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Glia in Pathological Pain: A Role for Fractalkine

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Abstract

Microglia and/or astrocytes play a significant role in the creation and maintenance of exaggerated pain states with inflammatory and/or neuropathic etiologies. The chemokine, fractalkine, has several functions, including the newly recognized role of mediating neuropathic pain conditions. Although constitutively expressed and released during inflammation, increased release of fractalkine binds to and activates microglia leading to pathological pain. We review the critical role of fractalkine in neuron-to-glia communication after peripheral nerve injury and inflammation and explore anti-inflammatory cytokines like interleukin-10 as a novel and effective approach for clinical pain control.

Keywords

Neuropathic pain; spinal cord; microglia; astrocytes; interleukin-10; gene therapy

Previously Understood Views of Pain

Traditionally, our understanding about neuronal signaling for pain transmission from the body to the central nervous system (CNS) occurs as a series of relay signals that are eventually processed in the brain. These relay signals are thought to serve protective and adaptive roles, with the first synaptic relay, between the first order (sensory) neuron and the second order neuron in the dorsal horn of the spinal cord. Neurons that receive and respond to pain information are primarily located in anatomically discrete regions of the spinal cord, that is, laminae I, II and V of the spinal cord dorsal horns. From the spinal cord dorsal horn, pain projection neurons relay their information to higher pain centers, such as to the brainstem and various relevant pain areas in the brain. Interestingly, the dorsal horn of the spinal cord can strongly modulate pain signaling (Millan, 1999). Although the neuronal functioning of these pain pathways has been examined for decades, the induction and maintenance of non-adaptive, pathological pain is poorly understood. However, since the early 1990's, there has been mounting evidence that glial cells (both astrocytes and microglia), in addition to neurons, also play a critical role in pain modulation (DeLeo et al., 2007).

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Glial Modulation of Neuronal Activity

Glia act to dynamically regulate neuronal synaptic communication (Haydon, 2001), and can lead to pathological pain via the release of a variety of neurotransmitters, neuromodulators (McMahon et al., 2005), proinflammatory cytokines and chemokines (Rostene et al., 2007). Astrocytes are closely associated with neuronal somas, dendrites, and pre- and post-synaptic sites, as well as with other glia (microglia), and their functions are now understood to include active, integrative roles during synaptic transmission (Fellin et al., 2006, Haydon, 2001). The intimate contact astrocytes have with neurons can lead to enhanced synaptic transmission. A variety of functional neurotransmitter receptors, which include ionotropic non-NMDA and NMDA receptors (Porter and McCarthy, 1997, Schipke et al., 2001, Seifert and Steinhauser, 2001), as well as metabotropic glutamate (mGluR3 & mGluR5), purinergic, and substance P receptors (Porter and McCarthy, 1997) are expressed on astrocytes and neurons. Glial activation can be initiated by neurotransmitters released from central terminals of activated peripheral nerves that leads to the production and release of factors that activate neurons in addition to activating neighboring glia (astrocytes and microglia). Microglia, the resident immune cells of the CNS, are not limited to responses during immunological challenges, but are now recognized to dynamically modulate neuronal and astrocyte function. Microglia express many of the same receptors and receptor subtypes that neurons and astrocytes express (Pocock and Kettenmann, 2007), and upon activation, produce many substances that activate surrounding neurons and glia (Scholz and Woolf, 2007).

In terms of an immunological role that can lead to pathological pain conditions, both astrocytes and microglia express receptors for pathogens, such as viruses (i.e. glycoprotein 120 [gp120], coat protein expressed on the human immunodeficiency virus) and bacteria (Hanisch, 2002, Keane and Hickey, 1997). Glia bind pathogens, become activated and release substances known to excite pain transmission neurons including nitric oxide (NO), leukotrienes, arachidonic acid, prostaglandins, and excitatory amino acids (EAAs) such as glutamate. These substances can further enhance substance P and EAA release from primary afferent pain transmission terminals (Aimar et al., 1998, Guo and Schluesener, 2007, Inoue et al., 1999, Malcangio et al., 1996, Southall et al., 1998). Additionally, glia produce and release proinflammatory cytokines, including interleukin-1 (IL1), tumor necrosis factor-alpha (TNF) and interleukin-6 (IL6) (Benveniste, 1997), that further stimulate glial cells and neurons (Halassa et al., 2007, Nguyen et al., 2002). In the CNS, astrocytes and microglia express receptors for IL1, TNF and IL6 (Halassa et al., 2007, Hanisch, 2002), and respond to these cytokines by releasing NO (Burgher et al., 1997, Murphy and Grzybycki, 1996), EAAs and prostaglandins (Halassa et al., 2007, Pocock and Kettenmann, 2007), increasing substance P release from primary afferent neurons (Inoue et al., 1999), and by up-regulating enzymes such as cyclooxygenase-2 characterized to produce prostaglandins that facilitate pain transmission (Samad et al., 2001). Thus, understanding pain mechanisms from a perspective that includes spinal cord glial stimulation has contributed to our knowledge about potential mechanisms that underlie the shift from adaptive pain to pathological pain (DeLeo et al., 2007).

Evolving Views of Pain Modulation: Glia Play a Significant Role in Pathological Pain States

Diverse animal models of nerve injury or inflammation, either peripherally or centrally, produce pathological pain states such as thermal hyperalgesia (exaggerated responses to painful heat) and mechanical allodynia (non-painful touch/pressure stimuli that become painful) (Zimmermann, 2001). These models have demonstrated the involvement of spinal cord glia which become activated with the development of pain enhancement. For example,

pain produced by chronic constriction injury (CCI), a well characterized rodent model of partial sciatic nerve injury (Bennett and Xie, 1988), was correlated with spinal cord glial activation, as measured by enhanced immunoreactivity of activated astrocytes (glial fibrillary acidic protein) in the spinal segments that receive input from the damaged sciatic nerve terminals (Garrison et al., 1991). Various models of inflammation such as subcutaneous irritants, intra-peritoneal inflammation, sciatic epineural immune activation and spinal immune activation (Watkins et al., 2001) lead to glial activation that parallels enhanced pain responses. Glial activation has also been demonstrated in diverse nerve-injury models such as adjuvant-induced arthritis, bone cancer, sciatic inflammation, spinal cord trauma, spinal root trauma, and sciatic nerve injury (DeLeo et al., 2007). Together, these studies support that examination by immunohistochemical techniques of glial activation during many different conditions leading to pathological pain is indicative of the *potential* glial involvement in pain.

However, many studies on spinal cord glial activation have gone beyond simply being correlated with exaggerated pain. Glial activation can be causal to pathological pain. As briefly reviewed above, a variety of functional receptors characterized on neurons are also expressed on glia, which bind pain transmission substances initially released from neurons (Pocock and Kettenmann, 2007). Most information about glial activation with subsequent release of pain-enhancing substances (NO, EAAs, substance P, prostaglandin E₂) has been gathered from *in vitro* studies of brain glia (Pocock and Kettenmann, 2007) and not of spinal glia. However, *in vivo* studies have shown that blocking the activation of EAA receptors also blocks pathological pain in neuropathic rodents. Indeed, the NMDA receptor antagonist, MK-801, attenuates CCI-induced astrocyte activation in dorsal spinal cord in addition to attenuating neuropathic pain (Garrison et al., 1994). Thus, the possibility that activation of astrocyte NMDA receptors leads to glial activation and contributes to consequent pain enhancement is suggested in this pioneering study. Alternatively, activation of NMDA receptors on neurons could induce the release of glial-activating substances, such as nitric oxide. This highlights a problem for interpreting *in vivo* glial receptor activation and associated behavioral responses (allodynia and hyperalgesia) because administration of agonists or antagonists to pain-relevant receptor substances simultaneously affects neurons as well as glia. Alternatively, a glial role in pathological pain processing can be examined by suppressing glial activation.

The role of *glia* in pathological pain can be explored by intrathecal administration of pharmacological agents (e.g., fluorocitrate, propentofylline, minocycline) that selectively disrupt spinal glial activation (De Leo et al., 2006, Watkins and Maier, 2003). For example, fluorocitrate at low peri-spinal (intrathecal; i.t.) doses, preferentially inhibits aconitase in the tricarboxylic acid cycle in glial cells (Fonnum et al., 1997, Hassel et al., 1992), thus inhibiting glial metabolism. The development of thermal and mechanical hyperalgesia in several animal models of pathological pain is attenuated or blocked by fluorocitrate (Watkins and Maier, 2003). Support that *microglia* mediate neuropathic pain in several animal models (nerve trauma, sciatic nerve inflammation, and spinal cord inflammation) is provided by the observation that spinal cord administration of minocycline, a drug that inhibits microglial activation, attenuates thermal hyperalgesia and/or allodynia, decreases microglial activation, and suppresses proinflammatory cytokine synthesis in spinal cord (Raghavendra et al., (in press)) (Ledeboer et al., 2005, Obata et al., 2007, Raghavendra et al., 2003b). Combined, such studies show strong support of an underlying glial mechanism in pain processing.

Once glia are activated, they release IL1, TNF and IL6 that are directly implicated in the development and maintenance of pathological pain (DeLeo et al., 2007). Indeed, spinal cord glia produce and release IL1, TNF and IL6 quite rapidly from dorsal spinal cord, as

evidenced by its detection in the surrounding cerebrospinal fluid (CSF) after immune stimulation with a peri-spinal injection of gp120 (Milligan et al., 2001a). Nitric oxide was found to be key in mediating elevations of IL1, TNF and IL6 mRNA and protein in lumbar spinal cord leading to allodynia in gp120-treated animals (Holguin et al., 2004). In addition, IL-1 was shown to be important for the maintenance of neuropathic pain states because established neuropathic pain can be reversed either by proinflammatory cytokine antagonists (Milligan et al., 2003) or by the anti-inflammatory cytokine interleukin-10 (IL10) (Abraham et al., 2004, Johnston et al., 2004, Ledeboer et al., 2007, Milligan et al., 2005a, Milligan et al., 2005b, Milligan et al., 2006a, Milligan et al., 2006b, Plunkett et al., 2001, Sloane et al., submitted, Yu et al., 2003) that inhibits the production and activity of proinflammatory cytokines (Moore et al., 2001). Such results are important when one is considering the potential of targeting proinflammatory cytokines for clinical pain control.

Neuron-to-glia Signals: Neurotransmitters and Neuromodulators

While a number of factors released spinal cord neurons and glia are known to sensitize neuron-glia interactions leading to pathological pain, the spinal glial response to trauma at distant locations is not fully understood. What are the neuron-to-glia signals that induce subsequent glial activation after peripheral inflammation or nerve trauma? As reviewed above, glia express functional receptors for many neurotransmitters and are able to respond to these signals (Pocock and Kettenmann, 2007). That is, glia are activated by substance P, EAA, purinergic ATP, and in response, release these same neurotransmitters as well as the neuromodulators cyclooxygenase-2 and prostaglandin E₂ (Pocock and Kettenmann, 2007). All of these factors are well-characterized to mediate pain facilitation (Woolf and Salter, 2000). From this body of literature, a number of candidate signals exist that are released from primary afferent terminals or intrinsic spinal neurons capable of communicating to surrounding microglia and astrocytes in pain-relevant areas of the spinal cord.

Neuron-to-glia signals: fractalkine

Neurotransmitters and neuromodulators are not the only substances neurons release that can trigger the activation of glia. Fractalkine is another factor released by neurons of the pain pathway, which has been documented to mediate pathological pain (Clark et al., 2007, Lindia et al., 2005, Milligan et al., 2004, Milligan et al., 2005c, Zhuang et al., 2007). Fractalkine, first discovered as neurotactin (Pan et al., 1997) is a chemokine, a term which refers to a family of over 50 proteins classically known as *chemotactic cytokines* (Asensio and Campbell, 1999) that induces leukocyte migration and facilitates inflammation (Thomson and Lotze, 2003). While chemokines typically bind multiple receptors and conversely, chemokine receptors bind multiple chemokines, this does not hold true for fractalkine (Rostene et al., 2007). A unique action of fractalkine as a chemokine is that it binds only one receptor and this receptor only binds fractalkine (Hesselgesser and Horuk, 1999). Structurally, fractalkine (CX3CL1) is a multi-domain protein composed of an extracellular chemokine motif with a long mucin stalk attached to a transmembrane spanning domain and a cytoplasmic C-terminus (Bazan et al., 1997). Under normal conditions, fractalkine is constitutively expressed in high abundance on the extracellular surface of neurons (Harrison et al., 1998), and the fractalkine receptor, CX3CR1, has been mostly characterized on microglial cells (Harrison et al., 1998, Lindia et al., 2005, Verge et al., 2004). One report demonstrated CX3CR1 expression on hippocampal neurons (Meucci et al., 2000). Strong neuronal excitation induces the cleavage of the extracellular chemokine domain to form a soluble, diffusible signal (Chapman et al., 2000). During pathological pain conditions, a state induced by strong neuronal excitation in the spinal cord, fractalkine receptor expression is increased in microglia in pain relevant areas (Verge et al., 2004). This cellular distribution is particularly intriguing as microglial cells are suspected to be the glial

cell type to respond first to incoming signals after peripheral inflammation or injury (Raghavendra et al., 2003a), in further support of fractalkine being a neuron-to-glia signal.

While microglial-derived fractalkine is implicated in the initiation of pathological pain, endogenous fractalkine mediates ongoing neuropathic pain. During chronic pathological pain states, an intrathecal injection of a fractalkine antagonist (a neutralizing antibody to the fractalkine receptor) attenuates both thermal hyperalgesia and mechanical allodynia after either chronic constriction injury or sciatic inflammatory neuropathy (Milligan et al., 2004, Milligan et al., 2005c). After induction of two different chronic pain models in the rat, that is unilateral intraplantar complete Freund's adjuvant, which is a model of inflammatory pain, or lumbar 5 spinal nerve ligation, which is a model of nerve trauma, CX3CR1 expression was strongly increased in microglia located in pain relevant lamina of the spinal cord (Lindia et al., 2005). This study was also the first to identify increased fractalkine expression in astrocytes during ongoing pathological pain such as mechanical hyperalgesia and low threshold mechanical allodynia.

In further support of a role for fractalkine underlying pathological pain states, simply the application of exogenous fractalkine is sufficient to produce exaggerated pain responses by activating microglia. An intrathecal injection of exogenous fractalkine produces both thermal hyperalgesia and mechanical allodynia (Milligan et al., 2004, Milligan et al., 2005c), and does so via the actions of activated glia. For example, the known glial product, IL-1, is strongly implicated in fractalkine-induced mechanical allodynia because intrathecal pretreatment with an IL-1 receptor antagonist blocks these pain changes (Milligan et al., 2005c). In support of this latter observation, we have demonstrated that IL-1 is released *in vitro* from dorsal spinal cord explants stimulated with fractalkine (Johnston et al., 2004), and fractalkine over-expression in the spinal cord leads to a pronounced thermal hyperalgesia and IL-1 protein release in CSF (Langer et al., 2006). We and others have demonstrated that fractalkine's pain enhancing effects are mediated by microglial-derived p38 mitogen-activated protein kinase (p38-MAPK), a factor characterized to mediate neuropathic pain (Zhuang et al., 2007), and are also blocked by disrupting microglial activation with minocycline (Milligan et al., 2005c, Owolabi and Saab, 2006), by blocking NO production, and by blocking the actions of IL-6 (Milligan et al., 2004). Lastly, activated dorsal horn microglia expressing the lysosomal cysteine protease cathepsin S (CatS), an enzyme that may act to induce fractalkine cleavage, is increased during neuropathic pain states (Clark et al., 2007). Exogenous intrathecal injection of CatS produced hyperalgesia, allodynia, and increased expression of activated microglial-derived p38-MAPK (Clark et al., 2007). This group further demonstrated that intrathecal application of fractalkine produced hyperalgesia and allodynia, supporting prior reports, and that intrathecal pretreatment with anti-fractalkine neutralizing antibody blocked both ongoing neuropathic pain and activated p38MAPK (Clark et al., 2007).

As discussed, the extracellular domain of fractalkine can be proteolytically cleaved from the cell surface, a process known as shedding (Hundhausen et al., 2007), and occurs via cleavage of its mucin stalk. *In vitro* exposure of neuronal mixed cultures and/or brain slices to glutamate, TNF, or interferon-gamma is sufficient to induce fractalkine shedding (Chapman et al., 2000, Erichsen et al., 2003). Matrix metalloproteinases are implicated in the process of shedding. Transmembrane fractalkine is constitutively shed from the cell surface by the activity of a disintegrin and metalloproteinase 10 (ADAM 10), and enhanced cleavage rapidly occurs by activation of the closely related enzyme ADAM17 (also known as tumor necrosis converting enzyme; TACE) (Garton et al., 2001, Ludwig et al., 2005). While *constitutive* shedding occurs primarily via ADAM10, inflammatory &/or excitotoxic conditions *induce* increased shedding, which is mediated primarily by ADAM17 (Ludwig et al., 2005). The inducible shedding is not reflected by an increase in fractalkine mRNA

(Hundhausen et al., 2003). Thus, evidence to date suggest that inducible cleavage of fractalkine is regulated post-translationally by matrix metalloproteases and CatS from glia (Cross and Woodroffe, 1999). In brain, strong neuronal excitation releases fractalkine, which then binds to and activates nearby glia (Chapman et al., 2000). As noted previously, in pain sensory relevant areas of the spinal cord (dorsal horn), fractalkine is expressed by neurons while its receptors are expressed mostly on microglia. During chronic neuropathic pain known to involve inflammatory mediators like TNF, excited neurons expressing fractalkine may provide a reservoir of soluble active fractalkine via inducible shedding by TACE or CatS, which can readily facilitate further fractalkine release to act on nearby glial cells.

Of importance is that fractalkine, via signaling through its receptor, CX3CR1, may be involved in modulating pathogenesis of several inflammatory neurodegenerative diseases such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) in rodent models (experimental autoimmune encephalomyelitis [EAE] for models of MS; transgenic mutant superoxide dismutase-1 [SOD1-G93A] for models of ALS) (Ransohoff et al., 2007). Fractalkine mediated neuroprotection was attributed to recruitment of the natural killer cell (subtype, NK1.1) a specific kind of innate immune cell that expresses CX3CR1. Other innate immune cells like dendritic cells or monocytes that also express CX3CR1 were not important for cellular recruitment or neuroprotection (Huang et al., 2006). CX3CR1 signaling during EAE or during motor neuron degeneration by SOD1-G93A was important for attenuating gliosis and neurotoxicity (Ransohoff et al., 2007). Interestingly, while CX3CR1 may underlie neuropathic sensory changes like allodynia, CX3CR1 also appears to be important for neuroprotection. Thus, CX3CR1 signaling preserves neurons, for example both motor and sensory neurons in the spinal cord, during highly excited (inflamed) conditions to not only increase neuronal survival, but also leads to enhanced pain sensory signaling.

Toll-like Receptor Signaling in Chemokine-Mediated Pathological Pain

Although fractalkine is responsible for the recruitment of natural killer cells during neurodegenerative conditions in animal models for MS or ALS, as well as mediates pain in animal models for neuropathic pain, other innate immune cells (leukocytes) and lymphocytes (T cells) are characterized to migrate into pain relevant areas of the spinal cord during neuropathic pain conditions (Cao and DeLeo, 2008, Sweitzer et al., 2002). The chemoattractant(s) responsible for spinal cord lymphocyte and leukocyte trafficking specifically during neuropathic pain conditions remains unknown. However, many well-characterized chemokine receptors and their ligands in the normal and diseased CNS are good candidates and have been reviewed previously (Rostene et al., 2007). Combined, this literature supports the notion that during spinal glial activation in neuropathic pain conditions (as discussed above), immune-cell trafficking may occur via chemokine signaling in the CNS. This cell trafficking takes place in an organized fashion. The activation of astrocytes, microglia and infiltrating immune cells is coordinated by pattern-recognition receptors (PRR) that these immune cells express (Guo and Schluesener, 2007). Toll-like receptors (TLRs) are a family of phylogenetically conserved PRRs, which recognize pathogen-associated molecular patterns that allow immune cells to distinguish 'self' molecules from non-self associated signals. Both leukocyte and lymphocyte activation are regulated by TLRs in the CNS during inflammation and neuronal injury (Guo and Schluesener, 2007), and astrocytes and microglia express TLRs (Aravalli et al., 2007). However, TLRs are also stimulated by several non-pathogen factors, for example, the cell stress proteins, heat-shock proteins (Guo and Schluesener, 2007). Indeed, without CNS pathogen stimulation, the role for TLR4 was identified in initiating spinal nerve trauma-induced neuropathic pain because mice lacking the TLR4 receptor did not develop

neuropathic pain nor activation of spinal innate immune cells or glial activation (Tanga et al., 2005). Further, blocking spinal cord TLR4 production in neuropathic rats also blocked pathological pain and microglial/macrophage activation (Tanga et al., 2005), supporting that TLR4 signaling in the spinal cord is important for mediating neuropathic pain. A recent report showed spinal TLR4 antagonism during sciatic nerve damage that leads to neuropathic pain in rats potentially alleviated pathological pain (Hutchinson et al., 2007). In addition, this study was the first to demonstrate that, like neuropathic pain, opioids activate glia via TLR4 activation that leads to opioid-induced tolerance (Hutchinson et al., 2007). These findings have profound implications on future treatment strategies for clinically utilized opioids, as TLR activation appears to play a significant role in neuropathic pain. Thus a review of the literature supports the notion that in spinal cord diseases like neuropathic pain, robust activation of microglia, astrocytes and innate immune cells coordinate inflammatory mediators via TLR and chemokine signaling (Jack et al., 2005, Kigerl et al., 2007, White et al., 2005).

Targeting Activated Glia with Anti-inflammatory Cytokines to Resolve Pathological Pain

Given chemokines like fractalkine act to attract cells to the site inflammatory activity, anti-inflammatory factors may dampen such signaling. Spinal anti-inflammatory treatment with interleukin-2 (IL-2), interleukin-4 (IL-4), transforming growth factor-beta (TGF- β) (Schafers and Sommer, 2007), and IL-10 (Moore et al., 2001) may prove beneficial. One approach for achieving sustained suppression of proinflammatory cytokine action is by utilizing gene delivery to the spinal cord. Gene-encoding vectors, such as viral and non-viral vectors to deliver the IL-2, IL-4 or IL-10 gene to the spinal cord to control pain in neuropathic rodent models have been examined and show promise (Hao et al., 2006, Ledebor et al., 2007, Milligan et al., 2005a, Milligan et al., 2005b, Yao et al., 2003, Yao et al., 2002a, Yao et al., 2002b). For example, spinal cord over-expression of IL-10 controls pain produced by sciatic inflammatory neuropathy and chronic constriction injury (Milligan et al., 2005a, Milligan et al., 2007, Milligan et al., 2005b). The duration of pain control by spinal IL-10 gene therapy was observed from 1-3 months (Milligan et al., 2006a, Sloane et al., submitted). Furthermore, the possibility that long-term IL-10 protein expression in the spinal could lead to IL-10 receptor insensitivity was examined and not supported. That is, neuropathic rats treated with spinal IL-10 gene therapy at a dose that suppressed allodynia for 30 days, and which had returned thereafter to a stable allodynia (~21 days), were fully responsive to a bolus injection of IL-10 protein. Allodynia was completely reversed for several days (Fig 1). Additionally, the therapeutic benefit of spinal IL-10 over expression via gene therapy extended to other clinically relevant pain problems such as a rodent model for cancer chemotherapeutic pain which attenuated allodynia for greater than 35 days (Ledebor et al., 2007). Thus, pathological pain states can be controlled by spinal application of anti-inflammatory cytokines that act block glial proinflammatory activity.

Preventing the activation of microglia and/or astrocytes prior to or during pain facilitation has contributed further to our understanding of the important role that glia play in the creation and maintenance of exaggerated pain states. The chemokine, fractalkine, exerts cell migratory roles as well as increases pain sensitivity. Fractalkine, released from neurons via enzymes that increased during inflammatory processes, leads to glial activation producing pathological pain. Thus fractalkine may play a critical role in how peripheral nerve injury and inflammation signals the spinal cord about damaging events in the body. Interleukin-10 (IL-10) decreases many proinflammatory factors and can both prevent and reverse enhanced pain states. If anti-inflammatory cytokines can be exploited as drug therapies, they may prove to be a novel and effective approach for clinical pain control.

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Figure 1. IL-10 protein reinstatement of reversal of neuropathic pain

Our prior work has shown that sensitization to IL-10 protein injections is evident in a time dependent manner. Plasmid DNA injections were delivered at a concentration of 5-6 ug/uL. The plasmid construct coding for rat interleukin-10 (pDNA-IL-10F129S) was identical to that previously described (Milligan et al 2006). Briefly, this plasmid contains cDNA encoding rat IL-10 under the control of a hybrid cytomegalovirus (CMV) enhancer/chicken beta actin promoter. A previously undetected point mutation, resulting in an amino acid change (F129S), has been identified in the rat IL-10 gene expressed in these studies. The effect of this point mutation on IL-10 bioactivity *in vitro* as well as *in vivo* is currently under investigation. For those previous studies, two pDNA-IL-10F129S injections were spaced 3 days apart (100 ug followed by 25 ug dose; normally leads to >3 month pain reversal). If IL-10 protein is administered 4 days following re-expression of pain (at a dose that normally reverses pain in the CCI model for less than 24 hr), a 30-day reversal of pain is observed (Sloane et al, *submitted*). Here, we illustrate that 3 weeks following the second pDNA-IL-10F129S injection, IL-10 neutralizing antibody was injected to elicit a long term re-expression of pain behavior. That is, if IL-10 protein at the same dose previously examined (Sloane et al, *submitted*) is administered 3 weeks following re-expression of pain, an ~8 day reversal of pain is observed, suggesting a sensitized spinal cord environment and not that IL-10 receptors have become desensitized to long-term gene expression. This approach for long-term anti-inflammatory protein delivery to the spinal cord may be a clinically viable means for pain control. Behavioral tests of allodynia were analyzed by repeated measures ANOVA followed by Fisher's protected least significant difference posthoc comparisons, where appropriate. Results were determined to be significant at $p < 0.05$.