang, Z., & Liu, X. (2005)
Suppression of morphine withdrawal syndrome by interleukin-2 and its gene.
Molecular Neuroscience, 16: 387-391.

Guadagno, J., Xu, X., Karajgikar, M., Brown, A., & Cregan, S.P. (2013). Microglia-
derived TNFα induces apoptosis in neural precursor cells via transcriptional
activation of the Bcl-2 family member Puma. Cell Death & Disease, 4: e538.

Gwak, Y.S. & Hulsebosch, C.E. (2011). Neuronal hyperexcitability: a substrate for
central neuropathic pain after spinal cord injury. Curr Pain Headache Rep, 15
(3): 215-222.

Gwak, Y.S., Kang, J., Unabia, G.C., & Hulsebosch, C.E. (2012). Spatial and temporal activation of spinal glial cells: role of gliopathy in central neuropathic pain following spinal cord injury in rats. Exp Neurol, 234 (2): 362-372.

Hains, B.C., Black, J.A., & Waxman, S.G. (2003). Primary cortical motor neurons undergo apoptosis after axotomizing spinal cord injury. J Comp Neurol, 462 (3): 328-341.

Hama, A. & Sagen, J. (2012). Combinations of intrathecal gamma-amino-butyrate receptor agonists and N-methyl-d-aspartate receptor antagonists in rats with neuropathic spinal cord injury pain. Eur J Pharmacol, 683 (1-3): 101-108.

Hameed, H., Hameed, M., & Christo, P.J. (2010). The effect of morphine on glial cells as a potential therapeutic target for pharmacological development of analgesic drugs. Curr Pain Headache Rep, 14 (2): 96-104.

Harden, N. & Cohen, M. (2003). Unmet needs in the management of neuropathic pain. J. Symptom Manage, 25 (5 Suppl): S12-S17.

Harris, J.A., Corsi, M., Quartaroli, M., Arban, R., & Bentivoglio, M. (1996). Upregulaiton of spinal glutamate receptors in chronic pain. Neuroscience, 74 (1): 7-12.

Hauser, K.F., Foldes, J.K., & Turbek, C.S. (1999). Dynorphin A (1-13) neurotoxicity in vitro: opioid and non-opioid mechanisms in mouse spinal cord neurons. Exp Neurol, 160 (2): 361-375.

Headrick, J.P., Faden, A.I., & Vink, R. (1995). Intrathecal dynorphin-A infusion in rat spinal cord causes energy depletion, edema and neurologic dysfunction.

Neurochem Int, 25 (5), 489-495.

Heinemann, A.W., McGraw, T.E., Brandt, M.J., Roth, E. & Dell'Oliver, C. (1992). Prescription medication misuse among persons with spinal cord injuries. Int J Addict, 27: 301-316.

Heinemann, A.W., Schnoll, S., Brandt, M., Maltz, R., & Keen, M. (1988). Toxicology screening in acute spinal cord injury. Alcohol Clin Exp Res, 12: 815-819.

Hemstapat, K., Le, L., Edwards, S.R., & Smith, M.T. (2009). Comparative studies of the neuro-excitatory behavioural effects of morphine-3-glucuronide and dynorphin A(2-17) following spinal and supraspinal routes of administration. Pharmacol Biochem Behav, 93: 498-505.

Heutink, M., Post, M.W., Bongers-Janssen, H.M., Dijkstra, C.A., Snoek, G.J., Spijkerman, D.C., & Lindeman, E. (2012). The CONESCI trial: results of a randomized controlled trial of a multidisciplinary cognitive behavioral program for coping with chronic neuropathic pain after spinal cord injury. Pain, 153 (1): 120-128.

Hojsted, J. & Sjorgren, P. (2007). Addiction to opioids in chronic pain patients: A literature review. Eur J Pain, 11: 490-518.

Hook, M.A., Ferguson, A.R., Garcia, G., Washburn, S.N., Koehly, L.M., & Grau, J.W. (2004). Monitoring recovery after injury: procedures for deriving the optimal test window. J Neurotrauma, 21 (1): 109-118.

Hook, M.A., Liu, G.T., Washburn, S.N., Ferguson, A.R., Bopp, A.C., Huie, J.R., & Grau, J.W. (2007). The impact of morphine after a spinal cord injury. Behav

Brain Res, 179: 281-293.

Hook, M.A., Moreno, G.A., Woller, S.A., Puga, D.A., Hoy, K.C., Jr., Balden, R. & Grau, J.W. (2009). Intrathecal morphine attenuates recovery of function after a spinal cord injury. J Neurotrauma, 26: 741-752.

Hook, M.A., Washburn, S.N., Moreno, G.L., Woller, S.A., Puga, D.A., Lee, K.H., & Grau, J.W. (2011). An IL-1 receptor antagonist blocks a morphine-induced attenuation of locomotor recovery after spinal cord injury. Brain Behav Immun, 25: 349-359.

Hulsebosch, C.E. (2002). Recent advances in pathophysiology and treatment of spinal cord injury. Adv Physiol Educ, 26 (1-4): 238-255.

Hulsebosch, C.E. (2008). Gliopathy ensures persistent inflammation and chronic pain after spinal cord injury. Exp Neurol, 214 (1): 6-9.

Hulsebosch, C.E., Hains, B.C., Crown, E.D., & Carlton, S.M. (2009). Mechanisms of chronic central neuropathic pain after spinal cord injury. Brain Res Rev, 60 (1): 202-213.

Hutchinson, M.R., Bland, S.T., Johnson, K.W., Rice, K.C., Maier, S.F., & Watkins, L.R. (2007). Opioid-induced glial activation: mechanisms of activation and implications for opioid analgesia, dependence, and reward. ScientificWorldJournal 7: 98–111.

Hutchinson, M.R., Coats, B.D., Lewis, S.S., Zhang, Y., Sprunger, D.B., Rezvani, N., Baker, E.M., Jekich, B.M., Wieseler, J.L., Somogyi, A.A., Martin, D., Poole, S., Judd, C.M., Maier, S.F., & Watkins, L.R. (2008b). Pro-inflammatory cytokines

oppose opioid-induced acute and chronic analgesia. Brain Behav Immun, 22 (8): 1178-1189.

Hutchinson, M.R., Northcutt, A.L., Chao, L.W., Kearney, J.J., Zhang, Y., Berkelhammer, D.L., Loram, L.C., Rozeske, R.R., Bland, S.T., Maier, S.F., Gleeson, T.T., & Watkins, L.R. (2008a). Minocycline suppresses morphine-induced respiratory depression, suppresses morphine-induced reward, and enhances systemic morphine-induced analgesia. Brain Behav Immun, 22 (8): 1248–1256.

Hutchinson, M.R., Shavit, Y., Grace, P.M., Rice, K.C., Maier, S.F., & Watkins, L.R. (2011). Exploring the neuroimmunopharmacology of opioids: An integrative review of mechanisms of central immune signaling and their implications for opioid analgesia. Pharmacol Rev, 63 (3): 772-810.

Hutchinson, M.R., Zhang, Y., Brown, K., Coats, B.D., Shridhar, M., Sholar, P.W., Patel, S.J., Crysdale, N.Y., Harrison, J.A., Maier, S.F., et al. (2008c). Non-steroselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). Eur J Neurosci, 28: 20-29.

Hutchinson, M.R., Zhang, Y., Shridhar, M., Evans, J.H., Buchanan, M.M., Zaho, T.X., Slivka, P.F., Coats, B.D., Rezvani, N., Wieseler, J., et al. (2010). Evidence that opioids may have toll-like receptor 4 and MD-2 effects. Brain Behav Immun, 24: 88-95.

Jacquet, Y.F. (1988). The NMDA receptor: central role in pain inhibition in rat periaqueductal gray. Eur J Pharmacol, 154 (3): 271-276.

Jarvis, M.F. (2010). The neural-glial purinergic receptor ensemble in chronic pain states. Trends Neurosci, 33: 48-57.

Johnston, I.N., Milligan, E.D., Wieseler-Frank, J., Frank, M.G., Zapata, V., Campisi, J., Langer, S., Martin, D, Green, P., Fleshner, M., Leinwand, L., Maier, S.F., Watkins, L.R. (2004). A role for pro-inflammatory cytokines and fractalkine in analgesia, tolerance, and subsequent pain facilitation induced by chronic intrathecal morphine. J Neurosci, 24 (33): 7353-7365.

Kajander, K.C., Shara, Y., Iadarola, M.J., Bennett, G.J. (1990). Dynorphin increases in the dorsal spinal cord in rats with a painful peripheral neuropathy. Peptides, 11 (4): 719-728.

Kang, S.M., Hoffmann, A., Le, D., Springer, M.L., Stock, P.G., & Blau, H.M. (1997). Immune response and myoblasts that express Fas ligand. Science, 278 (5341): 1322-13324.

Kawai, T., & Akira, S. (2007). Signaling to NF-κB by toll-like receptors. Trends in Molecular Medicine, 13 (11): 460-469.

Keane, R.W., Davis, A.R., & Dietrich, W.D. (2006). Inflammatory and apoptotic signaling after spinal cord injury. J Neurotrauma, 23: 335-344.

Kigerl, K.A. & Popovich, P.G. (2009). Toll-like receptors in spinal cord injury. Curr Top Microbiol Immunol, 336: 121-136.

Kigerl, K.A., Lai, W., Rivest, S., Hart, R.P., Satoskar, A.R., & Popovich, P.G. (2007). Toll-like receptor (TLR)-2 and TLR-4 regulate inflammation, gliosis, and myelin sparing after spinal cord injury. J Neurochem, 102 (1): 37-50.

Kim, Y., Cho, H.Y., Ahn, Y.J., Kin, J., & Yoon, Y.W. (2012). Effect of NMDA NR2B antagonist on neuropathic pain in two spinal cord injury models. Pain, Epub ahead of print.

Kim, Y.J., Hwang, S.Y., Oh, E.S., Oh, S., & Han, I.O. (2006). IL-1beta, an immediate early protein secreted by activated microglia, induces iNOS/NO in C6 astrocytoma cells through p38 MAPK and NF-kappa B pathways. J Neurosci Res, 84 (5): 1037-1046.

King, T. et al., (2009). Unmasking the tonic-aversive state in neuropathic pain. Nat Neurosci, 12: 1364-1366.

Koob, G.F., Lloyd, G.K., & Mason, B.J. (2009). Development of pharmacotherapies for drug addiction: a Rosetta Stone approach. Nat Rev Drug Discov, 8: 500-515.

Koyyalagunta, D., Bruera, E., Aigner, C., Nusrat, H., Driver, L., & Novy, D. (2013). Risk stratification of opioid misuse among patients with cancer pain using the SOAPP-SF. Pain Med, [Epub ahead of print].

Kreutzberg, G.W. (1996). Microglia: a sensor for pathological events in the CNS. Trends Neurosci, 19 (8): 312-318.

Krumins, S.A. & Faden, A.I. (1986). Traumatic injury alters opiate receptor binding in rat spinal cord. Ann Neurol, 19 (5): 498-501.

Lai, S.L., Gu, Y., Huang, L.Y. (1998). Dynorphin uses a non-opioid mechanism to potential N-methyl-D-aspartate currents in single rat periaqueductal gray neurons. Neurosci Lett, 247 (2-3): 115-118.

Langer, S., Martin, D., Green, P., Fleshner, M., Leinwand, L., Maier, S.F., &

Laulin, J.P., Maurette, P., Corcuff, J.B., Rivat, C., Chauvin, M., & Simonnet, G. (2002). The role of ketamine in preventing fentanyl-induced hyperalgesia and subsequent acute morphine tolerance. Anesth Analg, 94: 1263-1269.

Lee, C.Y., Perez, F.M., Wang, W., Guan, X., Zhao, X., Fisher, J.L., Guan, Y., Sweitzer, S.M., Raja, S.N., Tao, Y.X. (2011). Dynamic temporal and spatial regulation of mu opioid receptor expression in primary afferent neurons following spinal nerve injury. Eur J Pain, 15 (7): 669-675.

Lee, M., Silverman, S.M., Hansen, H., Patel, V.B., & Manchikanti, L. (2011). A comprehensive review of opioid-induced hyperalgesia. Pain Physician, 14 (2): 145-161.

Lepore, A.C., O'Donnell, J., Bonner, J.F., Paul, C., Miller, M.E., Rauck, B., Kushner, R.A., Pothstein, J.D., Fischer, L., & Maragakis, N.J. (2011). Spatial and temporal changes in promoter activity of the astrocyte glutamate transporter GLT1 following traumatic spinal cord injury. J Neurosci Res, 89: 1001-1017.

Letellier, E., Kumar, S., Sancho-Martinez, I., Krauth, S., Funke-Kaisser, A., Laudenklos, S., Konecki, K., Klussmann, S., Corsini, N.S., Kleber, S., Drost, S., Neumann, A., Lévi-Strauss, M., Brors, B., Gretz, N., Edler, L., Fischer, C., Hill, O., Thiemann, M., Biglari, B., Karray, S., & Martin-Villalba, A. (2010). CD95-ligand on peripheral myeloid cells activates Syk kinase to trigger their recruitment to the inflammatory site. Immunity, 32 (2): 240-252.

Levine, A.S., Grace, M., Billington, C.J., Krahn, D.D., Brown, D.M., & Morley, J.E. (1988). Effect of morphine and nalmefene on energy balance in diabetic and

non-diabetic rats. Pharmacol Biochem Behav, 29 (3): 495-500.

Lewis, S.S., Hutchinson, M.R., Rezvani, N., Loram, L.C., Zhang, Y., Maier, S.F., Rich, K.C., & Watkins, L.R. (2010). Evidence that intrathecal morphine-3-glucuronide may cause pain enhancement via toll-like receptor 4/MD-2 and interleukin-1beta. Neuroscience, 165: 569-583.

Li, Y., Sun, X., Zhang, Y., Huang, J., Hanley, G., Ferslew, K.E., Peng, Y., & Yin, D. (2009). Morphine promotes apoptosis via TLR2, and this is negatively regulated by beta-arrestin 2. Biochem Biophys Res Commun, 378 (4): 857-861.

Liang, D., Shi, X., Qiao, Y., Angst, M., Yeomans, D.C., & Clark, J.D. (2008). Chronic morphine administration enhances nociceptive sensitivity and local cytokine porduction after incision. Mol Pain, 4, np.

Lieberman, A.P., Pitha, P.M., Shin, H.S., & Shin, M.L. (1989). Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotrophic virus. Proc Natl Acad Sci, 86: 6348-6352.

Lim, G., Wang, S., zeng, Q., Sung, B., Yang, L., & Mao, J. (2005). Expression of spinal NMDA receptor and PKCgamma after chronic morphine is regulated by spinal glucocorticoid receptor. J Neurosci, 25: 11145-11154.

Liu, S., Xu, G.Y., Johnson, K.M., Echetebu, C., Ye, A., Hulsebosch, C.E., & McAdoo, D.J. (2008). Regulation of interleukin-1beta by the interleukin-1 receptor antagonist in the glutamate-injured spinal cord: endogenous neuroprotection. Brain Res, 1231: 63-74.

Long, J.B., Kinney, R.C., Malcolm, D.S., Graeber, G.M., & Holaday, J.W. (1987).

Intrathecal dynorphin A1-13 and dynorphin A3-13 reduce rat spinal cord blood

 flow by non-opioid mechanisms. Brain Res, 436: 374-379.

Lonjon, N., Kouyoumdjian, P., Prieto, M., Bauchet, L., Haton, H., Gaviria, M., Privat,

 A., & Perrin, F.E. (2010). Early functional outcomes and histological analysis

 after spinal cord compression injury in rats. J Neurosurg Spine, 12: 106-113.

Lu, M., Wang, S., Han, X., & Lv, D. (2013). Butein inhibits NF- κB activation and

 reduced infiltration of inflammatory cells and apoptosis after spinal cord injury

 in rats. Neurosci Lett, 542: 87-91.

Lutz, P.-E. & Kieffer, B.L. (2013). The multiple facets of opioid receptor function:

 implications for addiction. Curr. Opin Neurobio. (In Press)

Lynch, J.L. & Banks, W.A. (2008). Opiate modulation of IL-1alpha, IL-2, and TNF-

 alpha transport across the blood-brain barrier. Brain Behav Immun, 22 (7):

 1096-1102.

Ma, Y.Y., Guo, C.Y., Yu, P., Lee, D.Y., Han, J.S., & Cui C.L. (2006a). The role of

 NR2B containing NMDA receptor in place preference conditioned with

 morphine and natural reinforcers in rats. Exp Neurol, 200 (2): 343-355.

Ma, Y.Y., Yu, P., Guo, C.Y., & Cui, C.L. (2011). Effects of ifenprodil on morphine-

 induced conditioned place preference and spatial learning and memory in rats.

 Neurochem Res, 36 (3): 383-391.

Magrinelli, F., Zanette, G., & Tamburin, S. (2013). Neuropathic pain: diagnosis and

 treatment. Pract Neurol, 0: 1-16.

Majno, G. & Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell

death. Am J Pathol, 146 (1): 3015.

Malan, T.P., Ossipov, M.H., Gardell, L.R. Ibrahim, M., Bian, D., Lai, J., & Porreca, F. (2000). Extraterritorial neuropathic pain correlates with multisegmental elevation of spinal dynorphin in nerve-injured rats. Pain, 86 (1-2): 185-194.

Mao, J. & Mayer, D.J. (2001). Spinal cord neuroplasticity following repeated opioid exposure and its relation to pathological pain. Ann N Y Acad Sci, 933: 175-184.

Mao, J., Gold, M.S., & Backonja, M.M. (2011). Combination drug therapy for chronic pain: a call for more clinical studies. J Pain, 12 (2): 157-166.

Mao, J., Price, D.D., & Mayer, D.J. (1994). Thermal hyperalgesia in association with the development of morphine tolerance in rats: roles of excitatory amino acid receptors and protein kinase C. J Neurosci, 14 (4): 2301-2312.

Mao, J., Price, D.D., & Mayer, D.J. (1995a). Mechanisms of hyperalgesia and morphine tolerance: a current view of their possible interactions. Pain, 62 (3): 259-274.

Mao, J., Price, D.D., Zhu, J., Lu, J., & Mayer, D.J. (1997). The inhibition of nitric oxide-activated poly(ADP-ribose) synthetase attenuates transsynaptic alteration of spinal cord dorsal horn neurons and neuropathic pain in the rat. Pain, 72 (3): 355-366.

Mao, J., Sung, B., Ji, R.R., & Lim, G. (2002a). Chronic morphine induces downregulation of spinal glutamate transporters: implications in morphine tolerance and abnormal pain sensitivity. J Neurosci, 22 (18): 8812-823.

Martin, T.J., Kin, S.A., Buechlet, N.L., & Porreca, F. (2007). Opioid self-

administration in the nerve-injured rat: relevance of antiallodynic effects to drug consumption and effects of intrathecal analgesics. Anesthesiology, 106: 312-322.

Mayer, D.J., Mao, J., Holt, J., & Price, D.D. (1999). Cellular mechanisms of neuropathic pain, morphine tolerance, and their interactions. Proc Natl Acad Sci USA, 96: 7731-7736.

McAdoo, D.J., Hughes, M.G., Nie, L., Shah, B., Clifton, C., Fullwood, S., & Hulsebosch, C.E. (2005). The effect of glutamate receptor blockers on glutamate release following spinal cord injury. Lack of evidence for an ongoing feedback cascade of damage --> glutamate release --> glutamate release --> damage --> glutamate release --> etc. Brain Res, 1038 (1): 92-99.

McLaughlin, J.P., Land, B.B., Li, S., Pintar, J.E., Chavkin, C. (2006). Prior activation of kappa opioid receptors by U50-488, mimics repeated forced swim stress to potential cocaine place preference conditioning. Neuropsychopharmacology, 31 (4): 787-794.

McLaughlin, J.P., Marton-Popovici, M., & Chavkin, C. (2003). Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. J Neurosci, 23 (13): 5674-5683.

McTigue, D.M. (2008). Potential therapeutic targets for PPARgamma after spinal cord injury. PPAR Res, 2008: 517-162.

Mehta, A., Halder, S., Khanna, N., Tandon, O., & Sharma, K. (2011). Antagonism of stimulation-produced analgesia by naloxone and N-methyl-D-aspartate: role of

opioid and N-methyl-D-aspartate receptors. Hum Exp Toxicol, 31 (1): 51-56.

Meisner, J.G., Marsh, A.D., & Marsh, D.R. (2010). Loss of GABAergic interneurons in laminae I-III of the spinal cord dorsal horn contributes to reduced GABAergic tone and neuropathic pain after spinal cord injury. J Neurotrauma, 27 (4): 729-737.

Mellon, R.D. & Bayer, B.M. (1998). Evidence for central opioid receptors in the immunomodulatory effects of morphine: review of potential mechanism(s) of action. J Neuroimmunol, 83 (1-2): 19-28.

Migliorini, C.E., New, P.W., Tonge, B.J. (2009). Comparison of depression, anxiety and stress in persons with traumatic and non-traumatic post-acute spinal cord injury. Spinal Cord, 47 (11): 783-788.

Mika, J. (2008). Modulation of microglia can attenuate neuropathic pain symptoms and enhance morphine effectiveness. Pharmacol Rep, 60 (3): 297-307.

Mika, J., Zychowska, M., Popiolek-Barczyk, K., Rojewska, E., & Przewlocka, B. (2013). Importance of glial activation in neuropathic pain. Eur J Pharmacology (in press).

Milligan, E.D. & Watkins, L.R. (2009). Pathological and protective roles of glia in chronic pain. Nat Rev Neurosci, 10 (1): 23-36.

Min, K.J., Jou, I., & Joe, E. (2003). Plasminogen-induced IL-1beta and TNF-alpha production in microglia is regulated by reactive oxygen species. Biochem Biophys Res Commun, 312 (4): 969-974.

Min, K.J., Pyo, H.K., Yang, M.S., Ji, K.A., Jou, I., & Joe, E.H. (2004). Gangliosides

activate microglia via protein kinase C and NADPH oxidase. Glia, 48 (3): 197-206.

Minozzi, S., Amato, L., & Davoli, M. (2013). Development of dependence following treatment with opioid analgesics for pain relief: a systematic review. Addiction, 108: 688-698.

Moore, R.A. & McQuay, H.J. (2005). Prevalence of opioid adverse events in chronic non-malignant pain: systematic review of randomized trials of oral opioids. Arthritis Res Ther, 7 (5): R1046-1051.

Morasco, B.J. & Dobscha, S.K. (2008). Prescription medication misuse and substance use disorder in VA primary care patients with chronic pain. Gen Hosp Psychiatry 30, 93-99.

Nahin, R.L., Hylden, J.L., Iadarola, M.J., & Dubner, R. (1989). Peripheral inflammation is associated with increased dynorphin immunoreactivity in both projection and local circuit neurons in the superficial dorsal horn of the rat lumbar spinal cord. Neurosci Lett, 96 (3): 247-252.

Narita, M., Aoki, T., & Suzuki, T. (2000). Molecular evidence for the involvement of NR2B subunit containing N-methyl-D-aspartate receptors in the development of morphine induces place preference. Neuroscience, 101 (3): 601-606.

Navratilova, E., Xie, J.Y., King, T., & Porreca, F. (2013). Evaluation of reward from pain relief. Annals NY Acad Sci.

Nesic, O., Xu, G.Y., McAdoo, D., High, K.W., Hulsebosch, C., & Perez-Pol, R. (2001). IL-1 receptor antagonist prevents apoptosis and caspase-3 activation after spinal

cord injury. J Neurotrauma, 18 (9): 947-956.

Nicotra, L., Loram, L.C., Watkins, L.R., & Hutchinson, M.R. (2012). Toll-like receptors in chronic pain. Exp Neurol, 234 (2): 316-329.

Niikura, K., Narita, M., Butelman, E.R., Kreek, M.J., & Suzuki, T. (2010). Neuropathic and chronic pain stimuli downregulate central mu-opioid and dopaminergic transmission. Trends in Pharmacological Science, 31: 299-305.

Niikura, K., Narita, M., Narita, M., Nakamura, A., Okutsu, D., Ozeki, A., Kurahashi, K., Kobayashi, Y., Suzuki, M., & Suzuki, T. (2008). Direct evidence for the involvement of endogenous beta-endorphin in the suppression of the morphine-induced rewarding effect under a neuropathic pain-like state. Neurosci Lett, 435 (3): 257-262.

Nogueiras, R., Omero-Pico, A., Vazquez, M.J., Novelle, M.G., Lopez, M., & Dieguez, C. (2012). Thr opioid system and food intake: Homeostatic and hedonic mechanisms. Obes Facts, 9: 196-207.

Nottingham, S.A. & Springer, J.E. (2003). Temporal and spatial distribution of activated caspase-3 after subdural kainic acid infusions in rat spinal cord. J Comp Neurol, 464 (4): 463-471.

O'Connor, A.B., & Dworkin, R.H. (2009). Treatment of neuropathic pain: an overview of recent guideline. Am J Med. 122, (10 Suppl.), S22-S32.

Obreja, O., Rathee, P.K., Lips, K.S., Distler, C., & Kress, M. (2002). IL-1 beta potentiates heat-activated currents in rat sensory neurons: involvement of IL-1RI, tyrosine kinase, and protein kinase C. FASEB J, 16 (12): 1497-1503.

Odunayo, A., Dodam, J.R., Kerl, M.E., DeClue, A.E. (2010). Immunomodulatory
effects of opioids. J Vet Emerg Crit Care (San Antonio), 20 (4): 376-385.

Okun, E., Griffioen, K.J., Lathia, J.D., Tang, S.C., Mattson, M.P., & Arumugam, T.V.
(2009). Toll-like receptors in neurodegeneration. Brain Res Rev, 59 (2): 278-
292.

One degree of separation: paralysis and spinal cord injury in the United States. (2009).
Christopher & Dana Reeve Foundation.

Opree, A. & Kress, M. (2000). Involvement of the pro-inflammatory cytokines tumor
necrosis factor-alpha, IL-1 beta, and IL-6 but not IL-8 in the development of
heat hyperalgesia: effects on hear-evoked calcitonin gene-related peptide release
from rat skin. J Neurosci, 20 (16): 6289-6293.

Ozaki, S., Narita, M., Narita, M., Iinom, M., Miyoshi, K. & Suzuki, T. (2003).
Suppression of the morphine-induced rewarding effect and G-protein activation
in the lower midbrain following nerve injury in the mouse: involvement of G-
protein-coupled receptor kinase 2. Neuroscience, 116 (1): 89-97.

Ozaki, S., Narita, M., Narita, M., Ozaki, M., Khotib, J, & Suzuki, T. (2004). Role of
extracellular signal-regulated kinase in the ventral tegmental area in the
suppression of the morphine-induced rewarding effect in mice with sciatic nerve
ligation. J Neurochem, 88 (6): 1389-1397.

Ozinsky, A., Underhill, D.H., Fontenot, J.D., Hajjar, A.M., Smith, K.D., Wilson, C.B.,
Schroeder, L., & Aderem, A. (2000). The repertoire for pattern recognition of
pathogens by the innate immune system is defined by cooperation between toll-

like receptors. Proc Natl Acad Sci USA, 97: 13766-13771.

Papadopoulos, G., Weinberg, E.O., Massari, P., Gibson, F.C. 3rd., Wetzler, L.M., Morgan, E.F., & Genco, C.A. (2013). Macrophage-specific TLR2 signaling mediates pathogen-induced TNF-dependent inflammatory oral bone loss. J Immunol, 190 (3): 1148-1157.

Pineau, I. & Lacroix, S. (2007). Pro-inflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression patter and identification of the cell types involved. J Comp Neurol, 500 (2): 267-285.

Popa, C., Popa, F., Grigorean, V.T., Onose, G., Sandu, A.M., Popescu, M., Burnei, G., Stambu, V., Sinescu, C. & Davila, C. (2010). Vascular dysfunctions following spinal cord injury. J Med and Life, 3 (3): 275-285.

Popovich, P.G., Wei, P., & Stokes, B.T. (1997). Cellular inflammatory response after spinal injury in Sprague-Dawley and Lewis rats. J Comp Neurol, 377 (3): 443-464.

Porter, J.T. & McCarthy, K.D. (1997). Astrocytic neurotransmitter receptors in situ and in vivo. Prog Neurobiol, 51 (4): 439-455.

Prescription drugs: abuse and addiction (2011). National Institute on Drug Abuse Research Report Series.

Priller, J., Haas, C.A., Reddington, M. & Kreutzberg, G.W. (1995). Calcitonin gene-related peptide and ATP induce immediate early gene expression in cultured rat microglial cells. Glia, 15: 447-457.

Przewlocki, R., & Przewlocki, B. (2005). Opioids in neuropathic pain. Curr Pharm Des

11, 3013-3025.

Przewlocki, R., Hassan, A.H., Lason, W., Epplen, C., Herz, A., & Stein, C. (1992).
Gene expression and localization of opioid peptides in immune cells of
inflamed tissue: functional role in antinociception. Neuroscience, 48: 491-500.

Rabchevsky, A.G. (2006). Segmental organization of spinal reflexes mediating
autonomic dysreflexia after spinal cord injury. Prog Brain Res, 152: 265-274.

Radnitz, C.L. & Tirch, D. (1995). Substance misuse in individuals with spinal cord
injury. Int J Addict 30, 1117-1140.

Rafati, D.S., Geissler, K., Johnson, K., Unabia, G., Hulsebosch, C., Nessic, O., &
Perez-Polo, J.R. (2008). Nuclear factor-kappa B decoy amelioration of spinal
cord injury-induced inflammation and behavior outcomes. J Neurosci, 86: 566-
580.

Raghavendra, V, Rutkowski, M.D., & DeLeo, J.A. (2002). The role of spinal
neuroimmune activation in morphine tolerance/ hyperalgesia in neuropathic and
sham-operated rats. J Neurosci, 22 (22): 9980-9989.

Raghavendra, V., Tanga, F., & DeLeo, J.A. (2003). Inhibition of microglial activation
attenuates the development but not existing hypersensitivity in a rat model of
neuropathy. J Pharmacol Exp Ther, 306 (2): 624–630.

Raigorodsky, G. & Urca, G. (1987). Intrathecal N-methyl-D-aspartate (NMDA)
activates both nociceptive and antinociceptive systems. Brain Res, 422: 158-
162.

Ravenscroft, A., Ahmed, Y.S., & Burnside, I.G. (2000). Chronic pain after SCI. A

patient survey. Spinal Cord, 38: 611-614.

Re, K. & Dubner, R. (2010). Interactions between the immune system and nervous

systems in pain. Nat Med, 16 (11): 1267-1276.

Rischke, R. & Krieglstein, J.J. (1991). Postischemic neuronal damage causes astroglial

activation and increase in local cerebral glucose utilization of rat hippocampus.

Cereb Blood Flow Metab, 11: 106-113.

Ruda, M.A., Iadarola, M.J., Cohen, L.V., & Young, W.S. 3rd. (1988). In situ

hybridization histochemistry and immunocytochemistry reveal an increase in

spinal dynorphin biosynthesis in a rat model of peripheral inflammation and

hyperalgesia. Proc Natl Acad Sci U S A, 85 (2): 622-666.

Sah, R., Galeffi, F., Ahrens, R., Jordan, G., & Scwartz-Bloom, R.D. (2002).

Modulation of the GABAA-gated chloride channel by reactive oxygen species.

J Neurochem, 80: 383-391.

Sanchis-Segura, C. & Spanagel, R. (2006). Behavioral assessment of drug

reinforcement and addictive features in rodents: an overview. Addic Biol. 11, 2-

38.

Schäfers, M., Svensson, C.I., Sommer, C., & Sorkin, L.S. (2003). Tumor necrosis

factor-alpha induces mechanical allodynia after spinal nerve ligation by

activation of p38 MAPK in primary sensory neurons. J Neurosci, 23:2517-2521.

Schindler, A.G., Li, S., Chavkin, C. (2010). Behavioral stress may increase the

rewarding valence of cocaine-associated cues through a dynorphin/kappa-opioid

receptor-mediated mechanism without affecting associative learning or memory

retrieval mechanisms. Neuropsychopharmacology, 35 (9): 1932-1942.

Schindler, A.G., Messinger, D.I., Smith, J.S., Shankar, H., Gustin, R.M., Schattauer, S.S., Lemos, J.C., Chavkin, N.W., Hagan, C.E., Neumaier, J.F., & Chavkin, C. (2012). Stress produces aversion and potentiates cocaine reward by releasing endogenous dynorphins in the ventral striatum to locally stimulate serotonin reuptake. J Neurosci, 32 (49): 17582-17596.

Schneider, J.P. & Kirsh, K.L. (2010). Defining clinical issues around tolerance, hyperalgesia, and addiction: a quantitative and qualitative outcome study of long-term opioid dosing in a chronic pain practice. J Opioid Manag, 6: 385-395.

Schnell, L., Fearn, S., Klassen, H., Schwab, M.E., & Perry, V.H. (1999). Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord. Eur J Neurosci, 11 (10): 3648-3658.

Scholz, J. & Woolf, C.J. (2007). The neuropathic pain triad: neurons, immune cells, and glia. Nat Neurosci, 10 (11): 1361-1368.

Seino, K., Kayagaki, N., Takeda, K., Fukao, K., Okumura, K., & Yagita, H. (1997). Contribution of Fas ligand to T cell-mediated hepatic injury in mice. Gastroenterology, 113 (4): 1315-1322.

Sekhon, L.H., & Fehlings, M.G. (2001). Epidemiology, demographics, and pathophysiology of acute spinal cord injury. Spine, 26 (24 Suppl): S2–S12.

Shaham Y, Erb S, and Stewart J (2000) Stress-induced relapse to heroin and cocaine seeking in rats: a review. Brain Res Rev 33:13–33.

Sharma, H.S. & Sjoquist, P.O. (2002). A new antioxidant compound H-290/51

modulate glutamate and GABA immunoreactivity in the rat spinal cord following trauma. Amino Acids, 23: 261-272.

Sharma, H.S., & Ali, S.F. (2006). Alterations in blood-brain barrier function by morphine and methamphetamine. Ann N Y Acad Sci, 1074: 198-224.

Shippenberg TS, Chefer VI, Zapata A, and Heidbreder CA (2001) Modulation of the behavioral and neurochemical effects of psychostimulants by kappa-opioid receptor systems. Ann N Y Acad Sci 937:50–73.

Siddall P.J., McClelland J.M., Rutkowski S.B., Cousins M.J. (2003). A longitudinal study of the prevalence and characteristics of pain in the first 5 years following spinal cord injury. Pain, 103: 249-257.

Siddall, P.J., & Loeser, J.D. (2001). Pain following spinal cord injury. Spinal Cord, 39: 63-73.

Silverman, S.M. (2009). Opioid0induced hyperalgesia: clinical implications for the pain practitioner. Pain Physician, 12 (3): 679-684.

Sindrup, S.H. & Jensen, T.S. (1999). Efficacy of pharmacological treatments of neuropathic pain: an update and effect related to mechanism of drug action. Pain, 83: 389-400.

Skoubis, P.D., Matthes, H.W., Walwyn, W.M., Kieffer, B.L. & Maidment, N.T. (2001). Naloxone fails to produce conditioned place aversion in mu-opioid receptor knowckout mice. Neuroscience, 106: 757-763.

Smith, H.S. & Meek, P.D. (2011). Pain responsiveness to opioids: central versus peripheral neuropathic pain. J Opioid Manage, 7 (5): 391-400.

Smith, H.S. & Meek, P.D. (2012). Pain responsiveness to opioids: central versus peripheral neuropathic pain. J Opioid Manage, 391-401.

Soulet, D. & Rivest, S. (2008). Microglia. Curr Biol, 18 (12): R506-508.

Spanagel, R., Herz, A., & Shippenberg, T.S. (1990). The effects of opioid peptides on dopamine release in the nucleus accumbens: an in vivo microdialysis study. J Neurochem 55: 1734-1740.

Springer, J.E., Azbill, R.D., & Knapp, P.E. (1999). Activation of the caspase-3 apoptotic cascade in traumatic spinal cord injury. Nat Med, 5 (8): 943-946.

Stanley, L.C., Mrak, R.E., Woody, R.C., Perrot, L.J., Zhang, S., Marshak, D.R., Nelson, S.J., & Griffin, W.S. (1994). Glial cytokines as neuropathogenic factors in HIV infection: pathogenic similarities to Alzheimer's disease. J Neuropathol Exp Neurol, 53: 231-238.

Störmer, S., Gerner, H.J., Grüninger, W., Metzmacher, K., Föllinger, S., Wienke, C., Aldinger, W., Walker, N., Zimmermann, M., & Paeslack, V. (1997). Chronic pain/ dysaesthesiae in spinal cord injury patients: results of a multicentre study. Spinal Cord, 35 (7): 446-455.

Suter, M.R., Wen, Y.R., Decosterd, I., & Ji, R.R. (2007). Do glial cells control pain? Neuron Glia Biol, 3 (3): 255-268.

Suzuki, T., Kishimoto, Y., Misawa, M., Magase, H., & Takeda, F. (1999). Role of the kappa-opioid system in the attenuation of the morphine-induced place preference under chronic pain. Life Sci, 64 (1): PL1-7.

Svensson, M., Eriksson, N.P., & Aldskogius, H. (1993). Evidence for activation of

astrocytes via reactive microglial cells following hypoglossal nerve transection. J Neurosci Res, 35: 373-381.

Tanga, F.Y., Nutile-McMenemy, N., & DeLeo, J.A. (2005). The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. Proc Natl Acad Sci U S A, 102 (16): 5856–5861.

Tawfic, Q.A., Faris, A.S., & Date, R.R. (2013). The dilemma of opioid-induced hyperalgesia and tolerance in chronic opioid therapy. Sultan Qaboos Univ Med J, 13 (1): 185-187.

Teasell, R.W., Mehta, S., Aubut, J.A., Foulon, B., Wolfe, D.L., Hsieh, J.T., Townson, A.F., Short, C., & Spinal Cord Injury Rehabilitation Evidence Research Team. (2010). A systematic review of pharmacologic treatments of pain after spinal cord injury. Arch Phys Med Rehabil, 91 (5): 816-831.

Trang, T., Beggs, S., & Salter, S. (2011). Brain-derived neurotrophic factor from microglia: a molecular substrate for neuropathic pain. Neuron Glia Biology, 7 (1): 99-108.

Trescot, A.M., Helm, S., Hansen, H., Benyamin, R., Glaser, S.E., Aslaka, R., Patel, S., &Manchikanti, L. (2008). Opioids in the management of chronic non-cancer pain: an update of American Society of the Interventional Pain Physicians' (ASIPP) guidelines. Pain Physician, 11: S5-S62.

Vallejo, R., Tilley, D.M., Vogel, L., & Benyamin, R. (2010). The role of glial and the immune system in the development and maintenance of neuropathic pain. Pain Pract, 10: 167-184.

Vanderah, T.W., Gardell, L.R., Burgess, S.E., Ibrahim, M., Bogrul, A., Zong, C.M., Zhang, E.T., Malan Jr., T.P., Ossipov, M.H., Lai, J., & Porreca, F. (2000). Dynorphin promotes abnormal pain and spinal opioid antinociceptive tolerance. J Neurosci, 20 (18), 7074-7079.

Vanderah, T.W., Ossipov, M.H., Lai, J., Malan, Jr. T.P., Porreca, F. (2001). Mechanisms of opioid-induced pain and antinociceptive tolerance: descending facilitation and spinal dynorphin. Pain, 92 (1-2): 5-9.

Wang, C.C., Fang, K.M., Yang, C.S., & Tzeng, S.F. (2009). Reactive oxygen species-induced cell death of rat primary astrocytes through mitochondria-mediated mechanism. J Cell Biochem, 107: 933-943.

Wang, X.M. et al., (1999) Acute intermittent morphine increases preprodynorphin and kappa opioid receptor mRNA levels in the rat brain. Brain Res Mol Brain Res 66, 184-187.

Wang, Y-H., Sun, J-F., Chi, Z-Q., Liu, J-G (2010). The role of k-opioid receptor activation in mediating antinociception and addiction. Acta Pharmacologica Sinica 31: 1065-1070.

Wang, Y-H., Sun, J-F., Chi, Z-Q., Liu, J-G (2010). The role of k-opioid receptor activation in mediating antinociception and addiction. Acta Pharmacologica Sinica 31: 1065-1070.

Wang, Z., Gardell, L.R., Ossipov, M.H., Vanderah, T.W., Brennan, M.B., Hochgeschwender, U., Hruby, V.J., Malan, T.P., Jr., Lai, J., & Porreca, F. (2001). Pronociceptive actions of dynorphin maintain chronic neuropathic pain.

J Neurosci, 21 (5): 1779-1786.

Warms, C.A., Turner, J.A., Marshall, H.M. & Cardenas, D.D. (2002) Treatments for
chronic pain associated with spinal cord injuries: many are tried, few are
helpful. Clin J Pain 18, 155-163.

Watkins, L.R. (2004). A role for pro-inflammatory cytokines and fractalkine in
analgesia, tolerance, and subsequent pain facilitation induced by chronic
intrathecal morphine. J Neurosci, 24 (33): 7353-7365.

Watkins, L.R., Hutchinson, M.R., Ledeboer, A., Wieseler-Frank, J., Milligan, E.D., &
Maier, S.F. (2007). Glia as the "bad guys": implications for improving clinical
pain control and the clinical utility of opioids. Brain Behav Immun, 21 (2): 131-
146.

Watkins, L.R., Milligan, E.D., & Maier, S.F. (2003). Glial pro-inflammatory cytokines
mediate exaggerated pain states: implications for clinical pain. Adv Exp Med
Biol, 521: 1-21.

Weaver, L.C., Marsh, D.R., Gris, D., Brown, A., & Dekaban, G.A. (2006). Autonomic
dysreflexia after spinal cord injury: central mechanisms and strategies for
prevention. Prog Brain Res, 152: 245-263.

Weaver, L.C., Marsh, D.R., Gris, D., Meakin, S.O., & Dekaban, G.A. (2002). Central
mechanisms for autonomic dysreflexia after spinal cord injury. Prog Brain Res,
137: 83-95.

Wee, S., Specio, S.E., & Koob, G.F. (2007). Effects of dose and session duration on
cocaine self-administration in rats. J Pharmacol Exp Ther, 320 (3): 1134-1143.

Weed, M.R., Carruth, L.M., Adams, R.J., Ator, N.A., & Hienz, R.D. (2006). Morphine withdrawal dramatically reduced lymphocytes in morphine-dependent macaques. J Neuroimmune Pharmacol, 1: 250-259.

Wetering, E.J., Lemmens, K.M., Neiboer, A.P., & Huijsman, R. (2010). Cognitive and behavioral interventions for the management of chronic neuropathic pain in adults- a systematic review. Eur J Pain, 14 (7): 670-681.

Widerstron-Noga, E.G., & Turk. D.C. (2003). Types and effectiveness of treatments used by people with chronic pain associated with spinal cord injuries: influence of pain and psychosocial characteristics. Spinal Cord, 41: 600-609.

Woller, S.A. & Hook, M.A. (2013). Opioid administration following spinal cord injury: implications for pain and locomotor recovery. Exp Neurol, [Epub ahead of print]

Woller, S.A. Moreno, G.L., Hart, N., Wellman, P.J., Grau, J.W., & Hook, M.A. (2012). Analgesia or addiction: implications for morphine use after spinal cord injury. J Neurotrauma, 29 (8): 1650-1662.

Woods, A. & Zangen, A. (2001). A direct chemical interaction between dynorphin and excitatory amino acids. Neurochem Res, 26 (4): 395-400.

Xu & Yaksh, T.L. (2011). A brief comparison on the pathophysiology of inflammatory versus neuropathic pain.

Xu, G-Y, Liu S., Hughes, M.G., & McAdoo, D.J. (2008) Glutamate-induced losses of oligodendrocytes and neurons and activation of caspase-3 in the rat spinal cord. Neuroscience, 153 (4): 1034-1047.

Xu, G.Y., Hughes, M.G., Ye, Z., Hulsbosch, C.E., & McAdoo, D.J. (2004).
Concentrations of glutamate release following spinal cord injury kill
oligodendrocytes in the spinal cord. Exp Neurol, 187 (2), 329-336.

Yao, A-h., Jia, L-y., Zhang, Y-k., Ma, Q-r., Cheng, P., Liu, L., Ju, G., & Kuang, F.
(2012). Early blockade of TLRs MyD88-dependent pathway may reduce
secondary spinal cord injury in the rats. Evidence-Based Complementary and
Alternative Medicine, 2012: 591298.

Yu, W., Hao, J., Xu, X., & Wiesenfeld-Hallin, Z. (1997a). Comparison of the anti-
allodynic and antinociceptive effects of systemic, intrathecal, and
intracerebocentricular morphine in a rat model of central neuropathic pain. Eur J
Pain 1, 17-29.

Yu, W., Hao, J., Xu, X., & Wiesenfeld-Hallin, Z. (1997b). The development of
morphine tolerance and dependence in rats with chronic pain. Brain Res, 756:
141-146.

Zhang L, Berta T, Xu ZZ, Liu T, Park JY, Ji RR (2011). TNF-α contributes to spinal
cord synaptic plasticity and inflammatory pain: distinct role of TNF receptor
subtypes 1 and 2. Pain, 152: 419-427.

Zhang, X., Bao, L. Arvidsson, U., Elde, R., & Hökfelt, T. (1998b). Localization and
regulation of the delta-opioid receptor in dorsal root ganglia and spinal cord of
the rat and monkey: evidence for association with the membrane of large dense-
core vesicles. Neuroscience, 82 (4): 1225-1242.

Zhang, X., Bao, L., Shi, T.J., Ju., G., Elde, R., & Hökfelt, T. (1998c). Down-regulation

of mu-opioid receptors in rat and monkey dorsal root ganglion neurons and spinal cord after peripheral axotomy. Neuroscience, 82 (1): 223-240.

Zhang, Y., Chen, Q., & Yu, L.-C. (2008). Morphine: a protective or destructive role in neurons? The Neuroscientist, 14: 561.