Morphine Following Peripheral Nerve Injury Potentiates the Magnitude and Duration of Subsequent Allodynia: A Role of Toll-like Receptor 4

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April 4, 2013
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Abstract

Opioids are a frontline clinical therapy for the treatment of neuropathic pain. Yet, opioid use is fraught with many negative side effects. This study investigates the effect of repeated morphine following peripheral nerve constriction on mechanical allodynia and central immune activation in rats. Mild chronic constriction injury (CCI) was performed on Sprague Dawley rats by tying one chromic gut suture around the sciatic nerve. Following surgery, morphine (5 mg/kg) was administered twice daily for 5 days. Mechanical allodynia was assessed using the von Frey method. The magnitude of CCI-allodynia was potentiated by morphine at 1 day post-dosing, and the effect remained at 35 days post-dosing (n=11-12, p<0.001). The duration of CCI-allodynia was also potentiated by subsequent morphine in Fischer 344 rats (n=6, p<0.001 at day 35 post-dosing). Immunohistochemistry was used in the dorsal horn of the lumbar spinal cord at 1 and 21 days post-dosing in Sprague Dawley rats. No interaction between CCI and morphine was found to increase expression of either CD11b or GFAP. In the Sprague Dawley rats, intrathecal blockade of TLR4 using (+)-naloxone during morphine administration inhibited subsequent potentiation of allodynia (n=6, p<0.05 at day 28 post-dosing). Additionally, acute intrathecal blockade of IL-1 receptors 44 days post-morphine transiently reversed established potentiation of allodynia (n=4, p<0.001 at 1, 2, 4, and 6 hours post-dosing). Together, these findings suggest that repeated morphine following nerve injury potentiates both the magnitude and duration of allodynia, at least in part via TLR4 and the enduring release of IL-1.
Introduction

Millions of people worldwide experience the impact of neuropathic pain (Dworkin et al., 2007). In the United States alone, there are over five million people affected, with a cost of over $100 billion annually in lost productivity and medical costs. (Tawfik et al., 2007, Levine 1998). Symptoms include hypersensitivity to non-noxious stimuli (allodynia) and to noxious stimuli (hyperalgesia). Patients with neuropathic pain may experience reduced mobility and reduced ability to work (O’Connor 2009). These debilitating effects result in a substantial economic burden on both individual patients and on society (O’Connor 2009, Dworkin et al., 2007). The treatment of chronic pain is complex due to psychological, biological, and sociological factors. All therapies currently available to patients with neuropathic pain are symptom-based, neuronally targeted, and include treatment with prescription, temporary pain-relief drugs.

Pain can be categorized as acute nociceptive, chronic inflammatory, or neuropathic. Nociception, the process by which noxious stimuli are detected by the periphery and processed by the central nervous system, is a critical evolutionary function that alarms an organism of potentially tissue-damaging conditions (Milligan and Watkins 2009). Acute nociceptive pain can be the response to mechanical, thermal, or chemical stimuli, which in turn can be external (such as a hot stove) or internal (such as arthritic joint pain). A key aspect of nociceptive pain is that it occurs in the presence, but not absence, of such stimuli. In contrast, chronic inflammatory pain results from ongoing excitation of primary nociceptive neurons after tissue injury or inflammation. Such pain is accompanied by peripheral sensitization, which involves decreased activation thresholds at pain-sensing terminals, and central sensitization, which involves neuronal and chemical changes in pain-processing afferents (Costigan et al., 2009). Neuropathic pain, the focus of the present study, is initiated by damage to, or disease of, the nervous system.
Insults to the nervous system which may produce neuropathic pain include lesion or compression of the spinal cord or peripheral nerves, painful diabetic neuropathy, and post-herpetic neuralgia (Campbell and Meyer 2006). The mechanisms of neuropathic pain development and maintenance will be discussed in greater detail below. Opioids are one of our frontline clinical therapies for both acute and chronic pain. However, despite the high efficacy of initial opioid analgesia, this therapy is fraught with a variety of serious problems. For instance, opioid use is associated with tolerance, dependence, constipation, respiratory depression, and opioid-induced hyperalgesia (Hutchinson et al 2011, Angst and Clark 2006). CNS neuroinflammation has recently been understood to underlie such deleterious effects (Hutchinson et al. 2007). Due to a shifting analysis of the risk-benefit ratio of clinical opioid use, the need for more systematic, long term studies of human safety and opioid use in neuropathic pain has been recognized (Dworkin et al., 2007). The present study aims to identify additional factors that may limit the clinical application of opioids.

**Background**

**Nociceptive Processing**

Nociceptors, the primary fibers that innervate the head and body, are responsible for the detection of noxious stimuli. The cell bodies, or somas, of nociceptors innervating the body lie in the dorsal root ganglion (DRG), while the somas of nociceptors for the head are in trigeminal ganglia. The sensation of pain is initiated by the peripheral terminals of these fibers, which detect the mechanical, thermal, or chemical aspects of noxious stimuli. Two types of primary fibers are involved in nociception: A∆ fibers, which are myelinated, rapidly conducting fibers associated
with the sharp “first pain” sensation, and C fibers, which are larger, unmyelinated, slow-conducting fibers associated with the dull, longer-lasting “second pain” sensation (Woolf and Ma, 2007, Purves et al., 2008). The somatosensory Aβ and Aα fibers, in contrast, are large diameter myelinated fibers that are activated by non-painful stimuli and do not normally contribute to pain. Stimulation of the peripheral terminals of nociceptors with a sufficient stimulus activates a variety of ion channel types, which initiates action potentials. These action potentials are conducted toward the dorsal horn of the spinal cord, where the first synapses of the sensory pathway lie.

Primary afferent fibers form the presynaptic terminal of a synapse with spinal second order neurons or interneurons. Spinal neurons receive primary afferent inputs and project via the spinothalamic tract, the main second order ascending pathway. The thalamus is the target of these projections, and it relays pain information to somatosensory cortical areas, the insula, the prefrontal cortex, and the cerebellum. It is in these areas that the intensity, context, pattern, and affective components of pain information are encoded (Price 2000, Purves et al., 2008).

After cortical, insular, and cerebellar pain processing occurs, information is relayed back to the spinal cord via complex tertiary descending pathways. Critical in these pathways are the periaqueductal grey, the rostral ventromedial medulla, and the locus coeruleus (Julius and Basbaum, 2001). These supraspinal regions are capable of providing both anti- and pro-nociceptive modulation to pain information, and ultimately project back to the second-order neurons of the dorsal horn of the spinal cord (Julius and Basbaum, 2001). Thus, the second-order spinal neurons provide a critical junction between primary ascending afferents and complex supraspinal descending modulation. The final perceived sensations of pain at the site of a noxious stimulus are the result of events in these processing and modulatory pathways.
Neuropathic pain: Neuronal mechanisms

Dysregulation of normal nociceptive signaling can give rise to the sensations of neuropathic pain. Such sensations include pain in the absence of any external stimuli, as well as the phenomena of allodynia and hyperalgesia. The neuronal mechanisms of neuropathic pain are generally well understood.

Abnormal patterns of activation within the nociception pathway contribute to the sensations of neuropathic pain. Such ectopic activation occurs via increased sodium channel expression. In dorsal root ganglia, upregulation of Nav1.3 sodium channels has been observed after nerve injury (Hains et al. 2003). Other sodium channels, such as Nav1.8 - which contributes to heightened membrane excitability - and Nav1.7 have been implicated in neuropathic pain phenotypes (Costigan et al., 2009). Additionally, increased phosphorylated Nav1.8 channels on nociceptive neurons contribute to membrane excitability in neuropathic pain (Costigan et al., 2009). RasGTPase signaling in injured nociceptive neurons contributes to altered expression of such ion channels (Yudin et al. 2008).

Lowered activation thresholds in Aβ fibers produce increased Aβ signaling in response to stimuli that these fibers would not normally detect. Allodynia is thought to be the consequence of such signaling. (Devigili et al. 2008). Decreased activity of γ-aminobutyric acid (GABA)-ergic inhibitory interneurons in neuropathic pain contributes to increased activation of second order spinal neurons in response to these primary afferents. Such disinhibition is persistent in neuropathic pain due to death and damage of spinal interneurons (Scholz et al. 2005, Costigan et al. 2009).
Synaptic changes in locations throughout the nociceptive pathway are implicated in the transition from normal to pathological pain processing. Both increases in ion channel density and decreased action potential thresholds are observed in peripheral nociceptor terminals. In the dorsal horn, increased NMDA (voltage-dependent glutamate receptors permeable to Na\(^+\), K\(^+\), and Ca\(^{2+}\) ions) receptor phosphorylation and trafficking of these receptors to the plasma membrane is observed (Ultenius et al. 2006, Costingan et al. 2009).

Structural changes in afferent pathways and in the dorsal horn occur after peripheral nerve injury. Nociceptive neurons are stimulated to grow following nerve injury due to increased expression of a host of pro-neuroregeneration genes (Costigan et al., 2002). The addition of Aβ synapses in the dorsal horn, which contributes to pain-like responses of non-painful stimuli (allodynia), may be a result of such gene expression (Costigan et al., 2009). In addition to axonal regeneration in nociceptive neurons, extensive neuron death also contributes to structural changes. Spinal neuron death may be the result of glutamate excitotoxicity following extensive activation of surrounding cells (Costigan et al., 2009).

Neuropathic Pain: Neuroimmune Mechanisms

Current pain theory indicates that investigating only the neuronal aspects of neuropathic pain is an overly-simplistic and generally incomplete approach (Scholz and Woolf, 2007). In recent years, research into the neuronal mechanisms of neuropathic pain has been complemented by a rapidly growing interest in neuroimmune mechanisms. Indeed, the largest advances in the understanding of neuropathic pain in the last decade have come from a neuroimmune perspective. Thus, the following sections describe the currently known important cell types
implicated in neuroimmune mechanisms of pain, the activation of these cell types, and the consequences of activation on inflammation and neuropathic pain.

**Glia**

The non-neuronal cells of the brain, called glia, vastly outnumber neurons themselves. There are many types of glial cells with a variety of morphologies and functions throughout the CNS. In the fields of neuroimmunology and neuropathic pain, the best studied glial sub-types are microglia and astrocytes. These cell types are therefore discussed in detail below.

Two categories of microglia are commonly distinguished: perivascular and resident microglia. Both are of hematopoietic origin (Neumann and Wekerle, 2013). While perivascular microglia can be replenished via the bone marrow, resident microglia are generally considered to be un-replenishable (however, monocytes may traffic into the CNS under specific conditions and later differentiate into microglia (Zhang et al., 2006)). Resident microglia are commonly referred to as the macrophages of the CNS. Indeed, microglia serve in healthy nervous tissue to recognize molecular patterns associated with non-pathological cell death, and to phagocytose cellular debris (Neumann and Wekerle, 2013). Although microglia are constantly surveying the extracellular space for the presence of pathogens, they are inactive in the release of neuroexcitatory substances in healthy tissue (Watkins et al., 2009, Davalos et al., 2005).

Astrocytes, the most common glial cell, arise from the neuroectoderm (Haydon, 2001). Astrocytes are critical in healthy nervous tissue for maintaining the delicate conditions of the synaptic environment. They are capable of both sending and receiving signals to neurons at synapses and to other glia. Thus, in contrast to microglia, astrocytes are considered active participants in neurotransmission under physiological conditions (Haydon 2001). Astrocytic
processes associated with axons and synapses are responsible for the intake of potassium ions from the extracellular space following an action potential (Weinstein et al., 1991). They are also critical for the uptake of excess neurotransmitters in the synaptic cleft. Astrocytes may form gap junctions with neurons, which may locally modulate neuronal membrane potential and action potential frequency (Haydon 2001). Astrocytes also express a variety of neurotransmitter receptors, including ionotropic and metabotropic glutamate receptors, purinergic receptors, and substance P receptors (Porter and McCarthy, 1997). Thus, astrocytes in close contact with synapses can be stimulated by neurotransmitter release.

**Glial activation**

Stimulation of microglia and astrocytes results in a cascade of activity commonly referred to as glial activation. Activation of these two types of cells is associated with different phases of neuropathic pain: microglial activation is more central to the initiation phase, while activation of astrocytes following microglial activation, is associated with the later maintenance phases (Raghavendra et al., 2003). Microglial activation frequently leads to the phosphorylation of the MAPK p38 and subsequent intracellular signaling (Ji and Suter 2007). In astrocytes, activation results in the activation of ERK and JNK intracellular signaling pathways (among others). Microglial activation is commonly quantified in tissue by the expression of the protein CD11b, which associates with CD18 on microglial cell membranes to form the complement receptor CR3 (Neumann and Wekerle, 2013). In the innate immune system, CR3 is involved in cell-mediated cytotoxicity and phagocytosis. Quantification of activated astrocytes is commonly done by measuring the expression of glial fibrillary acidic protein, or GFAP. GFAP has been found to be critical in the extension of astrocytic “arms,” or processes, which commonly
surround neurons and mediate the composition of the neuronal microenvironment (Weinstein et al., 1991).

Glial activation ultimately involves the release of pro-inflammatory molecules following activation of the transcription factor NFκB, increased cell motility, and increased expression and/or trafficking of cytokine receptors to the cell membrane (Hutchinson et al., 2011). Like peripheral macrophages, microglia are activated by pathogen-associated molecular patterns, or PAMPs, such as the bacterial endotoxin lipopolysaccharide (LPS). Other mechanisms of glial activation include activation via neuron-to-glia signaling and activation by damage/danger-associated molecular patterns (DAMPs). These mechanisms are discussed in detail below.

**Glial activation via neuron-to-glia signaling**

There are likely many forms of neuron-to-glia signals responsible for the onset, development, and maintenance of neuropathic pain. Some of the best characterized of these signals are matrix metalloproteinases, the chemokine fractalkine and its receptor, and the chemokine/receptor pair CCL2/CCR2. These signals are focused on below in the consideration of glia-activating factors released by neurons.

An important category of microglial-activating molecules are matrix metalloproteinases, or MMP’s, which are released in the CNS by neurons and which activate glia during neuropathic pain. MMP’s activate the microglial MAPK p38, which is involved in intracellular signaling cascades that result in pro-inflammatory cytokine expression in the dorsal horn of the spinal cord in models of peripheral nerve injury in rats (Svensson et al., 2005, Yong and Guoping 2008). The timing and isotype of MMP released is important in determining its consequence on glial cells (Kawasaki et al., 2008). Both MMP$_2$ and MMP$_9$ induce the cleavage of pro-interleukin-1 beta
(IL-1β), a critical mediator of the proinflammatory response, but MMP$_2$-induced cleavage results in astrocyte activation during the ongoing phases of neuropathic pain, while MMP$_9$-induced cleavage activates p38 in microglia during the onset phase (Kawasaki et al., 2008). Thus, MMPs contribute to both the onset and maintenance of neuropathic pain via distinct patterns of release and activity (Milligan and Watkins 2009).

Chemokines are an important component of neuron-to-glia signaling in the ongoing phases of chronic pain (Milligan and Watkins 2009). In the peripheral immune system, chemokine gradients serve to attract both leukocytes and lymphocytes toward sites of inflammation. In the CNS, chemokines and their receptors are expressed heterogeneously depending on the surrounding circuitry and on neuroinflammatory conditions (Hutchinson et al., 2011). Both chemokine release and the appropriately timed expression of chemokine receptors on the intended recipient cells are critical in the mediation of this process. Importantly, chemokines and their receptors are variably expressed by both neurons and glia in the CNS (Tran and Miller 2003). The chemokine CX$_3$CL1, also known as fractalkine, is an example of a chemokine released by neurons and received by glia following peripheral nerve injury (Milligan and Watkins 2009). Fractalkine is constitutively expressed by neurons in the CNS, where it is bound to the extracellular membrane by a mucin stalk (Verge et al., 2004). When microglia are activated following nerve injury, they increase the expression and release of the protease cathepsin S (Clark et al., 2007). Cathepsin S serves to cleave membrane-bound fractalkine. This cleavage of fractalkine induces further microglial activation via the fractalkine receptor CX$_3$CR1. Thus, release of cathepsin S by activated microglia initiates a positive feedback loop in the onset of neuropathic pain, later contributing to pain maintenance (Clark et al., 2007). In rats, intrathecal cathepsin S leads to an increase in activated p38 MAPK in spinal microglia, and is
sufficient to induce allodynia and hyperalgesia in naïve rats (Clark et al., 2007). Fractalkine expression in the spinal cord and dorsal root ganglion is unchanged following peripheral nerve injury in rats. Expression of CX3CR1, on the other hand, is upregulated in microglia in DRGs and spinal tissue in response to such injury (Milligan et al., 2005). CX3CR1 expression on microglia is upregulated along circuits of normal nociception in spinal tissue, suggesting a mechanism of dysregulation of normal nociception systems in neuropathic pain (Verge et al., 2004). Intrathecal administration of fractalkine produces both mechanical allodynia and increased phosphorylation of p38 MAPK in spinal microglia (Milligan et al., 2004).

Another chemokine, CCL2, is expressed in DRG and spinal neurons following peripheral nerve injury but not under healthy conditions (Zhang et al., 2007). CCL2 and its receptor CCR2 are both expressed in sensory neurons following peripheral nerve injury (Zhang et al., 2007). CCR2 is also expressed on spinal microglia (Jung et al., 2008). CCL2 released by neurons in the dorsal horn activates microglia and induces the infiltration of monocytes into the central nervous system, which may then differentiate into microglia (Zhang et al., 2007). CCL2-induced microglial activation in the dorsal horn occurs in the early phase of neuropathy following peripheral nerve injury (Zhang and De Koninck, 2006). Astrocytes are also activated by CCL2 release in the dorsal horn, and such activation persists significantly longer than CCL2-induced microglial activation (Zhang and De Koninck, 2006). CCL2 activation of astrocytes is therefore proposed to be involved in the maintenance of the neuropathic pain state, while similarly activated microglia may be involved in the early onset phases.
Glial activation via endogenous danger signals

A second mechanism by which glia are activated is via response to endogenous danger signals. Damage/danger-associated molecular pattern molecules, or DAMPs, are produced endogenously, and can initiate or maintain an immune response in the absence of infectious agents.

Damage, apoptosis, or necrosis of cells can produce massive amounts of DAMPs. DAMPs can include misfolded proteins, heat-shock proteins, extracellular DNA, nuclear protein HMGB1, and other components of cells that are not accessible to other cells under normal conditions (Bianchi 2007). Microglia, which are constantly surveying the extracellular fluid for abnormal molecular patterns, become activated upon the binding of DAMPs to their extracellular receptors (Davalos et al., 2005).

ATP, released in mass quantity by apoptotic cells following tissue damage, is a microglial-activating DAMP. Microglia, but not neurons or astrocytes, express the ionotropic purinergic receptors P2X$_4$R and P2X$_7$R (Tsuda et al., 2003, Bartlett et al., 2013). Expression of these receptors becomes significantly upregulated in spinal cord microglia following peripheral nerve injury (Tsuda et al., 2003). ATP binding on P2X$_4$R induces microglia to release brain-derived neurotrophic factor (BDNF), which induces spinal disinhibition in neuropathic pain (Coull et al., 2005). Although considered a DAMP, ATP is also released by neurons as a neurotransmitter (Milligan and Watkins 2009). Neuronal release of ATP stimulates both microglia and astrocytes, and such stimulation has effects on both neurons and on other glia nearby (Milligan and Watkins 2009).
The presence of some DAMPs is detected by the innate immune receptors called toll-like receptors (TLRs). TLRs can be expressed on the plasma membrane of cells, or on the membranes of endosomes. Of these receptors, TLR4 is the best studied in the context of neuropathic pain (Watkins et al., 2009). TLR4 classically binds endotoxin, or lipopolysaccharide (LPS), and is important in the recognition of extracellular bacterial infections. In the CNS, TLR4 is primarily expressed by microglia (Hutchinson et al., 2008a). When LPS or molecules signifying tissue damage bind TLR4 and TLR4 accessory molecules on microglia, a signaling cascade is initiated that causes microglial activation (Laird et al., 2009). TLR4 signaling through the adaptor protein myeloid differentiation primary response gene 88 (MyD88) or through TIR-domain-containing adapter-inducing interferon-β (TRIF) ultimately leads to the liberation of the transcription factor NFκB from its cytosolic inhibitor IκB, allowing for the transcription and release of proinflammatory mediators – in particular, proinflammatory cytokines (Laird et al., 2009). Thus, microglial activation via TLR4 serves an important role in the early response to tissue damage and in the onset phase of neuropathic pain. Astrocytes may express TLR4 in the presence of established neuroinflammation (Hutchinson et al., 2008a). As will be discussed in greater detail below, several pharmacological compounds have been identified which bind and activate TLR4, including some opioids and their metabolites (Lewis et al., 2010, Hutchinson et al., 2011). Blockade of TLR4 signaling can attenuate the duration of alldynia in rodent models of peripheral nerve injury, demonstrating that TLR4 is central in the development of neuroinflammation (Hutchinson et al., 2008a).

**Glial activation by cytokines**

Cytokines released by glia and immune cells can also serve to further activate glia. The p38 MAPK and ERK signaling cascades in microglia and astrocytes can become activated by IL-
1β and tumor-necrosis factor alpha (TNFα) (Scholz and Woolf 2007). This cytokine-mediated glial activation leads to a further increase in cytokine production as well as increases in phagocytic activity (Milligan and Watkins et al., 2009).

**Key ways glial-derived mediators alter neuronal signaling**

Cytokines and other molecules released by activated glia interact with neurons to alter neuronal signaling, and, in the case of neuropathic pain, change the way pain is processed in the nervous system. Known effects include enhanced neuronal excitability, increased number and conductance of AMPA (ionotropic glutamate receptors permeable to Na\(^+\) ions, and sometimes permeable to Ca\(^{2+}\) ions) and NMDA receptors, downregulation of GABA receptors (either ionotrophic or metabotropic receptors that are generally inhibitory), downregulation of glutamate transporters on astrocytes, and increased sensory afferent neurotransmitter release (Milligan and Watkins, 2009).

The cytokine TNFα, released by activated glia in the CNS, is responsible for sustained changes in synaptic strength. Hippocampal neurons in culture treated with TNFα exhibit a significant increase in surface expression of AMPA receptors, implicating a role of this cytokine in AMPAR trafficking (Beattie et al., 2002). The increase in AMPA receptors observed following TNFα stimulation of hippocampal neurons is accompanied by a change in the subunit composition of some of these receptors. Membrane expression of AMPA receptors lacking the GluA2 subunit is increased following TNFα stimulation. These AMPA receptors are permeable to Ca\(^{2+}\) ions, and may contribute to increased excitotoxicity in response to excess synaptic glutamate (Ogoshi et al. 2005, Yin et al. 2012). TNFα stimulation also induces an increase in the frequency of miniature excitatory post-synaptic potentials (mEPSCs), the change in post-
synaptic membrane potential caused by the release of a single synaptic vesicle) in cultured neurons (Beattie et al., 2002). Research suggests that astrocytes are particularly important in TNFα-induced synaptic modulation (Stellwagen and Malenka 2006). Indeed, reports indicate that levels of glial TNFα play a major role in the development and maintenance of LTP and LTD in vitro (Stellwagen and Malenka 2006). Such changes in synaptic plasticity may play a role in the dysregulation of normal nociceptive circuits in neuropathic pain.

IL-1β release in the spinal cord has been shown to phosphorylate the NMDA receptor subunit NR-1, which is known to facilitate the transmission of nociceptive information in neuropathic pain (Zhang et al., 2008). IL-1β also induces an increase in Ca^{2+} influx through NMDA receptors, perhaps via the phosphorylation of NR2A/B receptor subunits (Viviani et al., 2003). Enhancement of NMDAR function via IL-1β may be critical to the maintenance of neuropathic pain. Increased NMDAR opening is associated with an increase in nitric oxide and prostaglandin E_2 production in neurons, which amplify neuronal excitability, as well as an increase in neuronal plasticity (Zhang et al., 2008).

As discussed in above sections, astrocytes mediate synaptic transmission in part by eliminating glutamate from the synapse following its release. In this way, astrocytes play a role in preventing prolonged neuronal activation and glutamate excitotoxicity. Prolonged cytokine release by chronically activated astrocytes is implicated in downregulating the glutamate transporters GLT-1 (L-glutamate transporter) and GLAST (glutamate-aspartate transporter) on astrocytes, allowing for an increase in excitatory synaptic transmission (Sung et al., 2003).

As mentioned above, spinal microglia become activated by neuronally-released ATP via the purinergic ionotropic receptor P2X_4 to release BDNF (Coull et al., 2005). BDNF activity on
spinal pain-processing neurons inhibits GABA signaling by inverting the polarity of GABA currents, causing GABA stimulation to result in depolarization rather than hyperpolarization (Coull et al., 2005). Delivery of ATP-activated microglia to the lumbar spinal cord is sufficient to produce allodynia in naïve rats (Coull et al., 2005). Recent findings suggest that BDNF from ATP-stimulated microglia signals on neurons to cause a downregulation of the $K^{+}/Cl^{-}$ co-transporter KCC2. Downregulation of KCC2 results in an altered anion equilibrium potential, which impairs the normally inhibitory response of neurons to GABA (Ferrini et al., 2013). Thus, BDNF-induces disinhibition, which plays an important role in the development of neuropathic pain (Coull et al., 2005).

**Pain and Opioids**

As mentioned above, opioids are one of the frontline therapies in the treatment of both acute and chronic pain. For millennia, opioids have been used for their analgesic properties. The efficacy of opioid analgesia is provided by opioid activity on neuronal receptors. Recently, opioids have also been shown to activate glia, with the proinflammatory consequences discussed above. The activity of opioids on both neurons and glia are discussed below, as well as the mechanisms by which opioids produce both analgesic and proinflammatory responses.

**Opioids: A neuronal perspective**

Three opioid receptor types are commonly distinguished: $\mu$, $\kappa$, and $\delta$. Classical opioid receptors are expressed on neurons in primary afferent pain fibers, the dorsal horn of the spinal cord, insula, amygdala, hypothalamus, hippocampus, and rostral ventromedial medulla (Hutchinson et al., 2011). Opioid receptors in the periaqueductal gray region of the midbrain, where integration of nociceptive information from the cortex and other areas occur, are thought
to be critical in the mediation of analgesia (Hutchinson 2011, Pavlovic et al., 1996). The rostral ventromedial medulla is a key player in modulating the effects of opioids on dorsal horn neurons. Interestingly, opioids in the rostral ventromedial medulla can have inhibitory or excitatory consequences (Hutchinson et al., 2011). Opioid binding on classical µ opioid receptors on neurons results in reduced excitatory amino acid and neuropeptide release and direct post-synaptic inhibition of neurons activated by harmful stimuli. Such activity is responsible for the potent efficacy of morphine analgesia. Classical µ opioid receptors are G-protein coupled, and bind opioids with high affinity. Classical opioid receptor antagonists such as naloxone and naltrexone inhibit opioid binding, and thus block analgesia.

Despite their efficacy in treating immediate pain symptoms and their widespread use, opioids come with a number of adverse effects, including tolerance, dependence, constipation, and respiratory depression. Additionally, a paradoxical opioid response has recently been well-characterized: opioid induced hyperalgesia (OIH). OIH refers to a change in pain sensitivity following opioid use, and has been clinically studied outside of the context of pain management (Angst and Clark, 2006). Patients undergoing methadone dependence therapy exhibit lower than expected pain thresholds (Doverty et al., 2001). While the mechanisms of OIH are not fully understood, it has been generally considered a neuronal phenomenon.

**Non-neuronal Opioid Activity**

Until recently, opioid pharmacology has been limited to the discussion of binding on classical neuronal receptors, discussed above. Yet, another critical action of morphine is its role in activating receptors on glia. Morphine has been demonstrated to bind to the TLR4 accessory protein myeloid differentiation protein 2 (MD2) (Wang et al, 2012). MD2 is an extracellular
protein which interacts with the extracellular domain of TLR4, and is critical for the recognition of TLR4 via LPS (Saitoh et al., 2004). Once bound to MD2, LPS is capable of interacting with TLR4 and initiating the proinflammatory TLR4 signaling cascade, leading to NFκB activation. Therefore, morphine binding of MD2 and the consequent activation of TLR4 provides a mechanism by which morphine induces production of pro-inflammatory mediators. Morphine activation of NFκB in microglia has been demonstrated in vitro, and inhibition of TLR4/MD2 interaction is sufficient to suppress such activation (Wang et al., 2012).

Clinically, the (-)-isomer of morphine is administered to produce analgesia. (-)-morphine activates both the MD2/TLR4 complex and µ-opioid receptors, while the (+)-isomer of morphine activates TLR4 signaling but has no activity on µ-opioid receptors (Jacquet et al., 1977, Hutchinson et al., 2010). Thus, unlike µ-opioid receptors, TLR4 activation is non-stereoselective.

The finding that TLR4 is activated by opioids in a non-stereoselective manner has important implications for the study and prevention of opioid-induced inflammation. Inhibition of TLR4 using (+)-opioid antagonists can inhibit opioid activation of TLR4 and prevent subsequent proinflammation, while maintaining the efficacy of opioid analgesia at µ-opioid receptors. Additionally, deleterious effects of opioids such as tolerance, dependence, constipation, and respiratory depression are mediated, at least in part, by TLR4 (Hutchinson et al., 2007, Hutchinson et al., 2012). The TLR4 antagonists (+)-naloxone and (+)-naltrexone are actively being researched in pre-clinical models as a method for inhibiting morphine-induced inflammation and improving analgesia.
In addition to tolerance and dependence, morphine has been shown to exacerbate allodynia and hyperalgesia in rodent models of pain. Morphine administration prior to surgical incision injury potentiates post-operative hyperalgesia and elevates p38 MAPK and ERK activation in dorsal horn microglia (Hovarth et al., 2010). Of particular interest are reports indicating that morphine administration has negative consequences in models of neuropathy. Repeated morphine prior to peripheral nerve injury has been shown to potentiate allodynia, perhaps via elevated TNFα release in the spinal cord (Loram et al., 2012a). Additionally, acute intrathecal morphine following spinal cord contusion increased allodynia three weeks later (Hook et al., 2009). Indeed, accumulating evidence implicates that opioid treatment of spinal cord injury has a multitude of negative consequences, and that opioids and SCI may synergize to produce particularly poor outcomes (Woller and Hook et al. 2013).

Given the increasing evidence that opioids activate proinflammatory signaling via glial TLR4 and produce negative outcomes in rodent models of neuropathic pain, it is becoming apparent that the clinical application of opioids in the treatment of neuropathy may have additional complications beyond those previously considered. The present study investigates the role of opioids in the potentiation of neuropathic pain following peripheral nerve injury, and the possible mechanisms by which such pain is maintained.

**Summary and Objectives**

Glial activation is a central component of both the onset and ongoing phases of neuropathic pain. Glial activation occurs via neuron-to-glia signaling following nervous system injury, as well as via endogenous danger signaling via toll-like receptors. In particular, TLR4 signaling on microglia, and later on astrocytes, is critical for the development and maintenance
of neuropathic pain. Antagonism of TLR4 in rodent models of chronic constrictive nerve injury (CCI) is sufficient to reverse neuropathic pain (Hutchinson et al., 2008a).

Importantly, TLR4 signaling in the CNS is also activated by opioids in a non-stereoselective fashion (Watkins et al. 2009, Lewis et al. 2010). Such activation, which induces glial activation and the release of proinflammatory substances into the CNS, may be responsible for a broad range of adverse effects of clinical opioid use. Thus, opioid-induced glial activation is central in the opposition of the efficacy of clinical opioid analgesia (Watkins et al., 2009).

As TLR4 mediates both neuropathic pain and opioid-induced glial activation, it is likely to play a critical role in patients receiving opioid treatment for neuropathic pain syndromes. Thus, the study of opioid administration in neuropathic pain is currently an area of intense research interest. Acute intrathecal morphine following spinal cord contusion has already been shown to induce a long term increase in allodynia in rats (Hook et al., 2009). I hypothesize that morphine and the products of nerve injury (DAMPs) interact at TLR4 in the CNS to produce enduring potentiation of allodynia. The objectives are as follows:

1. To determine if repeated opioid administration following peripheral nerve injury potentiates and prolongs mechanical allodynia compared to vehicle controls,
2. To determine if morphine-potentiated, CCI-induced allodynia corresponds with increased microglial and astrocyte activation in the dorsal horn,
3. To determine if TLR4 antagonism in the CNS during morphine administration inhibits subsequent morphine-potentiation of CCI-induced allodynia, and
4. To determine if proinflammatory cytokine signaling in the CNS contributes to morphine-potentiation of CCI-induced allodynia.
Methods

Subjects

Pathogen-free adult male Sprague–Dawley rats (300–325 g upon arrival; Harlan Labs, Denver, CO) were used for Experiment 1 \((n = 11\text{-}12 \text{ rats/group})\), Experiment 2 \((n = 5\text{-}6 \text{ rats/group})\), Experiment 3 \((n = 3\text{-}4 \text{ rats/group})\), and Experiment 4 \((n = 7\text{-}8 \text{ rats/group})\). Pathogen-free adult male Fischer 344 rats (200–225 g upon arrival; Harlan Labs, Denver, CO) were used for Experiment 5 \((n = 6 \text{ rats/group})\). For all experiments, rats were housed one, two or four per cage in a temperature-controlled environment \((23 \pm 2^\circ\text{C})\) with a 12 hr light/dark cycle (lights on at 07:00 hr), with standard rat chow and water available \textit{ad libitum}. All procedures occurred in the light phase. Rats were allowed 1 week of acclimation to the colony room before experimentation. The Institutional Animal Care and Use Committee of the University of Colorado at Boulder approved all procedures.

Drugs

\((-\text{-})\)-Morphine sulfate was a gift from Mallinckrodt, Inc. (St. Louis, MO). 10-14 days following surgery, a free base concentration of 5 mg/kg morphine dose (or equivolume saline) was administered twice daily between 9:00-11:00 am and 4:00-6:00 pm for five consecutive days via subcutaneous injection, using a 25-gauge sterile needle. The duration and dose of morphine administration was selected as it has been shown previously to potentiate inflammatory and neuropathic-like pain (Loram et al. 2012a). \((+\text{-})\)-Naloxone (gifted by Dr. Kenner Rice), a TLR4 antagonist, was administered via intrathecal osmotic minipump (Alzet, 2001, Cupertino, CA, USA) for the duration of morphine dosing (5 days) at a rate of 60 \(\mu\text{g/h}\), in order to inhibit the action of morphine on TLR4 (Hutchinson 2008a). This dose rate was selected based on a
previous study using chronically infused (+)-naloxone in the CCI model (Hutchinson et al., 2008a). Previously, acute intrathecal administration of 100 µg of an IL-1 receptor antagonist (IL-1ra) following intrathecal morphine administration has been shown to attenuate opioid-induced neuroinflammation (Hutchinson et al., 2008b). In the present study, IL-1ra (Amgen CA, USA) was chosen to examine the contribution of IL-1 signaling on morphine-enhanced CCI allodynia. IL-1ra was administered intrathecally using an acutely placed catheter (see below) 44 days following morphine administration. 100 µg of IL-1ra in 1 uL of saline was injected, followed by a 5 uL saline flush (Hutchinson et al. 2008b, Ledeboer et al. 2005).

**Chronic constriction injury (CCI) surgery**

The CCI model of nerve injury was performed aseptically at the mid-thigh level of the left hindleg. Briefly, animals were anesthetized with isoflurane, the skin of the hindquarters was shaved and the sciatic nerve gently elevated. Mild CCI was performed by loosely tying one sterile chromic gut suture (cuticular 4-0 chromic gut; Ethicon, Somerville, NJ) around the sciatic nerve (Grace et al., 2010). The classic model of CCI was performed by loosely tying four sterile chromic gut sutures around the isolated sciatic nerve (Bennett and Xie 1988). Sham surgery consisted of nerve exposure and elevation alone. The superficial muscle overlying the nerve was sutured with silk and the incision in the skin was surgically stapled.

**Mechanical allodynia**

Behavioral testing was conducted blind with respect to group assignment. Rats received three 60 min habituations to the test environment prior to commencement of behavioral testing. The von Frey test (Chaplan et al., 1994) was performed within the region of the hind paws innervated by the sciatic nerve, as previously described in detail (Milligan et al., 2000, 2001; Chacur et al.,
Assessments were made prior to (baseline) and at specific times after experimental manipulations. A logarithmic series of 10 calibrated Semmes–Weinstein monofilaments (von Frey hairs; Stoelting, Wood Dale, IL, USA) was applied in random sequence to the left and right hind paws to define the threshold stimulus intensity required to elicit a paw withdrawal response. Log stiffness of the hairs was determined by $\log_{10}$ (milligrams X 10) and ranged from manufacturer designated 3.61 (0.407 g) to 5.18 (15.136 g) filaments. An absolute threshold (the 50% paw withdrawal threshold) was calculated by fitting a Gaussian integral psychometric function, using a maximum-likelihood fitting method (Harvey, 1986; Treutwein and Strasburger, 1999) as described (Milligan et al., 2000, 2001). This fitting method allows parametric analyses that otherwise would not be appropriate (Milligan et al., 2000, 2001).

**Acute and chronic catheter implantation, and intrathecal drug administration**

The method of acute intrathecal drug administration and the construction and implantation of the indwelling intrathecal catheters was based on that described previously (Milligan *et al.*, 1999). Briefly, intrathecal catheterizations were conducted under isoflurane anesthesia by threading sterile polyethylene-10 tubing (PE-10 Intramedic Tubing; Becton Dickinson Primary Care Diagnostics, Sparks, MD, USA) guided by an 18-gauge needle between the L5 and L6 vertebrae. The catheter was inserted such that the proximal catheter tip lay over the lumbosacral enlargement. For indwelling catheters, the catheters were preloaded with the drug treatment so as to avoid the injection of air, and so as not to delay the delivery of drug with a large volume of vehicle following completion of surgery. The catheters were 12 cm in length, and were attached to a pre-loaded osmotic minipump (Alzet, 2001, Cupertino, CA, USA). To ensure that (+)-naloxone was present at the commencement of morphine dosing, the pumps were implanted at 17:00 h on the day before.
Immunohistochemistry

Rats were administered an overdose (0.90 mL) of pentobarbital (Abbott Laboratories, Abbott Park, IL, USA) and then perfused with saline, followed by 4% paraformaldehyde. All spinal cord tissue was post-fixed for four hours in 4% paraformaldehyde, and then stored in a 22% sucrose solution. Lumbar spinal cord sections L4 and L5 were sliced onto gelatin-substituted slides at a thickness of 20 μM. Slides were rinsed in phosphate buffered saline (PBS) and permeabilized in 0.1% Triton X-100 (St. Louis, MO, USA) in PBS. Slides were incubated in antibody solutions against CD11b (polyclonal antibody, mouse anti-rat) (BD-Pharmigen) and GFAP (monoclonal antibody, mouse anti-rat) (MP Biomedical, Sonon, OH, USA) in Odyssey Buffer (LI-COR, Lincoln, NE, USA) overnight at a dilution of 1:100. The next day, slides were rinsed in 0.1% Tween-20 (Sigma) in PBS and incubated in a biotinylated anti-mouse secondary antibody in Odyssey Blocking Buffer for two hours at a dilution of 1:500. For densitometry, integrated intensity of staining was quantified using the Odyssey Infrared Imaging System (LI-COR). Integrated intensity was examined in spinal dorsal horn laminae 1-6, ipsilateral to nerve injury, as previously described (Loram et al., 2012b).

Statistics

Statistical analyses were performed using Graphpad Prism 4 (GraphPad Software Inc., La Jolla, CA, USA). Except for in Experiment 5, data were analyzed using repeated measures 2 way ANOVA and Bonferroni posttests where appropriate. In Experiment 5, data were analyzed using 1 way ANOVA and Dunnet’s posttest. A statistically significant reduction in paw withdrawal threshold was interpreted as an increase in allodynia. P< 0.05 was considered statistically significant.
Experimental Design

Experiment 1. Does repeated morphine following mild peripheral nerve constriction potentiate allodynia?

One-suture CCI surgery was performed as described on Sprague–Dawley rats to induce a mild nerve injury. At 10 to 14 days following surgery, subcutaneous morphine was administered as described. Allodynia was assessed using the von Frey method prior to surgery (baseline), at 14 days following CCI surgery (pre-morphine), and at 1, 3, 5, 7, 10, 14, 21, 28, 35 days post morphine.

Experiment 2. Does morphine prolong the duration of CCI-induced allodynia?

In order to investigate the duration of morphine potentiation of allodynia, Fischer 344 rats, which experience CCI-induced allodynia for approximately 5 weeks, were used (in contrast with the Sprague Dawley strain, which are allodynic for approximately 12 weeks post-CCI) (Herradon et al., 2007, Herradon et al., 2008). The reasons for these strain differences are under investigation. Current data suggest that pleiotrophin, a growth factor which is involved in nerve regeneration, is upregulated in the DRGs of Fischer 344 rats, but not of Sprague Dawley rats, following CCI (Ezquerra et al., 2008). Additionally, differences in hypothalamic-pituitary-adrenal (HPA) axis responses between the two strains may contribute to the difference observed in CCI duration. Fischer 344 rats exhibit higher diurnal and stress corticosterone levels than do Sprague Dawley rats (Dhabhar et al., 1993). As corticosterone has been implicated as anti-inflammatory, such differences may contribute to a shorter-lasting CCI allodynia in the Fischer 344 rats. As the mild model of CCI does not produce consistent allodynia in the Fischer 344 strain, four-suture CCI surgery was performed as described on these rats to induce a nerve injury. Fourteen days following surgery, subcutaneous morphine was administered as described. Allodynia was
assessed using the von Frey method prior to surgery (baseline), at 14 days following CCI surgery (pre-morphine), and at 1, 7, 14, 21, 28, and 35 days post morphine.

**Experiment 3. Does morphine-potentiated, CCI-induced allodynia correlate with markers for glial activation?**

One-suture CCI surgery was performed on Sprague–Dawley rats as described to induce a mild nerve injury. Subcutaneous morphine was administered as described. One day (n=7-8 rats per group) or 21 days (n=7-8 rats per group) days after cessation of morphine dosing, rats were perfused with saline, followed by 4% paraformaldehyde. Lumbar spinal cord tissue was isolated, sliced, and stained for CD11b and GFAP as described.

**Experiment 4. Does intrathecal administration of the TLR-4 antagonist (+)-naloxone during morphine dosing interfere with subsequent potentiation of allodynia?**

One-suture CCI surgery was performed on Sprague–Dawley rats as described to induce a mild nerve injury. At 10-14 days following surgery, indwelling intrathecal catheters attached to osmotic minipumps pre-loaded with (+)-naloxone were implanted as described. Pumps were implanted the afternoon before the beginning of subcutaneous morphine dosing to ensure that the (+)-naloxone was being delivered concurrently with morphine. Chronic intrathecal (+)-naloxone was administered for 5 days. Subcutaneous morphine was then administered as described. Allodynia was assessed using the von Frey method prior to surgery (baseline), 12 days following CCI surgery (pre-[+]naloxone/morphine), and 1, 3, 5, 7, 10, 14, 21, 28, 35, 42, and 49 days post morphine.
Experiment 5. Does IL-1 signaling contribute to morphine potentiation of CCI-induced allodynia?

One-suture CCI surgery was performed on Sprague–Dawley rats as described to induce a mild nerve injury. At 10-14 days post-surgery, morphine (5 mg/kg) or equivolume saline was administered subcutaneously twice a day for five consecutive days (n=4 per group). At 44 days after cessation of drug dosing, 100 µg of IL-1ra was intrathecally administered to all rats, as described. Allodynia was assessed using the von Frey method prior to surgery (baseline), 14 days prior to IL-1ra dosing, 7 days prior to IL-1ra dosing, just before IL-1ra dosing, and 1, 2, 4, 6, and 24 hours following IL-1ra dosing.

Experiment 6. Does ex vivo morphine stimulation of the lumbar spinal cord following mild CCI induce the release of pro-inflammatory cytokines?

One-suture CCI surgery was performed on Sprague–Dawley rats as described to induce a mild nerve injury. At 10-14 days post-surgery, rats were lightly anesthetized with isoflurane and decapitated. A 5 mm section of lumbar spinal cord L4/L5 was isolated and rinsed first with 70% ethanol, then with sterile Dulbecco’s phosphate buffered saline (DPBS, Invitrogen, Grand Island NY, USA), then with sterile Hank’s Buffered Salt Solution (HBSS, Invitrogen). Tissue was incubated in supplemented Dulbecco’s Modified Eagle Medium (DMEM, 2 nM L-glutamine, 100 U penicillin, 100 µg streptomycin, 10 nM HEPES; Invitrogen) with 0µM, 1µM, or 10µM morphine for 24 hours. Media was collected and assayed for rat IL-1β and rat TNFα using commercially available enzyme-linked immunoassay (ELISA) kits specific for these proteins (R&D Systems, Minneapolis, MN, USA). ELISAs were performed according to the manufacturer’s instructions. The assay kit sensitivity for both the rat IL-1β and rat TNFα assays is 5 pg/mL.
Results

Experiment 1. Repeated morphine following mild peripheral nerve constriction potentiates allodynia.

Morphine has been shown to attenuate recovery and increase allodynia following spinal cord injury (Hook et al., 2009). In order to determine if morphine potentiates allodynia following peripheral nerve injury, Sprague Dawley rats underwent either modified CCI (to create mild allodynia by ligating the sciatic nerve with one chromic gut suture) or sham surgery at 10-14 days prior to morphine administration. Morphine (5 mg/kg) or equivolume saline was administered subcutaneously twice daily for five consecutive days (n=11-12 per group). Quantification of mechanical allodynia began before CCI surgery and continued through 35 days post morphine dosing conclusion. Absolute thresholds of rats receiving CCI surgery were significantly lower than those of rats receiving sham surgery in both ipsilateral and contralateral hind paws prior to morphine dosing (p<0.001, Figure 1). One day post-morphine, rats who received sham surgery followed by morphine had significantly lower absolute thresholds in both hind paws than sham rats who received the vehicle (p<0.001, Figure 1). This effect remained significant until seven days post-morphine in both hind paws. Rats that received CCI surgery and morphine had significantly lower thresholds in both paws at one day post-morphine than those who received CCI alone (p<0.001, Figure 1). This effect was also significant at 21, 28, and 35 days post-morphine (p<0.001, Figure 1).

Experiment 2. Repeated morphine following peripheral nerve constriction prolongs CCI-induced allodynia.

In contrast to the Sprague Dawley strain, the duration of CCI-induced allodynia is markedly shorter in the Fischer 344 strain (Herradon et al. 2007; Ezquerra et al. 2008; Herradon et al.
Thus, the Fischer 344 strain may be well positioned to determine whether repeated morphine following surgery prolongs CCI-induced allodynia. Fischer 344 rats underwent CCI surgery in order to induce allodynia by loosely tying 4 chromic-gut sutures around the sciatic nerve. At 14 days post-surgery, either subcutaneous morphine or equivolume saline was administered twice daily at the morphine dose of 5 mg/kg for 5 days (n=6/group). Absolute thresholds in the ipsilateral hind paws of rats in both groups were not statistically different at days 1, 7, or 14 days post-dosing (Figure 2). At day 21 post-dosing, rats receiving morphine had significantly lower absolute thresholds in both ipsilateral (p<0.05, Figure 2a) and contralateral (p<0.001, Figure 2b) paws. At days 28 and 35 post-dosing, rats receiving morphine had significantly lower absolute thresholds in both hind paws (p<0.001, Figure 2).

**Experiment 3. Changes in expression of markers for glial activation in the morphine-potentiated CCI-induced allodynia paradigm.**

As discussed previously, glial activation is a central component of the development and maintenance of neuropathic pain. To determine whether CCI and/or subsequent morphine increases the activation of microglia and/or astrocytes, the expression of activation markers for these cells was examined in the dorsal horn of the lumbar spinal cord. As in Experiment 1, Sprague Dawley rats underwent either modified CCI or sham surgery to create mild allodynia by ligating the sciatic nerve with one chromic gut suture two weeks prior to morphine administration. 5 mg/kg morphine (or equivolume saline) was administered subcutaneously twice daily for five consecutive days. Lumbar spinal tissue was isolated at 1 (n = 7-8 rats/group) or 21 (n = 7-8 rats/group) days post morphine, and section L4 and L5 were sliced at a thickness of 20 µm. In order to assess astrocyte and/or microglial activation, tissue sections were stained for the astrocyte activation marker GFAP or for the microglial activation marker CD11b.
Integrated intensity was examined in the dorsal horn ipsilateral to nerve injury. At 1 day post morphine, there was an effect of CCI on CD11b expression, but not an effect of morphine (P<0.005, Figure 3a). There was no effect of either CCI or morphine on GFAP expression at 1 day post morphine (Figure 3b). At 21 days post morphine, there was no effect of either CCI or morphine on either CD11b expression or GFAP expression (Figure 3c-d).

Experiment 4. Co-administration of intrathecal (+)-naloxone with morphine inhibits potentiated CCI-induced allodynia.

Chronic intrathecal TLR4 antagonism has been shown to reverse CCI-induced allodynia (Hutchinson et al., 2008a). As morphine has been shown to activate TLR4 (Wang et al., 2012), we hypothesized that chronic intrathecal infusion of the TLR4 antagonist (+)-naloxone during morphine administration would inhibit subsequent potentiation of CCI-induced allodynia. Sprague Dawley rats underwent modified CCI surgery to create mild allodynia by ligating the sciatic nerve with one chromic gut suture. At 12 days following surgery, indwelling intrathecal catheters attached to osmotic minipumps preloaded with either (+)-naloxone or saline were implanted (n=6/group). The catheters were preloaded with the drug treatment so as not to delay the delivery of drug following completion of surgery. To ensure that (+)-naloxone was centrally present at the commencement of morphine dosing, the pumps were implanted at 17:00 h on the day before morphine dosing. Morphine was then injected subcutaneously twice daily for 5 days at the dose of 5 mg/kg, as in Experiments 1 and 2. In the ipsilateral hind paw, rats co-treated with (+)-naloxone and morphine had significantly higher absolute thresholds than rats receiving morphine but no (+)-naloxone at days 1, 3, 5, 7, 14, and 28 post-dosing. (p<0.05, Figure 4a). In the contralateral hind paw, rats co-treated with (+)-naloxone and morphine had significantly
higher absolute thresholds at days 21 post-dosing (p<0.05, Figure 4b), 28 post-dosing (p<0.01, Figure 4b), and 35 days post-dosing (p<0.05, Figure 4b).

**Experiment 5. Intrathecal IL-1ra reverses CCI and morphine potentiation of allodynia.**

IL-1 receptor antagonism prior to morphine has been shown to inhibit morphine attenuation of recovery following spinal cord injury. (Hook et al., 2011). This experiment investigates the effect of IL-1 receptor blockade following morphine administration after CCI. Sprague Dawley rats underwent modified CCI surgery to create mild allodynia by ligating the sciatic nerve with one chromic gut suture two weeks prior to morphine administration. Morphine (5mg/kg) or equivolume saline was administered subcutaneously twice daily for five consecutive days (n=4 per group). At 44 days post-dosing, 100 µg of IL-1ra was intrathecally administered to all rats. In both the ipsilateral and contralateral hind paws, rats receiving CCI, morphine, and IL-1ra had a reversal of allodynia beginning at 1 hour post dosing compared to allodynia measured just prior to dosing (p<0.001, Figure 5). The reversal was significant in both paws at 2, 4, and 6 hours post-IL-1ra (p<0.001, Figure 5). The reversal was no longer significant at 24 hours post-dosing. In rats receiving CCI, saline, and IL-1ra, allodynia in the ipsilateral and contralateral hind paws was significantly decreased at 2 hours post-IL-1ra (p<0.05, Figure 5), and significantly decreased in the contralateral paw at 4 hours post IL-1ra (p<0.001, Figure 5b) but at no other time points.

**Experiment 6. Ex vivo morphine stimulation of the lumbar spinal cord following mild CCI and subsequent pro-inflammatory cytokine release.**

As discussed previously, cytokines released in the CNS can alter neuronal signaling and contribute to the potentiation of pain. Therefore, this experiment seeks to investigate whether
morphine administration ex-vivo increases cytokine release in the spinal cord following CCI. Sprague Dawley rats underwent either modified CCI or sham surgery to create mild allostynia by ligating the sciatic nerve with one chromic gut suture. At 10-14 days post-surgery, a 5 mm section of lumbar spinal cord L4/L5 was isolated and incubated in supplemented DMEM media with 0, 1, or 10 µM morphine for 24 hours. ELISA of the media for IL-1β and TNFα were performed. Although the concentration of protein was in range of the standard curves, no statistical differences in IL-1β or TNFα release were seen between groups (n=3/group, data not shown).

Discussion

The present study has shown that repeated, systemic morphine following CCI of the sciatic nerve significantly potentiates both the magnitude and duration of mechanical allostynia. In the Sprague-Dawley strain of rats, we have demonstrated that morphine potentiates CCI- and sham- allostynia over two vastly different profiles. Rats receiving sham surgery followed by morphine treatment experienced significantly increased allostynia in both ipsilateral (Figure 1A) and contralateral (Figure 1B) hind paws, compared to sham-vehicle controls (such referred pain is consistently observed following CCI in our laboratory – see Grace et al., 2010). This effect, while robust, disappeared at seven days post-morphine. In contrast, rats receiving morphine treatment following CCI surgery experienced significantly increased allostynia compared to CCI-vehicle controls, and this effect continued for the duration of the study (35 days post morphine dosing) (Figure 1). Because the allostynia in the CCI-morphine rats was at the lower limit of the detection range, the magnitude of allostynia at time-points at which the difference between this group and the CCI-saline group did not meet statistical significance may have been found to be
significantly different using a more sensitive measure. This longer lasting form of allodynia, compared to the short-lasting allodynia in sham-morphine subjects, indicates an interaction between morphine and CCI to produce significantly potentiated allodynia. It is possible that the shorter sham-morphine allodynia is an effect of minor pain potentiation as a result of sciatic nerve exposure alone, which was undetectable without potentiation via morphine.

Morphine potentiation of CCI-induced allodynia was also demonstrated in the Fischer 344 strain of rats (Figure 2), which were chosen for the study due to the shorter duration of their CCI-induced allodynia (Herradon et al., 2007, Herradon et al., 2008). As mentioned above, reasons for this difference between strains is under investigation, but may be due to differences in neurotrophin expression following CCI, or to differences in HPA-axis responses (Ezquerra et al. 2008, Dhabhar et al. 1991). Due to this shorter-lasting CCI-induced allodynia, the Fischer 344 strain is a unique tool for the practical examination of the allodynia-potentiating effect of morphine following CCI resolution. As both CCI-morphine and CCI-saline groups were at the limit of detection prior to morphine dosing, no potentiation of allodynia by morphine was observable. However, rats receiving CCI and morphine demonstrated significantly lower absolute thresholds for the duration of the study, while CCI-vehicle controls began demonstrating a reversal of allodynia at 21 days post-injections. Thus, in the Fischer 344 model of CCI in which allodynia is normally reversed at approximately five weeks post-surgery, morphine causes a significant potentiation in allodynia duration.

Because morphine induces such long-lasting, significant potentiation of allodynia, it stands to reason that systemic morphine has altered the microenvironments of the nociceptive pathway to produce long-term changes in pain processing. In Sprague-Dawley rats, the resolution of CCI-induced allodynia around 10 weeks post-surgery was accompanied by the
apparent simultaneous resolution of morphine-potentiated pain (Figure 4). Thus, in this strain, morphine potentiation of pain may be reliant on endogenous danger signals produced by CCI. However, as described above, allodynia in the Fischer 344 strain was continually potentiated by morphine even following the resolution of the effects of CCI. This difference may be reflective of as yet un-explained strain differences.

In examining astrocyte and microglial activation in the dorsal horn of the spinal cord, few significant differences were observed between groups (Figure 3). CCI had a highly significant effect on CD11b, a marker of microglial activation, in the dorsal horn at one day post-morphine, and the effect was no longer significant at 21 days. This finding is consistent with other reports that microglia become activated early in the development of neuropathic pain (Raghavendra et al., 2003). However, there was no significant effect of morphine on CD11b expression. The astrocyte activation marker GFAP expression was not significantly affected by either CCI or morphine at either one or 21 days post-morphine dosing. Before this study, no one had investigated CD11b and GFAP expression in the dorsal horn following repeated systemic morphine during established peripheral nerve injury. Our findings are consistent with one previous study showing that chronic morphine prior to surgical incision injury does not increase activation markers of microglia and astrocytes in the dorsal horn (Hovarth et al., 2010). However, increased p38 and ERK activation have been observed in the morphine/surgical incision injury model (Hovarth et al., 2010). Thus, it seems that only examining glial activation markers in the dorsal horn is an overly simplistic approach to the study of glial contributions to pain in our model. Rather, examining expression of proteins known to be active in neuroinflammation would perhaps be a more informative study. TLR4 receptor expression (which is difficult to study due to a lack of specific antibodies) and activation of downstream
components such as NFκB (and inactivation of the NFκB-sequestering protein IκB), as well as the MAPK proteins p38 and ERK, would be interesting targets of further research. Caspase-1, a component of the NLRP3 inflammasome which cleaves pro-IL-1β following inflammatory signaling, is also a potential target of future activation/gene expression studies.

Previous work implicates astrocytes for the long-term maintenance of neuropathic pain (Raghavendra et al., 2003) via downregulation of the glutamate transporters GLT-1 and GLAST (Sung et al., 2003). Investigating the role of our CCI-morphine model on GLT-1 and GLAST expression could elucidate the role of astrocytes in the allodynia-potentiating phenomenon we have observed. This measure of the role of astrocytes would focus more on astrocyte function than the GFAP approach taken in the present study. Pharmacologically inducing upregulation of GLT-1 using the antibiotic ceftriaxone has been shown to reverse CCI-induced allodynia (Ramos et al., 2010). Exploring the effect of ceftriaxone in the CCI-morphine model may also be a promising way to investigate the role of astrocytes.

Ex-vivo stimulation of lumbar spinal tissue with morphine following CCI did not produce increased release of proinflammatory cytokines compared to sham controls (Experiment 6, data not shown). These results were unexpected, given that CCI alone is associated with increased CNS inflammation (Milligan 2009). As the technique of ex-vivo isolation and stimulation of CCI tissue sections is relatively novel to our laboratory, experimental optimization may produce more informative results. For instance, because the media surrounding the tissue does not reflect the milieu of DAMPs present in vivo, supplementing the media in which the tissue sections lie with endogenous danger signaling molecules such as nuclear protein HMGB1 may provide an environment more similar to physiological conditions. Such danger signals may be critical in the regulation of pain-induced changes in the spinal cord in situ. Therefore, the effect of DAMP-
supplemented media on the experiment performed in this study should be examined in future research. Additionally, tissue sections in this experiment were treated with morphine for 24 hours, while the in vivo experiments in this study investigated the effect of repeated morphine administration for 5 days. Thus, another potential modification of this experiment would be to repeatedly dose CCI rats with morphine in vivo, isolate their spinal tissue, and incubate this tissue in media containing morphine prior to media analysis for cytokines.

Despite a general failure to detect increased cytokine release ex-vivo and increased dorsal horn CD11b/GFAP expression post CCI + morphine, this study still indicates that the phenomenon of morphine-potentiation of CCI-induced allodynia is, at least in part, centrally mediated. In the Sprague-Dawley strain, intrathecal administration of the TLR4-antagonist (+)-naloxone during morphine dosing post-CCI was sufficient to block subsequent morphine-potentiation of allodynia (Figure 4). Chronic intrathecal infusion of (+)-naloxone has previously been shown to reverse CCI-induced allodynia (Hutchinson et al., 2008). While allodynia was not tested during (+)-naloxone/morphine administration in this study, we have shown that morphine-potentiated allodynia is similarly blocked by TLR4 antagonism. Our findings in combination with Hutchinson et al.’s 2008 findings indicate that both CCI and morphine act at TLR4 during the development of the allodynia resulting from those factors. These results indicate that activity at TLR4 by either endogenous danger signals in the CCI model or by morphine in our model is a central and preventable mechanism by which CCI and morphine induce allodynia.

Intrathecal administration of an IL-1 receptor antagonist 44 days post-morphine was sufficient to reverse both CCI- and morphine- potentiation of allodynia in the Sprague-Dawley strain (Figure 5). Thus, the phenomenon of morphine potentiation of allodynia post-CCI is
apparently mediated by TLR4, while the maintenance of both CCI- and morphine- potentiation of such allodynia is mediated by IL-1 signaling.

The data suggest that two theories, which are not mutually exclusive, may explain the roles of CCI and morphine interacting via TLR4 and IL-1 signaling to potentiate allodynia. Firstly, DAMPs produced following CCI may combine with morphine to act at TLR4 in inducing activation of the inflammasome, which activates pro-IL-1 cleavage and IL-1 release. Ensuing autocrine and paracrine IL-1 signaling may induce a positive feedback loop, leading to sustained production and release of IL-1 even when morphine and its metabolites are no longer present. This effect may be maintained (at least in the Sprague Dawley strain) as long as DAMPs are being produced as a result of CCI. However, the observation that allodynia is maintained in the Fischer 344 rats receiving morphine following CCI after the resolution of CCI allodynia in vehicle rats suggests a second theory regarding the interaction of CCI and morphine: morphine increases the impact of CCI trauma by initiating an increased and enduring production of DAMPs. This action of morphine, mediated by TLR4, would delay healing following CCI by prolonging the inflammation invoked by DAMPs. Such a hypothesis gains support from prior observations with spinal cord injury where a single dose of morphine was sufficient to both increase neuronal cell death and increase the extent of the necrotic site (Hook et al., 2007). Such an amplification of cell injury and cell death would be perfectly aligned with prolonged release of DAMPs given how slowly over time damage within the CNS is resolved (Vargas and Barres 2007).

Considering these two possible roles of morphine in the interaction with CCI to produce an enduring potentiation of allodynia, a further experiment would be to intrathecally infuse IL-1ra for the duration of morphine administration, as was done using (+)-naloxone in this study.
Such antagonism of IL-1 signaling might not be as effective as TLR4 blockade in inhibiting subsequent potentiation of alldynia, as inflammasome/IL-1 activation is not the only consequence of TLR4 signaling. For example, TLR4-induced activation of ceramide formation and the production of sphingosine-1-phosphate (S1P) leads to increased activation of NFκB, increasing the production of cytokines such as TNFα and the expression of prostaglandins such as PGE2 (Salvemini et al., 2013). S1P also induces the activation of endothelial cells, increasing the permeability of the CNS to peripheral inflammatory factors (Salvemini et al., 2013). Thus, co-administration of IL-1ra with morphine as a follow-up to this study may be an informative way to examine the relative contributions of TLR4 signaling cascades and IL-1 signaling.

As a whole, the data presented here implicate morphine as having detrimental effects in the treatment of neuropathic pain following peripheral nerve injury. Not only has morphine been shown to potentiate the magnitude of alldynia in two strains of rats, but it has also been shown to significantly increase the duration of alldynia in the Fischer 344 strain. TLR4 and IL-1 have been shown to be central in the mediation of the phenomenon of increased magnitude of alldynia. Our findings suggest that clinical use of a TLR4 antagonist which does not alter morphine analgesia such as (+)-naloxone in combination with morphine treatment may be a promising way to prevent potentiation of pain in human patients. Expanding upon this study in the ways described above will allow us to further investigate the mechanisms by which morphine potentiates CCI-induced alldynia, and continue searching for ways to improve clinical opioid analgesia.
Contributions

Due to the wide range of expertise required to execute this project, I am grateful for the contributions of Dr. Linda Watkins, Dr. Peter Grace, and Keith Strand in this study. Dr. Watkins, Dr. Grace, and I contributed to the design of this study. I was responsible for the execution of the study. Dr. Grace contributed by conducting all von Frey testing. I, Dr. Grace, and Keith Strand all participated in drug dosing and tissue isolation. I was responsible for subcutaneous injections, while Keith Strand performed intrathecal injections. I was also responsible for all animal handling, drug preparation, catheter preparation, CCI surgeries, tissue preservation and slicing, immunohistochemistry, densitometry, tissue culture, ELISA, data organization and entry, statistical analysis, scheduling, and writing associated with the project.

Acknowledgements

This research project would not have been possible without the support of my thesis advisor, Dr. Linda Watkins, and my direct supervisor and mentor, Dr. Peter Grace. I would also like to thank Dr. Khara Ramos for her mentorship and Keith Strand for teaching lab protocols and aiding in experiments. This honors thesis was supported by funds from the Bioscience Undergraduate Research Skills and Training Program, the National Institute on Drug Abuse Summer Research Program and the Undergraduate Research Opportunities Program at the University of Colorado at Boulder.
Figure Legends

**Figure 1.** At 10-14 days after mild sciatic nerve injury or sham surgery, Sprague Dawley rats were dosed with morphine 5 mg/kg or vehicle twice daily for five days. Absolute thresholds were determined using the von Frey method in the ipsilateral (Figure 1A) and contralateral (Figure 1B) paws. Morphine treatment after mild nerve injury resulted in significant exaggeration of allodynia, compared with nerve-injured rats receiving saline. Morphine treatment induced transient allodynia in sham-operated rats, compared to those receiving saline. $n=11-12$/group. CCI+Morphine vs. CCI+Saline: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Sham+Morphine vs. Sham+Saline: † † † $P < 0.001$.

**Figure 2.** Nerve injury was performed on Fischer 344 rats using the classic 4 suture CCI model. At 10-14 days post-surgery, rats were dosed with morphine 5 mg/kg or vehicle twice daily for five days. Absolute thresholds were determined using the von Frey method in the ipsilateral (Figure 2A) and contralateral (Figure 2B) paws. Morphine treatment after nerve injury resulted in significantly lowered absolute thresholds at 21, 28, and 35 days post-morphine in the ipsilateral paw, and at 14, 21, 28, and 35 days post-morphine in the contralateral paw. $n=6$/group. *$p<0.05$; ***$p<0.001$.

**Figure 3.** At 10-14 days after mild sciatic nerve injury or sham surgery, Sprague Dawley rats were dosed with morphine 5 mg/kg or vehicle for five days. At 1 (n = 7-8 rats/group) or 21 (n = 7-8 rats/group) days post-dosing, spinal tissue was isolated. Spinal sections L4 and L5 were analyzed using immunohistochemistry for expression of GFAP and CD11b in the ipsilateral dorsal horn. At 1 day post morphine, there was an effect of CCI on CD11b expression but not of
morphine (P<0.005). There was not an effect of either CCI or morphine at 1 day post morphine. At 21 days post morphine, neither CCI nor morphine had an effect on either CD11b or GFAP.

**Figure 4.** At 10-14 days after mild sciatic nerve injury or sham surgery, Sprague Dawley rats were implanted with indwelling intrathecal catheters attached to osmotic minipumps pre-loaded with (+)-naloxone, and dosed with morphine 5 mg/kg or vehicle twice daily for five days. Absolute thresholds were determined using the von Frey method. Co-treatment of (+)-naloxone significantly attenuated the exaggeration of CCI-allodynia by morphine, compared to saline. n = 6/group. *P < 0.05; **P < 0.01; ***P < 0.001.

**Figure 5.** At 10-14 days after mild sciatic nerve injury surgery, Sprague Dawley rats were dosed with morphine 5 mg/kg or vehicle twice daily for five days. At 44 days post-dosing, 100 µg of IL-1ra was intrathecally administered. In both the ipsilateral and contralateral hindpaws, rats receiving CCI, morphine, and IL-1ra had a reversal of allodynia beginning at 1 hour post dosing compared to allodynia measured just prior to dosing. The reversal was significant in both paws at 2, 4, and 6 hours post-IL-1ra. The reversal was no longer significant at 24 hours post-dosing. In rats receiving CCI, saline, and IL-1ra, allodynia in the ipsilateral and contralateral hindpaws was significantly decreased at 2 hours post-IL-1ra, and significantly decreased in the contralateral paw at 4 hours post IL-1ra, but at no other time points. n= 3-4/group. *P < 0.05; ***P < 0.001.
Figures

A  Ipsilateral

B  Contralateral

Figure 1
Figure 2
Figure 3

A. CD11b

B. GFAP

Day 1

C. Intensity

D. Intensity

CCI: P<0.005

Morphine  Vehicle
Figure 4
Figure 5

A

Ipsilateral

Absolute Threshold (g)

CCl+morphine

CCl+saline

Hours post injection

B

Contralateral

Absolute Threshold (g)

CCl+morphine

CCl+saline

Hours post injection
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