

Exploring the Glial Mechanisms Underlying Central Neuropathic Pain in a Novel
Rat Model of Spinal Cord Injury

by

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A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirement of the degree of
Doctor of Philosophy
Department of Psychology and Neuroscience
2014

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Cord Injury
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Of scholarly work in the above mentioned discipline

Abstract

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Glial Mechanisms of Central Neuropathic Pain

Thesis directed by Distinguished Professor Linda Watkins

Neuropathic pain is a debilitating condition that is usually intractable to treatment. Spinal cord injury (SCI) is the leading cause of central neuropathic pain (CNP), and patients often describe their pain as severe and intolerable. Although most of the focus in the pain literature has been on neurons, it is now well known that non-neuronal cells of the central nervous system called microglia and astrocytes (herein referred to as “glial cells” or “glia”) play a key role in the induction and maintenance of neuropathic pain. Glia are immune cells that are normally in a surveying/quiescent state, but upon activation by an inflammatory stimulus, they release a host of proinflammatory mediators that can sustain neuropathic pain for weeks, months, and even years. Interestingly, glia can also release anti-inflammatory mediators, making them ideal for therapeutic targeting. The glial mechanisms underlying CNP have not been fully explored and understood, and Chapter II is a set of studies undertaken to determine the involvement of glia in a novel rat model of thoracic-13/lumbar-1 (T13/L1) dorsal root avulsion SCI termed Spinal Neuropathic Avuision Pain (SNAP). SNAP induced a robust, reliable, long-lasting below-level hind paw allodynia that was reversed by 3 different glial activation inhibitors. These studies confirmed that glia are involved in the mechanism underlying SNAP and that use of therapies that target glial cells could provide a better route for treating CNP. The studies in Chapter III determined that CNP could be attenuated by a *single* injection of an adenosine A_{2A} receptor agonist for at least 6 weeks. This attenuation is due to a decrease in glial activation and proinflammatory cytokine expression, and at least in part dependent on the anti-inflammatory cytokine IL-10. The studies in Chapter IV detail a phenomenon in which administering opioids

shortly after SNAP increases the magnitude of allodynia and potentiates the expression of proinflammatory mediators. This mechanism is at least in part dependent on toll-like receptor 4 and the proinflammatory cytokine IL-1 β . The evidence from this dissertation suggests that glial cells are critically involved in the mechanisms underlying CNP, and that they can be manipulated to attenuate or potentiate CNP depending on their microenvironment and pattern of receptor activation.

Dedication

This dissertation is dedicated to my true love, soul mate, and husband Shane Michael, my other half and beautiful sister Anjelica “Jeli” Ellis, my wonderful parents Ben and Jeanne Ellis, my amazing grandma Jeannette Veltrie, my furry four legged children Shelby and Iacocca (Cocca), and to all the rats, especially Sneezy.

Acknowledgments

I would first like to thank my mentors Linda Watkins and Steve Maier. I am honored to have been a graduate student under you, and I couldn't imagine having a better mentor. Linda, you have taught me so much and push me to be a better scientist every day. You care about me not just as a scientist, but as a person, and I will be forever grateful to have been a part of your lab.

Thank you to my committee: Linda, Steve, Serge, Ben, and Mike for taking the time out of your busy lives and schedules to mentor me through this process.

I would like to also thank my post-doc mentors Lisa Loram, Khara Ramos, Peter Grace, and especially Julie Wieseler.

I must of course give a shout out to all my fellow grad students and lab mates who have been on this journey with me, especially my office mate Mike Weber who had to put up with everything from me buying a house, getting married, and writing my dissertation, and "Friend" Alexis Northcutt without whom there is NO WAY I would have gotten through grad school without.

Last but not least I want to thank my husband and family. You have all been there with me from the beginning and have whole-heartedly supported me and showered me with love. There was never any doubt in my mind that I could do this because you all told me I could! I love you so very much Shane, Jeli, Mom, Dad, and Grammy Bear!

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Chapter I

Introduction

Preface

One of the most dynamic sensory systems in the body is pain. Pain in a normal, healthy sensory system is important as it is both protective and adaptive; pain is a warning signal to get you out of danger and begin the healing process. It is when you have a dysregulation of this system that pain becomes pathological. Acute, non-pathological pain activates peripheral nerve endings called nociceptors, which send signals to the dorsal horn of the spinal cord that are relayed all the way up to the cortex. This pain is referred to as “nociceptive pain”.⁵⁴ However, when neural tissue of the central nervous system (CNS) becomes damaged, a form of pathological pain referred to as central neuropathic pain can occur.²⁰⁸ Such neuropathic pain is characterized by a sensory deficit, a burning sensation, widespread pain extending beyond the area of original injury, pain caused by light touch (allodynia), or spontaneous pain in the absence of noxious stimuli.³⁶ Central neuropathic pain can last for months or even years, and arises in a variety of CNS disorders including spinal cord injury (SCI), traumatic brain injury (TBI), multiple sclerosis (MS), stroke, and Parkinson’s disease. Unfortunately, this type of pain is often severe and intolerable and is intractable to treatment. There is an urgent need to develop more efficacious therapeutics in order to help the millions of people that suffer from chronic pain disorders.

Our understanding of pathological pain has previously centered on neuronal mechanisms. However, it is now accepted that glial cells, here referred to as microglia and astrocytes, are important pain modulators and contribute to both the induction and maintenance of neuropathic pain, including central neuropathic pain.³²⁶ Glia are the non-neuronal immune-

like cells of the CNS and constitute nearly 70% of the total cell population in the brain and spinal cord.²¹¹ There are several subtypes of glia, including microglia, astrocytes, and oligodendrocytes. Although they all have some overlap in function, they play different roles in pain processing. For this dissertation, only microglia and astrocytes will be discussed.

Microglia only make up 5-10% of the total glial population and are split into two subtypes, resident microglia and perivascular microglia. Resident microglia are bone-marrow or yolk sac derived hematopoietic cells that migrate to the CNS during embryonic or early post-natal development, and are never replenished.¹²⁹ Perivascular microglia are derived from bone-marrow precursors in adulthood and are continuously replenished, especially after peripheral nerve damage and CNS inflammation.^{129, 176, 362} Resident microglia have long processes that are constantly surveying their environment and upon activation can exert both pro- and anti-inflammatory effects. Perivascular microglia can alter the blood brain barrier (BBB) and both resident and perivascular microglia can produce proinflammatory mediators upon activation that can go on to activate nearby neurons, astrocytes, and other microglia. Microglia are normally in a quiescent surveillance state and function to support, insulate, and protect neurons. Microglial cells also mediate innate immunity in the CNS,¹²² along with other mononuclear phagocytes. In addition, microglia and macrophages are thought to be on a continuum of activation anywhere between a classically activated proinflammatory state and an alternatively activated anti-inflammatory state.²⁸¹

Astrocytes are derived from the neuroectoderm and make up more than 50% of the total glial population in the CNS. Astrocytes help to support neurons and develop and maintain the BBB³⁴ and are also unique in that they can release neurotransmitters. Astrocytes can both synthesize D-serine for glutamate production and deactivate glutamatergic activity by taking up

extracellular glutamate.³² Astrocytes also encapsulate synapses and are in close contact with neurons, which allows them to directly alter neuronal communication.¹²⁶ Astrocytes express multiple neurotransmitter receptors including N-methyl-D-aspartate receptors (NMDAR), metabotropic glutamate receptors, substance P receptors, and purinergic receptors.²⁵⁰ During chronic inflammation, microglia become activated first and astrocytes become activated later and can remain activated for long periods of time in response to the release of proinflammatory mediators from the microglia.¹⁴⁷

Glia become activated in response to neurotransmitters, calcitonin gene related peptide (CGRP), chemokines, cytokines, substance P, matrix metalloproteinases (MMPs), prostaglandins, nitric oxide, adenosine triphosphate (ATP), and their exposure to endogenous danger signals that are released by stressed, damaged, and dying/dead cells that are injured due to trauma, ischemia, cellular and metabolic stress, inflammation, or infection.¹⁶⁶ Upon activation, glial cells undergo a set of functional and morphological changes.⁵⁹ They retract their processes, increase soma size, display activation markers, and release a host of proinflammatory molecules that can lead to neuronal hyperexcitability, pain amplification, and chronic inflammation.³⁴⁵ Markers of microglial activation include complement receptor 3 (CD11b/c), actin binding protein Iba1, the pattern recognition receptor toll-like receptor 4 (TLR4), and major histocompatibility complex II, while the major marker of astrocyte activation is glial fibrillary acidic protein (GFAP).³²⁶ Of note, there are no markers that are exclusive to microglia. Interestingly, glia can also release anti-inflammatory molecules, which work to ameliorate neuropathic pain and restore normal pain signaling.³⁴⁴ Since glia can be both pathological and protective (see below for discussion), they are excellent therapeutic targets for neuropathic pain. However, central neuropathic pain is complex and the underlying mechanisms have still not been fully elucidated,

nor are there effective treatments available. The purpose of this dissertation is to help try and elucidate some of these mechanisms, specifically focused on glia, and provide novel therapeutic targets.

The Role of Glia in Pain Processing

Glial cells are now recognized to play a very important role in pathological pain processing and have been implicated in many models of both peripheral and central neuropathic pain. Neuropathic pain is extremely difficult to treat, and there are currently no effective treatments available. Targeting glial cells for neuropathic pain management could provide chronic pain patients with better therapies and allow for a better quality of life. The following sections describe the role of glia in pain processing and how they might be targeted for central neuropathic pain control.

Non-pathological pain processing

Acute pain signaling occurs when a stimulus, such as stepping on a nail, activates peripheral nerve terminals on first-order primary afferent neurons called nociceptors. Nociceptors contain transient receptor potential (TRPA, TRPM, and TRPV) channels, sodium channels (Nav), potassium channels (KCNK), and /or acid-sensing ion channels (ASICs). These primary sensory neurons convey noxious (thermal, mechanical, chemical) signals through the cell body in the dorsal root ganglion (DRG) to secondary pain projection neurons in laminae I and V of the dorsal horn of the spinal cord through three major types of axon fibers: A β (large, myelinated, fast conducting, non-noxious signals), A δ (smaller, myelinated, slower conducting, noxious signals), and C (smallest, unmyelinated, slowest conducting, noxious signals).²⁰⁸ The axon terminals of these fibers form the presynaptic element of the central synapses of the central sensory pathway in the spinal dorsal horn or trigeminal nuclei. Activation of these fibers by

noxious stimuli leads to glutamate, substance P, and/or CGRP release from these central presynaptic afferent nerve terminals in the spinal cord dorsal horn. The axons of second-order nociceptive pain projection neurons in the spinal cord dorsal horn decussate and join the ascending fibers of the anterolateral system where they project to and terminate in the rostral ventral medulla (RVM), the periaqueductal grey, or the thalamus, where they transfer information about the intensity and duration of the peripheral noxious stimulus. Third order neurons from thalamic nuclei including the ventral posterior, intralaminar, and parafascicular nuclei project to the primary and secondary somatosensory cortex, anterior cingulate cortex, amygdala, insular cortex, and pre-frontal cortex, which can encode different aspects of pain including sensory-discriminative, emotional, and cognitive aspects.¹³⁶

Pathological pain processing in neurons and glia

Neuropathic pain arises when there is ongoing nerve injury and inflammation in the spinal cord even after the original damage has resolved. Much more is known about neuropathic pain arising from peripheral nerve injury compared to central neuropathic pain, therefore this section will describe neuropathic pain in terms of peripheral nerve injury, although there are some known differences between mechanisms underlying peripheral and central neuropathic pain that will be discussed.

Neuropathy elicits changes in the activity and properties of neurons. Mechanoreceptors can become sensitized to applied mechanical stimuli,²⁶⁸ and damaged nerves can exhibit ongoing ectopic activity due to the accumulation of sodium channels around damaged axons. This aberrant activity can spread to the DRGs and nerve fibers can cross-excite each other, which is known as ephaptic transmission and can cause spontaneous pain and prime the spinal cord to amplify responses to subsequent stimuli.⁵⁵ As discussed above, many of the primary sensory

neurons utilize the excitatory neurotransmitter glutamate. Binding of glutamate on post-synaptic spinal neurons leads to depolarization via α -amino-3-hydroxy 5-methyl-4-isoxazelo-propionic acid (AMPA) receptors,²⁴⁶ NMDA receptors,²²¹ and the G-protein coupled metabotropic (mGluR) family of receptors.³⁵⁹ In addition, there are presynaptic kainate receptors for glutamate in the spinal cord.¹⁵⁷ AMPA receptor activation normally sets the baseline responses of dorsal horn neurons, but if there is repetitive high frequency stimulation of AMPAR on these neurons, there is an amplification and prolongation of the response which is known as wind-up and results from activation of NMDARs.⁶⁷ Acute or low frequency inputs to the spinal cord do not activate NMDARs because it is blocked by a magnesium ion (Mg^{2+}) plug. Only sustained membrane depolarization is enough to remove the Mg^{2+} block and activate NMDARs, resulting in a large calcium ion (Ca^{2+}) influx which can activate various effectors including nitric oxide synthase,²⁷ calcium/calmodulin-dependent kinases (CaMKI/II), and extracellular-related kinases (ERK), which can all lead to plasticity such as long-term potentiation (LTP).¹⁹⁶ NMDAR activation plays a key role in inflammation and chronic pain, especially hyperalgesia (amplified responses to painful stimuli).²⁹³

Descending pathways from the brainstem can also influence pain signaling in the dorsal horn. NK1 (neurokinin 1 receptor for substance P) positive neurons in the brainstem initiate descending facilitation through parabrachial-rostral ventral medulla connections down to serotonergic (5-HT₃) receptors in the spinal cord, promoting neuropathic pain.⁵⁵ Descending inhibition involves the release of norepinephrine from the brainstem (locus coeruleus) into the spinal cord where it acts at α_2 -adrenoceptors, inhibiting transmitter release from primary afferent terminals and suppressing firing of dorsal horn neurons.²⁰⁴ Another inhibitory pathway is through [gamma]-aminobutyric acid (GABA) interneurons in lamina II of the dorsal spinal cord.

Normally these GABAergic interneurons inhibit laminae I and V neurons, but following peripheral nerve injury, the ongoing activity from primary afferent neurons can cause a degeneration of these GABAergic interneurons. This results in decreased inhibition of the pain pathway and contributes to hypersensitivity, apoptosis, and chronic pain.²⁷⁸

It is also possible to switch neurons that normally respond to noxious stimuli to now respond to non-noxious stimuli. Lamina I neurons do not receive direct input from low-threshold A δ and A β afferents and they normally respond to noxious stimuli.¹⁸⁹ However, in neuropathic pain situations, there is a disruption of chloride anion (Cl⁻) homeostasis that weakens inhibition of these lamina I neurons and causes them to be activated in response to normally non-noxious stimuli.¹⁵⁶

Neurons in the spinal cord can become sensitized and amplify the normal pain response from any of a multitude of mechanisms including those reviewed above. This amplification of pain is referred to as central sensitization.³⁶⁸ Central sensitization results in increased responses to painful stimuli (hyperalgesia) and pain resulting from normally non-noxious stimuli (allodynia). Sensitized primary afferent neurons release an exaggerated amount of substance P, glutamate, CGRP, the chemokines fractalkine and monocyte chemoattractant protein 1 (MCP1), and ATP, which can now activate glia as well as feedback to activate other sensitized neurons. In addition, prolonged neuronal excitation can also activate mitogen-activated protein kinases (MAPKs) ERK, Jun N-terminal kinase (JNK), and p38 in glia, and each of these kinases can activate the transcription factor nuclear factor- κ B (NF κ B) which can synthesize a variety of different proinflammatory substances that can then go on to activate other neurons and surrounding glia, thereby amplifying pain and inflammation.²⁰⁸

Similarities and differences between peripheral and central neuropathic pain

Central neuropathic pain is defined as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system”.³²⁰ Secondary pain that arises from a central process, such as shoulder-hand syndrome that arises after stroke or painful spasticity after MS, is not considered central pain. In addition, changes in the CNS secondary to a peripheral lesion are also not considered changes arising from central pain.³⁴¹ In order to distinguish central neuropathic pain from peripheral neuropathic pain in SCI, a mechanism-oriented classification is used. Below-level pain is central pain as it is a direct consequence of spinal cord damage, whereas at-level pain is often due to peripheral nerve root injury and muscle spasms, which are not considered central pain.²⁸⁸ Although there is activation of ERK 1/2, p38 MAPK, and CaMKII in both at- and below-level pain, activation of JNK is specific to below-level pain.¹³⁸ Activated microglia in SCI with below-level pain produce and regulate prostaglandin E₂ via activation of pERK 1/2 MAPK, which maintains central neuropathic pain by inducing hyperexcitability in dorsal horn sensory neurons. This is mechanistically different than in peripheral neuropathic pain where microglial activation plays more of a role in the initiation rather than maintenance of pain and does not induce long-term hyperexcitability in neurons.³⁶⁵ In contrast to most peripheral neuropathies, central neuropathic pain can have a delayed onset.⁵ Furthermore, studies have shown that lesions within the central nociceptive pathways are necessary for the development of both peripheral and central neuropathic pain,³⁴¹ but are not sufficient to generate central neuropathic pain and must be accompanied by additional excitotoxic and inflammatory processes in order to initiate and maintain central neuropathic pain.⁹¹ Similar to peripheral neuropathic pain, both spontaneous and evoked pain can be present in central neuropathic pain.²³⁵ In addition, increased expression of voltage-gated Nav1.3 sodium

channels in the spinal cord triggered by inflammatory processes and glial activation can maintain both peripheral and central neuropathic pain.¹¹⁷

Glia activation in central neuropathy

It is well established that glia are activated by peripheral nerve injury,^{191, 309} but glia are also activated by direct CNS trauma. In both humans and in animal models of TBI, microglial activation persists for weeks to even years after the initial trauma, especially in moderate to severe cases.^{150, 219, 227} In animal models, chronic microglial activation increases expression of interleukin (IL)-1 β and tumor necrosis factor (TNF)- α and is implicated in post-TBI neurodegeneration.^{130, 190} In humans, chronic microglial activation is associated with cognitive dysfunction²⁵⁴ and post-mortem studies demonstrate that reactive microglia in the corpus callosum and frontal lobe are up-regulated years after TBI.²⁹⁸ Glial activation has also been implicated in different models of MS including experimental autoimmune encephalomyelitis (EAE),²⁵⁶ and the cuprizone and lysolecithin demyelinating models.^{212, 279} Glial activation is associated with inflammation-induced neurodegeneration, and the putative glial activation inhibitor minocycline decreases demyelination and improved motor coordination.^{241, 295} Both microglia and astrocytes are activated in animal models of Parkinson's³²² and stroke,^{236, 267} and switching microglia from a classical proinflammatory state to an alternatively activated anti-inflammatory state is associated with better functional outcomes following stroke.^{148, 287}

Glial activation and inflammation in spinal cord injury (SCI)

Finally, SCI, which is the focus of this dissertation, is one of the main causes of disability worldwide,²⁶¹ and more than 65% of SCI patients develop neuropathic pain.²⁸⁹ Glial activation is implicated in nearly every animal model of SCI including hemisection,³⁵⁵ rhizotomy,³⁰⁵ contusion,³⁸ and avulsion.⁴⁶ Furthermore, attenuating glial activation improves

functional outcomes and decreases SCI-induced central neuropathic pain.^{78, 111} During SCI, there is a large amount of tissue trauma, glial activation, recruitment and infiltration of peripheral immune cells, and break down of the BBB, which can be compromised for up to twenty-eight days post-SCI.²⁴⁸ Neutrophils signal the first inflammatory response after SCI, they infiltrate the spinal cord within the first hour after injury and peak at twenty-four hours post-injury,⁷³ but are gone by one week post-injury.³⁰³ Activated glia and macrophages are apparent fairly early following SCI and remain activated for about four weeks post-injury, but can be activated for much longer.^{112, 249} B and T lymphocytes infiltrate the injured spinal cord by six hours post-injury and are usually gone by one week.²⁷⁷ It is also important to note central neuropathy and inflammation takes much longer to resolve than that of peripheral origin. The spinal cord trauma and inflammation that results after SCI induces a massive release of endogenous danger signals that can be sustained across years after the original tissue damage due to the extremely slow neurodegeneration and clearance of damaged tissue that occurs in the CNS.³²⁹ This enduring inflammation and glial cell activation induces a sustained release of proinflammatory mediators that can sensitize neurons and amplify pain and inflammation for months or even years.

However, inflammation is not always pathological and the initial neuroinflammatory response is often adaptive and beneficial, and activated glia can actually be neuroprotective in certain cases. Macrophages and microglia promote regeneration of axons by scavenging myelin, and they can clear neuronal and other cellular debris produced by pathogens or apoptotic or necrotic cells during inflammation by phagocytosis.¹⁹⁹ Activated glia often down-regulate proinflammatory cytokines to limit damage to nearby healthy tissue.^{82, 201} Without clearance of this toxic cell debris by activated glia, toxicity could easily spread throughout the CNS.³²⁴ Activated glial cells can also produce the pro-regenerative cytokine, transforming growth factor-

beta (TGF- β) and enhance neurite outgrowth.³⁰³ IL-10 is an anti-inflammatory cytokine that is released in response to neuroinflammation, and microglia activated in response to CNS damage can release IL-10 in order to decrease inflammation.¹¹⁵ Activated microglia can also help with wound healing in the CNS.²⁸¹ Toll-like receptor 4 (TLR4) activation can also mediate microglial neuroprotection in certain situations.²⁰⁸ It has also been suggested that CD4+ T-cell mediated neuroprotection is dependent on microglial activation.³³ Therefore, it is important to recognize that glial activation can be protective in certain cases, and it is only when pain and inflammation becomes severe and chronic (ie. pathological) that glial activation is pathological and should be attenuated.

Targeting glia for treating central neuropathic pain

There are three main ways of targeting glia for therapeutic benefit: attenuating glial activation using glial activation inhibitors, using compounds that inhibit cytokine production or action, and administering anti-inflammatory compounds. Although there are very few current compounds in clinical trials or use (see chapter V for a discussion of those that are), there are a number of promising pre-clinical treatments being used in research settings.

One group of glial activation inhibitors is phosphodiesterase inhibitors (PDEs), which include propentofylline (PPF) and ibudilast. PDEs hydrolyze cAMP and/or cGMP, thereby inactivating these second messengers in the cyclic nucleotide signaling cascade.⁵¹ When PDEs are inhibited, the subsequent increase in cAMP decreases proinflammatory cytokines,²⁴³ reduces immune cell infiltration/activation and free radical formation,¹¹ and increases production of the anti-inflammatory cytokine IL-10,²⁹⁰ resulting in less pain and less glial activation. Another set of glial activation inhibitors are macrophage migration inhibitory factor (MIF) inhibitors which include ibudilast. MIF is a ubiquitously distributed protein that is now considered to be a

cytokine² and signals through ERK 1/2 and AKT.^{181, 193, 210} MIF increases firing in nociceptive neurons and induces proinflammatory gene transcription in microglia,² and MIF inhibition is thought to decrease both of these. MIF is increased in compression SCI¹⁶⁵ and intrathecal recombinant MIF elicits pain in naïve mice.² Mice with SCI deficient in MIF do not develop neuropathic pain, have better functional recovery, and have fewer apoptotic neurons.²²⁶ In contrast, MIF can reduce secondary damage following SCI.⁸³ A third type of glial activation inhibitors are drugs that block TLR4 activation, a glial receptor implicated in glial activation in response to a variety of endogenous danger signals. The (+)- isomers of naloxone and naltrexone attenuate glial activation in this manner.²⁶ PPF, ibudilast, and (+)-naltrexone all attenuate SCI-induced neuropathic pain,⁷⁸ suggesting that these compounds may have some clinical utility. Minocycline, a second-generation tetracycline antibiotic also attenuates glial activation, likely by preventing cytokine and nitric oxide (NO) production and reducing microglial trafficking to injured neurons.²⁶⁵ Minocycline does decrease macrophage/microglial activation in SCI,²⁴⁰ but its clinical utility is limited because of the many side effects and difficulty of administration.

Compounds that inhibit cytokine production include the IL-1 β antagonist anakinra (Kineret) as well as the TNF- α antagonist etanercept (Enbrel). Both of these are known to provide better functional recovery after SCI in rodents,^{133, 361} but one limitation of these compounds is that they do not cross the BBB, which could limit their therapeutic potential for central neuropathic pain.

Anti-inflammatory compounds include adenosine A_{2A} receptor agonists such as CGS21680 and ATL313, which are thought to treat pain by attenuating glial activation by decreasing TNF- α and increasing IL-10 production via increases in cAMP mediated production of protein kinase A and C.^{191, 192} IL-10 gene therapy is also successful in treating neuropathic

pain,²⁹⁷ and, very recently, in dogs with arthritis (unpub obs.). Sustained release of IL-10 is thought to be able to cause a shift in both immune cells and the microenvironment from proinflammatory to anti-inflammatory, thereby relieving neuropathic pain for a prolonged period of time.²⁹⁹

Although all of the compounds described above are thought to act primarily on glial cells, it is extremely important to note that none of them are truly specific to glial cells. Neurons have receptors for and express IL-1 β and TNF- α ,^{50, 233} they have adenosine A_{2A}Rs,²⁰² TLR4,⁷⁰ and can be modulated by PDE and MIF inhibition.⁹⁴ Co-administering PPF and the competitive NMDAR antagonist (3-(2-carboxipiperazin-4)1-propyl phosphonic acid) ($\pm$)-CPP had additive effects, resulting in a blockade of the calcium current and intracellular transduction process in neurons, leading to decreased neuropathic pain.²¹⁵ Ibudilast exerts neuroprotection on hippocampal neurons by inhibiting calcium influx and by sustaining a high level of cAMP.³¹⁹ Naltrexone can decrease neuronal firing rate in prefrontal cortex¹⁶⁹ and can decrease demyelination in EAE.²⁵² CGS21680 can increase sleep and the number of GABAergic neurons expressing Fos in the pre-optic hypothalamus¹⁷² and can exert survival activity of motor neurons via activation of the adenylate cyclase-cAMP-PKA pathway and the TrkB neurotrophin receptor.¹⁶⁷ However, upon a review of these literatures, it appears that, in large part, effects of the drugs under study in this dissertation have effects on neurons that largely arise secondarily from the effects of the drugs on glia.

Although they have distinct functions, macrophages behave very similarly to microglia and can infiltrate from the periphery where they can differentiate into microglia in the CNS.³²⁶ Macrophages and other immune cells express receptors for and release proinflammatory cytokines,³³⁰ IL-10,⁵⁰ TLR4,^{182, 286} have adenosine A_{2A}Rs,⁸⁹ and again like neurons can be

modulated by PDE and MIF inhibition.^{94, 181} PPF suppresses the production of TNF- α ¹⁵⁴ and reactive oxygen species in macrophages,⁹ and can also counteract neutrophil activation by blocking the removal of adenosine.³⁶³ Both PPF and ibudilast can prevent kainite-induced cell death in oligodendroglia,³⁵⁸ and ibudilast can also inhibit platelet aggregation in the presence of endothelial cells.²⁵⁹ Furthermore, ibudilast reduces inflammatory cell infiltration into the dorsal spinal cord in EAE. Naltrexone can suppress morphine-induced HIV replication in neonatal monocyte-derived macrophages.¹⁸⁵ CGS21680 can reduce the number of infiltrated granulocytes into ischemic tissue²⁰² and ATL313 can reduce inflammation in SCI via activation of A_{2A}R on bone-marrow derived cells.¹⁸⁶ Therefore, these compounds and all of those discussed in the chapters below could potentially be acting directly and/or indirectly on neurons and other immune cells as well as glia. However, there is a large body of literature that suggests that the primary actions of the compounds used in this dissertation are on glial cells.

Glia and Adenosine Signaling

Adenosine receptors are found on all cell types in the body, including glial cells. Adenosine agonists and antagonists have recently gained attention for the many therapeutic effects seen in neurodegenerative, autoimmune, and chronic pain disorders. Agonism of the adenosine A_{2A}R induces an up-regulation of the anti-inflammatory cytokine IL-10, which is released by multiple cell types including glial cells. The following sections will describe the role of adenosine, IL-10, and glial cells in pain and inflammation.

Adenosine signaling

A large role of adenosine receptors is to regulate the inflammatory processes that occur after injury and trauma, and adenosine receptors have been a recent focus for neuropathic pain targets.⁹⁷ Adenosine is a purine nucleoside that can signal through four different types of G-

protein-coupled-receptors, adenosine receptors (ARs), A_1 , A_{2A} , A_{2B} , and A_3 .³⁶⁹ All four types are found on glial cells and can modulate the release of cytokines.³⁰² AR signaling can occur through activation or inhibition of adenylyl cyclase, phospholipase C, and MAPKs, and activation of A_1 and A_3 decrease cAMP, while activation of A_{2A} and A_{2B} increase cAMP.¹⁴⁶ The main source of extracellular adenosine is ATP, which is released from all cells at a basal level and is significantly increased in response to inflammation, tissue damage, and nerve injury.^{71, 198} Of note, the spinal cord trauma and inflammation that results after SCI induces a massive release of endogenous danger signals, including ATP, and ATP can signal through P2 purinergic receptors which can activate spinal glial cells and induce neuropathic pain.²²² Other sources of extracellular adenosine include passive transport from intracellular pools and from intracellular cAMP or extracellular cAMP released from damaged cells.²⁷⁵

Extracellular adenosine levels are extremely variable depending on the tissue and degree of injury, and the effects of adenosine agonists and antagonists on central disorders are often confusing and opposing. The effects depend on the model, the timing, the tissue, the dose, and the specific cellular milieu surrounding the microenvironment. This is likely due to the ability of these receptors to adapt to and change within their local environment. For example, $A_{2A}R$ agonists are neuroprotective in acute brain damage following stroke, but are neurally detrimental in experiments looking at chronic effects of brain damage after stroke.³⁰² In a mouse model of TBI, when Dai et al. administered the $A_{2A}R$ agonist CGS21680 at post-TBI times when brain glutamate levels were low, behavioral deficits and mRNA expression of $TNF-\alpha$, $IL-1\beta$, and inducible nitric oxide (iNOS) were attenuated, but if CGS21680 was administered at post-TBI times when brain glutamate levels were high, behavioral deficits and cytokine expression were exaggerated beyond TBI plus vehicle.⁵⁶ Furthermore, if CGS21680 was administered during

high glutamate levels post-TBI, pre-TBI treatment with a glutamate release inhibitor had neuroprotective effects, suggesting that glutamate levels can influence whether or not A_{2A}R activation leads to a proinflammatory or an anti-inflammatory response. It has also been hypothesized that A_{2A}R agonism on immunocompetent cells such as microglia and macrophages results in anti-nociception, while A_{2A}R agonism of peripheral nerve cells is pro-nociceptive.¹³⁹

Role of A_{2A}R in SCI

In animal models of SCI, A_{2A}R agonists have multiple therapeutic effects. In mouse models of SCI, the selective A_{2A}R agonist ATL313 reduced motor deficits and demyelination,¹⁸⁶ and both ATL313 and CGS21680 decreased TNF- α expression in the spinal cord.¹⁰¹ A different A_{2A}R agonist, ATL146e, decreased paralysis in rabbits with contusion SCI twelve hours post-surgery compared to controls.⁴⁰ Although there are multiple studies that examine anti-allodynic effects of A_{2A}R agonism in peripheral neuropathic pain,^{28, 183, 191} the studies discussed in chapter III are the first to examine the anti-allodynic effects of A_{2A}R agonism in central neuropathic pain. One of the possible mechanisms for the anti-inflammatory and anti-allodynic effects of A_{2A}R agonism in SCI is a down-regulation of proinflammatory cytokines and an up-regulation of the anti-inflammatory cytokine IL-10.

IL-10 and alternative activation

IL-10 is a potent anti-inflammatory cytokine that is produced by glia and immune cells as well as neurons. IL-10 is released in response to inflammation in order to help control the proinflammatory response through negative feedback and also to help with wound healing.¹⁶³ The IL-10 receptor is a heterotetramer comprised of four chains, two IL-10R1 and two IL-10R2 where IL-10R1 is critical for IL-10 binding and signal transduction and has a high affinity for the IL-10R complex, and IL-10R2 is only required for signaling and can be part of the

receptor complexes of other cytokines.^{168, 360} When IL-10 binds IL-10R1, IL-10R1 then interacts with IL-10R2 to activate Jak1 and tyrosine kinase 2 of the Janus kinase family.²¹⁷ These phosphorylate IL-10R1 which allows phosphorylation and binding of signal transducer and activator of transcription 3 (STAT3), which ultimately leads to anti-inflammatory activity. IL-10 can inhibit NF κ B and MAPK signaling, thereby suppressing proinflammatory cytokine production.^{48, 206}

There is both clinical and pre-clinical evidence that IL-10 is therapeutic in SCI. Human neuropathic pain patients have decreased levels of IL-10 in cerebrospinal fluid (CSF) compared to healthy controls, and these decreased levels correlate to higher pain scores.⁸ Acute systemic administration of IL-10 in a rat contusion model of SCI reduced expression of TNF- α in spinal cord and increased motor recovery and decreased lesion size two months post-injury.²⁰ However, there are also instances where IL-10 in SCI can be deleterious. Interestingly, chronic systemic administration of IL-10 is detrimental to recovery in SCI and can even increase peripheral neuropathy.³¹⁷ Also, if IL-10 is administered intrathecally immediately after SCI, indices of injury are exacerbated.²⁵ It is thought that a single immediate intrathecal injection or chronic systemic injections are detrimental because they do not allow recruitment of monocytes to the injury site where they would be able to release local IL-10 and participate in phagocytosis and wound healing.²⁸⁵

It is now accepted that macrophages can exist in a spectrum of different states of activation. The definition of the different activation states of these cells was originally based on *in vitro* studies of peripheral monocytes/macrophages, which was later confirmed *in vivo* in rodents.¹⁰⁷ Classical activation of macrophages, also known as the M1 phenotype, is caused by the soluble cytokine interferon (IFN)- γ and results in the expression of high levels of

proinflammatory cytokines and increased microbicidal capacity.⁵⁷ Alternative activation of macrophages, also known as the M2 phenotype, is split into two different categories based on function. This first is wound healing, which is stimulated by the release of IL-4 and IL-13 from T helper 2 (Th2) lymphocytes and results in the up-regulation of mannose receptor and arginase-1.²² The other category is regulatory/anti-inflammatory, and is stimulated by the release of IL-10 from microglia and macrophages, resulting in further increases in IL-10.²¹⁷ It is thought that the same macrophage can adopt either a more proinflammatory M1-like or more anti-inflammatory M2-like phenotype depending on the stimulus, and can switch back and forth between the phenotypes as the microenvironment changes.²¹⁶ The question remains whether microglia can also adopt these different activation states/phenotypes. Part of the difficulty in assessing microglial activation states is the lack of a specific marker for simply identifying microglia, as macrophages also express the same markers, and the mechanisms of how these phenotypes are regulated in microglia are still poorly understood.²² However, it is clear that microglia do express differential phenotypes depending on the stimulus and environment.

Microglia can respond differently to the same stimulus depending on what, if any, signals precede or follow the initial stimulus. For example, microglia can be “primed”, where the first stimulus primes the cell to have an exaggerated response upon activation by a second stimulus.⁹⁶ Microglia can also become activated by small amounts of T-cell-derived TNF- α without releasing TNF- α themselves.^{281, 284} Small doses of TNF- α or IFN- γ can make microglia exert neuroprotective effects, whereas larger doses then cause a switch in the microglia to now become cytotoxic.^{19, 31} When microglia are exposed to a proinflammatory environment and then subsequently encounter the anti-inflammatory Th2 cell-derived cytokine IL-4, the microglia can switch to a more anti-inflammatory/protective phenotype.³⁰ Finally, there is a group of microglial

cells that are thought to be quiescent up to a certain age, but become increasingly dysfunctional with the aging process.²⁸¹

Glia and the Immune System

The way that the body responds to invading pathogens is by activating the innate immune response. The very first line of defense is the physical barrier made up of the epithelium (skin, gut, lungs), but there are many other components that make up the innate immune system. These include pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), nucleotide-binding and oligomerization domain leucine-rich repeats containing (NOD)-like receptors (NLRs), scavenger receptors (SRs), and glial cells. The following sections will discuss the role of glia in innate immunity.

Glia as immune cells

Microglial cells are of myeloid origin¹⁰³ and both microglia and astrocytes, like other classical innate immune cells (macrophages, dendritic cells), become activated by recognizing specific molecular patterns on pathogens.²²⁵ These molecular patterns are termed pathogen-associated molecular patterns (PAMPs) and are recognized by PRRs on glia cells. In addition to recognizing pathogens, it is now known that the innate immune system also responds to endogenous “danger” signals that are released by damaged/dying cells such as that seen in SCI and other traumatic and inflammatory diseases.⁴³ These danger signals are termed danger associated molecular patterns (DAMPs) and include things like ATP, heat shock proteins, and high mobility group box 1 (HMGB1), although there are many others that have been identified.²³⁴ These DAMPs bind to and activate TLRs, which induce different immune-like intracellular signaling cascades.²⁰⁸ There are currently 13 different TLRs, and TLR2 and TLR4 are important in neuropathic pain and are expressed predominately on microglia (although can

also be expressed on astrocytes and neurons).^{39, 336} Furthermore, DAMPs activate glia by binding to TLR4 which results in the release of the proinflammatory cytokines IL-1 β , TNF- α , and IL-6.²⁶

TLR4 signaling

Glial activation via TLR4 is heavily implicated in both peripheral and central neuropathic pain.^{26, 144} Although the exact details of how different DAMPs activate TLR4 is not entirely known, it is likely similar to activation of TLR4 by gram-negative bacteria such as lipopolysaccharide (LPS). The TLR4 signaling cascade will be described using LPS as the example ligand. TLR4 is a membrane bound receptor and LPS binding leads to intracellular activation of the enzyme acid sphingomyelinase which then generates ceramide (a bioactive lipid). Ceramide induces the generation of a lipid raft containing the co-receptor myeloid differentiation factor 2 (MD2) and TLR4 (there are other elements of the lipid raft that will not be discussed here). Ceramide also activates NADPH oxidase, leading to peroxynitrite formation, which is important in the development of many pain disorders including pain after chronic opioid use.²⁷³ CD14 then transfers the ligand to MD2, leading to both MD2-TLR4 heterodimerization and then homodimerization of the MD2-TLR4 pairs. Ensuing intracellular signaling occurs through toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) to at least three parallel pathways: the IP3K/Akt pathway (cell motility/survival/apoptosis/generation of NO and TNF- α), the NF κ B pathways (proinflammatory cytokine transcription), and MAPK pathways.³⁴⁵ In order to activate a proinflammatory response, the adaptor proteins myeloid differentiation primary response 88 (MyD88) and the TIR-domain-containing adapter-inducing interferon- β (TRIF) must bind to the intracellular domain of TLR4. Together these proteins activate the transcription factors NF κ B, AP-1, and IRF-3, which leads to the expression of proinflammatory cytokines, including IL-1 β and TNF- α .²⁴⁵ Also of importance for neuropathic pain, TLR4 is

activated by every clinically relevant class of opioids.^{141, 142, 340, 345} This is important because many neuropathic pain patients, and virtually all SCI pain patients, are treated with opioids at some point and there is evidence that opioids can have deleterious effects on health and recovery following injury.^{132, 270}

Although TLR4 is found predominately on glial cells in the spinal cord, it is also expressed on neurons in the brain and on peripheral cells.^{182, 367} Peripheral blood mononuclear cells (PBMCs) that were isolated from chronic pain patients and stimulated *ex vivo* with LPS released increased IL-1 β compared to healthy controls,¹⁷³ and trigeminal sensory neurons and hippocampal neurons express TLR4 and CD14 and can respond to LPS.^{336, 364} Diogenes et al. show that, *in vitro*, TLR4 co-localizes with capsaicin-sensitive TRPV1 in trigeminal sensory neurons and LPS increases capsaicin-induced CGRP release via TRPV1 and TLR4, causing a sensitization of these trigeminal neurons.⁷⁰ Zhao et al. showed that cultured hippocampal neurons stimulated with LPS lead to increased expression of TNF- α and IL-1 β via NF κ B activation.³⁶⁴ TLR4 may also be able to activate the PKC- ϵ signaling pathway, and PKC- ϵ leads to activation of sensory neurons via its expression in nociceptors.²²⁸ TLR4 is also found on non-nociceptive, non-myelinated, neurofilament H (NF-H) expressing neurons,³³⁶ and spinal nerve ligation can induce small diameter neurons to express NF-H.¹²⁰ TLR4, MyD88, and TRIF are also up-regulated in DRG neurons seven days after paclitaxel-related chemotherapy-induced peripheral neuropathy (CIPN), at which time rats were also allodynic. However, the up-regulation in TLR4, MyD88, and TRIF was gone by day fourteen even though rats were still allodynic at day twenty one, suggesting that TLR4 expression in DRG neurons is only contributing to acute, not chronic, neuropathic pain.¹⁸⁷

Cytokine processing and inflammasomes

TNF- α is important for initiating the proinflammatory response and neuropathic pain,^{6, 304} especially in mediating thermal hyperalgesia.³⁰⁰ TNF- α has two receptors, the constitutively expressed TNFR1-p55R and the inducible (under inflammatory conditions) TNFR2-p75R, and binding to one or the other induces a differential response.⁵⁰ Binding to the p55 subunit induces programmed cell death, but binding to the p75 subunit induces translocation of NF κ B into the nucleus and stimulates the production of IL-1 β .³²⁶ TNF- α and IL-1 β released by activated glia can then produce and amplify pain by activating other surrounding glial cells, by acting directly on their receptors on neurons or by phosphorylating NMDA receptors on neurons, thereby increasing neuronal excitability by releasing other substances like D-serine that enhance NMDA channel function, and by activating p38 and ERK MAPK cascades.²⁰⁸

IL-1 β is also importantly involved in neuropathic pain and it is considered the “gatekeeper of inflammation”, and is therefore very tightly regulated.¹⁷⁷ The way in which IL-1 β is processed is different than most other cytokines; it must be cleaved from a precursor protein (pro-IL-1 β) into a mature protein that can then be released.³²⁷ The molecule that cleaves the pro-form of IL-1 β into the mature form is the protease caspase-1, although caspase-1 must also be cleaved from a precursor protein, pro-caspase-1.¹⁷⁴ The way that pro-caspase-1 is cleaved is through the formation and activation of a group of intracellular proteins collectively called the inflammasome.²¹³ The inflammasome complex is made up of a sensor molecule, the adaptor protein apoptotic speck protein (ASC), and the effector protein pro-caspase-1.¹⁷⁷ The majority of the inflammasomes also contain a nucleotide-binding oligomerization receptor (Nod-like receptor, NLR), which acts as the intracellular sensor of alarmins.⁶⁶ There are six NLR inflammasomes (NLRP1, 2, 3, 6, 7, and NLRC4), and three of these (NLRP1, 2, 3) have been

identified in the CNS.^{121, 200, 209} NLRP1 is found mainly on neurons, including motor neurons in the spinal cord,⁶³ and NLRP2 is found mainly on astrocytes.²⁰⁹ However, NLRP3 is thought to be expressed only in myeloid cells and is the only one known to be expressed on microglia.¹²¹

It is thought that the NLRP1 inflammasome in the CNS, unlike in the periphery, is preassembled, allowing for rapid activation of the innate immune response following trauma.⁶³ The NLRP1 inflammasome in the brain and spinal cord is a multi-protein complex made up of NLRP1, caspase-1, ASC, caspase-11, and the inhibitor of apoptosis proteinX-linked inhibitor of apoptosis protein (XIAP).⁶³ SCI-induced activation of the NLRP1 inflammasome is associated with cleavage of XIAP, which is thought to reduce the threshold for activation of caspase-1, leading to amplified expression of IL-1 β .⁶² Of note, XIAP is not associated with NLRP1 on peripheral immune cells.¹⁷⁰ Pannexin-1 is thought to link P2X7 and P2X4 to the NLRP1 inflammasome only in neurons, and deletion of P2X4 in mice following SCI significantly decreases NLRP1 inflammasome activation, production of IL-1 β , and infiltration of immune cells into the injury site.⁶¹ NLRP2 consists only of NLRP2, ASC, and caspase-1 and normally inhibits NF κ B.¹⁸⁸ However, if NLRP2 in astrocytes is activated by a DAMP such as ATP, it interacts with P2X7 and pannexin-1 to initiate rapid cleavage of caspase-1 and production of mature IL-1 β .⁶²

The NLRP3 inflammasome is the most studied and is unique in that basal NLRP3 protein expression is too low for actual formation/activation of the inflammasome.¹⁴ In order for NLRP3 inflammasome formation to occur, the NLRP3 gene must first be transcribed and translated into protein. This step is thought to require TLR2 or 4 ligation, and is referred to as the “priming” step. After enough NLRP3 protein has accumulated, a second signal is required for NLRP3 to interact with ASC. NLRP3 is unusual in that it responds to a wide variety of second signals,

which include ATP, reactive oxygen species, and other alarmins.¹³⁵ ASC can then recruit pro-caspase-1, which results in the cleavage/activation of pro-caspase-1 which then cleaves pro-IL-1 β into its mature protein form. Although inflammasome-dependent processing of IL-1 β is most common, there are also inflammasome-independent mechanisms for processing IL-1 β .

Although caspase-11 can directly process pro-IL-1 β into mature IL-1 β in the absence of caspase-1, it is very slow and inefficient.³³⁹ It is more likely that Caspase-11 is enhancing caspase-1 activation independently of inflammasome activation. Caspase-11 also directly controls the activation of the effector caspases 3 and 7 of the pyroptosis apoptotic pathway independent of caspase-1.³³² Caspase-8 can also process pro-IL-1 β into its mature form independent of inflammasome activation by activating a MALT1–caspase-8–ASC complex.¹¹⁰ IL-1 β release in human PBMCs induced by autophagy is also inflammasome-independent and is thought to involve the inhibition of p38 MAPK phosphorylation.⁵³ Stimulation of microglia with ATP in acidic conditions induces the release of the 20-kDa form of IL-1 β rather than the more commonly reported 17-kDa form,⁷⁶ and the cleavage of the 20-kDa form does not require caspase-1 but instead depends on the protease cathepsin D.³¹⁰ Both a cathepsin B inhibitor and caspase-1 inhibitor administered to cultured neonatal microglia from NLRP3 knock out (KO) mice that were stimulated with *Staphylococcus aureus* (*S. aureus*) bacteria attenuated IL-1 β release, providing further evidence of inflammasome-independent processing of mature IL-1 β .¹²¹ Lastly, there are other non-caspase proteases that can cleave IL-1 β into its mature form,⁸⁸ including proteinase-3, which is implicated in acute arthritis.¹⁵³

Theoretical Considerations and Thesis Overview

There are more than a quarter million Americans suffering from chronic pain, a problem that costs millions of dollars and for which there are no truly effective treatments.³⁶ Many of

these treatments target neurons, but it is important to consider other cell types that could be contributing to pain, including macrophages, other peripheral immune cells, and glial cells.

Glial activation has been implicated in many different central pain disorders, however, this dissertation will focus on SCI, as it is one of the most prevalent and debilitating disorders. Furthermore, we have developed a novel rat model of unilateral T13/L1 avulsion SCI specifically to study the mechanisms, both glial and otherwise, that underlie central neuropathic pain.^{261, 349} Avulsion literally means “to rip out”, and dorsal root avulsions often happen in vehicle accidents, especially motorcycle accidents. Our avulsion model, termed Spinal Neuropathic Avulsion Pain (SNAP), causes actual spinal cord dorsal horn damage, and results in robust bilateral below-level central neuropathic pain that lasts for about two months.³⁴⁹ Our model is also unique in that it does not cause paralysis, autotomy, or urinary tract infections, which can confound the study of pain behavior. In addition, we know that blocking glial activation and the subsequent release of proinflammatory cytokines attenuates SNAP.³⁴⁹ Interestingly, SNAP does not induce thermal hyperalgesia. However, thermal hyperalgesia is rarely seen in central neuropathic pain disorders because it usually indicates sensitization of peripheral afferents.³⁴¹ It is well accepted in the pain literature that dorsal spinal cord glial activation and the consequent release of neuroexcitatory and neuroinflammatory mediators are necessary and sufficient for the induction and maintenance of chronic pain.³⁴³ This is perhaps why neuronally targeted treatments are ineffective; the glia are still activated and are able to maintain the pain state. We hypothesize that glia, and their release of inflammatory mediators, amplify chronic SCI pain. Therefore, studying the behavioral and molecular changes associated with SCI pain and using the results to develop treatments that target glia could potentially yield much better results in SCI pain patients. The studies presented in chapter II discuss the effects of

administering three different glial activation inhibitors, PPF, ibudilast, and (+)-naltrexone in SNAP. Each of these compounds has a distinct mechanism of action but have each been documented to exert their primary effects on glial cells.

Adenosine is found on every cell type throughout the body and is best known for its interaction with caffeine, which is a non-specific adenosine antagonist.²⁶¹ There are four different types of adenosine receptors, and there is an expanding body of literature that shows the ability of adenosine A_{2A}R agonists to decrease inflammation by suppressing proinflammatory and increasing anti-inflammatory cytokines in peripheral,¹²⁵ and central,¹⁹¹ immune cells. Because of this, there has been a recent interest in developing A_{2A}R agonists, such as CGS21680 and ATL313, for clinical use. The studies in chapter III discuss the effects of giving a single acute intrathecal injection of an adenosine A_{2A}R agonist in SNAP.

The most effective and widely used therapy for central neuropathic pain is opioid treatment. However, recent studies in both clinical and preclinical models have shown that opioid treatment is actually detrimental to recovery.^{131, 328} Opioids amplify the magnitude and duration of chronic pain and suppress motor recovery,¹³² and opioid treatment can also cause constipation,²²³ which is a major problem in SCI. These deleterious effects of opioid administration impact almost every single SCI patient since they are universally treated with opioids, whether en route to the hospital, in the hospital during the acute phase of trauma, or long after the initial injury when chronic pain develops. Opioids activate TLR4 on glial cells,¹⁴⁵ which in the presence of other endogenous danger signals that are massively released by damaged cells in the spinal cord during SCI; can actually amplify pain and neuroinflammation. The studies presented in chapter IV discuss the deleterious effects of administering a week-long course of systemic morphine shortly after SNAP.

Taken all together, the complete set of experiments described in this dissertation strongly suggest that spinal glia are significant contributors to the mechanisms underlying central neuropathic pain and neuroinflammation and are a promising therapeutic target for pain control.

Chapter II

Systemic Administration of Propentofylline, Ibudilast, and (+)-Naltrexone Each Reverses Mechanical Allodynia in a Novel Rat Model of Central Neuropathic Pain

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Abstract

Central neuropathic pain (CNP) is a debilitating consequence of central nervous system (CNS) damage for which current treatments are ineffective. To explore mechanisms underlying CNP, we developed a rat model involving T13/L1 dorsal root avulsion. The resultant dorsal horn damage creates bilateral below-level (L4-6) mechanical allodynia. This allodynia, termed spinal neuropathic avulsion pain (SNAP), occurs in the absence of confounding paralysis. To characterize this model, we undertook a series of studies aimed at defining whether SNAP could be reversed by any of 3 putative glial activation inhibitors, each with distinct mechanisms of action. Indeed, the phosphodiesterase inhibitor propentofylline, the macrophage migration inhibitory factor (MIF) inhibitor ibudilast, and the toll-like receptor 4 (TLR4) antagonist (+)-naltrexone each reversed below-level allodynia bilaterally. Strikingly, none of these impacted SNAP upon first administration but required 1-2 weeks of daily administration before pain reversal was obtained. Given reversal of CNP by each of these glial modulatory agents, these results suggest that glia contribute to the maintenance of such pain and enduring release of MIF and endogenous agonists of TLR4 is important for sustaining CNP. The markedly delayed efficacy of all 3 glial modulatory drugs may prove instructive for interpretation of apparent drug failures after shorter dosing regimens.

Introduction

Central neuropathic pain (CNP) is a common and debilitating consequence of a variety of different central nervous system (CNS) traumas including spinal cord injury (SCI), stroke, traumatic brain injury (TBI), and multiple sclerosis (MS). The percentage of patients that develop central pain following trauma range from 8% in stroke, 25% in MS, 50% in TBI, and up to 66% in SCI.^{23, 224, 289} Mechanisms underlying CNP are poorly understood, and current pharmacotherapies for treating this type of pain are not effective.

CNP is extremely difficult to treat, and current therapies have limited response rates, provide only minor pain relief, and often have intolerable side effects. Classes of drugs commonly used to treat CNP include tricyclic antidepressants (TCAs), calcium channel trafficking inhibitors, anticonvulsants, and opioids.⁹⁰ Using SCI as an example, a clinical SCI study found that gabapentin, which inhibits calcium channel trafficking,^{128, 171} was no more effective than the active placebo and that the TCA amitriptyline was slightly more effective than active placebo but resulted in undesirable side effects (nausea, bladder problems, constipation).²⁶⁰ Another clinical study found that the anticonvulsant drug lamotrigine did not relieve central pain in patients with MS.²⁴ Although opioids are one of the most widely used and effective central pain treatments,³⁵² the side effects and addiction problems that can arise from chronic use cause many pain patients to discontinue treatment.¹⁷ In addition, recent literature has shown that morphine given shortly after SCI can have deleterious effects on recovery.¹³¹ One commonality between all of these drugs is that their mechanisms of action are thought to be largely neuronal, but it is well known that glial cells that become activated after a traumatic inflammatory event play a large role in the induction and maintenance of a variety of chronic pain states of peripheral origin.^{35, 109} Examining the potential role of glia in mechanisms

underlying CNP may potentially lead to the development of more efficacious CNP treatments as well as improve our understanding of the mechanisms that underlie this type of pain.

Since the complexities of CNS traumas make them extremely difficult to treat, it is important to consider not only neuronal targeting compounds, but also those treatments that target other cell types such as glia and macrophages. The current series of studies examined the effects of administering three different putative glial activation inhibitors, propentofylline (PPF; a phosphodiesterase (PDE) inhibitor),^{220, 308} ibudilast (some PDE actions but considered predominantly as a MIF inhibitor),⁴⁹ and (+)-naltrexone (a non-opioid TLR4 antagonist),¹⁴⁴ in a novel model of unilateral T13/L1 dorsal root avulsion SCI. Dorsal root avulsions are common after automobile and motorcycle accidents^{18, 37} and can cause below-level pain.^{75, 85} Our avulsion model creates a discrete dorsal horn spinal cord lesion resulting in bilateral below-level neuropathic pain (termed spinal neuropathic avulsion pain [SNAP]), but does not cause paralysis, gross white matter damage, or urinary tract infections that are inherent, at least in part, in most models of stroke, TBI, MS, and contusion and hemisection SCI.³⁴⁹ We developed this avulsion model in order to study pain behavior in isolation from such complicating factors so that we may better understand the specific mechanisms underlying CNP.

Materials and Methods

Animals

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado at Boulder. The care and use of the animals also conformed to guidelines of the International Association for the Study of Pain. Pathogen-free male Sprague-Dawley rats (325-350g; Harlan Laboratories, Madison, WI, USA) were used for all experiments. Rats were pair-housed prior to surgery and then single-housed after surgery with standard rat

chow and water available *ad libitum*. Housing was in a temperature-controlled room that was maintained at 23 $\pm$ 2°C with a 12hr light/dark cycle (lights on at 0700 hr). The rats were allowed a minimum of 1 week to habituate to the colony room before initiating the experiment. All procedures were done during the light cycle.

Drugs

Propentofylline (PPF; a gift from MediciNova, San Diego, CA, USA and Solace/Patheon UK Ltd, Wiltshire, England) was dissolved in sterile endotoxin-free isotonic saline (Abbott Laboratories, Abbott Park, IL, USA) and administered at dose of 10 mg/kg per intraperitoneal (i.p.) injection. Controls received i.p. equivolume (1 ml/kg) saline. Ibudilast (MN-166; a gift from MediciNova, San Diego, CA, USA) was dissolved in 100% corn oil (Mazola) and administered at 10 mg/kg per subcutaneous (s.c.) injection. Controls received s.c. equivolume (0.5 ml/kg) corn oil. (+)-Naltrexone (a gift from NIDA and NIAAA, Rockville, MD, USA) was dissolved in sterile endotoxin-free isotonic saline (Abbott Laboratories, Abbott Park, IL, USA) and administered at 6 mg/kg per s.c. injection. Controls received equivolume (1 ml/kg) saline. All drugs were given systemically as they are all known to cross the blood brain barrier.^{95, 144, 178}

Spinal Neuropathic Avulsion Pain (SNAP) Surgery

Unilateral (left) T13/L1 dorsal root avulsion was performed under isoflurane anesthesia, as previously described in detail.³⁴⁸ Briefly, laminectomy was performed at the T12 vertebral level and the dura mater was incised over the dorsal root entry zone. The T13 and L1 dorsal rootlets were carefully isolated and then clamped at the dorsal root entry zone and briskly pulled out (avulsed). Sterile saline-moistened surgical sponge was placed over the exposed spinal cord to protect it, the muscle was sutured in layers with sterile 3-0 silk, and the skin was closed with stainless steel wound clips. Immediately following surgery, rats were single-housed in a cage

with foam padding for a few hours to protect their spinal cord from further trauma due to the brief ataxic period that follows recovery from anesthesia. Sham operated rats were treated identically, except for avulsing of the rootlets. Combi-Pen-48 antibiotic (0.2 ml; Bimeda, Inc., Le Sueur, MN, USA) was administered at the time of surgery and daily for 4 days after surgery.

Low Threshold Mechanical Allodynia Testing

Prior to surgery, rats were habituated to the testing environment for 4 consecutive days prior to recording of behavioral responses. All von Frey assessments were performed blind with respect to drug and surgery assignments. Assessment of von Frey thresholds occurred before surgery (baseline) and across a timecourse beginning two weeks after surgery. The von Frey test was performed on the plantar surface of each hind paw as previously described in detail.²⁰⁵ A logarithmic series of 10 calibrated Semmes–Weinstein monofilaments (Stoelting) were sequentially applied to the left and right hind paws in random order, each for 8 s at constant pressure to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. Log stiffness of the hairs is determined by $\log_{10}$ (milligrams $\times 10$). The range of monofilaments used in these experiments (0.407–15.136 g) produces a logarithmically graded slope when interpolating a 50% response threshold of stimulus intensity (expressed as $\log_{10}$ (milligrams $\times 10$)).⁴¹ The stimulus intensity threshold to elicit a paw withdrawal response was used to calculate the 50% paw withdrawal threshold (absolute threshold) using the maximum-likelihood fit method to fit a Gaussian integral psychometric function.¹²⁴ This method normalizes the withdrawal threshold for parametric analyses.¹²⁴ The general von Frey testing rubric is as follows. Rats are baselined before surgery and are allowed a 14 day recovery period. Rats are tested on a weekly basis on days 14, 21, and 28 post-surgery. Drug administration begins after the day 28 behavioral test, with one exception ((+)-naltrexone) where drug

administration began on day 32 post-surgery due to extenuating circumstances. Behavioral testing then occurs once per week until the conclusion of the study, except for when a more detailed timecourse was necessary to define when reversal of enhanced mechanical reactivity (allodynia) began to occur. In these studies, behavioral testing occurs every 2-3 days after drug administration.

Pharmacological Manipulations

Propentofylline (PPF) Timecourses

Daily PPF dosing began after development of SNAP was confirmed by von Frey testing 14, 21, and 28 days after surgery. PPF (10 mg/kg) or equivolume vehicle (saline) was administered once daily for either 14 days or 35 days. PPF was administered between 1600-1800 hr, and behavioral testing occurred approximately 15 hr later (0700-0900 hr), so to parallel previous publications.³¹⁴ This delayed testing timepoint is standard when looking at glial effects of PPF in order to allow time for second messenger signaling cascades to exert their effects.^{114, 215, 251, 309, 315} Rats were behaviorally tested to define their mechanical response thresholds every 2-7 days until the final behavioral test 15 hr after the last dose of PPF or vehicle.

Ibuprofen Timecourse Later in the Development of SNAP

Daily ibuprofen dosing began after development of SNAP was confirmed by von Frey testing 14, 21, and 28 days after surgery. Ibuprofen (10 mg/kg) or equivolume vehicle (corn oil) was administered once daily for 35 days. Ibuprofen was administered between 0800-1000 hr each morning and testing occurred 1-2 hr later, so to parallel previous publications.¹⁷⁹ Rats were behaviorally tested to define their mechanical response thresholds every 7 days until the final behavioral test 1-2 hr after the last dose of ibuprofen or vehicle.

Ibutilast Timecourse Early in the Development of SNAP

Daily ibutilast dosing began after development of SNAP was confirmed by von Frey testing 14 days post-surgery. Ibutilast (10 mg/kg) or equivolume vehicle (corn oil) was administered once daily for 21 days. Ibutilast was administered between 0800-1000 hr each morning and testing occurred 1-2 hr later, as described above. Rats were behaviorally tested to define their mechanical response thresholds every 7 days until the final behavioral test after the last dose of ibutilast or vehicle.

(+)-Naltrexone Timecourse

Daily (+)-naltrexone dosing began after development of SNAP was confirmed by von Frey testing 14, 21, 28, and 32 days after surgery. Given its relatively short half-life compared to ibutilast and PPF, (+)-naltrexone (6 mg/kg) or equivolume vehicle (saline) was administered three times a day for 14 days. (+)-Naltrexone was administered at approximately 0900, 1200, and 1500 hr and testing occurred 1 hr after the second injection, based on our previous studies.¹⁴⁴ Rats were behaviorally tested to define their mechanical response thresholds every 7 days until the final behavioral test after the last dose of (+)-naltrexone or vehicle.

(+)-Naltrexone Drug Cessation Timecourse

Daily (+)-naltrexone dosing began after development of SNAP was confirmed by von Frey testing 14, 21, and 28, days after surgery. Given its relatively short half-life compared to ibutilast and PPF, (+)-naltrexone (6 mg/kg) or equivolume vehicle (saline) was administered three times a day for 6 days. (+)-Naltrexone was administered at approximately 0900, 1200, and 1500 hr and testing occurred 1 hr after the second injection, based on our previous studies. Rats were behaviorally tested to define their mechanical response thresholds daily until the final

behavioral test after the last dose of (+)-naltrexone or vehicle on day 33. Rats were then behaviorally tested every 2-6 days, at approximately 1300hr, until they returned back to pre-drug allodynia levels. Since we already observed that (+)-naltrexone had no effect on sham operated rats in the previous (+)-naltrexone study, and to conserve drug, sham groups were not included in this study.

Statistical Analysis

All data were expressed as mean $\pm$ standard error of the mean (SEM). Ipsilateral and contralateral behavioral data were analyzed individually. Behavioral measures were normalized as described above and group differences were analyzed by comparing area under the curve (AUC), as previously described by Jones and Sorkin.¹⁵² AUC values (GraphPad Prism 5.01; GraphPad software Inc., San Diego, CA) were calculated from absolute threshold values, from 3.56 (the lowest threshold response value in the data set) up to the threshold response of each rat across time. Decreased AUC reflects an increase in mechanical allodynia. For all studies, the AUC measures across time were collapsed into a single timepoint for each animal, thus there is not a repeated measurement. For baseline measurements, a two-way ANOVA at that single timepoint was the statistic used. For pre-drug statistics, a t-test was performed on the AUC values as there were only two groups, sham and SNAP. For statistics during the drug administration period, a two-way ANOVA was then performed on the AUC values, with the exception of the studies examining the effects of (+)-naltrexone on SNAP. For the (+)-naltrexone drug timecourse in Figure 5, a one-way ANOVA was used because it is not a 2x2 design. For the study looking at behavior after stopping (+)-naltrexone administration in Figure 6, a t-test was used because there are only two groups, SNAP+Vehicle and SNAP+(+)-Naltrexone. The appropriate AUC statistic was performed on days 35-63 for Figure 1, days 35-42 for Figure 2,

days 35-63 for Figure 3, days 14-28 for Figure 4, days 35-46 for Figure 5, days 29-33 for Figure 6B, and days 35-42 for Figure 6C. A Bonferroni *post hoc* test for multiple comparisons was used where appropriate. For all tests, $p < 0.05$ was considered statistically significant.

Results

Effect of Administering Propentofylline on SNAP

In this first study, once daily PPF was administered i.p. at 10 mg/kg for 35 days beginning 28 days post-surgery. No differences were observed between groups in the response thresholds recorded for the hindpaw ipsilateral (Fig 2.1A) or contralateral (Fig 2.1B) to the avulsion injury pre-surgery [baseline (BL)]. No differences were observed between the SNAP groups on either the ipsilateral or contralateral hindpaw pre-drug, recorded 14, 21, and 28 days after surgery; that is, prior to initiation of PPF treatment. PPF had no effect on the response thresholds of sham operated rats, which showed mild and transient allodynia compared to avulsion. The SNAP group was significantly more allodynic than the sham group pre-drug (days 14-28) on both the ipsilateral ($t_{30} = 4.396$; $p < 0.001$) and contralateral ($t_{29} = 2.9$; $p < 0.01$) hindpaw. The two-way ANOVA comparing the AUC of Sham+Vehicle, Sham+PPF, SNAP+Vehicle, and SNAP+PPF over the drug treatment timecourse (days 35-63) showed a significant interaction in both the ipsilateral ($F_{1,32} = 16.29$; $p < 0.001$; Fig 2.1C) and contralateral ($F_{1,32} = 15.78$; $p < 0.001$; Fig 2.1D) hindpaw. There was also a significant main effect of surgery in both the ipsilateral ($F_{1,32} = 16.57$; $p < 0.001$) and contralateral ($F_{1,32} = 11.94$; $p < 0.01$) hindpaw as well as a significant main effect of drug treatment in both the ipsilateral ($F_{1,32} = 8.603$; $p < 0.01$) and contralateral ($F_{1,32} = 4.232$; $p < 0.05$) hindpaw. Bonferroni *post hoc* analysis of the AUCs revealed that the SNAP+Vehicle group was significantly more allodynic than all other groups ($p < 0.05$) in both the ipsilateral and

contralateral hindpaw. Furthermore, there were no significant differences between the other three groups in both the ipsilateral and contralateral hindpaw ($p>0.05$).

Figure 2.1: Effect of administering propentofylline on SNAP

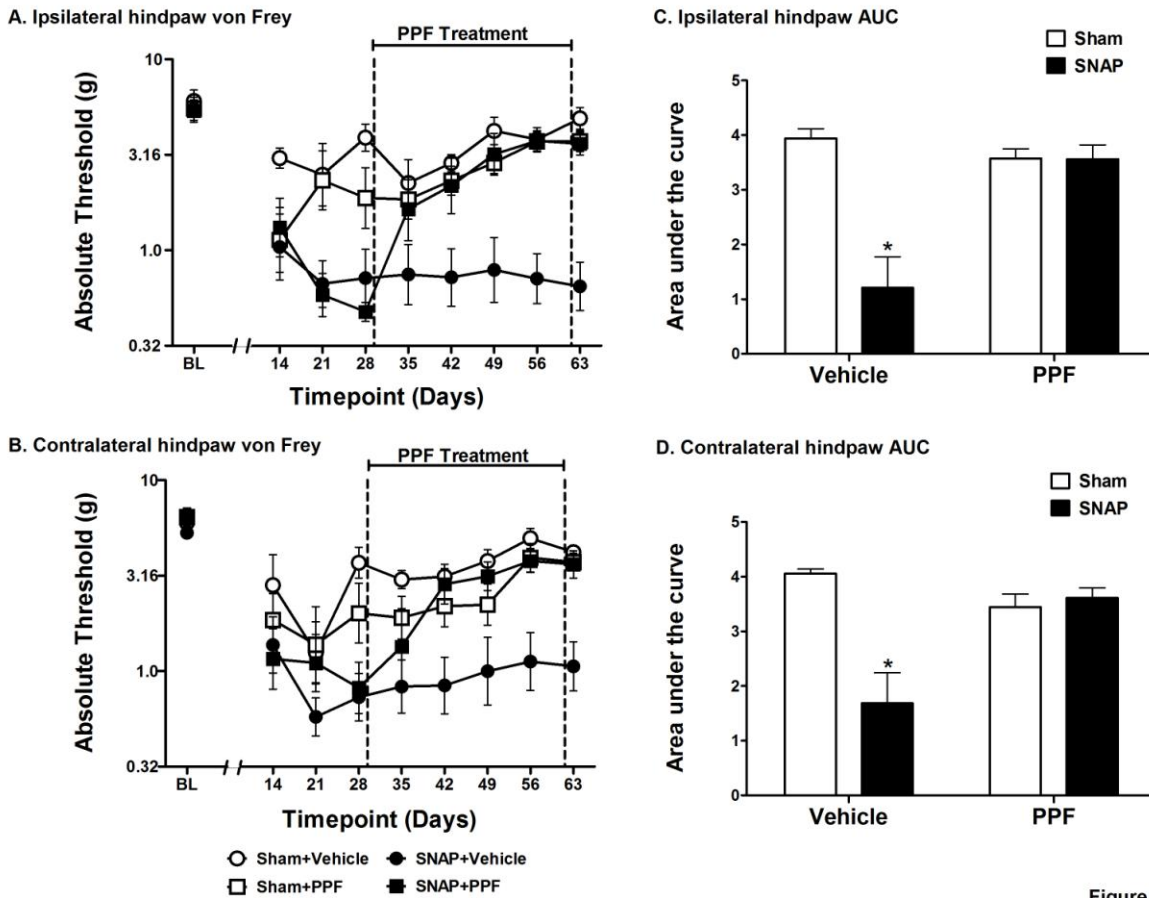


Figure 2.1. Assessment of the effects of propentofylline (PPF) on SNAP. Rats were tested for mechanical allodynia across a timecourse on both the ipsilateral (**A**) and contralateral (**B**) hindpaw. Rats that received PPF (10 mg/kg, i.p.) for 35 days beginning 28 days after surgery were significantly less allodynic than rats that received vehicle in both the ipsilateral (**C**) and contralateral (**D**) hindpaw. Data are presented as mean $\pm$ SEM and analyzed using a two-way ANOVA on the AUCs, $n=6-8$ /group. * $p<0.05$ compared to all other groups.

Detailed Timecourse of Administering Propentofylline on SNAP

Given that PPF was able to reverse SNAP, the next step was to determine how quickly PPF reversed the pain. To answer this question and to provide a replication of the full reversal of allodynia reported above, PPF was administered for 14 days and tested every 2-4 days over the

drug timecourse. No differences were observed between groups in the response thresholds recorded for the hindpaw ipsilateral (Fig 2.2A) or contralateral (Fig 2.2B) to the avulsion injury pre-surgery [baseline (BL)]. No differences were observed between the SNAP groups on either the ipsilateral or contralateral hindpaw pre-drug, recorded 14, 21, and 28 days after surgery; that is, prior to initiation of PPF treatment. PPF had no effect on the response thresholds of sham operated rats, which showed mild and transient allodynia compared to SNAP. The SNAP group was significantly more allodynic than the sham group pre-drug (days 14-28) on both the ipsilateral ($t_{22} = 4.155$; $p < 0.001$) and contralateral ($t_{22} = 4.154$; $p < 0.001$) hindpaw. The two-way ANOVA comparing the AUC of Sham+Vehicle, Sham+PPF, SNAP+Vehicle, and SNAP+PPF over the drug treatment timecourse (days 35-42) showed a significant interaction in both the ipsilateral ($F_{1,20} = 7.854$; $p < 0.05$; Fig 2.2C) and contralateral ($F_{1,20} = 6.996$; $p < 0.05$; Fig 2.2D) hindpaw. There was also a significant main effect of surgery in both the ipsilateral ($F_{1,20} = 40.65$; $p < 0.0001$) and contralateral ($F_{1,20} = 67.61$; $p < 0.0001$) hindpaw as well as a significant main effect of drug treatment in both the ipsilateral ($F_{1,20} = 7.442$; $p < 0.05$) and contralateral ($F_{1,20} = 9.668$; $p < 0.01$) hindpaw. Bonferroni *post hoc* analysis of the AUCs revealed that SNAP+Vehicle group was significantly more allodynic than all other groups ($p < 0.05$) in both the ipsilateral and contralateral hindpaw. Furthermore, there were no significant differences between the other three groups in both the ipsilateral and contralateral hindpaw ($p > 0.05$). Replicating the effects reported above, PPF again completely reversed allodynia, such that response thresholds for the SNAP+PPF group were comparable to those of sham controls.

Figure 2.2: Detailed timecourse of administering propentofylline on SNAP

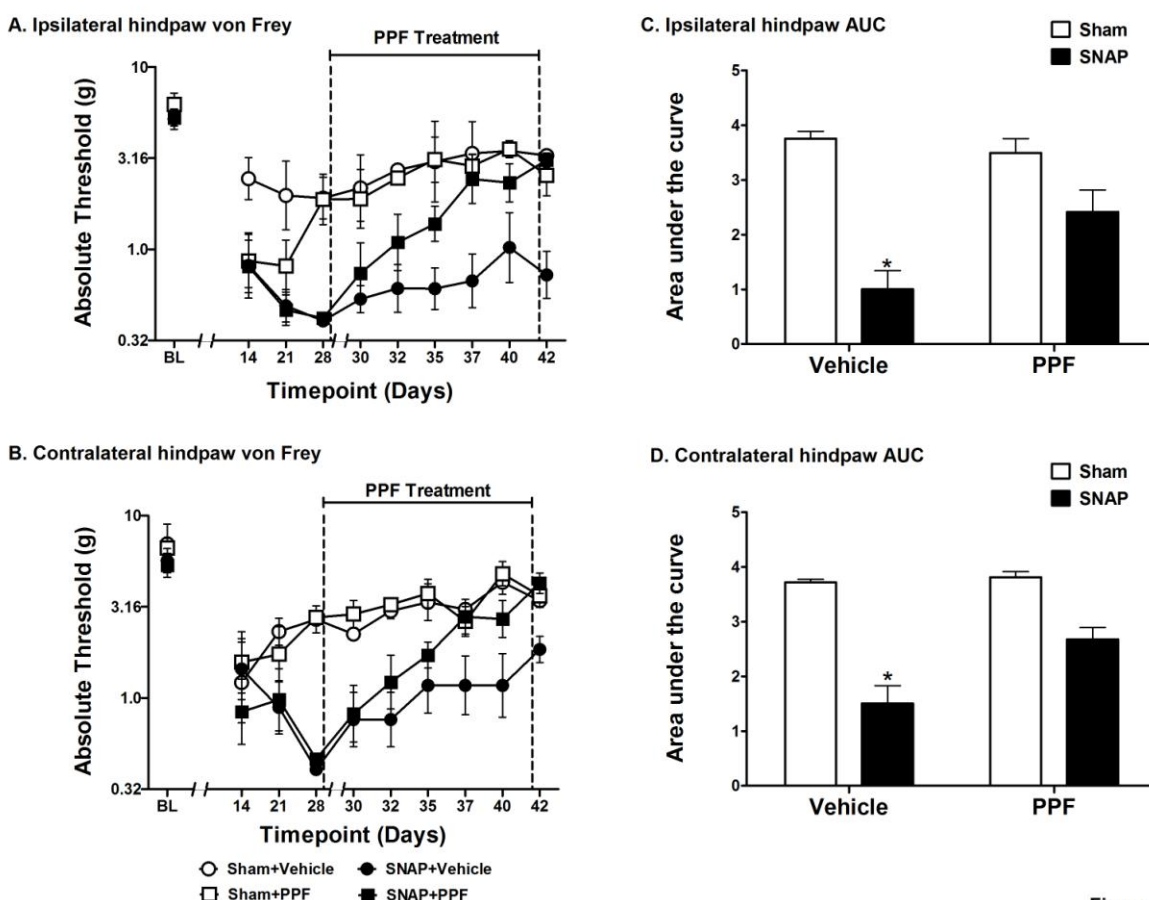


Figure 2

Figure 2.2. Detailed timecourse of the effects of PPF on SNAP. Rats were tested for mechanical allodynia across a timecourse on both the ipsilateral (**A**) and contralateral (**B**) hindpaw. Rats that received PPF (10 mg/kg, i.p.) for 14 days beginning 28 days after surgery were significantly less allodynic than rats that received vehicle in both the ipsilateral (**C**) and contralateral (**D**) hindpaw. Data are presented as mean $\pm$ SEM and analyzed using a two-way ANOVA on the AUCs, $n=6$ per group. $*p<0.05$ compared to all other groups.

Effect of Administering Ibudilast Late in the Development of SNAP

To define whether similar results could be achieved using a different putative glial activation inhibitor with a distinct mechanism of action, ibudilast was chosen for test. Ibudilast was studied here as it is known to have MIF inhibitor and toll like receptor 4 (TLR4) inhibitor mechanisms of action for neuropathic pain reversal²⁶⁴ beyond its action as a PDE inhibitor.¹⁷⁹

Ibuprofen was administered s.c. once daily at 10 mg/kg for 35 days beginning 28 days post-surgery. No differences were observed between groups in the response thresholds recorded for the hindpaw ipsilateral (Fig 2.3A) or contralateral (Fig 2.3B) to the avulsion injury pre-surgery [baseline (BL)]. No differences were observed between the SNAP groups on either the ipsilateral or contralateral hindpaw pre-drug, recorded 14, 21, and 28 days after surgery, prior to initiation of ibuprofen dosing. Ibuprofen had no effect on the response thresholds of sham operated rats, which showed mild and transient allodynia compared to SNAP. The SNAP group was significantly more allodynic than the sham group pre-drug (days 14-28) on both the ipsilateral ($t_{26} = 6.366$; $p < 0.0001$) and contralateral ($t_{27} = 3.767$; $p < 0.001$) hindpaw. The two-way ANOVA comparing the AUC of Sham+Vehicle, Sham+Ibuprofen, SNAP+Vehicle, and SNAP+Ibuprofen over the drug treatment timecourse (days 35-63) showed a significant interaction in both the ipsilateral ($F_{1,28} = 11.01$; $p < 0.01$; Fig 2.3C) and contralateral ($F_{1,28} = 9.033$; $p < 0.01$; Fig 2.3D) hindpaw. There was also a significant main effect of surgery in both the ipsilateral ($F_{1,28} = 28.92$; $p < 0.0001$) and contralateral ($F_{1,28} = 13.03$; $p < 0.01$) hindpaw as well as a significant main effect of drug treatment in both the ipsilateral ($F_{1,28} = 20.97$; $p < 0.0001$) and contralateral ($F_{1,28} = 8.975$; $p < 0.01$) hindpaw. Bonferroni *post hoc* analysis of the AUCs revealed that SNAP+Vehicle group was significantly more allodynic than all other groups ($p < 0.05$) in both the ipsilateral and contralateral hindpaw. Furthermore, there were no significant differences between the other three groups in both the ipsilateral and contralateral hindpaw ($p > 0.05$).

Figure 2.3: Effect of administering ibudilast late in the development of SNAP

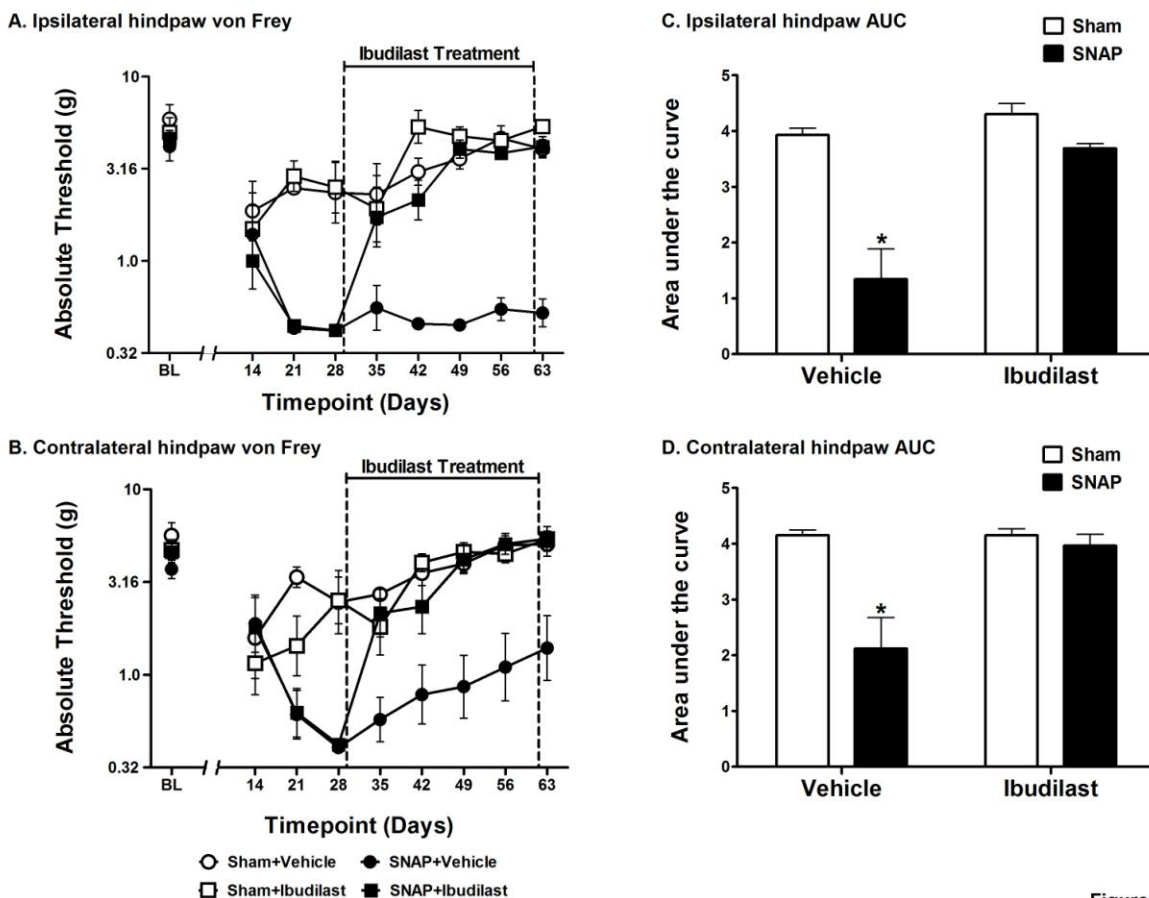


Figure 3

Figure 2.3. Assessment of the effects of ibudilast administered late in the development of SNAP. Rats were tested for mechanical allodynia across a timecourse on both the ipsilateral (**A**) and contralateral (**B**) hindpaw. Rats that received ibudilast (10 mg/kg, s.c.) for 35 days beginning 28 days after surgery were significantly less allodynic than rats that received vehicle in both the ipsilateral (**C**) and contralateral (**D**) hindpaw. Data are presented as mean $\pm$ SEM and analyzed using a two-way ANOVA on the AUCs, $n=6-8$ per group. $*p<0.05$ compared to all other groups.

Effect of Administering Ibutilast Early in the Development of SNAP

Since two different putative glial activation inhibitors could reverse established chronic SNAP, here it was tested whether ibudilast would prove effective when administration began at an earlier timepoint after surgery. In order to examine this issue, ibudilast was administered once daily s.c. at 10 mg/kg for 21 days beginning 14 days post-surgery. No differences were observed

between groups in the response thresholds recorded for the hindpaw ipsilateral (Fig 2.4A) or contralateral (Fig 2.4B) to the avulsion injury pre-surgery [baseline (BL)]. Ibudilast had no effect on the response thresholds of sham operated rats, which showed mild and transient allodynia compared to SNAP. The two-way ANOVA comparing the AUC of Sham+Vehicle, Sham+Ibudilast, SNAP+Vehicle, and SNAP+Ibudilast over the drug treatment timecourse (days 14-35) showed a significant interaction in both the ipsilateral ($F_{1,20} = 12.79$; $p < 0.01$; Fig 2.4C) and contralateral ($F_{1,20} = 7.268$; $p < 0.05$; Fig 2.4D) hindpaw. There was also a significant main effect of surgery in both the ipsilateral ($F_{1,20} = 58.24$; $p < 0.0001$) and contralateral ($F_{1,20} = 55.06$; $p < 0.001$) hindpaw as well as a significant main effect of drug treatment in both the ipsilateral ($F_{1,20} = 15.18$; $p < 0.001$) and contralateral ($F_{1,20} = 10.20$; $p < 0.01$) hindpaw. Bonferroni *post hoc* analysis of the AUCs revealed that SNAP+Vehicle group was significantly more allodynic than all other groups ($p < 0.05$) in both the ipsilateral and contralateral hindpaw. Furthermore, there were no significant differences between the other three groups in the ipsilateral hindpaw ($p > 0.05$). While the ipsilateral and contralateral hindpaws of the SNAP+Ibudilast group did not statistically differ, the contralateral hindpaw of the SNAP+Ibudilast group, given its tighter SEMs, was found to be statistically different from both the Sham+Vehicle group and the Sham+Ibudilast group ($p < 0.05$), supportive of a modestly reliable but incomplete reversal of allodynia contralaterally.

Figure 2.4: Effect of administering ibudilast early in the development of SNAP

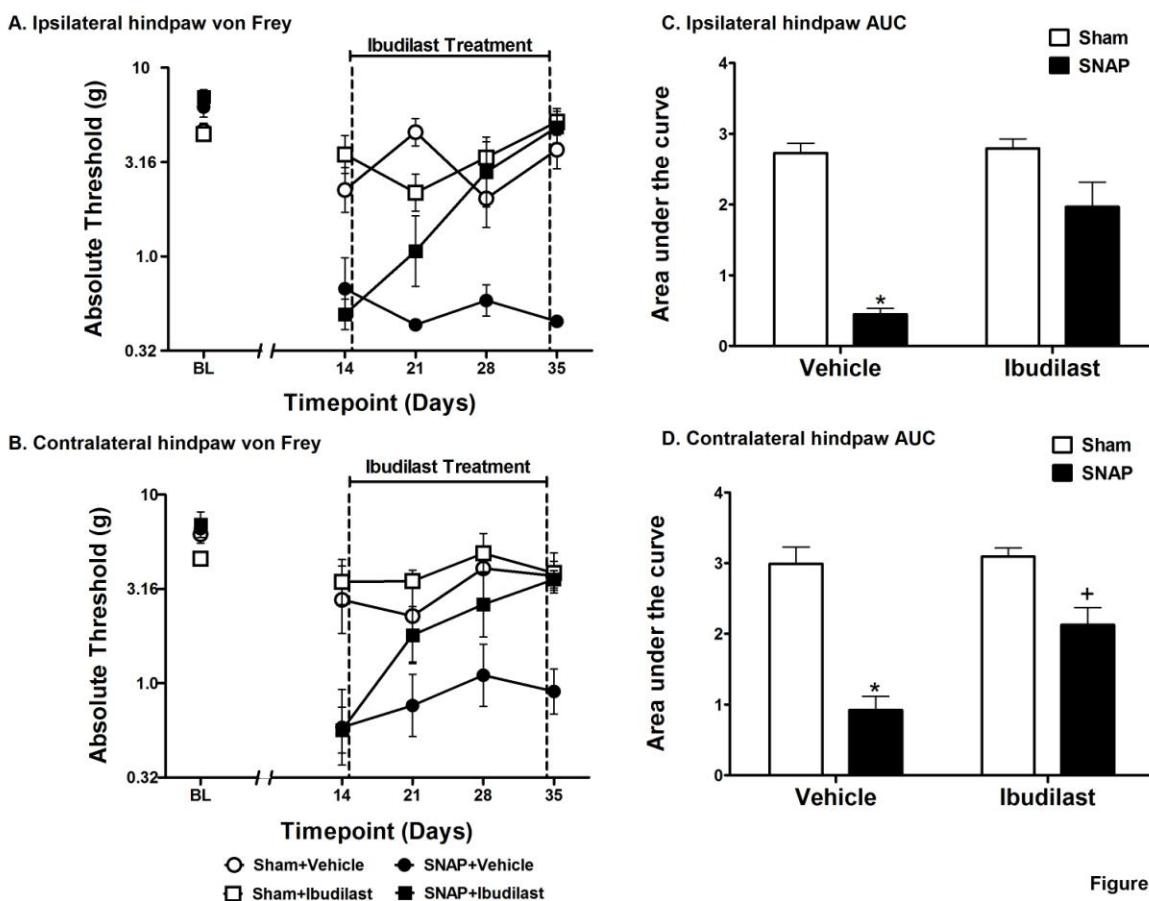


Figure 2.4. Assessment of the effects of ibudilast administered early in the development of SNAP. Rats were tested for mechanical allodynia across a timecourse on both the ipsilateral (**A**) and contralateral (**B**) hindpaw. Rats that received ibudilast (10 mg/kg, s.c.) for 21 days beginning 14 days after surgery were significantly less allodynic than rats that received vehicle in both the ipsilateral (**C**) and contralateral (**D**) hindpaw. Data are presented as mean $\pm$ SEM and analyzed using a two-way ANOVA on the AUCs, $n=5-6$ per group. * $p<0.05$ compared to all other groups, + $p<0.05$ compared to Sham+Vehicle and Sham+Ibudilast.

Effect of Administering (+)-Naltrexone on SNAP

The success of ibudilast and PPF, above, raises the question of whether a glial modulator with no known PDE activity can also resolve SNAP. As one of the mechanisms of action of ibudilast is as a TLR4 inhibitor,²⁶⁴ this study sought to define whether TLR4 inhibition would be sufficient to resolve SNAP. Given that TLR4 is activated by endogenous substances released by

cellular stress, damage and death,^{26, 142} inhibition of TLR4 was chosen from the known ibudilast mechanisms of action for test here given the neuropathology associated with SNAP. (+)-Naltrexone was chosen for test as it is a non-opioid, blood-brain barrier permeable, highly selective TLR4 antagonist³¹⁶ that has been shown to reverse peripheral neuropathic pain.¹⁴⁴ Here we administered 3x daily (+)-naltrexone s.c. at 6 mg/kg for 14 days beginning 32 days post-surgery. To extend the detailed timecourse reported for PPF above, behavior was again recorded every 3-4 days of (+)-naltrexone dosing so to define the rapidity with which allodynia reversal would occur in the absence of PDE inhibition. No differences were observed between groups in the response thresholds recorded for the hindpaw ipsilateral (Fig 2.5A) or contralateral (Fig 2.5B) to the avulsion injury pre-surgery [baseline (BL)]. No differences were observed between the SNAP groups on either the ipsilateral or contralateral hindpaw pre-drug recorded 14, 21, 28, and 32 days after surgery; that is prior to initiation of (+)-naltrexone dosing. (+)-Naltrexone had no effect on response thresholds of sham operated rats, which showed mild and transient allodynia compared to SNAP. The SNAP group was significantly more allodynic than the sham group pre-drug (days 14-32) on both the ipsilateral ($t_{16} = 6.928$; $p < 0.0001$) and contralateral ($t_{16} = 7.444$; $p < 0.0001$) hindpaw. The one-way ANOVA comparing the AUC of Sham+Vehicle, SNAP+Vehicle, and SNAP+(+)-Naltrexone over the drug treatment timecourse (days 35-46) was significant in both the ipsilateral ($F_{2,15} = 11.76$; $p < 0.001$; Fig 2.5C) and contralateral ($F_{2,15} = 16.82$; $p < 0.001$; Fig 2.5D) hindpaw. Bonferroni *p post hoc* analysis of the AUCs revealed that SNAP+Vehicle group was significantly more allodynic than all other groups ($p < 0.05$) in both the ipsilateral and contralateral hindpaw. Furthermore, there were no significant differences between the other two groups in both the ipsilateral and contralateral hindpaw ($p > 0.05$).

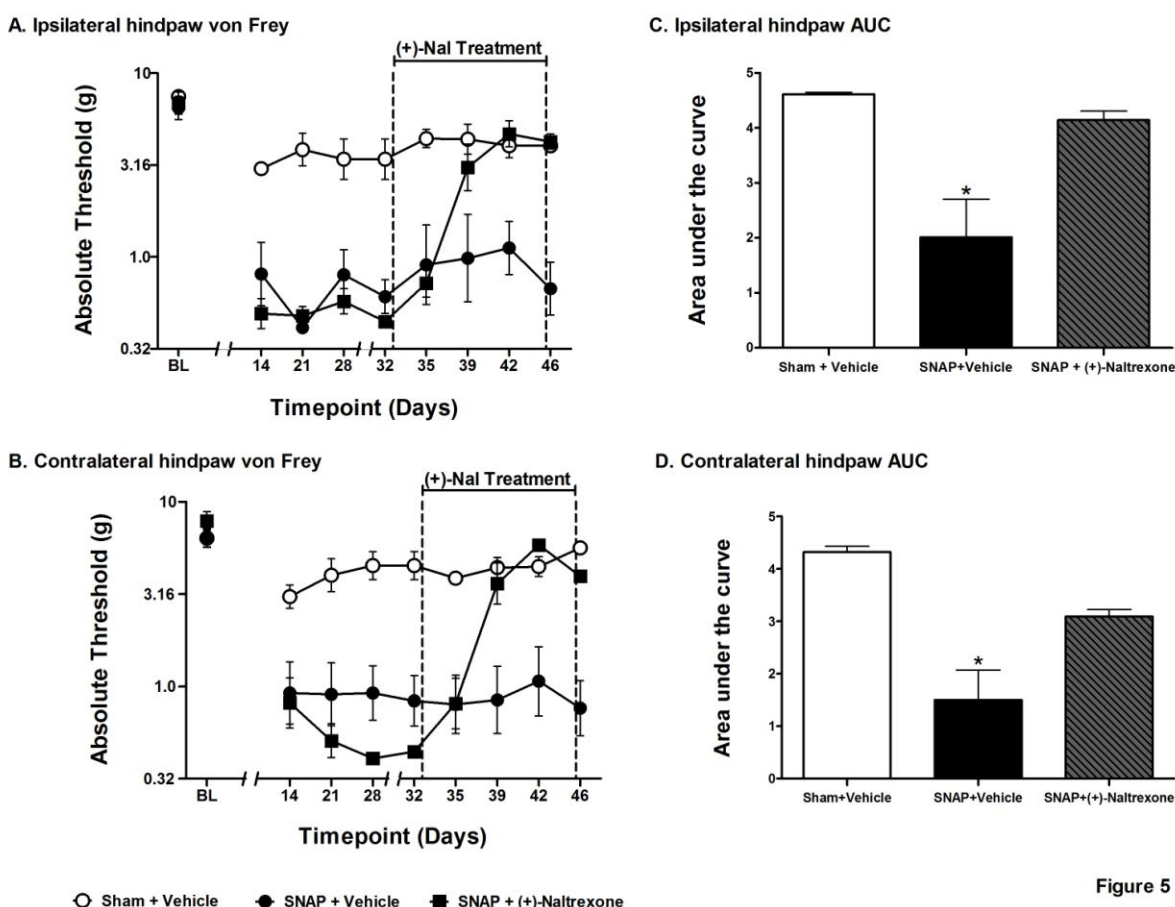
Figure 2.5: Effect of administering (+)-naltrexone on SNAP**Figure 5**

Figure 2.5. Assessment of the effects of (+)-naltrexone on SNAP. Rats were tested for mechanical allodynia across a timecourse on both the ipsilateral (**A**) and contralateral (**B**) hindpaw. Rats that received (+)-naltrexone (6 mg/kg, s.c.) for 14 days beginning 32 days after surgery were significantly less allodynic than rats that received vehicle in both the ipsilateral (**C**) and contralateral (**D**) hindpaw. Data are presented as mean $\pm$ SEM and analyzed using a one-way ANOVA on the AUCs, $n=6$ per group. * $p<0.05$ compared to all other groups.

Effect of Ceasing (+)-Naltrexone Administration on SNAP

In order to determine whether the reversal of allodynia observed with such glial modulators may be sustained after elimination of the drug, a final study was undertaken using (+)-naltrexone as a test compound for this purpose. Here we administered 3x daily (+)-naltrexone s.c. at 6 mg/kg for 6 days beginning 28 days post-surgery, then stopped administering the drug after day 33 and continued to record behavior until the rats were back at pre-drug

allodynia pain thresholds. No differences were observed between groups in the response thresholds recorded for the hindpaw ipsilateral (Fig 2.6A) or contralateral (Fig 2.6B) to the avulsion injury pre-surgery [baseline (BL)]. No differences were observed between the SNAP groups on either the ipsilateral or contralateral hindpaw pre-drug recorded 14, 21, and 28 days after surgery; that is prior to initiation of (+)-naltrexone dosing. The t-test comparing the AUC of SNAP+Vehicle and SNAP+(+)-Naltrexone over the drug treatment timecourse (days 29-33) was significant in both the ipsilateral ($t_9 = 3.267$; $p < 0.01$; Fig 2.6C) and contralateral ($t_9 = 2.833$; $p < 0.05$; Fig 2.6D) hindpaw. The t-test comparing the AUC of SNAP+Vehicle and SNAP+(+)-Naltrexone over the drug cessation period (days 35-42) was not significant in either the ipsilateral ($p > 0.05$; Fig 2.6E) or contralateral ($p > 0.05$; Fig 2.6F) hindpaw.

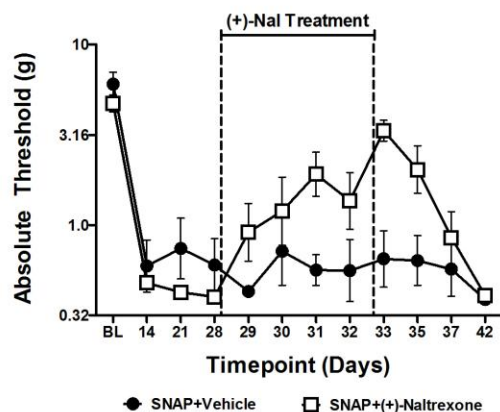
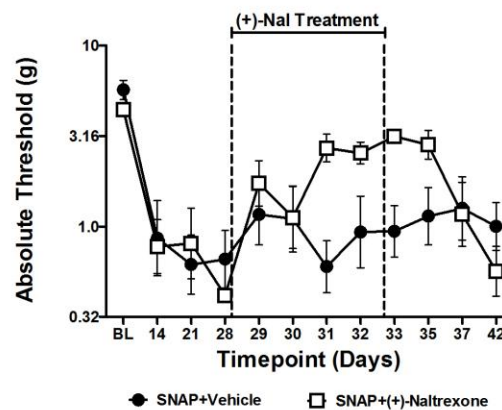
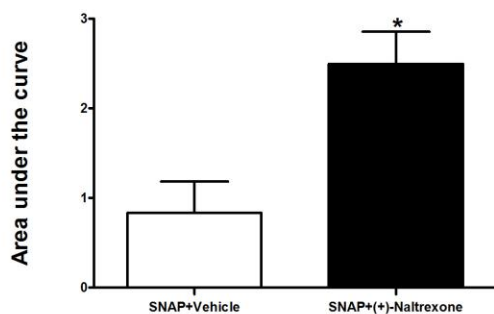
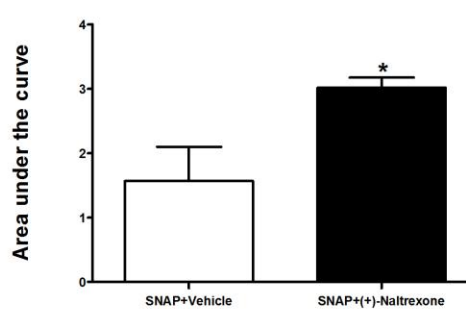
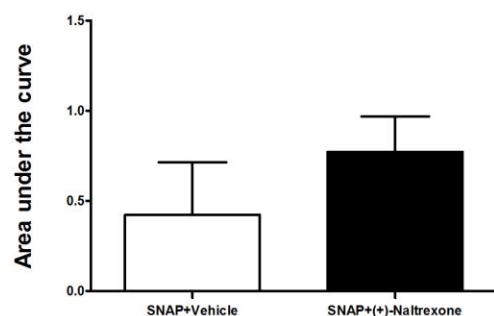
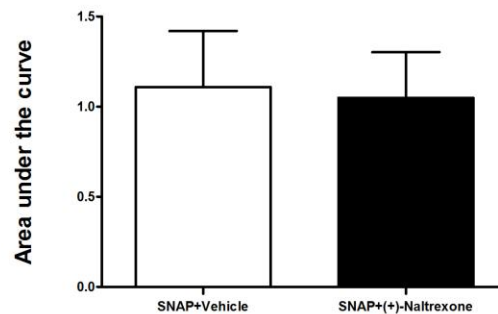
Figure 2.6: Effect of ceasing (+)-naltrexone administration on SNAP**A. Ipsilateral hindpaw von Frey****B. Contralateral hindpaw von Frey****C. Ipsilateral hindpaw AUC over drug timecourse****D. Contralateral hindpaw AUC over drug timecourse****E. Ipsilateral hindpaw AUC after drug cessation****F. Contralateral hindpaw AUC after drug cessation**

Figure 2.6. Assessment of the effects of ceasing (+)-naltrexone on SNAP. Rats were tested for mechanical allodynia across a timecourse on both the ipsilateral (**A**) and contralateral (**B**) hindpaw. Rats that received (+)-naltrexone (6 mg/kg, s.c.) for 6 days beginning 28 days after surgery were significantly less allodynic than rats that received vehicle in both the ipsilateral (**C**) and contralateral (**D**) hindpaw. After stopping (+)-naltrexone administration on day 33, rats that were receiving (+)-naltrexone returned to pre-drug allodynia thresholds in both the ipsilateral (**E**) and contralateral (**F**) hindpaw. Data are presented as mean $\pm$ SEM and analyzed using a t-test on the AUCs, n=6 per group. * $p < 0.05$ compared to all other groups.

Discussion

Here we show that three different putative glial inhibitors with distinct mechanisms of action, PPF, ibudilast, and (+)-naltrexone, are all able to reverse SNAP. Thus, converging lines of evidence from testing multiple inhibitors commonly assumed to have some glial mechanisms of action suggest a role for glia in this phenomenon. Importantly, none of these inhibitors reversed allodynia upon first administration, but required multiple days of treatment to achieve full reversal. Taken together, these data suggest that treating CNP with inhibitors that have some action on glia may prove to be a fruitful strategy for improving clinical pain control in such cases.

One strategy for controlling CNP is increasing cyclic adenosine monophosphate (cAMP) by inhibiting phosphodiesterases (PDEs) which hydrolyze cAMP and/or cyclic guanosine monophosphate (cGMP).²¹ PDE inhibitors reverse CCI-induced allodynia and hyperalgesia,³²⁵ and rolipram has been shown to decrease proinflammatory cytokine levels in TBI⁷ and SCI.²⁴³ Further, selective PDE4 inhibitors decrease hindpaw allodynia from compression SCI by reducing immune cell infiltration/activation and free radical formation.¹¹ PPF is a methyl-xanthine selective PDE4 inhibitor,³⁰⁹ an isoform expressed in both microglia and astrocytes.¹⁹⁵ Although neurons express PDE4, PDE4 is the major isoform expressed in immune and inflammatory cells.¹⁴⁹ Further, microglial activation is regulated by PDE4²⁸³ and PPF decreases release of proinflammatory cytokines in microglia.²⁸⁰ In addition, systemic PPF decreases both

microglia and astrocyte activation in a rat peripheral neuropathy model.³⁰⁹ The present study is the first to show that *systemic* PPF is effective in reversing allodynia in a CNP model. The only other neuropathic pain studies using PPF administered it intrathecally in a rat model of hemisection SCI, which also reversed mechanical allodynia.¹¹¹ Notably, the half life of PPF and its active metabolite is very short, ~1 hr,³⁰⁹ therefore the anti-allodynic effects observed in the current studies at ~15 hr post administration suggest that persistent intracellular alterations have occurred, in keeping with the conclusions of Sweitzer et al.³⁰⁹ Studies have shown that increases in cAMP results in increases of the anti-inflammatory cytokine IL-10,^{3, 77, 290} including from microglia.³⁵⁷ Notably, IL-10 has been recognized for its therapeutic potential in SCI.^{317, 366} The resulting ratio of decreased proinflammatory signaling to increased anti-inflammatory signaling could explain the sustained pain reversal observed even when propentofylline is not present at the time of testing.

Although ibudilast, like PPF, is a PDE inhibitor (inhibits PDE 1, 2, 3, and 4 in rat),¹⁷⁸ PDE inhibition alone cannot account for the anti-inflammatory effects of ibudilast.⁴⁹ Therefore, there must be an alternative mechanism.²⁶⁴ Ibudilast is now known to also inhibit macrophage migration inhibitory factor (MIF)²⁶⁴ and is effective in treating SNL,¹⁷⁹ SCI (present data),¹¹⁹ and CCI pain.¹¹⁹ MIF stimulates the release of IL-1 and TNF and is required for IL-1 and TNF-induced MAPK activation.³¹⁸ MIF itself is now recognized as a pro-inflammatory cytokine² and has been implicated in multiple central neuropathies including stroke,³³⁸ MS,¹¹⁶ TBI,³²³ and SCI.²²⁶ In studies examining the role of MIF in SCI, MIF knock-out mice with compression SCI recovered motor function faster than wild-type controls,²²⁶ and a clinical pilot study found that SCI patients with chronic pain had higher levels of serum MIF compared to both non-injured controls as well as SCI patients who did not have a history of chronic pain.³⁰¹ It is important to

note that these increased circulating levels of MIF were detected long after the initial injury, providing evidence that there is enduring release of MIF that could then be sustaining the CNP state we observe in SNAP which is reversed by ibudilast administration. Taken together, these studies suggest that since MIF is up-regulated in multiple CNP models, MIF inhibitors are potential candidates for successful CNP therapeutics.

Another potential CNP treatment that has not been as well studied is (+)-naltrexone, a selective TLR4 antagonist, as evidenced by in vivo and in vitro studies as well as in silico modeling.^{141, 144, 145, 316} In contrast to PPF and ibudilast, (+)-naltrexone has no known PDE or MIF inhibitory actions. Although we have shown that (+)-naltrexone reverses peripheral CCI pain,¹⁴⁴ no one has shown reversal of CNP using (+)-naltrexone until now. (+)-Naltrexone is unique compared to the (-)-naltrexone isomer in that it does not bind classical μ -opioid receptors.³¹⁶ As a result, (+)-naltrexone does not cause many of the undesirable side effects observed with many other opiate-based analgesics,^{144, 316} making it a more desirable treatment option. Since TLR4 in the CNS is found predominantly on glial cells¹⁴¹ and blocking TLR4 reverses allodynia, it is very likely that the pain reversal seen here after (+)-naltrexone administration is glially mediated. Importantly, as observed with PPF and ibudilast, it took multiple days of (+)-naltrexone administration to observe full reversal, indicating that acute dosing with glial modulators may not be ideal for treating established CNP. In addition, the anti-allodynic effects of (+)-naltrexone gradually dissipated once the drug was no longer being administered, which has also been observed previously for PPF¹¹² and ibudilast.¹⁷⁹ This suggests that although these drugs are perhaps able to attenuate glial activation during administration, they are not inducing permanent changes that can overcome the proinflammatory environment and persistent glial activation caused by the original injury and subsequent central sensitization.

One reason that many drugs fail clinically could be that they are not given for a sufficiently long duration, and there are examples in the literature of this with all three of the drugs tested here. Reversal of spinal nerve ligation (SNL)-induced allodynia failed to occur when PPF was administered for only 7 days,²³¹ whereas it took 10 days of daily PPF administration to both prevent and reverse SNL-induced allodynia.³⁰⁹ In the CCI and spinal nerve ligation models, only transient pain reversal occurred with one day of systemic ibudilast, and ~5 days of dosing was required for full reversal of CCI allodynia.¹⁷⁹ Similarly, only transient reversal (~1 hr) of allodynia occurs with a single intrathecal injection of (+)-naltrexone, whereas chronic intrathecal infusion of (+)-naltrexone completely reversed allodynia only when administered for 4 days.¹⁴⁴ In the current studies it took multiple days of systemic administration to achieve significant allodynic reversal with all 3 of the drugs tested, which suggests that had these drugs been administered as a single injection or for only a couple of days they would have failed.³⁴²

Furthermore, treatments are often more effective if they are administered early on in pain development. For instance, disease modifying anti-rheumatic drugs for rheumatoid arthritis are only effective for treating pain and other symptoms if administered within the first three months symptoms appear.¹⁸⁴ Another example of successful early pharmacological intervention is in migraine patients, where administering triptans within the first 20-120 minutes after symptom onset can actually prevent allodynia from developing.²⁹ Unfortunately, there are many cases where pain patients do not or are not able to seek treatment until they have been in chronic pain for months. Thus, it is important to find therapeutics that are effective not only when administered upon first pain development but also after the symptoms have become chronic. Here we gave ibudilast in the early stages of pain development as well as in a later stage of chronic pain development, and saw complete reversal of allodynia in both cases. These data

suggest that glia are likely involved both in the early stages of neuropathic pain development as well as the long-term maintenance of chronic neuropathic pain, and that at least ibudilast has the potential to treat patients at various stages of their pain progression. Ibudilast has recently been identified as a toll-like receptor 4 (TLR4) antagonist¹⁴⁵ (Johnson, unpub. obs., 2012), and ibudilast as well as PPF and (+)-naltrexone were able to treat below-level contralateral pain as well, increasing their attractiveness as therapeutics.

Although the studies here focus on the *glial* mechanisms underlying CNP, we cannot rule out the fact that neurons and other cell types are also likely involved. Chew et al. report increased ipsilateral bilateral glial activation in the dorsal horn following L3-L6 dorsal root avulsion⁴⁶ and increased ipsilateral mechanical hypersensitivity following L5 dorsal and ventral root avulsion,⁴⁷ which lends support to what we observe in the current studies. However, they also report significant increases in bilateral infiltrating macrophages in the dorsal horn, which could also be helping to maintain the neuropathic pain.^{46, 47} Inhibiting AMPA receptors attenuates mechanical allodynia and neuronal hyperexcitability following SCI.¹¹³ In addition, injecting NMDA near a SCI contusion injury site delays functional recovery⁸⁴ and administering NMDA antagonists shortly after SCI can improve functional recovery and increase pain thresholds.¹⁶² A recent study found that co-administering PPF and a neuronal NMDA receptor inhibitor had additive effects on attenuating peripherally induced-chronic pain.²¹⁵ It is thought that MIF triggers ERK/NMDA dependent plasticity in sensory neurons² and it has been shown that ibudilast suppresses apoptosis in nerve cells during ischemic brain injury.¹⁸⁰ Lastly, there are studies that report that TLR4 is expressed in primary sensory neurons and can contribute to neuropathic pain.⁷⁰

Since the drugs used here were administered systemically and are putative glial inhibitors, we recognize that their actions may not be exclusively on glia and could be interacting with other cell types, including resident peripheral immune cells or cells recruited to the injured spinal tissue. PPF suppresses the production of $\text{TNF-}\alpha$ ¹⁵⁴ and reactive oxygen species in macrophages,⁹ and can also counteract neutrophil activation by blocking the removal of adenosine.³⁶³ Both PPF and ibudilast can prevent kainite-induced cell death in oligodendroglia,³⁵⁸ and ibudilast can also inhibit platelet aggregation in the presence of endothelial cells.²⁵⁹ Furthermore, ibudilast reduces inflammatory cell infiltration into the dorsal spinal cord in EAE,⁹⁹ which could also be happening in SNAP, along with attenuating resident microglial activation. Since TLR4 can also be expressed on neurons⁷⁰ and peripheral^{182, 203} or recruited immune cells,²⁸⁶ (+)-naltrexone could be exerting some effects on these cells as well. Since we use a pain model of central origin and multiple pain studies using these compounds suggest the anti-allodynic effects are glially mediated,^{119, 144, 179, 252, 309, 314, 346} it is likely that these drugs exert at least some of their anti-allodynic effects by inhibiting glial activation. However, these observations highlight the importance of considering the contributions of neurons, peripheral/infiltrating immune cells, and glia to CNP.

In conclusion, it is clear that glia play an important role in the pathogenesis of CNP. We were able to show, using our dorsal root avulsion model of SCI (SNAP), that administering putative glial inhibitors reverses SCI-induced allodynia both at the onset of pain as well as after chronic neuropathic pain had developed, although it takes at least a week of daily dosing to achieve full reversal. Treating CNP with inhibitors that have some mechanistic action on glial cells has the potential to optimize treatment and dramatically increase the quality of life for thousands of chronic pain patients.

Chapter III

Sustained Reversal of Central Neuropathic Pain Induced by a Single Intrathecal Injection of Adenosine A_{2A} Receptor Agonists

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Abstract

Central neuropathic pain (CNP) is a debilitating outcome of spinal cord injury (SCI) and current treatments are ineffective. A growing body of literature suggests that activating adenosine A_{2A} receptors (A_{2A}Rs) decreases the release of proinflammatory cytokines and increases the release of anti-inflammatory cytokines. We investigated the effects of administering intrathecal (i.t.) A_{2A}R agonists on CNP by measuring hindpaw mechanical allodynia in a rat model of SCI termed spinal neuropathic avulsion pain (SNAP). Other models of SCI cause extensive damage to the spinal cord, which cause paralysis and health problems. SNAP rats with unilateral T13/L1 avulsion do not have motor or health problems and develop below-level bilateral allodynia. A single i.t. injection of the A_{2A}R agonist 2-*p*-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamido adenosine HCl (CGS21680) reversed SCI-induced allodynia for at least 6 weeks. The reversal is in part mediated by interleukin (IL)-10, as administering neutralizing IL-10 antibodies 1 week after CGS21680 abolished the anti-allodynic effect of CGS21680. Dorsal spinal cord tissue from the ipsilateral site of SCI (T13/L1) was assayed 1 week and 6 weeks after CGS21680 for IL-10, CD11b, and TNF- α gene expression. CGS21680 treatment did not change IL-10 gene expression but did significantly decrease CD11b and TNF- α gene expression at both timepoints. A single i.t. injection of another A_{2A}R agonist, 4-(3-(6-amino-9-(5-cyclopropylcarbamoyl-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)prop-2-ynyl)piperidine-1-carboxylic acid methyl ester (ATL313), was also able to

significantly prevent and reverse SCI-induced allodynia. The enduring pain reversal after a single i.t. injection of A_{2A}R agonists suggests that A_{2A}R agonists could be exciting new candidates for treating SCI-induced CNP.

Introduction

Central neuropathic pain is a common and debilitating consequence of spinal cord injury (SCI). More than 66% of people with SCI develop chronic pain,²⁸⁹ and of that population ~30% develop below-level central neuropathic pain.²⁸⁸ Below-level pain is defined as pain occurring at least 2 dermatomes away from the original injury. Patients with below-level pain describe their pain as severe,³⁵³ and current pharmacotherapies for treating central neuropathic pain aren't effective, with only ~50% pain relief in 1 out of 2-3 people.¹⁶ We developed a dorsal root avulsion animal model we termed spinal neuropathic avulsion pain (SNAP) to study SCI-induced central neuropathic pain. Current animal models of SCI, such as contusion and hemisection, cause extensive damage to the spinal cord and animals develop paralysis, urinary tract infections, and autotomy, which can complicate assessments of pain behavior. SNAP rats do not have compromised motor function or health problems and unilateral T13/L1 avulsion induces robust, reliable, bilateral below-level mechanical allodynia.³⁴⁹

It is known that spinal glia (microglia, astrocytes) play a key role in facilitating chronic pain states in animal models of peripheral neuropathic pain.³⁴³ During an inflammatory event, glia become activated and release neuroexcitatory substances that include proinflammatory cytokines, which drive neuropathic pain.²⁰⁸ Recent studies have shown that glia are activated in models of SCI including hemisection¹¹² and contusion,³¹² and persistent glial activation can both induce and maintain below-level SCI neuropathic pain.¹³⁷ Conversely, glial cells can also release anti-inflammatory mediators that attenuate neuropathic pain.²⁰⁶ Pharmacological therapies that modulate glia in such a way that they express a less proinflammatory phenotype could prove to be an ideal treatment for central neuropathic pain in SCI.

Adenosine is an endogenous substance in the central nervous system (CNS) and can bind 4 different G protein-coupled receptors: adenosine 1 receptor (A_1R), $A_{2A}R$, $A_{2B}R$, and A_3R .²⁷⁵ $A_{2A}R$ s are unique because they are found not only on neurons, but on immune cells in the peripheral and central nervous systems.⁵⁸ The anti-inflammatory effects of adenosine are generally attributed to occupancy of the A_{2A} receptor,¹²⁵ and there is an expanding body of literature that documents the ability of $A_{2A}R$ agonists to decrease inflammation by suppressing proinflammatory cytokines and increasing anti-inflammatory cytokines in peripheral¹²⁵ and central^{191, 192} immune cells. Furthermore, a single intrathecal (i.t.) administration of either of two different $A_{2A}R$ agonists (CGS21680, ATL313) reversed peripheral neuropathy-induced allodynia for more than 4 weeks.¹⁹¹ Whether or not this enduring reversal from a single administration of an $A_{2A}R$ agonist can also be observed in a model of central neuropathic pain, and what the mechanisms might be, is currently unknown. The aim of this study was to provide an initial characterization of $A_{2A}R$ agonism and potential underlying mechanisms in central neuropathic pain.

Our hypothesis was that $A_{2A}R$ agonists would attenuate SNAP by decreasing glial activation and subsequent release of proinflammatory cytokines while also increasing the release of anti-inflammatory cytokines. Therefore, our first goal was to administer CGS21680 at a single timepoint and record a behavioral timecourse and then attempt to block the anti-allodynic effect. We next wanted to determine whether there were changes in pro- and anti-inflammatory gene expression in the spinal cord at different timepoints following CGS21680 administration. Lastly, we wanted to define whether the effects were either agonist or timing-specific. Thus, we administered another $A_{2A}R$ agonist, ATL313, both early and late in the development of SNAP.

Materials and Methods

Animals

Pathogen-free male Sprague-Dawley rats (325-350g; Harlan Laboratories, Madison, WI, USA) were used for all experiments. Rats were pair-housed prior to surgery and then single-housed after surgery with standard rat chow and water available *ad libitum*. Housing was in a temperature-controlled room that was maintained at 23 $\pm$ 2°C with a 12 hr light/dark cycle (lights on at 0700 hr). The rats were allowed a minimum of 1 week to habituate to the colony room before initiating the experiment. All procedures were performed during the light cycle. Following surgery, the animals were singly housed. All protocols were approved by the University of Colorado at Boulder Institutional Animal Care and Use Committee.

Drugs

The A_{2A}R agonist 2-*p*-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamido adenosine HCl (CGS21680) was purchased from Sigma-Aldrich (St Louis, MO, USA). The A_{2A}R agonist 4-(3-(6-amino-9-(5-cyclopropylcarbamoyl-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)prop-2-ynyl)piperidine-1-carboxylic acid methyl ester (ATL313) was a gift from PGxHealth, a division of Clinical Data (New Haven, CT, USA). All drugs were dissolved in 100% DMSO to create a 10 mM stock solution and were stored at -20°C. Fresh aliquots were diluted in sterile endotoxin-free isotonic saline (Abbott Laboratories, Abbott Park, IL, USA). The vehicle for all drugs was 0.01% DMSO saline solution given the dilution of the drugs from stock was 1:10,000 to yield a 10 μ M dose for CGS21680 and a 1 μ M dose for ATL313, based on our past studies of their relative efficacies in reversing pain induced by peripheral neuropathy.^{191, 192} All vehicle injections were administered equivolume to CGS21680 and ATL313. Rat IL-10 neutralizing antibodies were raised in sheep at the National Institute of Biological Standards and

Control (South Mimms, Hertfordshire, UK), purified by Avigen (Alameda, CA, USA), and administered in saline. An equal dose of normal sheep IgG in saline (Sigma-Aldrich, St Louis, MO, USA) was used as the control.

Spinal Neuropathic Avulsion Pain (SNAP) Surgery

Unilateral (left) T13/L1 dorsal root avulsion was performed under isoflurane anesthesia. Briefly, laminectomy was performed at the T12 vertebral level and the dura mater was incised over the dorsal root entry zone. The T13 and L1 dorsal rootlets were carefully isolated and then clamped at the dorsal root entry zone and briskly pulled out (avulsed). The dura was then sutured closed with sterile 6-0 silk. Sterile saline-moistened surgical sponge was placed over the exposed spinal cord to protect it, the muscle was sutured in layers with sterile 3-0 silk, and the skin was closed with stainless steel wound clips. Immediately following surgery, rats were single-housed in a cage with foam padding for a few hours to protect their spinal cord from further trauma due to the brief ataxic period that follows recovery from anesthesia. Sham operated animals were treated identically, including dura suturing, except for avulsing of the rootlets. Combi-Pen-48 antibiotic (Bimeda, Inc., Le Sueur, MN, USA) was administered at the time of surgery and daily for 4 days after surgery.

Acute intrathecal (i.t.) injections

Rats were anesthetized for no more than 5 min under isoflurane. The lumbar region was shaved and cleaned with 70% EtOH. A sterile 18 gauge guide needle with the hub removed was inserted into the L5/L6 vertebral space. A PE-10 catheter was inserted into the guide needle, pre-marked such that the proximal end of the PE-10 tubing rested over L4–L6 lumbar spinal cord. All drugs were administered over 20 s (1 μ l of drug followed by 2 μ l of sterile saline flush) with

a 30 s delay before removing the catheter and guide needle. No animals incurred observable neurological damage from the procedure.

Low Threshold Mechanical Allodynia Testing

Prior to surgery, rats were habituated to the testing environment for 4 consecutive days prior to recording of behavioral responses. All von Frey assessments were performed blind with respect to drug and surgery assignments. Assessment of von Frey thresholds occurred before surgery (baseline) and across a timecourse beginning two weeks after surgery. The von Frey test was performed on the plantar surface of each hind paw as previously described in detail.²⁰⁵ A logarithmic series of 10 calibrated Semmes–Weinstein monofilaments (Stoelting) were sequentially applied to the left and right hind paws in random order, each for 8 s at constant pressure to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. Log stiffness of the hairs is determined by $\log_{10}$ (milligrams $\times 10$). The range of monofilaments used in these experiments (0.407–15.136 g) produces a logarithmically graded slope when interpolating a 50% response threshold of stimulus intensity (expressed as $\log_{10}$ (milligrams $\times 10$)).⁴¹ The stimulus intensity threshold to elicit a paw withdrawal response was used to calculate the 50% paw withdrawal threshold (absolute threshold) using the maximum-likelihood fit method to fit a Gaussian integral psychometric function.¹²⁴ This method normalizes the withdrawal threshold for parametric analyses.¹²⁴ SNAP rats require a 2 week recovery period after surgery before reliable von Frey testing can begin.

Processing of tissue for PCR

RNA isolation and cDNA synthesis. Total RNA from the 5 mm piece of the T13/L1 thoracic spinal cord was extracted using the standard phenol/chloroform extraction with TRIzol Reagent (Invitrogen) according to the manufacturer's guidelines. Total RNA was reverse

transcribed into cDNA using Superscript II First-Strand Synthesis System (Invitrogen). First-strand cDNA was synthesized using total RNA, random hexamer primer (5 ng/μl) and 1 mM dNTP mix (Invitrogen) and incubated at 65°C for 5 min. After 2 min incubation on ice, a cDNA synthesis buffer (5 x reverse transcription (RT) buffer; Invitrogen) and dithiothreitol (10 mM) was added and incubated at 25°C for 2 min. Reverse transcriptase (Superscript II; 200 U; Invitrogen) was added to a total volume of 20 μl and incubated for 10 min at 25°C, 50 min at 42°C, and deactivating the enzyme at 70°C for 15 min. cDNA was diluted twofold in nuclease-free water and stored at -80°C until real-time PCR (RT-PCR) was performed.

RT-PCR. Primer sequences were obtained from the GenBank at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and are displayed in Table 3.1. Primers were generated to span an intron to eliminate genomic interference. Amplification of the cDNA was performed, in a blinded procedure, using Quantitect SYBR Green PCR kit (QIAGEN) in iCycler iQ 96-well PCR plates (Bio-Rad) on a MyiQ single Color Real-Time PCR Detection System (Bio-Rad). The reaction mixture (26 μl) was composed of Quanti-Tect SYBR Green (containing fluorescent dye SYBR Green I, 2.5 mM MgCl₂, dNTP mix, and Hot Start Taq polymerase), 10 nM fluorescein, 500 nM each forward and reverse primer (Invitrogen), nuclease-free water, and 1 μl of cDNA from each sample. Each sample was measured in duplicate. The reactions were initiated with a hot start at 95°C for 25 min, followed by 40 cycles of 15 s at 94°C (denaturation), 30 s at 55–60°C (annealing), and 30 s at 72°C (extension). Melt curve analyses were conducted to assess uniformity of product formation, primer–dimer formation, and amplification of nonspecific products. The PCR product was monitored in real-time, using the SYBR Green I fluorescence, using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). Threshold for detection of PCR product was set in the log-linear

phase of amplification and the threshold cycle (*CT*) was determined for each reaction. The level of the target mRNA was quantified relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the comparative *CT* (ΔCT) method. The expression of GAPDH was not significantly different between treatments.

Table 3.1. Primer Sequences

Gene	Primer Sequence (5'-3')	GenBank accession no.
<i>GAPDH (i-s)</i>	TCTTCCAGGAGCGAGATCGC (forward) TTCAGGTGAGCCCCAGCCTT (reverse)	NG_028301.1
<i>IL-10 (i-s)</i>	GGACTTTAAGGGTTACTTGGG (forward) AGAAATCGATGACAGCGTCG (reverse)	NM_012854.2
<i>CD11b (i-s)</i>	CTGGTACATCGAGACTTCTC (forward) TTGGTCTCTGTCTGAGCCTT (reverse)	NM_012711.1
<i>TNF-α (i-s)</i>	CAAGGAGGAGAAGTTCCCA (forward) TTGGTGGTTTGCTACGACG (reverse)	NM_012675.3

i-s, intron-spanning primers generated to reduce genomic interference

Pharmacological Manipulations

Effect of administering the A_{2A}R agonist CGS21680 on SNAP-induced mechanical allodynia

Baseline measures on the von Frey test were collected prior to surgery. SNAP or sham surgery was performed and behavioral responses to a mechanical stimulus were recorded 2, 3, and 4 weeks post-surgery. At 4 weeks post-surgery, one acute i.t. injection of CGS21680 or equivolume vehicle was administered (n = 6-8 per group). Behavioral testing occurred once a week for 6 weeks after drug treatment.

Effect of administering neutralizing IL-10 antibody on the CGS21680 effect in SNAP allodynia

SNAP was conducted and behavioral responses to mechanical stimuli were tested as described above. At 4 weeks post-surgery, an acute i.t. administration of CGS21680 (10 μ M) or

equivolume vehicle was administered. One week later, a single i.t. injection of either sheep anti-rat neutralizing IL-10 IgG antibodies (0.2 µg/ml; 10 µl) or equivolume and equidose sheep IgG (0.2 µg/ml; 10 µl) was administered (n = 9 rats per group). Behavioral responses were measured 1, 2, 3, 6, 24, 48 h, and 1, 2, and 3 weeks after drug administration.

Effect of administering the A_{2A}R agonist CGS21680 on gene expression

CD11b, IL-10, and TNF-α were chosen for analysis because pilot and published studies from our lab,¹⁹¹ as well as in the literature,^{72, 232, 333} identify these markers as being specifically modulated by A_{2A}R agonists. After recording behavioral responses at the 1 week or 6 week post-drug treatment timepoint, SNAP rats and a group of naïve control rats were overdosed with sodium pentobarbital (0.8 ml) and transcardially perfused with ice-cold 0.9% saline for 2 min. The spinal cord was dissected and a 5 mm piece of ipsilateral dorsal spinal cord, with the meninges removed, was dissected out to include the T13/L1 spinal level. All tissue was flash frozen in liquid nitrogen. Samples were then stored at -80°C until analysis.

Effect of administering the A_{2A}R agonist ATL313 on SNAP-induced mechanical allodynia

SNAP or sham surgery was conducted and behavioral responses to mechanical stimuli were tested as described above. At either 1 week or 7 weeks post-surgery, one acute i.t. injection of ATL313 (1 µM) or equivolume vehicle was administered (n = 6-7 per group). Behavioral testing occurred once a week for the duration of the experiment.

Statistical Analysis

All data were analyzed using GraphPad Prism version 5.04 for Windows Vista (GraphPad Software, San Diego, CA, USA). Ipsilateral and contralateral behavioral data were analyzed individually. Behavioral measures were normalized as described above and analyzed

using unpaired t-test or repeated-measures two-way ANOVA with time and treatment as main effects. The RT-PCR data was analyzed using a one-way ANOVA. Bonferroni *post hoc* tests were used where appropriate, and $p < 0.05$ was considered statistically significant.

Results

Effect of administering the A_{2A}R agonist CGS21680 on SNAP-induced mechanical allodynia

In this first study, a single i.t. injection of 10 μ M CGS21680 was administered 4 weeks post-SNAP or sham surgery. This timepoint was chosen because SNAP rats have been stably allodynic for at least 2 weeks and sham rats are no longer allodynic from the intense traumatic nature of the sham surgery. No differences were observed between groups in the response thresholds recorded for the hindpaw ipsilateral (Fig 3.1A) or contralateral (Fig 3.1B) to the avulsion injury pre-surgery [baseline (BL)]. CGS21680 had no effect on the response thresholds of sham operated rats. The two-way repeated measures ANOVA from the time of injection (4 weeks post-SNAP) until the end of the experiment (10 weeks post-SNAP) showed a significant interaction in both the ipsilateral ($F_{15,105} = 1.776$; $p < 0.05$; Fig. 3.1A) and contralateral ($F_{15,105} = 2.258$; $p < 0.01$; Fig. 3.1B) hindpaw. There was also a significant main effect of time in both the ipsilateral ($F_{5,105} = 13.56$; $p < 0.0001$) and contralateral ($F_{5,105} = 5.823$; $p < 0.0001$) hindpaw as well as a significant main effect of drug treatment in both the ipsilateral ($F_{3,105} = 80.42$; $p < 0.0001$) and contralateral ($F_{3,105} = 64.81$; $p < 0.0001$) hindpaw. Bonferroni *post hoc* analysis revealed that SNAP+Vehicle group was significantly more allodynic than all other groups ($p < 0.05$) in both the ipsilateral and contralateral hindpaw. Furthermore, there were no significant differences between the other three groups in both the ipsilateral and contralateral hindpaw ($p > 0.05$).

Figure 3.1: Effect of administering the A_{2A}R agonist CGS21680 on SNAP

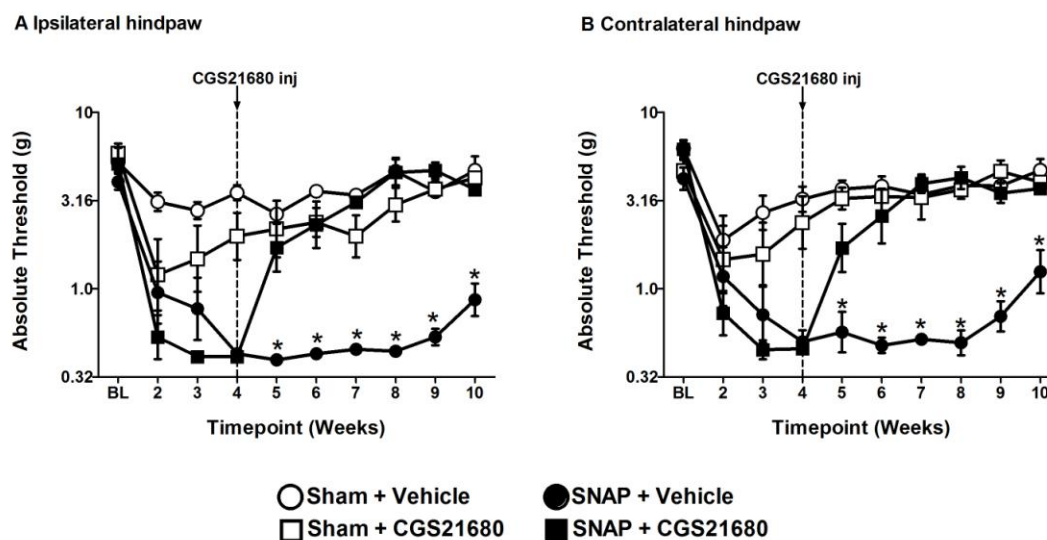


Figure 3.1. Assessment of the effects of administering CGS21680 on SNAP. Rats were tested for mechanical allodynia across a timecourse on both the ipsilateral (**A**) and contralateral (**B**) hindpaw. SNAP rats that received a single i.t. injection of CGS21680 (10 μ M) 4 weeks after surgery were significantly less allodynic than SNAP rats that received vehicle in both the ipsilateral (**A**) and contralateral (**B**) hindpaw. CGS21680 had no effect on sham rats. Data are presented as mean $\pm$ SEM and analyzed using a repeated measures two-way ANOVA, $n = 6$ per group. * $p < 0.05$ compared to all other groups.

Effect of administering neutralizing IL-10 antibody on the CGS21680 effect in SNAP

allodynia

Based on previous work in our lab showing the involvement of IL-10 in this mechanism,¹⁹¹ which was done in a peripheral neuropathic pain model (CCI), we wanted to define if IL-10 was involved in a central neuropathic pain model. A single i.t. injection of CGS21680 (10 μ M) was administered 4 weeks post-SNAP (shams were not included in this study because we know that CGS21680 has no effect on sham rats) to parallel the above study. One week later, a single i.t. injection of sheep anti-rat neutralizing IL-10 IgG antibodies (0.2 μ g/ml; 10 μ l) or control IgG was administered and rats were tested over a timecourse for

mechanical allodynia. No differences were observed between groups in the response thresholds recorded for the hindpaw ipsilateral (Fig 3.2A) or contralateral (Fig 3.2B) to the avulsion injury pre-surgery [baseline (BL)]. CGS21680 significantly decreased pain thresholds (4-5 week timepoint) in both the ipsilateral ($t_{34} = 8.648$; $p < 0.0001$) and contralateral ($t_{34} = 9.696$; $p < 0.0001$) hindpaw. The two-way repeated measures ANOVA from the time of anti-IL-10 injection (5 weeks post-SNAP) until the end of the experiment (3 weeks post-anti-IL-10 inj.) showed a significant interaction in both the ipsilateral ($F_{8,128} = 7.224$; $p < 0.0001$; Fig. 3.2A) and contralateral ($F_{8,128} = 5.638$; $p < 0.0001$; Fig. 3.2B) hindpaw. There was also a significant main effect of time in both the ipsilateral ($F_{8,128} = 10.82$; $p < 0.0001$) and contralateral ($F_{8,128} = 7.354$; $p < 0.0001$) hindpaw as well as a significant main effect of drug treatment in the ipsilateral ($F_{1,128} = 7.676$; $p < 0.05$) but not the contralateral ($p > 0.05$) hindpaw. Bonferroni *post hoc* analysis revealed that the groups were significantly different beginning 4 hr post-anti-IL-10 injection through the 48 hr timepoint post-anti-IL-10 injection in both the ipsilateral ($p < 0.01$) and contralateral ($p < 0.05$) hindpaw.

Figure 3.2: Effect of administering neutralizing IL-10 antibody on the CGS21680 effect in SNAP

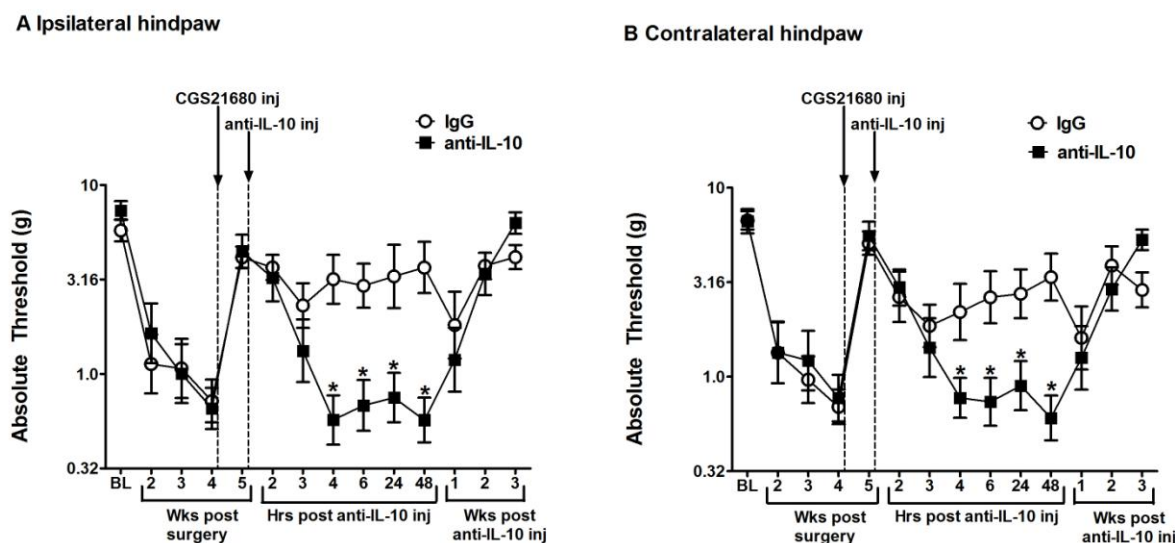


Figure 3.2. Assessment of the effects of administering anti-IL-10 on CGS21680-reversed SNAP rat allodynia. Rats were tested for mechanical allodynia across a timecourse on both the ipsilateral (**A**) and contralateral (**B**) hindpaw. SNAP rats that received a single i.t. injection of anti-IL-10 (0.2 $\mu\text{g/ml}$; 10 μl) 1 week after a single i.t. CGS21680 were significantly more allodynic than SNAP rats that received vehicle in both the ipsilateral (**A**) and contralateral (**B**) hindpaw. Data are presented as mean $\pm$ SEM and analyzed using a repeated measures two-way ANOVA, $n = 9$ per group, $*p < 0.05$.

Effect of administering the $A_{2A}R$ agonist CGS21680 on mRNA gene expression

Since neutralizing IL-10 significantly abolished the anti-allodynic effect of CGS21680, we next wanted to look at the mRNA gene expression profile at two different timepoints following CGS21680 of the anti-inflammatory cytokine IL-10 as well as the microglial activation marker CD11b and the proinflammatory cytokine TNF. We chose these specific markers because they are known to be modulated by $A_{2A}R$ agonists as stated above. CGS21680 did not significantly increase IL-10 mRNA in the ipsilateral T13/L1 dorsal spinal cord at either the 1 week ($p > 0.05$; Fig. 3.3A) or 6 week ($p > 0.05$; Fig. 3.3B) timepoint post-injection. CGS21680 did significantly decrease CD11b mRNA at both the 1 week ($F_{2,20} = 12.99$; $p < 0.001$;

Fig. 3.3C) and 6 week ($F_{2,14} = 8.123$; $p < 0.01$; Fig. 3.3D) timepoint post-injection. Bonferroni *post hoc* analysis revealed that CD11b mRNA was significantly increased in the SNAP+Vehicle group ($p < 0.01$) at both timepoints compared to the other 2 groups. CGS21680 also significantly decreased TNF- α mRNA at both the 1 week ($F_{2,21} = 9.251$; $p < 0.01$; Fig. 3.3E) and 6 week ($F_{2,17} = 19.32$; $p < 0.0001$; Fig. 3.3F) timepoint post-injection. Bonferroni *post hoc* analysis revealed that TNF mRNA was significantly increased in the SNAP+Vehicle group ($p < 0.05$) at both timepoints compared to the other 2 groups.

Figure 3.3: Effect of administering the A_{2A}R agonist CGS21680 on mRNA gene expression

T13/L1 Injury Site mRNA Gene Expression

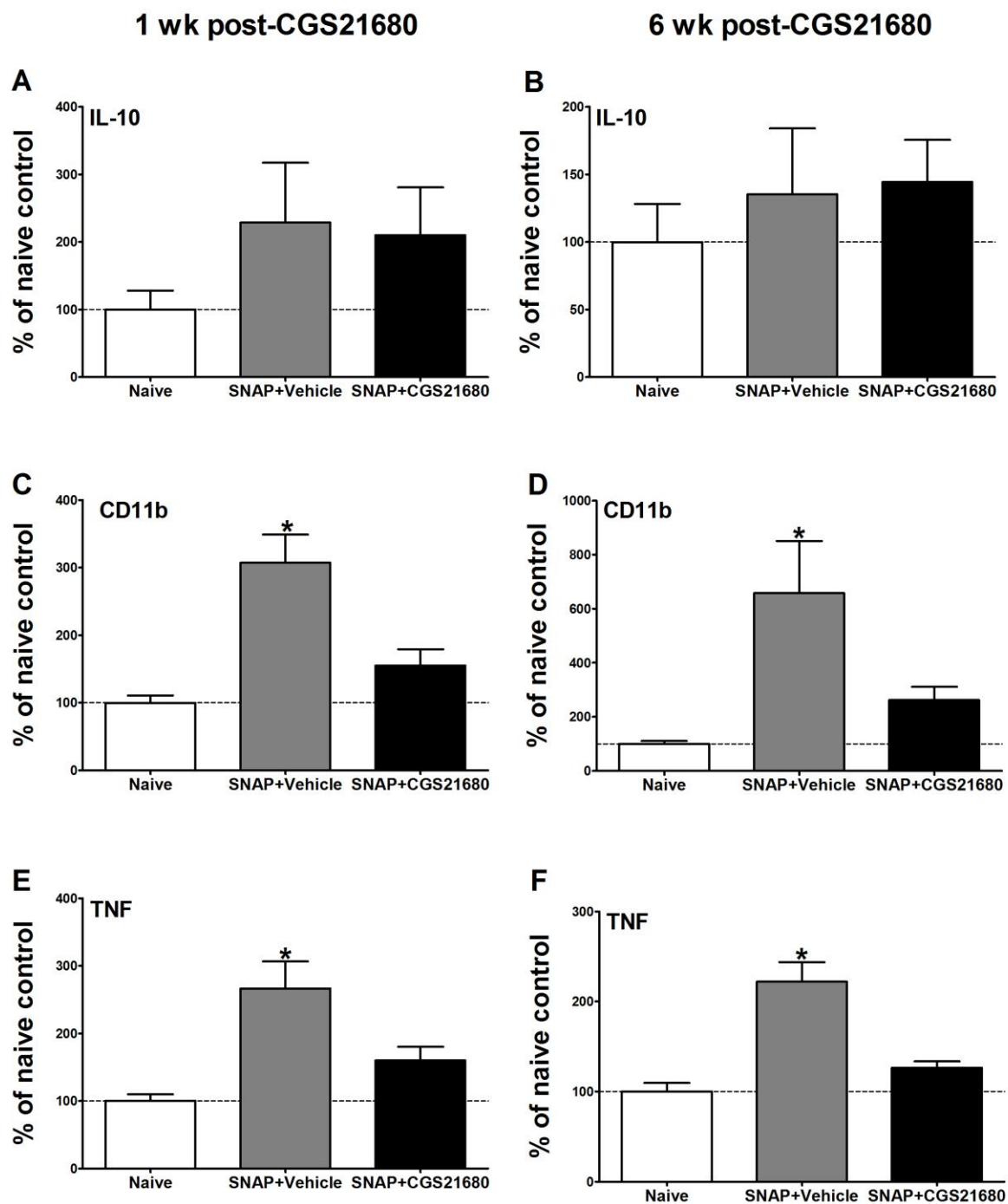


Figure 3.3. Assessment of the effects of administering CGS21680 on mRNA gene expression in the T13/L1 dorsal spinal cord. CGS21680 did not significantly increase IL-10 mRNA at either the 1 week (A) or 6 week (B) timepoint post-injection. CGS21680 significantly decreased CD11b mRNA at both the 1 week (C) and 6 week (D) timepoint post-injection. CGS21680 also significantly decreased TNF mRNA at both the 1 week (E) and 6 week (F) timepoint post-injection. Data are presented as mean $\pm$ SEM and analyzed using a one-way ANOVA, $n = 6$ to 9 per group. * $p < 0.05$ compared to all other groups.

Effect of administering the $A_{2A}R$ agonist ATL313 on SNAP-induced mechanical

allodynia

Lastly, we wanted to test the efficacy of another $A_{2A}R$ agonist, ATL313, on SNAP-induced allodynia. ATL313 was provided as a gift (Dr. J. Rieger) and was not available for use when the previous studies using CGS21680 were undertaken. ATL313 was administered either early in the development of SNAP or after SNAP had been maintained for at least 6 weeks. No differences were observed between groups in the response thresholds recorded for the hindpaw ipsilateral (Fig 3.4A,C) or contralateral (Fig 3.4B,D) to the avulsion injury pre-surgery [baseline (BL)]. CGS21680 had no effect on the response thresholds of sham operated rats, which showed mild and transient allodynia compared to SNAP. When ATL313 was administered 1 week post-SNAP, the two-way repeated measures ANOVA from 1 week after the injection (2 weeks post-SNAP) until the end of the experiment (9 weeks post-SNAP) showed a significant interaction in both the ipsilateral ($F_{21,147} = 2.551$; $p < 0.001$; Fig. 3.4A) and contralateral ($F_{21,140} = 2.175$; $p < 0.01$; Fig. 3.4B) hindpaw. There was also a significant main effect of time in both the ipsilateral ($F_{7,147} = 6.212$; $p < 0.0001$) and contralateral ($F_{7,140} = 4.627$; $p < 0.001$) hindpaw as well as a significant main effect of drug treatment in both the ipsilateral ($F_{3,147} = 27.85$; $p < 0.0001$) and contralateral ($F_{3,140} = 15.53$; $p < 0.0001$) hindpaw. Bonferroni *post hoc* analysis revealed that SNAP+Vehicle group was significantly more allodynic than all other groups ($p < 0.05$) in both the ipsilateral and contralateral hindpaw from 3-9 weeks post-ATL313 injection. Furthermore, there were no

significant differences between the other three groups in both the ipsilateral and contralateral hindpaw ($p > 0.05$). When ATL313 was administered 7 weeks post-SNAP, the two-way repeated measures ANOVA from 1 week after the injection (8 weeks post-SNAP) until the end of the experiment (9 weeks post-SNAP) showed a significant interaction in both the ipsilateral ($F_{3,120} = 3.948$; $p < 0.05$; Fig. 3.4C) and contralateral ($F_{3,20} = 6.649$; $p < 0.01$; Fig. 3.4D) hindpaw. There was also a significant main effect of time in both the ipsilateral ($F_{1,20} = 4.451$; $p < 0.05$) and contralateral ($F_{1,20} = 15.92$; $p < 0.001$) hindpaw as well as a significant main effect of drug treatment in both the ipsilateral ($F_{3,20} = 20.56$; $p < 0.0001$) and contralateral ($F_{3,20} = 7.364$; $p < 0.01$) hindpaw. Bonferroni *post hoc* analysis revealed that SNAP+Vehicle group was significantly more allodynic than all other groups ($p < 0.01$) in the ipsilateral hindpaw from 8-9 weeks post-ATL313 and only at 8 weeks post-ATL313 injection ($p < 0.05$) in the contralateral hindpaw. Furthermore, there were no significant differences between the other three groups in both the ipsilateral and contralateral hindpaw ($p > 0.05$).

Figure 3.4: Effect of administering the A_{2A}R agonist ATL313 on SNAP

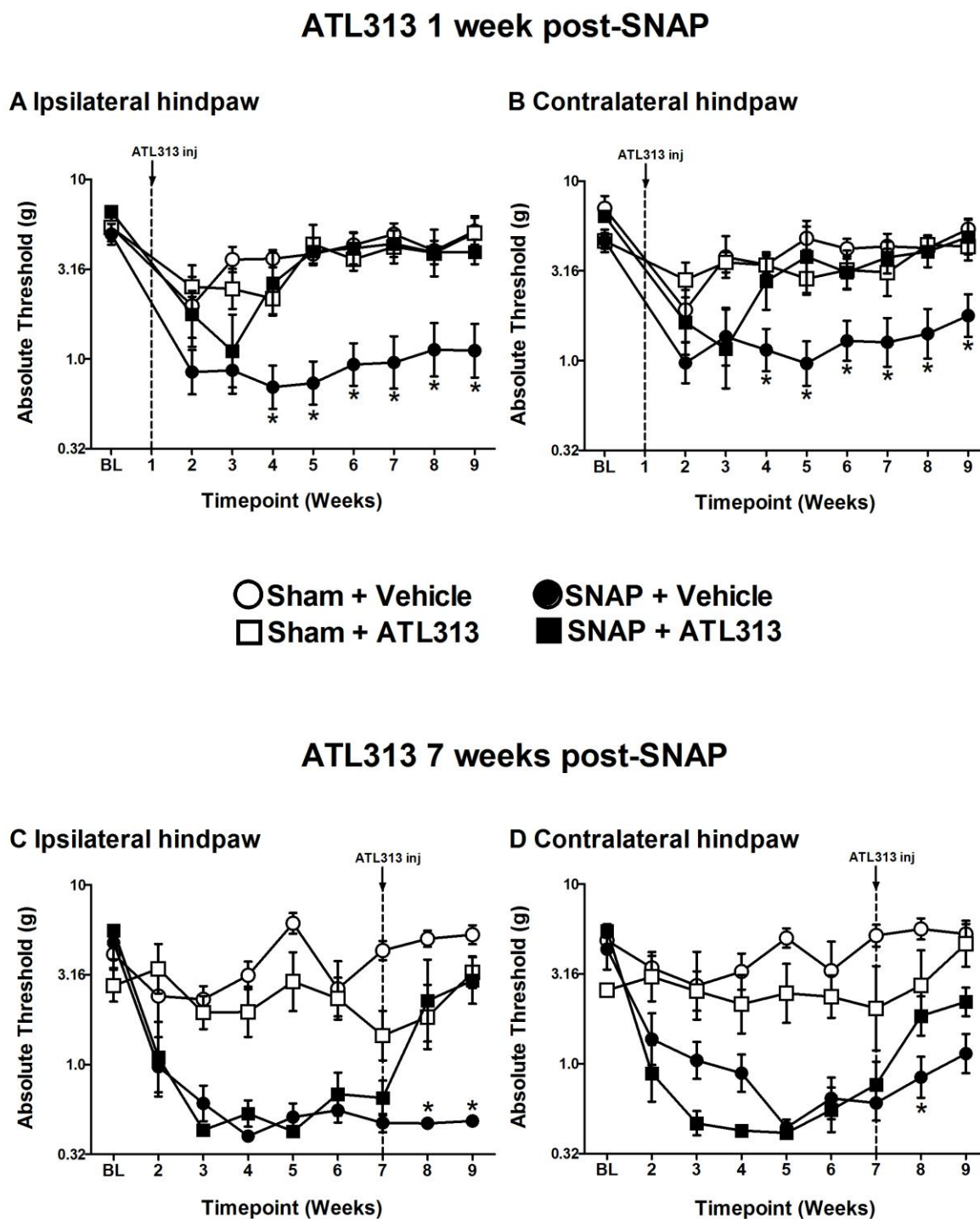


Figure 3.4. Assessment of the effects of administering ATL313 on SNAP. Rats were tested for mechanical allodynia across a timecourse on both the ipsilateral (A,C) and contralateral (B,D) hindpaw. SNAP rats that received a single i.t. injection of ATL313 (1 μ M) 1 week after surgery

were significantly less allodynic than SNAP rats that received vehicle in both the ipsilateral (**A**) and contralateral (**B**) hindpaw. ATL313 had no effect on sham rats. SNAP rats that received a single i.t. injection of ATL313 (1 μ M) 7 weeks after surgery were significantly less allodynic than SNAP rats that received vehicle in both the ipsilateral (**C**) and contralateral (**D**) hindpaw. ATL313 had no effect on sham rats. Data are presented as mean $\pm$ SEM and analyzed using a repeated measures two-way ANOVA, $n = 6$ per group. $*p < 0.05$ compared to all other groups.

Discussion

The present series of studies demonstrate that SNAP SCI creates a profound mechanical allodynia below the spinal level of the injury that was resolved by i.t. delivery of an $A_{2A}R$ agonist. A *single* i.t. injection of the $A_{2A}R$ agonists CGS21680 and ATL313 administered weeks after surgery reversed SNAP-induced below-level central neuropathic pain for up to 6 weeks, and this is at least partially mediated by IL-10. The fact that $A_{2A}R$ agonists are able to reverse established central neuropathic pain (4-7 weeks post-SCI) is important mechanistically, as it implies that $A_{2A}R$ sensitive pathways remain importantly involved in the long term maintenance of the pain, suggestive that $A_{2A}R$ s may prove to be a realistic therapeutic target for clinical pain control. This is the first time anyone has shown a long-lasting, single injection $A_{2A}R$ agonist-induced reversal of central neuropathic pain; most pharmacological therapies require multiple days of systemic dosing to reverse neuropathic pain. In addition to reversing SNAP SCI-induced allodynia in the present experiments, treatment with CGS21680 also significantly decreased mRNA expression of the microglial activation marker CD11b and the proinflammatory cytokine TNF- α at both 1 and 6 weeks following CGS21680 administration.

The effects of $A_{2A}R$ agonism observed in the present studies are likely due in part to suppression of microglial activation and the subsequent release of TNF- α , leading to a shift in the microenvironment of the spinal cord from proinflammatory to more anti-inflammatory. It is known that anti-inflammatory cytokines attenuate neuropathic pain,²⁰⁷ and also that certain therapies, such as IL-10 gene therapy, can shift CNS cells from classically activated (more

proinflammatory) to alternatively activated (less proinflammatory).²⁹⁶ Other studies have shown that A_{2A}R agonists decrease TNF- α in spinal cord glia,¹⁰¹ monocytes and macrophages,²¹⁴ and CSF cells,¹⁹¹ as well as decrease both microglial and astrocyte activation in the spinal cord dorsal horn following nerve injury.¹⁹¹ Taken together, these data suggest that the ability of A_{2A}R agonists to reduce inflammation is partially responsible for the long-lasting reversal of SNAP-induced allodynia.

A_{2A}R agonists are also known to potentiate the production of the anti-inflammatory cytokine IL-10 *in vivo*.¹⁹¹ IL-10 is neuroprotective in SCI and can delay the onset of SCI-induced pain behavior,¹ as well as reduce TNF- α production.²⁰ Here we administered a neutralizing IL-10 antibody (anti-IL-10) 1 week after CGS21680 in SNAP rats and saw that the pain-relieving effects of CGS21680 were abolished, but only for 48 hours post-anti-IL-10. This suggests that A_{2A}R agonists induce sustained release of IL-10, and this contributes to the enduring effects of the A_{2A}R agonist. However, we did not observe any significant changes at any timepoint in IL-10 gene expression in the T13/L1 spinal cord following CGS21680 administration. This lack of significant change in IL-10 mRNA in the spinal cord is not entirely unexpected, as we have previously reported A_{2A}R agonist-induced increases of IL-10 mRNA only in cells of the CSF, not in the actual spinal cord or meninges.¹⁹¹ We also do not observe significant changes in spinal IL-10 protein following A_{2A}R agonism in SNAP (data not shown). Furthermore, we have shown that IL-10 protein is not up-regulated in cultured neonatal microglia or astrocytes following ATL313 administration.¹⁹² A possible reason for increased IL-10 mRNA in the CSF but not in the actual spinal cord or cultured glial cells after A_{2A}R agonist administration could be that A_{2A}R agonists are shifting the inflammatory phenotype of other resident and/or recruited immunocompetent cells to an alternative activation state.

In addition to neurons and glia, endothelial cells, neutrophils, and macrophages all express A_{2A} receptors,¹²⁵ and both neutrophils and macrophages are heavily influenced by their microenvironments and can be phenotypically shifted or polarized from one immune state to another.⁹³ However, since neutrophils are short-lived cells⁹³ and endothelial cells are classically thought to be more involved with trafficking and recruiting other immune cells,¹²⁵ the most likely infiltrating immune cells exerting effects on spinal glia are monocytes/macrophages. At SCI lesion sites, microglia and resident/recruited macrophages assume a proinflammatory, or classical M1, phenotype.¹⁵⁹ This state is characterized by increased proinflammatory mediators including TLR2 and 4, TNF- α , IL-1 β , and IL-6.¹⁹⁷ Additionally, M1 macrophages cause neurotoxicity, release oxidative metabolites, and slow axonal growth. The M1 phenotype is rapidly induced and can be maintained at sites of SCI for at least 1 month.¹⁵⁹ Our lab has also demonstrated that activated meningeal immune cells can produce proinflammatory cytokines,³⁴⁷ and that inflammatory mediators in cells of the CSF can penetrate the spinal cord and exert effects on glia.²⁹⁷ In contrast to the M1 cells, microglia/macrophages can take on an alternative (M2) activation state when the microenvironment is less proinflammatory. This phenotype is characterized by increased anti-inflammatory mediators including IL-10 and arginase.¹⁹⁷ M2 microglia/macrophages promote tissue healing and axonal growth following SCI. At sites of SCI, the M1 phenotype dominates and there are very few microglia/macrophages expressing the M2 phenotype. However, if there is a change in the microenvironment from more proinflammatory to less proinflammatory, M1 microglia/macrophages can phenotypically shift to a predominately M2 phenotype.¹⁵⁹ It has been argued that microglia/macrophages possess an M2 phenotype in the intact CNS.²⁴⁷ This phenomenon is also observed in IL-10 gene therapy where i.t. administration of plasmid IL-10 causes cells in the CSF to shift from a classically activated ED1 phenotype to a

more alternatively activated ED2 phenotype.²⁹⁶ In the present studies we measured arginase-1 mRNA and protein in the spinal cord following ATL313 administration (data not shown) as a marker of alternative activation, but again we did not observe any significant up-regulation in either arginase-1 mRNA or protein, suggesting that if there is alternative activation it is likely coming from resident/recruited immunocompetent cells.

The anti-inflammatory effects of adenosine agonists are not always adenosine receptor specific. A_{2A}R and A_{2B}R agonists increase cyclic adenosine monophosphate (cAMP) while A₁R and A₃R agonists decrease cAMP.⁹⁸ A_{2A}R agonist-induced increases of intracellular cAMP have been shown to reduce inflammation and apoptosis following SCI,¹⁰¹ and A_{2B}R agonists have been shown to produce similar effects on mechanical allodynia compared to A_{2A}R agonists, although the reversal is not as long-lasting.¹⁹² In contrast, A₁R and A₃R agonists show very transient pain reversal.^{44, 192} Central administration of A₁R agonists have anti-hyperalgesic effects in models of SCI and peripheral neuropathy, but the behavior resolves within 2 hours;^{134, 257} not like the stable, enduring allodynic reversal defined here with A_{2A}R agonists. Additionally, activation of A₃Rs elicit nociceptive and proinflammatory responses in rats.²⁷⁶ Taken together, this supports that the anti-inflammatory effects in the present studies are specific to the A_{2A}R and are not due to general adenosine activation.

One challenge in the SCI and pain fields is identifying treatments that don't require daily administration but still provide long-lasting pain relief. In the present studies, a *single* i.t. injection of an A_{2A}R agonist reverses central neuropathic pain for up to 6 weeks. We have shown in SNAP that in order to achieve full reversal of allodynia using three different systemic glial activation inhibitors (propentofylline, ibudilast, and (+)-naltrexone), they must be administered daily for at least 1 week.⁷⁸ Furthermore, once the drugs are no longer being administered the pain

returns. Others have shown that you need at least 3 weeks of daily dosing to fully reverse mechanical allodynia in both central and peripheral neuropathy.^{118, 314} Although ATL313 does not cross the blood brain barrier, the commercially available CGS21680 does²⁹¹ and so could be administered systemically, which would allow it to be used in a larger clinical population. Thus, the clinical potential of these agonists is promising for long-term control of central neuropathic pain with very few administrations.

Taken together, this set of studies demonstrates that a single i.t. injection of an adenosine A_{2a}R agonist reverses central neuropathic pain, and that this effect is long-lasting and at least partially dependent on IL-10. These agonists also decrease microglial activation and the proinflammatory cytokine TNF- α , and are also likely up-regulating IL-10 in immunocompetent cells. Although more work needs to be done on elucidating the mechanisms of these agonists in neuropathic pain states, these agonists have the potential to be successful in treating a variety of chronic pain disorders.

Chapter IV

Morphine Amplification of Central Neuropathic Pain and Neuroinflammation in a Rat Model of Spinal Cord Injury

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Abstract

Central neuropathic pain (CNP) is a pervasive, debilitating problem that impacts thousands of people living with central nervous system (CNS) disorders, including spinal cord injury (SCI). Unfortunately, current therapies for treating this type of pain are ineffective and often have intolerable side effects. Although opioids are one of the most commonly used CNP treatments, recent animal literature has indicated that administering opioids shortly after a traumatic injury can actually have deleterious effects on long-term health and recovery. This is important, as opioids are universally administered as a first-line intervention following a traumatic injury such as SCI. In addition, many SCI patients who develop chronic pain weeks or months after the initial injury are put on opioid regimens. In order to study the deleterious effects of administering morphine shortly after trauma, we used our T13 dorsal root avulsion model (Spinal Neuropathic Avulsion Pain, SNAP). Administering a week-long course of 10 mg/kg/day morphine beginning 24 hr post-SNAP amplified SNAP and gene expression of glial activation markers and toll-like receptor 4 (TLR4). Blocking TLR4 with the antagonist (+)-naltrexone prevented morphine amplification of SNAP. Furthermore, morphine also amplified IL-1 β and NLRP3 mRNA and IL-1 β protein. These data suggest that morphine can make CNP worse and that TLR4 and IL-1 β are involved in this mechanism and could be potential treatment targets for preventing the deleterious effects of administering opioids after traumatic injury.

Introduction

Spinal cord injury (SCI) is a debilitating condition that is the leading cause of central neuropathic pain, and unfortunately central neuropathic pain is usually intractable to treatment. Current therapies, including opioids, provide only 50% pain relief in 1 out of 2-3 patients.⁹² Although opioids are the most effective analgesic for central neuropathic pain,³⁵¹ recent rodent and clinical studies have shown that opioids administered soon after trauma can actually be detrimental to health and recovery.^{131, 132, 270}

In order to study central neuropathic pain in isolation from other potentially confounding effects of traumatic SCI, we have developed and optimized a unilateral thoracic (T) 13 and a unilateral T13/lumbar (L)1 avulsion (rapid rip of dorsal roots) model of SCI that does not cause paralysis, urinary tract infections/retention, autotomy, or other non-pain relevant aspects that can complicate the study of central neuropathic pain. Our model, termed SNAP (Spinal Neuropathic Avvulsion Pain), creates physical damage to the outer laminae of the sensory dorsal horn and robust and reliable below-level bilateral hindpaw mechanical allodynia that lasts for ~9 weeks.^{78,}
³⁴⁹ Importantly, glia (microglia and astrocytes) are activated in response to SNAP⁷⁹ and administering glial inhibitors attenuates SNAP.^{80, 81}

Glia (microglia and astrocytes) are non-neuronal, immune-like cells of the central nervous system (CNS) that are normally in a quiescent/surveillance state and are responsible for maintaining CNS homeostasis and monitoring tissue for trauma and foreign pathogens.³¹¹ When glia become activated, they undergo numerous phenotypic and morphological changes and release a host of proinflammatory and neuroexcitatory mediators that include the proinflammatory cytokines interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α). Persistent glial activation and the subsequent proinflammatory environment results in

neuropathic pain,³⁴⁴ including SCI-induced central neuropathic pain.^{47, 137, 161} Importantly, blocking glial activation in multiple classic models of SCI attenuates neuropathic pain.^{111, 119}

Glia become activated in response to their exposure to danger signals that are released by stressed, damaged, and dying/dead cells.¹⁶⁶ It has been recently recognized that endogenous danger signals activate glia (especially microglia²⁵⁵) by binding to toll-like receptor 4 (TLR4).²⁶ Importantly, we know that blocking TLR4 using the antagonist (+)-naltrexone reverses SNAP.⁷⁸

TLRs are pattern recognition receptors (PRRs) that are the primary sensors that recognize pathogen-associated molecular patterns (PAMPs), such as gram-positive and gram-negative bacteria.²³⁰ We have recently discovered that TLR4 is activated not only by alarmins, but also by *every clinically relevant class of opioids*.^{141, 142, 340, 345} This is important because virtually all SCI patients are treated with opioids soon after injury, whether *en route* to the hospital or during early care for acute trauma.¹³³ TLR4 activation can also prime the NLRP3 inflammasome, a multi-protein complex involved in processing IL-1 β from its pro- form to its active mature form.¹⁷⁷ Since there are now multiple clinical^{123, 270, 321, 328} and pre-clinical^{131, 132} examples of the deleterious effects of opioids given shortly after trauma, and given the significant clinical implications this could have, the goal of these studies was to first define whether morphine amplifies SNAP, and if so, to determine some of the potential underlying mechanisms.

Materials and Methods

Animals

Pathogen-free male Sprague-Dawley rats (325-350g; Harlan Laboratories, Madison, WI, USA) were used for all experiments. Rats were pair-housed prior to surgery and then single-housed after surgery with standard rat chow and water available *ad libitum*. Housing was in a temperature-controlled room that was maintained at 23 $\pm$ 2°C with a 12 hr light/dark cycle

(lights on at 0700 hr). The rats were allowed a minimum of 1 week to habituate to the colony room before initiating the experiment. All procedures were performed during the light cycle. Following surgery, the animals were singly housed. All protocols were approved by the University of Colorado at Boulder Institutional Animal Care and Use Committee.

Drugs

Where applicable, drugs were prepared and are reported as free base concentrations. (–)-Morphine sulfate was gifted by Mallinckrodt, Inc. (St. Louis, MO, USA) and was dissolved in sterile endotoxin-free isotonic saline (Abbott Laboratories, Abbott Park, IL, USA) and administered at a dose of 10 mg/kg per subcutaneous (s.c.) injection. Controls received s.c. equivolume (1 ml/kg) saline. (+)-Naltrexone (synthesized and gifted by Kenner Rice, NIDA and NIAAA, Rockville, MD, USA) was dissolved in sterile endotoxin-free isotonic saline (Abbott Laboratories) and administered at 6 mg/kg per s.c. injection. Controls received s.c. equivolume (1 ml/kg) saline. (+)-Naltrexone was given systemically as it is known to cross the blood brain barrier.¹⁴⁴

Spinal Neuropathic Avulsion Pain (SNAP) Surgery

Unilateral (left) T13 dorsal root avulsion was performed under isoflurane anesthesia, as previously described in detail.³⁴⁸ Briefly, laminectomy was performed at the T12 vertebral level and the dura mater was incised over the dorsal root entry zone. The T13 dorsal rootlets were carefully isolated and then clamped at the dorsal root entry zone and briskly pulled out (avulsed). Sterile saline-moistened surgical sponge was placed over the exposed spinal cord to protect it, the muscle was sutured in layers with sterile 3-0 silk, and the skin was closed with stainless steel wound clips. Immediately following surgery, rats were single-housed in a cage with foam padding for a few hours to protect their spinal cord from further trauma due to the brief ataxic

period that follows recovery from anesthesia. Rats are placed in hanging cages immediately after each morphine injection with access to food and water *ad libitum* for ~6 hr in order to ensure they do not choke on bedding and to make it easier to observe their breathing and behavior. Sham operated rats were treated identically, except for avulsing of the rootlets. Combi-Pen-48 antibiotic (0.2 ml; Bimeda, Inc., Le Sueur, MN, USA) was administered at the time of surgery and daily for 4 days after surgery.

Low Threshold Mechanical Allodynia Testing

Prior to surgery, rats were habituated to the testing environment for 4 consecutive days prior to recording of behavioral responses. All von Frey assessments were performed blind with respect to drug assignments. Assessment of von Frey thresholds occurred before surgery (baseline) and across a timecourse beginning two weeks after surgery. The von Frey test was performed on the plantar surface of each hind paw as previously described in detail.²⁰⁵ A logarithmic series of 10 calibrated Semmes–Weinstein monofilaments (Stoelting) were sequentially applied to the left and right hind paws in random order, each for 8 s at constant pressure to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. Log stiffness of the hairs is determined by $\log_{10}$ (milligrams $\times 10$). The range of monofilaments used in these experiments (0.407–15.136 g) produces a logarithmically graded slope when interpolating a 50% response threshold of stimulus intensity (expressed as $\log_{10}$ (milligrams $\times 10$)).⁴¹ The stimulus intensity threshold to elicit a paw withdrawal response was used to calculate the 50% paw withdrawal threshold (absolute threshold) using the maximum-likelihood fit method to fit a Gaussian integral psychometric function.¹²⁴ This method normalizes the withdrawal threshold for parametric analyses.¹²⁴

Processing of tissue for PCR

RNA isolation and cDNA synthesis. Total RNA from the 5 mm piece of the T13/L1 thoracic spinal cord was extracted using the standard phenol/chloroform extraction with TRIzol Reagent (Invitrogen) according to the manufacturer's guidelines. Total RNA was reverse transcribed into cDNA using Superscript II First-Strand Synthesis System (Invitrogen). First-strand cDNA was synthesized using total RNA, random hexamer primer (5 ng/μl) and 1 mM dNTP mix (Invitrogen) and incubated at 65°C for 5 min. After 2 min incubation on ice, a cDNA synthesis buffer (5 x reverse transcription (RT) buffer; Invitrogen) and dithiothreitol (10 mM) was added and incubated at 25°C for 2 min. Reverse transcriptase (Superscript II; 200 U; Invitrogen) was added to a total volume of 20 μl and incubated for 10 min at 25°C, 50 min at 42°C, and deactivating the enzyme at 70°C for 15 min. cDNA was diluted twofold in nuclease-free water and stored at -80°C until real-time PCR (RT-PCR) was performed.

RT-PCR. Primer sequences were obtained from the GenBank at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and are displayed in Table 4.1. Primers were generated to span an intron to eliminate genomic interference. Amplification of the cDNA was performed, in a blinded procedure, using Quantitect SYBR Green PCR kit (QIAGEN) in iCycler iQ 96-well PCR plates (Bio-Rad) on a MyiQ single Color Real-Time PCR Detection System (Bio-Rad). The reaction mixture (26 μl) was composed of Quanti-Tect SYBR Green (containing fluorescent dye SYBR Green I, 2.5 mM MgCl₂, dNTP mix, and Hot Start Taq polymerase), 10 nM fluorescein, 500 nM each forward and reverse primer (Invitrogen), nuclease-free water, and 1 μl of cDNA from each sample. Each sample was measured in duplicate. The reactions were initiated with a hot start at 95°C for 25 min, followed by 40 cycles

of 15 s at 94°C (denaturation), 30 s at 55–60°C (annealing), and 30 s at 72°C (extension). Melt curve analyses were conducted to assess uniformity of product formation, primer–dimer formation, and amplification of nonspecific products. The PCR product was monitored in real-time, using the SYBR Green I fluorescence, using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). Threshold for detection of PCR product was set in the log-linear phase of amplification and the threshold cycle (*CT*) was determined for each reaction. The level of the target mRNA was quantified relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the comparative *CT* (ΔCT) method. The expression of GAPDH was not significantly different between treatments.

Table 4.1. Primer Sequences

Gene	Primer Sequence (5'-3')	GenBank accession no.
<i>GAPDH (i-s)</i>	TCTTCCAGGAGCGAGATCGC (forward) TTCAGGTGAGCCCCAGCCTT (reverse)	NG_028301.1
<i>CD11b (i-s)</i>	CTGGTACATCGAGACTTCTC (forward) TTGGTCTCTGTCTGAGCCTT (reverse)	NM_012711.1
<i>GFAP (i-s)</i>	AGATCCGAGAAACCAGCCTG (forward) CCTTAATGACCTCGCCATCC (reverse)	NM_017009.2
<i>TLR4 (i-s)</i>	TCCCTGCATAGAGGTACTTC (forward) CACACCTGGATAAATCCAGC (reverse)	NM_019178.1
<i>TNF-α (i-s)</i>	CAAGGAGGAGAAGTTCCCA (forward) TTGGTGGTTTGCTACGACG (reverse)	NM_012675.3
<i>IL-1β (i-s)</i>	CCTTGTCGAAGTGTCTGAAG (forward) GGGCTTGGAAGCAATCCTTA (reverse)	NM_031512.2
<i>NLRP3 (i-s)</i>	AGAAGCTCCCCTTGGTGAATT (forward) GTTGTCTAACTCCAGCATCTG (reverse)	NM_001191642.1

i-s, intron-spanning primers generated to reduce genomic interference

Enzyme linked immunosorbant assay (ELISA)

IL-1 β protein in rat T13/L1 ipsilateral dorsal spinal cord was analyzed using a commercially available ELISA kit specific for rat IL-1 (R&D Systems, Minneapolis, MN, USA). The sensitivity of the IL-1 β assay is less than 5 pg/mL.

Western Blot

Thoracic T13/L1 ipsilateral dorsal spinal cord (~5 mm piece) was sonicated in a cocktail containing extraction buffer (Invitrogen, Carlsbad, CA, USA) and protease inhibitors (Sigma, St. Louis, MO, USA). Ice-cold tissue samples were centrifuged at 14,000 rpm at 4°C for 10 min. The resulting supernatant was removed and the protein concentration of each sample was quantified using the Bradford method. Protein samples were loaded into a standard polyacrylamide gel (8-12% 15 well Bis-Tris Gel, Invitrogen). Electrophoresis was performed in MOPS running buffer using the XCell SureLock Mini-Cell (Invitrogen). Protein was transferred onto a nitrocellulose membrane using the iBlot system (Bio-Rad, Hercules, CA, USA). The membrane was blocked with Odyssey blocking buffer (Li-COR Biosciences, Lincoln, NE, USA) for 1 hr and incubated with NLRP3 primary antibody (LifeSpan BioSciences, Seattle, WA, USA) in blocking buffer at a dilution of 1:500 and β -actin (Sigma) at a dilution of 1:1,000 overnight at 4 °C. The following day, the membrane was washed and then incubated in goat-anti-rabbit for NLRP3 and goat-anti-mouse for β -actin secondary antibody both at a dilution of 1:10,000 at RT for 1 hr. Protein expression was visualized and quantified using the Odyssey Infrared Imager (Li-COR Biosciences).

Pharmacological Manipulations

Effect of administering a week-long course of morphine beginning 24 hr post-surgery on SNAP

Morphine (10 mg/kg) or equivolume vehicle (saline) was administered once daily for 7 days beginning 24 hr post-SNAP (n = 6 per group). Sham rats are not the most appropriate controls because the traumatic nature of the sham surgery can also be potentiated by morphine. Instead, a group of surgically naïve rats were given the same course of morphine as the SNAP rats and placed in the hanging cages at the same time as the SNAP rats for 7 days. Rats were then given 1 week to fully recover from surgery and completely clear out the morphine. At 2 weeks post-surgery, rats were behaviorally tested to define their mechanical response thresholds once a week for 5 weeks post-surgery.

Effect of administering a week-long course of morphine beginning 24 hr post-surgery on glial activation marker, TLR4, and TNF- α gene expression

After recording behavioral responses at the 1 week or 5 week post-drug treatment timepoint, SNAP rats that received morphine (10 mg/kg s.c.) or vehicle and two groups of surgically naïve rats, one that received no manipulation and was kept in their home cage and one that received the same course of morphine as the SNAP rats (10 mg/kg s.c. once a day for 7 days; all groups n = 6-8 per group) and placed in the hanging cages, were overdosed with sodium pentobarbital (0.8 ml) and transcardially perfused with ice-cold 0.9% saline for 2 min. The spinal cord was dissected and a 5 mm piece of ipsilateral dorsal spinal cord, with the meninges removed, was dissected out to include the T13/L1 spinal level. All tissue was flash frozen in liquid nitrogen. Samples were then stored at -80°C until analysis.

Effect of co-administering a week-long course of morphine and the TLR4 antagonist (+)-naltrexone beginning 24 hr post-surgery on SNAP

(+)-Naltrexone (6 mg/kg s.c.) or equivolume vehicle (Saline) was administered three times a day at approximately 0900, 1200, and 1500 hr for 7 days beginning 24 hr post-surgery.

Immediately after the first (+)-naltrexone injection each day, morphine was injected once a day for 7 days at 10 mg/kg s.c. beginning 24 hr post-surgery. Beginning 2 weeks post-surgery, rats were behaviorally tested to define their mechanical response thresholds once a week for 10 weeks post-surgery.

Effect of administering a week-long course of morphine 24 hr post-surgery on IL-1 β and NLRP3 inflammasome marker protein and gene expression

After recording behavioral responses at the 1 week post-drug treatment (2 weeks post-SNAP) timepoint, SNAP rats that received morphine (10 mg/kg s.c.) or vehicle and a group of surgically and drug naïve rats (n = 6 per group, each condition repeated twice to obtain tissues for protein and mRNA), were overdosed with sodium pentobarbital (0.8 ml) and transcardially perfused with ice-cold 0.9% saline for 2 min. The spinal cord was dissected and a 5 mm piece of ipsilateral dorsal spinal cord, with the meninges removed, was dissected out to include the T13/L1 spinal level. All tissue was flash frozen in liquid nitrogen. Samples were then stored at -80°C until analysis.

Statistical Analysis

All data were analyzed using GraphPad Prism version 5.04 for Windows Vista (GraphPad Software, San Diego, CA, USA). Ipsilateral and contralateral behavioral data were analyzed individually. Behavioral measures were normalized as described above and group differences were analyzed by comparing area under the curve (AUC), as previously described by Jones and Sorkin.¹⁵² AUC values (GraphPad Prism 5.01; GraphPad software Inc., San Diego, CA) were calculated from absolute threshold values, from 3.56 (the lowest threshold response value in the data set) up to the threshold response of each rat across time. Then a one-way ANOVA was performed on the AUC values for each group. Decreased AUC reflects an increase

in mechanical allodynia. For all studies, the AUC measures across time were collapsed into a single timepoint for each animal, thus there is not a repeated measurement. For baseline measurements, a one-way ANOVA at that single timepoint was the statistic used. The RT-PCR, western blot, and ELISA data were analyzed using a one-way ANOVA. Bonferroni *post hoc* tests were used where appropriate, and $p < 0.05$ was considered statistically significant.

Results

Effect of administering a week-long course of morphine beginning 24 hr post-surgery on SNAP

In order to determine if morphine amplifies SNAP, morphine (10 mg/kg s.c.) or vehicle was administered once a day for one week beginning 24 hr post-surgery. No differences were observed between groups in the response thresholds recorded for the hindpaw ipsilateral (Fig 4.1A) or contralateral (Fig 4.1B) to the avulsion injury pre-surgery [baseline (BL)]. The one-way ANOVA comparing the AUC of Naïve+Morphine, SNAP+Vehicle, and SNAP+Morphine from 2-5 weeks post-surgery was significant in both the ipsilateral ($F_{2,15} = 31.21$; $p < 0.0001$; Fig 4.1C) and contralateral ($F_{2,15} = 25.15$; $p < 0.001$; Fig 4.1D) hindpaw. Bonferroni *post hoc* analysis of the AUCs revealed that the SNAP+Morphine group was significantly more allodynic than all other groups ($p < 0.05$) in both the ipsilateral and contralateral hindpaw. Furthermore, the SNAP+Vehicle group was significantly more allodynic than the Naïve+Morphine group in both the ipsilateral and contralateral hindpaw ($p < 0.05$).

Figure 4.1: Effect of administering a week-long course of morphine beginning 24 hr post-surgery on SNAP

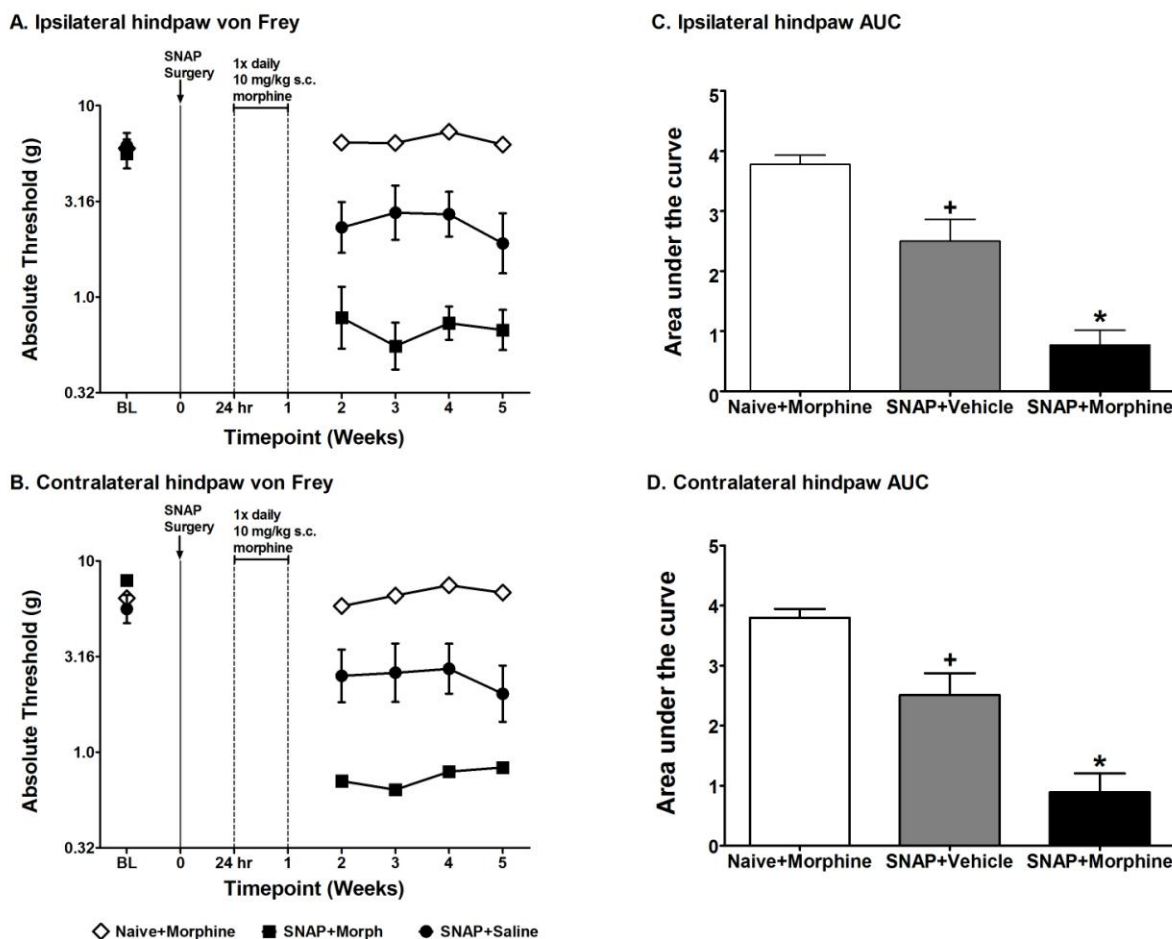


Figure 4.1. Assessment of the effects of morphine on SNAP. Rats were tested for mechanical allodynia across a timecourse on both the ipsilateral (A) and contralateral (B) hindpaw. SNAP rats that received morphine (10 mg/kg, s.c.) for 7 days beginning 24 hours after surgery were significantly more allodynic than SNAP rats that received vehicle and naïve rats that received morphine in both the ipsilateral (C) and contralateral (D) hindpaw. Data are presented as mean $\pm$ SEM and analyzed using a one-way ANOVA on the AUCs, $n=6$ per group. * $p<0.05$ compared to all other groups, $^+p<0.05$ compared to Naïve+Morphine group.

Effect of administering a week-long course of morphine beginning 24 hr post-surgery on glial activation marker, TLR4, and TNF- α gene expression

Since morphine amplified SNAP, we also wanted to determine if morphine amplified mRNA expression of the glial activation markers CD11b and GFAP as well as TLR4 and TNF-

α . The one-way ANOVA for CD11b mRNA was significant at both the 1 week ($F_{3,21} = 27.32$; $p < 0.0001$; Fig. 4.2A) and 4 weeks ($F_{3,19} = 25.02$; $p < 0.0001$; Fig. 4.2B) timepoint post-final morphine injection. Bonferroni *post hoc* analysis revealed that CD11b mRNA was significantly increased in the SNAP+Vehicle group ($p < 0.05$) at both timepoints compared to both naïve groups, and was further amplified by morphine at both timepoints. There was no significant difference between the naïve groups at either timepoint. The one-way ANOVA for GFAP mRNA was also significant at both the 1 week ($F_{3,15} = 23.91$; $p < 0.0001$; Fig. 4.2C) and 4 week ($F_{3,15} = 9.905$; $p < 0.001$; Fig. 4.2D) timepoint post-final morphine injection. Bonferroni *post hoc* analysis revealed that GFAP mRNA was significantly increased in the SNAP+Vehicle group ($p < 0.05$) compared to both naïve groups, and was further amplified by morphine only at the 1 week post-final morphine injection timepoint. At the 4 week post-final morphine injection timepoint, the SNAP+Morphine group was only different from the two naïve groups and there were no other differences between any groups. There was no significant difference between the naïve groups at either timepoint. The one-way ANOVA for TLR4 mRNA was significant at both the 1 week ($F_{3,20} = 18.67$; $p < 0.0001$; Fig. 4.3A) and 4 week ($F_{3,21} = 3.469$; $p < 0.05$; Fig. 4.3B) timepoint post-final morphine injection. Bonferroni *post hoc* analysis revealed that TLR4 mRNA was significantly increased in the SNAP+Vehicle group ($p < 0.05$) compared to both naïve groups, and was further amplified by morphine only at the 1 week post-final morphine injection timepoint. There were no significant differences between groups at the 4 weeks post-final morphine injection timepoint. There was no significant difference between the naïve groups at either timepoint. The one-way ANOVA for TNF- α mRNA was significant at both the 1 week ($F_{3,22} = 4.736$; $p < 0.05$; Fig. 4.3C) and 4 week ($F_{3,20} = 7.241$; $p < 0.01$; Fig. 4.3D) timepoint post-final morphine injection. Bonferroni *post hoc* analysis revealed that at the 1 week post-final

morphine injection timepoint, TNF- α was significantly increased only in the SNAP+Morphine group ($p<0.05$), although there was a strong trend for a significant increase in the SNAP+Vehicle group. At the 4 week post-final morphine injection timepoint, the SNAP+Vehicle group and the SNAP+Morphine group were not significantly different from each other, but were both significantly different from both naïve groups ($p<0.05$). There was no significant difference between the naïve groups at either timepoint.

Figure 4.2: Effect of administering a week-long course of morphine beginning 24 hr post-surgery on glial activation marker mRNA gene expression

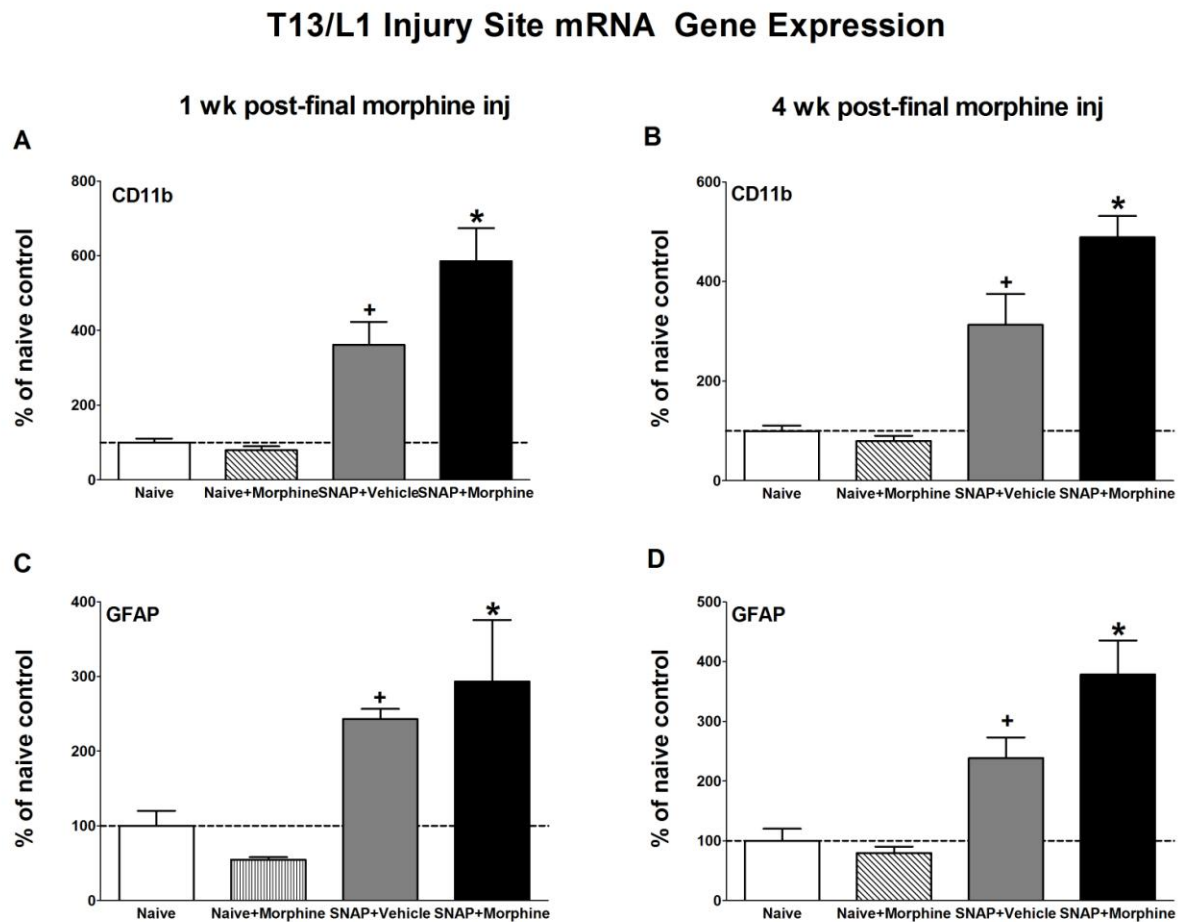


Figure 4.2. Assessment of the effects of administering morphine on CD11b and GFAP mRNA gene expression in the T13/L1 ipsilateral dorsal spinal cord. SNAP significantly increased CD11b mRNA at both the 1 week (A) and 4 week (B) timepoint post-last injection, and morphine further amplified CD11b at both timepoints. SNAP significantly increased GFAP

mRNA at both the 1 week (C) and 4 week (D) timepoint post-final morphine injection, and morphine further amplified GFAP only at the 1 week timepoint. Data are presented as mean $\pm$ SEM and analyzed using a one-way ANOVA, $n = 5$ to 7 per group. * $p < 0.05$ compared to all other groups, $^+p < 0.05$ compared to both naïve groups.

Figure 4.3: Effect of administering a week-long course of morphine beginning 24 hr post-surgery on TLR4 and TNF- α mRNA gene expression

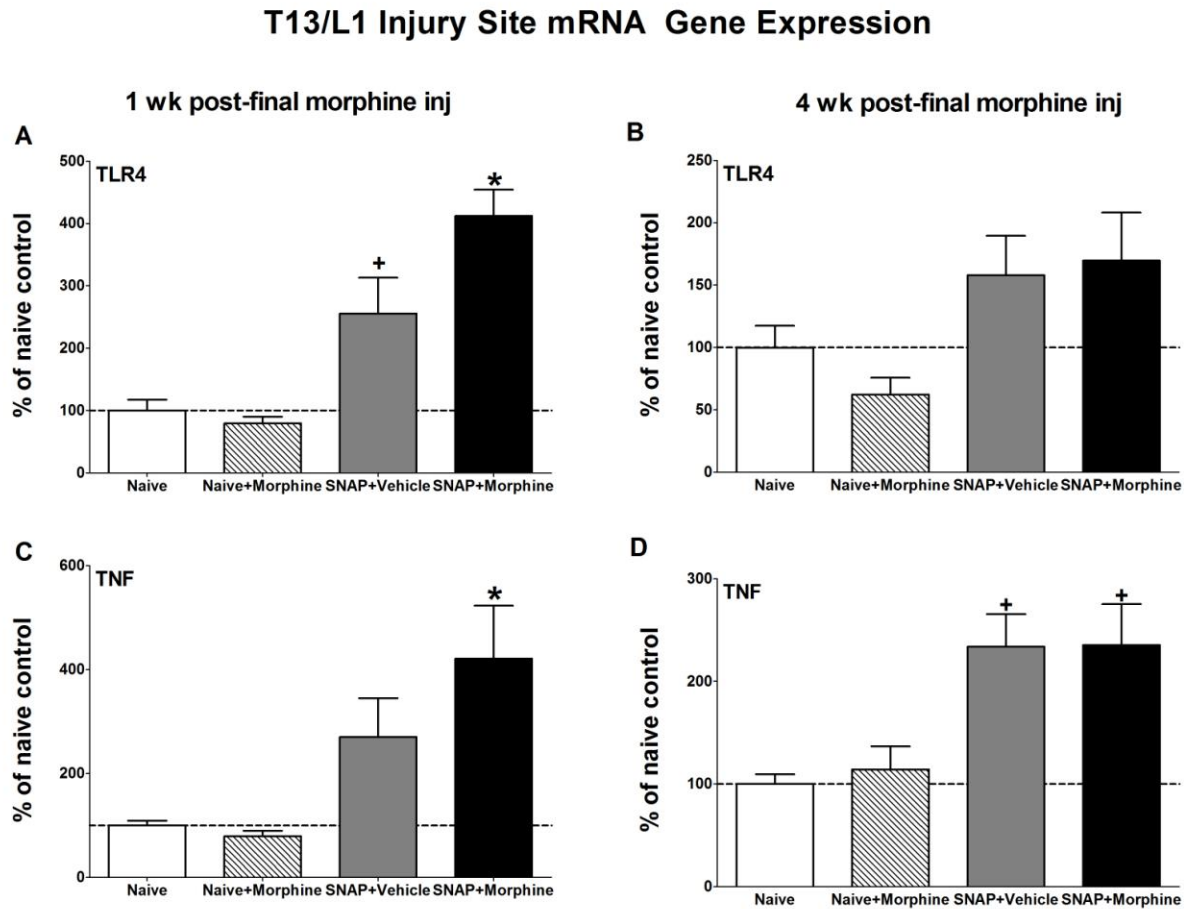


Figure 4.3. Assessment of the effects of administering morphine on TLR4 and TNF- α mRNA gene expression in the T13/L1 ipsilateral dorsal spinal cord. SNAP significantly increased TLR4 mRNA at both the 1 week (A) and 4 week (B) timepoint post-final morphine injection, but morphine only amplified TLR4 mRNA at the 1 week timepoint. Only morphine significantly increased TNF- α mRNA at the 1 week (C) timepoint post-final morphine injection, while both SNAP and morphine increased TNF- α mRNA at the 4 week (D) timepoint post-final morphine injection. Morphine did not further amplify TNF- α mRNA at the 4 week timepoint. Data are presented as mean $\pm$ SEM and analyzed using a one-way ANOVA, $n = 5$ to 7 per group. * $p < 0.05$ compared to all other groups, $^+p < 0.05$ compared to both naïve groups.

Effect of co-administering a week-long course of morphine and the TLR4 antagonist (+)-naltrexone beginning 24 hr post-surgery on SNAP

In order to determine if antagonizing TLR4 blocks morphine amplification of SNAP, the TLR4 antagonist (+)-naltrexone (6 mg/kg/inj s.c.) or vehicle was co-administered with morphine (single 10 mg/kg s.c. injection per day) or vehicle once a day and then administered alone two more times a day for one week beginning 24 hours post-surgery. All rats received SNAP surgery. No differences were observed between groups in the response thresholds recorded for the hindpaw ipsilateral (Fig 4.4A) or contralateral (Fig 4.4B) to the avulsion injury pre-surgery [baseline (BL)]. The one-way ANOVA comparing the AUC of Vehicle+Vehicle, Morphine+Vehicle, and Morphine+(+)-Naltrexone from 2-10 weeks post-surgery was significant in both the ipsilateral ($F_{2,15} = 9.892$; $p < 0.01$; Fig 4.4C) and contralateral ($F_{2,13} = 6.679$; $p < 0.05$; Fig 4.4D) hindpaw. Bonferroni *post hoc* analysis of the AUCs revealed that the Morphine+Vehicle group was significantly more allodynic than all other groups ($p < 0.05$) in both the ipsilateral and contralateral hindpaw. Furthermore, there were no significant differences between the other two groups in both the ipsilateral and contralateral hindpaw ($p < 0.05$).

Figure 4.4: Effect of co-administering a week-long course of morphine and the TLR4 antagonist (+)-naltrexone beginning 24 hr post-surgery on SNAP

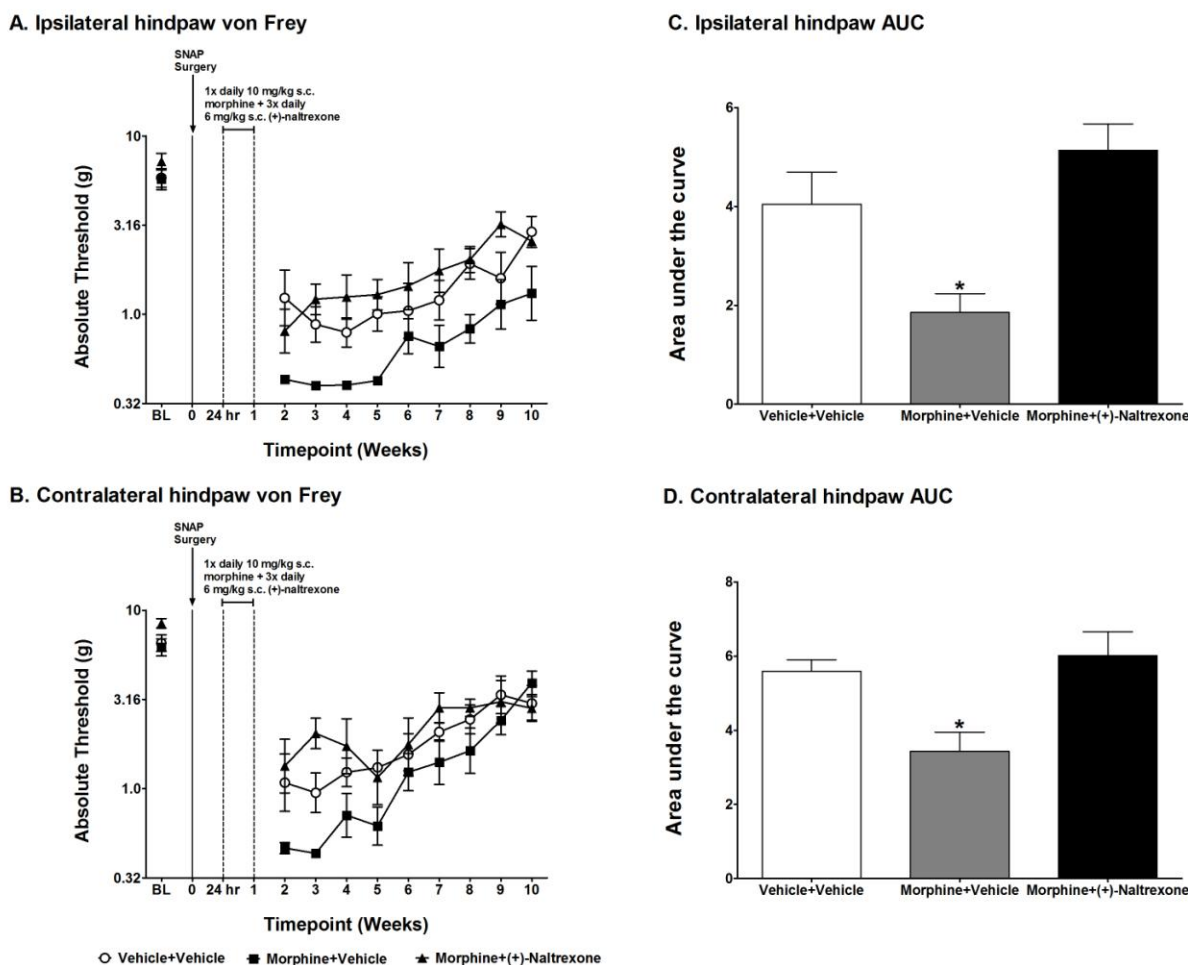


Figure 4.4. Assessment of the effects of morphine + (+)-naltrexone on SNAP. Rats were tested for mechanical allodynia across a timecourse on both the ipsilateral (**A**) and contralateral (**B**) hindpaw. SNAP rats that received (+)-naltrexone (3x 6 mg/kg, s.c.) + morphine (10 mg/kg, s.c.) for 7 days beginning 24 hours after surgery were significantly less allodynic than SNAP rats that received morphine + saline and were not significantly different than SNAP rats that received vehicle + vehicle in both the ipsilateral (**C**) and contralateral (**D**) hindpaw. Data are presented as mean $\pm$ SEM and analyzed using a one-way ANOVA on the AUCs, $n=6$ per group. * $p<0.05$ compared to all other groups.

Effect of administering a week-long course of morphine 24 hr post-surgery on IL-1 β and NLRP3 inflammasome marker protein and gene expression

Next we wanted to look at markers of the NLRP3 inflammasome and define if morphine amplified mRNA and protein at a single timepoint. Since the naïve+morphine group in the above studies were not statistically different from the naïve group for any marker analyzed, only a naïve group will be presented here. The one-way ANOVA for IL-1 β mRNA was significant ($F_{2,16} = 10.03$; $p < 0.01$; Fig 4.5A) and was also significant for IL-1 β protein ($F_{2,15} = 14.54$; $p < 0.001$; Fig. 4.5B). Bonferroni *post hoc* analysis revealed that IL-1 β mRNA and protein was significantly increased in the SNAP+Vehicle group ($p < 0.05$) compared to the naïve group, and was further increased by morphine. The one-way ANOVA for NLRP3 mRNA was significant ($F_{2,16} = 10.59$; $p < 0.01$; Fig 4.5C) and was also significant for NLRP3 protein ($F_{2,20} = 33.3$; $p < 0.0001$; Fig 4.5D). Bonferroni *post hoc* analysis revealed that NLRP3 mRNA was significantly increased in the SNAP+Vehicle group ($p < 0.05$) compared to the naïve group, and was further increased by morphine, and that NLRP3 mRNA was significantly increased in the SNAP+Vehicle group ($p < 0.05$) compared to the naïve group, but was not further amplified by morphine.

Figure 4.5: Effect of administering a week-long course of morphine 24 hr post-surgery on IL-1 β and NLRP3 inflammasome mRNA gene expression and protein

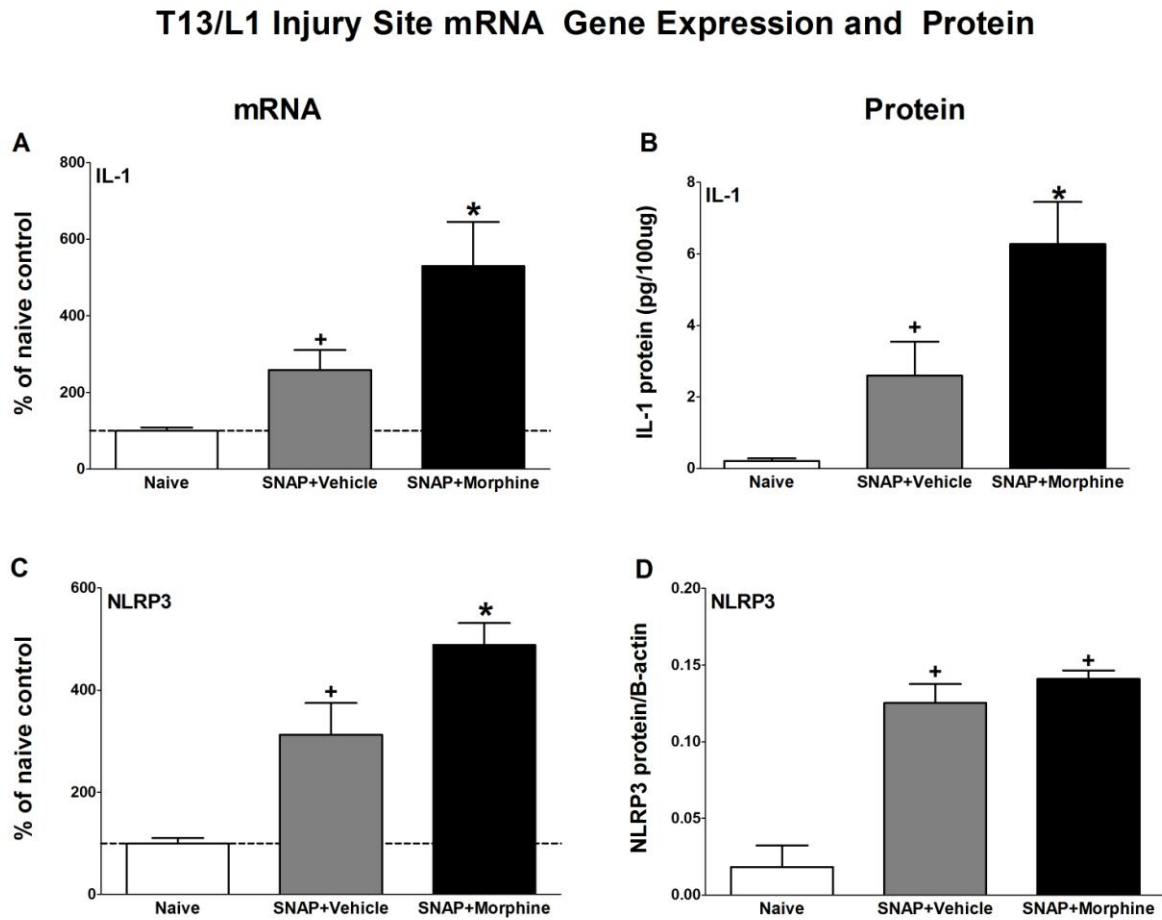


Figure 4.5. Assessment of the effects of administering morphine on IL-1 β and NLRP3 mRNA gene expression and protein in the T13/L1 dorsal spinal cord. SNAP significantly increased IL-1 β mRNA (A) and protein (B) which were both further amplified by morphine. SNAP significantly increased NLRP3 mRNA (C) and protein (D), but only the mRNA was further amplified by morphine. Data are presented as mean $\pm$ SEM and analyzed using a one-way ANOVA, n= 6-8 per group. *p<0.05 compared to all groups, ⁺p<0.05 compared to the naïve group.

Discussion

Although opioids have long been one of the most effective analgesics used for pain, it is important to recognize that there may also be detrimental effects of administering opioids for pain in certain situations. In the present set of studies, we provide evidence that a week-long course of morphine starting 24 hours after avulsion SCI amplifies central neuropathic pain for 4 weeks. This amplification of central neuropathic pain was blocked by administering the TLR4 antagonist (+)-naltrexone. Morphine also amplified SNAP-induced mRNA gene expression of markers of glial activation at both 1 and 4 weeks after the final morphine injection, and TLR4 mRNA 1 but not 4 weeks after the last morphine administration. Lastly, morphine amplified both SNAP-induced mRNA and protein expression of IL-1 β and amplified SNAP-induced mRNA but not protein expression of the NLRP3 inflammasome. It is important to note that all of these changes were observed in tissue collected 1 week after the final morphine dose, and thus cannot be attributed to active morphine metabolism. Furthermore, we know that morphine administration in naive rats does not induce pain and inflammation; there must already be a proinflammatory environment in order to observe morphine-potentiated effects.

We are not the first to describe deleterious effects of administering opioids shortly after trauma. Hook et al. reported that a single injection of intrathecal morphine 24 hours following contusion SCI attenuated weight gain and locomotor function and increased tissue loss around the SCI lesion site.¹³² Interestingly, they did not find any differences in mechanical reactivity at day 21 post-surgery, although this is not surprising because it was a single injection of intrathecal morphine rather than a more clinically relevant multi-day course of administration. We are thus the first group to show that chronic opioid administration shortly after surgery/traumatic injury amplifies central neuropathic pain for weeks after the initial injury. Hook et al. also suggest that

glia are involved in the mechanism by which morphine undermines health and recovery,¹³³ and our data in the present studies demonstrate that glia are playing a key role in morphine amplification of central neuropathic pain.

We have established that morphine binds to the non-classic opioid receptor TLR4, inducing glial activation and the release of proinflammatory cytokines.^{140, 143, 145, 345} Glia are activated in multiple models of SCI,^{38, 46, 350} and here we show that spinal mRNA of the microglial activation marker CD11b and astrocyte activation marker GFAP are increased in SNAP 2 weeks post-surgery and remain elevated 5 weeks post-surgery. Furthermore, morphine amplifies both of these markers at both timepoints, indicating that morphine is likely acting on glial cells and amplifying their activation via TLR4. In addition, it is known that TLR4 mRNA levels are increased by 72 hr in contusion injury,¹⁶⁰ and here TLR4 mRNA is still increased by SNAP 2 weeks post-surgery and is further increased by morphine. TNF- α is increased in the spinal cord after hemisection SCI,²³⁸ and TNF- α blockade decreases inflammation and improves motor recovery in an experimental mouse model of SCI.¹⁰⁰ Morphine increased TNF- α mRNA 2 weeks post-surgery, but surprisingly SNAP alone did not increase TNF- α (we believe that if we had the appropriate control group to allow us to run a 2-way ANOVA, SNAP+Vehicle would significantly increase TNF- α mRNA at this timepoint). However, TNF- α mRNA was significantly increased in the SNAP+Vehicle and SNAP+Morphine groups compared to the naïve groups at the later timepoint (although there is not a morphine-potentiated increase of TNF- α at this timepoint). TLR4 mRNA is no longer increased by SNAP or potentiated by morphine 5 weeks post-surgery, but both microglia and astrocyte activation markers (CD11b and GFAP, respectively) are. This suggests that both microglia and astrocytes are involved in both the initiation and the long-term maintenance of morphine amplification of SNAP.

Previous studies from our lab have shown that both peripheral and central neuropathic pain can be reversed by the TLR4 antagonist (+)-naltrexone.^{78, 144} Here, (+)-naltrexone was able to block morphine amplification of SNAP, which is more evidence that TLR4 is critically involved in the mechanism underlying morphine amplification of SNAP. Of note, the group of rats that received morphine plus (+)-naltrexone was trending toward being significantly less allodynic than the vehicle plus vehicle group even after 5 weeks post-surgery when morphine is no longer potentiating SNAP. We realize that opioids are often the least expensive and most effective treatment available for chronic pain, and are not advocating that opioids should be completely banned from clinical use. What is promising about the (+)-naltrexone data is that (+)-naltrexone could be co-administered with opioids in order to allow classical mu opioid binding for analgesia while blocking opioid binding at TLR4 and preventing glial activation and subsequent amplification of neuropathic pain and inflammation. Notably, based on prior studies of (+)-naltrexone and acute morphine, blocking TLR4 in this manner potentiates acute morphine analgesia, implying that (+)-naltrexone would provide improved morphine analgesia at the same time as preventing the long-term deleterious effects on chronic pain.

The proinflammatory cytokine IL-1 β is key to the inflammatory cascade and is therefore very tightly regulated.⁶⁸ IL-1 β mRNA and protein are up-regulated in SCI,^{237, 244, 337} and Hook et al. blocked morphine-induced attenuation of locomotor recovery by administering IL-1 receptor antagonist.¹³³ Both IL-1 β mRNA and protein are increased by SNAP 2 weeks post-surgery, and are both further amplified by morphine. These data demonstrate that IL-1 β is an important cytokine involved in morphine amplification of SNAP, and could provide a future therapeutic target. The way in which IL-1 β is processed is different than most other cytokines; it must be cleaved from a precursor protein (pro-IL-1 β) into a mature protein that can then be released.³²⁷

The molecule that cleaves the pro-form of IL-1 β into the mature form is the protease caspase-1, which must also be cleaved from a precursor protein, pro-caspase-1.¹⁷⁴ Pro-caspase-1 is cleaved through the formation and activation of a group of intracellular proteins collectively called the inflammasome.²¹³ There are 6 NLR inflammasomes (NLRP1, 2, 3, 6, 7, and NLRC4), and 3 of these (NLRP1, 2, 3) have been identified in the CNS.^{121, 200, 209} NLRP3 is the only one known to be expressed on microglia.¹²¹ The NLRP3 inflammasome is unique in that basal NLRP3 protein expression is too low for actual formation/activation of the inflammasome.¹⁴ In order for NLRP3 inflammasome formation to occur, the NLRP3 gene must first be transcribed and translated into protein. This step is thought to require TLR4 ligation, and is called the “priming” step. After enough NLRP3 protein has accumulated, a second signal is required for NLRP3 to become active, which results in the cleavage/activation of pro-caspase-1 which then cleaves pro-IL-1 β into its mature protein form. In the present studies, there is a significant increase in NLRP3 mRNA induced by SNAP and amplified by morphine but no significant increase in NLRP3 protein. This is indicative of NLRP3 priming, but since there does not appear to be significant morphine-induced amplification of NLRP3 inflammasome activation, this cannot fully explain the increase and further morphine-induced amplification of IL-1 β protein. One possibility is that since the IL-1 β mRNA and protein is measured from spinal cord tissue homogenates, we cannot be sure that the IL-1 β is mature IL-1 β and not pro-IL-1 β . There is also evidence that NLRP1 is involved in the inflammatory response in SCI⁶¹ and traumatic brain injury.⁶³ In addition, there are other inflammasome-independent mechanisms of IL-1 β processing that could be occurring and contributing to the inflammatory response, such as through activation of caspases-11 and/or 8.¹⁷⁷ Lastly, other proinflammatory mediators, such as TNF- α , could be playing a role in this

mechanism as well, since we know that TNF- α mRNA is up-regulated by SNAP and amplified by morphine.

The analgesic properties of opioids have been thoroughly studied for many years, but only recently have there been reports in the clinical literature of the deleterious effects of administering opioids. In one study, Salengros et al. found that a higher dose of the opioid remifentanyl resulted in worse post-operative pain in patients compared to a lower dose,²⁷⁰ which suggests that some of these detrimental effects could be dose-dependent. In addition, patients who received the higher dose of remifentanyl had a larger area of allodynia around the operation site 1 month after the surgery, which is mirrored in the current rat studies demonstrating morphine amplification of pain 5 weeks post-surgery. In other studies, van Gulik et al. found that remifentanyl administration during cardiac surgery dose-dependently predicted chronic thoracic pain in patients 1 yr later,³²⁸ and Hansen et al. found that patients given remifentanyl during abdominal surgery had worse post-operative pain compared to those who did not receive remifentanyl.¹²³ Furthermore, trauma patients who were on a chronic opioid regimen had significantly higher pain 4 months after the original injury compared to those who never received opioids.³²¹ Taken together, all of the animal and clinical literature suggests that it is important to understand the mechanisms underlying the deleterious effects of administering opioids in order to develop new treatments and/or to improve the efficacy of current treatments.

Chapter V

General Discussion

Summary of main experimental results

Central neuropathic pain is a pervasive problem, especially in spinal cord injury (SCI), and is unfortunately often intractable to treatment. It is now well known that in addition to neurons, glial cells are responsible for initiating and maintaining neuropathic pain states,³⁴⁴ and that by blocking glial activation, neuropathic pain can be prevented,³⁰⁹ attenuated,¹¹¹ and reversed.⁷⁸ However, the underlying mechanisms involved are still only partially understood. The goal of this dissertation was to characterize and elucidate some of these mechanisms in order to better understand central neuropathic pain and to help guide the development of better chronic pain treatments.

In chapter II, a series of studies was conducted in order to determine if our T13/L1 dorsal root avulsion model of SCI, SNAP, could be reversed by three different glial activation inhibitors, each with a distinct mechanism of action. In the first set of experiments, the phosphodiesterase (PDE) inhibitor propentofylline (PPF) was administered once daily intraperitoneally (i.p.) at 10 mg/kg for two weeks beginning four weeks post-surgery, and responses to a mechanical stimulus were recorded weekly in the first experiment. In the next experiment, responses were recorded every two to four days in order to provide a more detailed timecourse as to how quickly PPF reversed SNAP-induced allodynia. PPF reversed SNAP-induced allodynia in both the ipsilateral and contralateral hindpaws, and the complete reversal took about one week. These results suggested that glia were involved in SNAP and that PDE inhibition could be important in glial mechanisms for treating neuropathic pain. PDE activation has also been implicated in depression and anxiety,²⁴² stroke,¹²⁷ MS,⁶⁹ multiple models of

neuropathic pain,^{215, 355, 356} Huntington's disease,¹⁰⁴ schizophrenia,²⁹⁴ arterial hypertension,⁶⁰ renal disease,⁴⁵ and asthma/chronic obstructive pulmonary disease (COPD);⁸⁷ and inhibiting PDEs in these diseases is usually beneficial. We next wanted to explore whether or not a glial activation inhibitor that inhibited PDE but also had a well defined different mechanism of action would also attenuate SNAP. In addition, we wanted to know if we could reverse SNAP both early and late in the development of allodynia. Ibudilast is known to have PDE inhibitory action as well as macrophage migration inhibitory factor (MIF) inhibitory action.^{179, 264} Ibudilast was administered subcutaneously (s.c.) once daily at 10 mg/kg for five weeks beginning four weeks post-surgery (late in the development of SNAP) and for three weeks beginning two week post-surgery (early in the development of SNAP). In both studies ibudilast reversed SNAP in both the ipsilateral and contralateral hindpaws. These data indicated that PDE inhibition alone could not account for the anti-inflammatory effects of ibudilast and that in addition to PDE inhibition, MIF inhibition could also be important for glial mechanisms underlying chronic pain. MIF has been implicated in COPD,⁸⁶ asthma,³¹³ stroke,³³⁸ MS,¹¹⁶ TBI,³²³ and SCI,²²⁶ and ibudilast has been used to treat asthma,¹⁵⁵ COPD,¹⁰ stroke,¹⁸⁰ TBI,²⁶² SNL pain,¹⁷⁹ CCI pain,¹¹⁹ and SCI pain,⁷⁸ and has been studied in clinical trials for MS.²⁶⁴ Lastly, we wanted to determine if a glial activation inhibitor that had no known PDE or MIF activity could reverse SNAP. The selective TLR4 antagonist (+)-naltrexone was administered s.c. three times daily at 6 mg/kg/inj for two weeks beginning approx. four weeks post-surgery, and (+)-naltrexone reversed SNAP in both the ipsilateral and contralateral hindpaws. Furthermore, when (+)-naltrexone was given for five days and then stopped, the allodynia gradually returned over approximately one week. This suggests that (+)-naltrexone decreased proinflammatory signaling in glia and/or neurons and was able to maintain pain relief for a short time after drug clearance, but was not able to induce permanent

changes that could overcome the proinflammatory environment and persistent glial activation caused by the original SCI.

These studies indicated that the pain reversal by (+)-naltrexone was glially mediated via antagonism of TLR4 since TLR4 is found predominately on glial cells in the CNS.¹⁴¹ Since TLR4 activation can result in downstream proinflammatory cytokine and MIF release,^{106, 253} it is likely that glial activation via TLR4 is mechanistically important in SNAP and administration of PDE, MIF, and TLR4 inhibitors are acting either directly or indirectly on glial signaling via TLR4, resulting in decreased allodynia. Taken altogether, the studies in chapter II provide strong evidence for an important role of glia in the mechanisms underlying SNAP via PDE, MIF, and TLR4 actions. However, there are also other mechanisms and receptors to target for glially-mediated pain control.

Adenosine A_{2A} receptors have recently garnered much attention in the literature for anti-inflammatory effects exerted upon their activation in peripheral immune cells.^{125, 306} Such a profile is suggestive that they have the potential to be effective glial inhibitors as well. Chapter III details a set of studies aimed at exploring the effects of administering adenosine A_{2A} receptor agonists in SNAP. A single intrathecal (i.t.) injection of 10 μ M CGS21680 administered four weeks post-surgery reversed SNAP in both the ipsilateral and contralateral hindpaws for at least six weeks. If a single i.t. injection of neutralizing IL-10 antibody (0.2 μ g/ml in 10 μ L) was given one week post-CGS21680 (10 μ M), the anti-allodynic effect of CGS21680 was completely abolished for approximately forty-eight hours. This indicated that the anti-inflammatory cytokine IL-10 is important in the mechanism by which CGS21680 exerts its anti-allodynic effect. Furthermore, since the effect of neutralizing IL-10 only lasted approximately forty-eight hours, it seems as though CGS21680 induced a long-term alteration in cells releasing IL-10. Ipsilateral

T13/L1 dorsal spinal cord tissue collected one and six weeks post-CGS21680 was then analyzed for mRNA gene expression of IL-10, the microglial activation marker CD11b, and the proinflammatory cytokine TNF- α . CGS21680 decreased CD11b and TNF- α at both timepoints, but there were no differences in gene expression of IL-10 at either timepoint. These data suggested that CGS21680 attenuated inflammation in part by decreasing microglial activation and the subsequent release of TNF- α . Since there were no differences in IL-10 mRNA, this could indicate that the source of IL-10 is from infiltrating immune cells in the CSF as suggested in prior publications.^{191, 192} The last set of studies was undertaken in order to determine if adenosine A_{2A}R agonists would be able to prevent SNAP and reverse well-established SNAP. We also wanted to use a different agonist to provide converging lines of evidence that the effects were indeed a result of A_{2A}R activation, rather than being agonist-specific. A single i.t. injection of 1 μ M ATL313 administered one week post-surgery attenuated the development of SNAP in both the ipsilateral and contralateral hindpaws. ATL313 also reversed established SNAP when administered seven weeks post-surgery in both the ipsilateral and contralateral hindpaws. These last studies confirmed that the anti-allodynic effect was not agonist specific and that they could be administered at a variety of timepoints with similar success. Overall, chapter III demonstrated that A_{2A}R agonists are promising glial inhibitors for treating central neuropathic pain. In contrast, one of the most commonly used therapeutics for pain, opioids, which activate glia, may actually be detrimental to health and recovery.

Opioids are normally thought of as being excellent analgesics and helpful rather than harmful for controlling pain. However, the studies in chapter IV demonstrate a case where pain and inflammation can actually be *amplified* by administering a course of morphine shortly after injury. In the first set of experiments, a week-long course of morphine (10 mg/kg s.c.)

administered beginning twenty-four hours post-surgery amplified SNAP for four weeks. Importantly, this same course of morphine given to naïve rats did not produce amplification of pain, indicating that trauma/inflammation is necessary in order to observe this potentiated pain and would not occur in a normal healthy individual. Ipsilateral T13/L1 dorsal spinal tissue collected one and four weeks after the final morphine administration was analyzed for mRNA gene expression of CD11b, the astrocyte activation marker GFAP, toll-like receptor 4 (TLR4), and the proinflammatory cytokine TNF- α . CD11b and GFAP mRNA were increased by SNAP alone and were further amplified by morphine at both the one and four week timepoint, and TLR4 mRNA was increased by SNAP alone and further amplified by morphine only at the one week timepoint. TNF- α mRNA was amplified by morphine at the one week timepoint and was increased by SNAP and morphine compared to naïve controls at the four week timepoint, but was no longer amplified by morphine. These data suggested that morphine could be potentiating pain by amplifying glial activation and the subsequent release of proinflammatory mediators. Since TLR4 mRNA was amplified by morphine and also because we have previously shown that (+)-naltrexone reverses SNAP,⁷⁸ we hypothesized that TLR4 could be involved in the mechanism of morphine amplification of SNAP and that antagonizing TLR4 would block this amplification. In order to test this hypothesis, (+)-naltrexone (three times daily 6 mg/kg/inj s.c.) was administered with our typical course of morphine (once daily 10 mg/kg s.c. for seven days beginning twenty-four hours post-surgery). As expected, (+)-naltrexone blocked morphine amplification of SNAP in both the ipsilateral and contralateral hindpaws, suggesting that morphine is potentiating TLR4 activation on glial cells, thereby amplifying pain. The final study was designed to determine the possible mechanisms of morphine amplification of neuroinflammation. Spinal tissue collected one week after the last morphine dose was analyzed

for mRNA gene expression and protein expression of the proinflammatory cytokine IL-1 β and the NLRP3 inflammasome, which is partially responsible for processing IL-1 β . IL-1 β mRNA and protein were both increased by SNAP and further amplified by morphine, whereas NLRP3 mRNA was increased by SNAP and further amplified by morphine but NLRP3 protein was only increased by SNAP alone and was not further amplified by morphine. These results implied that increased processing of IL-1 β by the NLRP3 inflammasome may be partially responsible for the exaggerated IL- β release induced by morphine, but that there are likely other mechanisms involved and they need to be further explored. Taken together, this complete set of studies suggests that administering opioids after traumatic injury can be detrimental to long-term health and recovery, and that mechanisms underlying this phenomenon include amplification of glial activation, TLR4, and IL-1 β .

Central neuropathic pain is extremely difficult to treat and the underlying mechanisms are multi-faceted and complex. The failure of treatments that only target neuronal cells suggests that there are other cell types that are critically involved, and it is important to develop therapies that target more than just neurons. The current series of studies provide evidence that blocking glial activation and increasing anti-inflammatory mediators attenuate central neuropathic pain and neuroinflammation, while amplifying glial activation and proinflammatory mediators can potentiate central neuropathic pain and neuroinflammation. It is important to recognize this dichotomy, and by manipulating glia and other immune cells it may be possible to develop better, more efficacious therapies and fundamentally change the way neuropathic pain is treated.

The clinical utility of administering glial activation inhibitors for central neuropathic pain

A significant issue in the chronic pain field is the failure of the majority of available treatments in the clinical population. Although there are likely many different complex reasons for this, one reason could be that most of the current neuropathic pain treatments that are clinically available target neurons. The studies presented in this dissertation provide strong evidence that targeting glia and other immune cells could be a more effective way of treating chronic pain, and there is actual clinical evidence to support this pre-clinical data, as discussed below.

In order to be considered a good candidate for clinical applications, a drug for systemic administration should be small, blood brain-barrier permeable, and easy to administer. All three of the drugs discussed in chapter II (propentofylline (PPF), ibudilast, and (+)-naltrexone) fit these criteria and all three have been tested clinically. PPF has been previously tested in clinical trials for Alzheimer's and vascular dementia,^{164, 239} schizophrenia,²⁷² and multiple sclerosis.³⁰⁷ Although the results of these trials were not definitive, they did suggest improvements with PPF compared to controls. More recently, PPF was tested in a clinical trial to treat post herpetic neuralgia (PHN). Nearly half of PHN patients fail to respond or have intolerable side effects to neuronally-based medications including opioids, tricyclic anti-depressants, gabapentin, and pregabalin.^{74, 354} Unfortunately, the clinical trial using PPF for controlling PHN failed. However, there were major problems with how the trial was run. First, many of the side effects that patients experienced would not have required PPF to reach the CNS, therefore there are questions about whether the dosing was correct. Next, it is known that food intake prevents absorption of PPF, and the patients in this trial were not on food restrictions.³⁴² Finally, patients were only treated

with PPF for four weeks,¹⁷⁵ whereas most other clinical studies of chronic pain medications treat for at least six weeks.^{108, 266, 331} Chapter II discusses multiple examples of drugs failing with only a few administrations, but are then successful if given for a longer duration. If PPF was given for longer in the PHN clinical trial, it might have reached significance. Similar to PPF, ibudilast is also used clinically in a variety of applications including allergic rhinitis¹⁹⁴ and conjunctivitis,²⁶⁹ chronic obstructive pulmonary disease,¹⁰ and asthma.¹⁵⁵ It is also currently being tested in clinical trials for migraine and in MS, where it was discovered to have neuroprotective effects and potentially anti-inflammatory effects.¹³ Furthermore, ibudilast has been tested (both single and multiple dosing) in healthy volunteers and did not cause any serious adverse effects, had few side effects, and was well tolerated.²⁶³ Unlike PPF and ibudilast, the (+)-isomer of naltrexone has not been clinically tested. However, the (-)-isomer of naltrexone was approved by the FDA to treat opioid addiction in 1984 and was run through clinical trials and approved to treat alcohol use disorders in 1994.^{229, 334, 335} In addition, the Department of Defense has funded Xalud Therapeutics to move (+)-naltrexone to Investigational New Drug status, positioning it for future clinical trials in drug abuse and neuropathic pain. Moving to clinical trials for SCI neuropathic pain will be facilitated by Xalud Therapeutics' development of (+)-naltrexone toward FDA Investigational New Drug status, with all of the requisite toxicology, scale-up manufacturing, stability testing, etc. involved. Taken together, there is promising clinical data to back up the pre-clinical data discussed in chapter II suggesting that these glial activation inhibitors could be the future for treating central neuropathic pain.

Adenosine itself is antinociceptive in humans,²⁷⁴ but is problematic in that it can dramatically reduce heart rate and cause undesirable side effects.³⁶⁹ However, adenosine agonists are now being recognized as good candidates for treating neuropathic pain in addition to other

disorders including cancer, arthritis, and diabetes.^{64, 258, 333} Although there are also clinical antagonists for each of the adenosine receptor types, they will not be discussed here as there is little evidence of their efficacy in CNS disorders. A₁ agonists are the most difficult to develop because of off-target effects, receptor desensitization, and decreased heart rate.^{15, 158} Multiple pain clinical trials using A₁R agonists have been discontinued, likely due to these issues.²¹⁸ One study using the A₁R agonist GR79236 in migraine patients was successful with only minor adverse effects;¹⁰² however, the use of partial agonists and allosteric enhancers seems to be more promising, especially for pain. Allosteric enhancers have attracted attention in recent years given that they bind receptors at distinctly different sites from the primary ligand site (active site), and can induce a conformational change in the receptor that changes the potency or affinity of the primary ligand for the receptor.²⁸² Partial agonists are being tested clinically for treating type II diabetes,^{65, 158} and there is an A₁R allosteric enhancer, T-62, that was in clinical trials for PHN but was terminated due to transient increases in liver enzymes in some patients.^{12, 369} Perhaps the best candidate adenosine receptor for developing pain therapeutics is the A_{2A}R. There was one clinical trial completed by Biovitrum using the orally administered A_{2A}R agonist BVT.115959 to treat diabetic neuropathic pain.³⁶⁹ Although parkinsonian-like symptoms and vasodilatory effects of adenosine receptor activation have been linked to the A₂R when administered peripherally,¹⁴⁶ the drug was well-tolerated (three doses/day for four weeks), and there was a statistically significant slight improvement in pain symptoms, although the mechanism of action was assumed to be more peripherally rather than centrally mediated. Part of our hypothesis regarding the anti-inflammatory effects of A_{2A}R agonists is that they increase levels of IL-10. A current clinical study found that in cultured lymphocytes from rheumatoid arthritis patients, CGS21680 dose-dependently increased IL-10,³³³ which lends support to our hypothesis. Importantly,

CGS21680 did not affect cell viability and did not have cytotoxic effects on these human lymphocytes,³³³ suggesting that it could be well-tolerated in pain patients. The other $A_{2A}R$ agonist studied in chapter III, ATL313, is also being developed for clinical use by Clinical Data for acute inflammatory pain and ophthalmic disease.²¹⁸ In addition to the studies discussed in chapter III, we have tested ATL313 in a variety of pain models, and a single intrathecal injection reverses pain in chronic constriction injury (CCI), spinal nerve ligation (SNL), and sciatic inflammatory neuropathy (SIN).¹⁹² Of note, ATL313 does not cross the blood brain barrier, but it does reverse zymosan-induced allodynia when administered directly over the sciatic nerve in the SIN model, suggesting that peripheral administration could have some therapeutic effects (Ellis, unpub. obs.). Although the mechanisms underlying $A_{2A}R$ agonist-induced pain reversal are still poorly understood, we have evidence that PKA/PKC are involved,¹⁹² which could provide more therapeutic targets as there are multiple protein kinase inhibitors in clinical development for treating pain.^{4, 52, 105, 271} Although there are multiple examples of successful preclinical studies using $A_{2B}R$ agonists for pain,¹⁹² atherosclerosis,²¹⁸ and diabetes,¹⁵¹ there are currently none in clinical trials. Interestingly, $A_{2B}R$ activation increases cAMP similar to $A_{2A}R$ activation, suggesting that development of clinical agonists would be worthwhile. Like $A_{2B}R$ agonists, there are currently no A_3R agonists in clinical trials. However, there is preclinical evidence that A_3R agonists decrease neuropathic pain⁴⁴ and reduce ischemic brain injury,⁴² indicating that these agonists could be successful in future clinical trials. Although there are clear difficulties in developing adenosine agonists, the preclinical evidence here and in chapter III as well as the clinical data suggests that adenosine agonists, especially $A_{2A}R$ agonists, should be pursued at least for treating chronic pain if not the other central disorders discussed.

As discussed in chapter IV, there are very few clinical studies on the deleterious effects of administering opioids after trauma, and the majority of the studies actually examine post-operative pain rather than trauma pain (ie. SCI pain).^{123, 328} It is critical that more clinical studies designed specifically to examine the deleterious effects of administering opioids after trauma/surgery are undertaken because opioids are almost universally used as a first line treatment for trauma patients and for most surgical procedures in general. It is also imperative that we develop alternative compounds. Since opioids truly are the best analgesic available, it would be advantageous to be able to administer a drug along with the opioid to still allow for the analgesic effects while reducing the negative effects. (+)-Naltrexone would be an excellent candidate, and we are currently funded by the Department of Defense to explore these possibilities with (+)-naltrexone on enhancing morphine analgesia, decreasing amplification of pain, enhancing motor recovery, and decreasing injury lesion size and constipation. If (+)-naltrexone is successful in ameliorating some of these deleterious effects, it could change the way military personnel and civilians alike are treated after traumatic injury. Of note from chapter IV, morphine did not amplify pain or inflammation in naïve rats, suggesting that trauma/inflammation must already be present at the time of opioid administration in order to see the deleterious effects. This is important clinically because we would not expect to see these negative effects in healthy individuals who take opioids recreationally or acutely for a single procedure. The clinical implications of the deleterious effects of opioid administration are too large to ignore and more research needs to be done in order to elucidate the mechanisms and develop alternatives.

Chronic pain affects millions of people and is very complex and has been historically extremely difficult to treat. We now know that neuropathic pain is not just a product of neuronal

hyperexcitability, but is also the result of glial activation and subsequent release of proinflammatory substances. The evidence presented in this dissertation will hopefully help to guide the development of glially-targeted therapies in order to offer true relief to those suffering from chronic pain disorders.

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