Neuropathic pain: role of inflammation, immune response, and ion channel activity in central injury mechanisms

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ABSTRACT

Neuropathic pain (NP) is a significant and disabling clinical problem with very few therapeutic treatment options available. A major priority is to identify the molecular mechanisms responsible for NP. Although many seemingly relevant pathways have been identified, more research is needed before effective clinical interventions can be produced. Initial insults to the nervous system, such as spinal cord injury (SCI), are often compounded by secondary mechanisms such as inflammation, the immune response, and the changing expression of receptors and ion channels. The consequences of these secondary effects may complicate the clinical treatment of the primary injury and result in high comorbidity. In this review, we will describe physiological outcomes associated with SCI along with some of the mechanisms known to contribute to chronic NP development.

KEYWORDS: MMPs, Nitric Oxide, TRPV-1, NKCC-1, Cannabinoid Receptors, CB1/CB2, Microglia

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Introduction

Spinal Cord Injury and Neuropathic Pain

Spinal cord injury (SCI) often results in devastating motor and sensory deficits for which current therapy is largely ineffective. Additionally, SCI can induce the development of chronic neuropathic pain states and significantly worsen the quality of life of these patients. A lack of sufficient understanding of the mechanisms underlying NP has affected the development of effective analgesic and restorative therapies. Two of the most common clinical pain behaviors associated with NP syndrome are allodynia and hyperalgesia. Allodynia occurs when normally non-noxious stimuli produce pain and hyperalgesia is the condition of an exaggerated pain response produced by a normally mildly noxious stimulus. The development of some degree of central NP is believed to occur in up to 70% of SCI patients and causes significant discomfort and disability in many areas of a patient’s life.1 It is estimated that up to 1 percent of the population suffers from some degree of NP.2 SCI can produce marked changes in the synaptic circuits of the dorsal horn cells as well as in areas well rostral to the site of trauma through a variety of mechanisms.3 Of particular interest are changes in receptor and ion channel expression and activity, release of local inflammatory cytokines and reactive oxygen species, activation of the immune response in microglia and other immune cells, and the activation of specific intracellular cascades. These are some of the most commonly studied mediating factors that are known to be involved in NP following SCI.

Spinal Cord Contusion Models

A variety of animal models have been designed to study the development of NP following SCI. Some of these models include spinal cord contusion, spinal hemisection, spinal ischemic injury, quinacrine-induced excitotoxic lesions, clip-compression lesions, and argon laser induced lesions.4–6 These models attempt to produce NP symptoms that mimic those observed in a clinical setting, however, the more specific the lesion induced the less clinically relevant the results. Here we examine one of the most commonly employed models used to study central NP development, the contusive spinal cord injury model (cSCI). This is performed using a weight-drop impactor following a laminectomy that spares the dura mater.7 Following the procedure motor deficits and pain behaviors are measured over a set period of time. Common assessments include an open field locomotor test measuring hind limb performance8, a sciatic nerve function index measuring the various relationships between the toes and feet of the hind limbs,9 walking track evaluations10 and an extensor postural thrust measurement which measures the force generated by the hind limbs.11 Changes in response to sensory stimuli are also important measurement taken following spinal cord contusion.12 Thermal hyperalgesia is typically exhibited beginning about 21 days following injury. Assessments used to quantify the response to this type of noxious heat stimuli are hind paw withdraw latencies and heat threshold tests.13

Inflammatory Response

The inflammatory process is of great significance in the development of NP. Not only does inflammation produce a variety of changes in the extracellular environment, but it also induces profound intracellular changes. Some of the notable components of the inflammation process known to contribute to the manifestation of NP include the accumulation and recruitment of inflammatory cytokines, chemokines and prostaglandins, the modulation of extracellular proteins, changes in transmembrane receptor expression, immune cell infiltration, and intracellular changes modulated via ion channel activity and receptor signaling.14–17 Although the inflammatory response is observed within most tissues in the body following insult, the central nervous system is unique in several important ways. Several distinct cell types are found only in the central nervous system: neurons, astrocytes, oligodendrocytes, and microglia. Further, neurons are known to be limited in the ability to regenerate. The CNS also lacks a lymphatic drainage system and is therefore relatively limited in its ability to expand because of the presence of surrounding tissues such as the dura, spinal canal, and skull. Thus relatively small levels of swelling can have significant consequences. The blood-brain barrier also has a role in producing an inflammatory response unique to the CNS. Endogenous neurochemical mediators and growth
factors produced secondary to the injury contribute to trauma induced BSCB disruption, edema, and the subsequently observed motor deficits and NP.  

For our purposes in understanding the development of NP, this complex defense mechanism of inflammation can be separated into two classes: beneficial and detrimental. Inflammation in the CNS serves a function of eliminating pathogens, phagocytizing necrotic tissue, mediating repair mechanisms of some types of damaged tissues, and recruiting leukocytes to the damages area. Additionally, neurotrophic factors released by microglia and astrocytes in response to cytotoxic factors produced by damaged cells have been shown to have some neuroprotective effects. The inflammatory response, however, is also known to play a significant role in the development of NP syndromes. Many of the same neurotrophic factors that have shown neuroprotective effects, such as BDNF and TNF-α, also enhance the inflammatory response. In doing so, further damage is caused to injured and non-injured tissue alike and is often more severe than the damage caused by the primary insult. Mediators of this secondary damage are due to acute increases in proteases, nitric oxide, bradykinins, prostaglandins, and tumor necrosis factor alpha (TNF-α), among others. In addition, these extracellular factors influence a number of intracellular changes that further promote the development of NP and will be discussed below.

**MMPs**

Matrix metalloproteases (MMPs) are a family of zinc-dependent extracellular proteases that are involved in the digestion of extracellular matrix components as well as some cell surface proteins like adhesion molecules, receptors, growth factors and cytokines. MMP-9 has constitutive expression in uninjured states and is thought to mediate the cellular extracellular environment by assisting in growth and regenerative processes as it is expressed in migrating growth cones. In injured tissue, MMP-2 and MMP-9 play a key role in the inflammatory response by modulating the development of neuropathic pain. Reactive oxygen species, mechanical stimuli, and mitogen activated protein kinase (MAPK) pathways activate transcription factors such as activator protein 1 (AP-1) and nuclear factor–κB (NF-κB). These factors cause a direct increase in MMPs as well as the other powerful inflammatory mediators such as IL-1β and TNF-α. MMPs are thus overexpressed in injured spinal cord tissue and both are thought to mediate NP through IL-1β cleavage. One important difference between the aforementioned MMPs, however, is that MMP-9 and MMP-2 upregulation is correlated with early and later phases of NP respectively. Another notable difference between these two MMP profiles is that NP associated with MMP-9 expression is generally associated with microglia activation while MMP-2 related NP is seen to be maintained by astrocyte activation. A significant detrimental effect of the overexpression of MMP-9 following SCI is the disruption of the blood-spinal cord barrier. This disruption permits the infiltration of immune cells, such as neutrophils, and inflammatory components which contribute to secondary tissue damage and will interact with resident astrocytes and microglia, subsequently producing even more inflammatory cytokines at the injury site. Invading neutrophils will facilitate lesion expansion via necrotic mechanisms and also through the production of reactive oxygen species, proteases, and nitric oxide. Delayed upregulation of MMP-2 is observed beginning 5 days following cSCI and peaks 7-14 days post-injury. Less is known about the expression patterns of MMP-2 following injury, however, it appears that macrophages infiltrating the injury site and astrocytes surrounding the lesion contribute substantially to the induction of MMP-2. Knockout studies of MMP-9 deficient mice have demonstrated improved blood-spinal cord barrier integrity following SCI producing diminished neutrophil and macrophage infiltration. Treatment with atorvastatin, an MMP inhibitor, post-SCI produced similar results. This suggests that inhibition of MMP-9 has a neuroprotective role in following SCI. MMP activity is also implicated in glial scarring following SCI. While this scarring can help to reestablish the blood-spinal cord barrier following injury, the cells within the scar prevent neurogenesis by secreting inhibitory molecules. MMP-2 knockout mice show higher prevalence of glial scarring following injury suggesting MMP-2 may also have a beneficial role in improving functional recovery and reducing NP following SCI. Directed inhibition of MMPs may therefore offer an efficient and precise target for the treatment of NP at both early and later phases of presentation.

**TIMPs**

Matrix metalloprotease tissue inhibitor (TIMP) activity is critical in order to maintain a healthy balance of ECM turnover in the healthy state because it prevents rampant breakdown of ECM proteins by constitutively active MMPs. The most common mechanism of TIMP inhibition of MMP activity involves binding of the N-terminal amino acid of the TIMP protein and the zinc ion coordinated to the MMP. This interaction between TIMPs and the catalytic domain of MMPs results in conformational changes that prevent MMP proteolytic activity. In one study, TIMP-1 was shown to inhibit MMP-9 activity, suggesting its induction may have a neuroprotective function in CNS insults. As mentioned previously, MAPK activity is implicated in MMP regulation. It is also active in the upregulation of TIMP through the stimulation of c-Jun and c-Fos binding domains. Interestingly, elevated levels of TIMP expression, induced through activation of cannabinoid receptors, was shown to reduce cancer cell invasiveness. This suggests that cannabinoids may provide a therapeutic approach to the attenuation of NP development.

**CB receptors**

The cannabinoid (CB) system has been implicated as playing an important physiological role in analgesia. The endogenous CB system includes the CB receptors (CB1 and CB2) and natural ligands (endo-CBs) anandamide (AEA) and 2-arachidonylglycerol (2-AG). The cannabinoid receptors CB1 and CB2 are G-protein coupled receptors that have been shown to be upregulated following CNS insults. Their activities have also been implicated in the development of NP by promoting a decrease cAMP production, reducing intracellular Ca++ levels and modulating MAPK activity. CB receptors are found predominantly in the presynaptic membrane which allows them to effectively inhibit the release of neurotransmitters. In studies where cannabinoid receptor agonists were administered in animal models of NP, TIMP production was induced and a decrease in hyperalgesic behavior was observed. CB1 receptor activity is also implicated in the modulation of NP induced by the TRPV1 receptor through PKA activity. CB1 receptors have been observed in the spinal cord, dorsal root ganglia, and peripheral terminals of c-fibers and are usually found co-localized with TRPV1 receptors. It is
thought that CB1 receptor activity inhibits the Ca++ dependent MAPK signaling that promotes MMP-2 activity and subsequent NP. Additionally, enzymes that regulate the biosynthesis and catabolism of the endo-CBs are emerging as important modulators of CB system activity levels in NP development. One study demonstrated that CB2 receptor-mediated control of NP was IFN-gamma dependent. These studies suggest that the CB receptors attenuate behavior associated with NP through multiple mechanisms. However, NP pain attenuated by CB activation appears to be active only after CNS injury and not peripheral nerve injury. This has been demonstrated using CB1 knockout mice although the mechanism for this difference is not well understood.

Nitric Oxide

One of the most potent inflammatory mediators is nitric oxide (NO). It induces vasodilation, enhances the production of free radicals, and mediates important cellular activities through the induction of cyclic guanosine monophosphate (cGMP). High levels of NO are also neurotoxic. Because the half-life of NO is quite short, the level of NO synthase activity is usually the best measurement of NO induced inflammation. Of the three forms of NO synthase, endothelial, neuronal and inducible, inducible nitric oxide synthase (iNOS) is the primary form active during the chronic inflammatory process. The primary cytokines responsible for iNOS upregulation are thought to be TNF-α and interleukin-1b (IL-1β), both of which act through the nuclear transcription factor NF-κB and are induced several hours to several days following injury [49,50]. Constitutively expressed forms of NOS, neuronal and endothelial, play a more significant role in the inflammatory response immediately following injury as they are released by the damaged cells. Administration of iNOS inhibitors has been shown to improve function and alleviate NP in chronic constrictive models of SCI. Further, exogenously administered agmatine, a general inhibitor of NOS, has been shown effective at reducing inflammation, hyperalgesia, and allodynia following excitotoxic SCI. Interestingly, evidence also exists to suggest that some endogenous NO activity may have neuroprotective effects following SCI. Rats treated with a novel NO-releasing gabapentin derivative following injury demonstrated reduction in pain behavior, although the mechanism is unclear. The role of NO in NP is complex and the multiple actions mediated by NO must be considered when seeking to develop therapeutic strategies targeting NOs role in NP development.

Prostaglandin

The prostaglandins are another important mediator of the inflammatory response. These lipid compounds are derived from arachidonic acid through the cyclooxygenase (COX) pathway. Two different isoforms of COX exist, COX-1 and COX-2, and both are implicated in pain and inflammation. Inducible COX-2 expression is known to be involved in the inflammatory response 2 hours following SCI and inhibition of COX-2 activity has been shown to improve functional recovery. This is thought to be due, at least in part, to a reduction in inflammation. COX-2 is also thought to be induced in the onset of NP following SCI through the production of prostaglandin E2 (PGE2) and the subsequent activation of microglia signaling.

Bradykinin

Bradykinin (BK), a peptide generated by the kinin system, is vasoactive and known to have hyperalgesic effects thought to be mediated through interaction with the B1 receptor. B1 knockout mice show less NP behavior than their wild-type counterparts. Interestingly, BK is also thought to have a moderate neuroprotective role through its attenuation of cytokine release from activated microglia. The B1 receptor has been shown to sensitize another receptor implicated in NP, the vanilloid receptor 1 (TRPV-1). The upregulation of both the B1 and TRPV-1 receptors in rats demonstrating hyperalgesic behavior following contusive SCI has been observed. Additionally, B1 and TRPV-1 antagonists were shown to have antihyperalgesic effects, even reversing TH behavior, following cSCI. Interestingly, B1 attenuation was shown to reduce NP behavior only in rats undergoing cSCI, suggesting that the B1 receptor may be uniquely upregulated following SCI.

Ion Channel Activity

TRPV-1

As mentioned previously, the TRPV-1 ionotropic receptor is implicated in the hyperalgesic response associated with pain. The TRPV-1 receptor is highly expressed in c-fiber sensory neurons and functions in both thermal and mechanical nociception. It is also activated by capsaicin and indirectly by lipoxigenase products. Following cSCI, TRPV-1 expression is significantly increased in the dorsal horn in animals exhibiting NP behavior. Endogenous TRPV1 agonists have been shown to play a role in increasing NKCC1 activity following painful conditions leading to allodynia. Further, TRPV-1 antagonists, such as AMG 9810, have notable antihyperalgesic effects in cSCI rat models. Interestingly, cooperative interactions of CB1, CC chemokines, and TRPV1 are likely to play a role in SCI induced changes and NP development at brain level.

NMDA Receptors

Excitatory glutamatergic receptors are implicated in synaptic plasticity and excitotoxicity involved in the development of NP syndromes. N-methyl-D-aspartate (NMDA) receptors are permeable to monovalent ions and calcium and blocked by extracellular magnesium. Glutamate also functions as a coagonist with glutamate in activating this channel. Direct protein kinase A (PKA) mediated phosphorylation of NMDA receptors is implicated in hyperalgesia and allodynia in central but not peripheral NP syndromes. In SCI models, administration of an NMDA antagonist, gacyclidine, leads to improved motor function recovery, and reduced astrogliosis. Similarly, administration of a different antagonist, MK-801, produces dose dependent reduction in thermal hyperalgesia but slower analgesic effects. Another NMDA antagonist, CHF3381, showed reduced hyperalgesia in improving pain in humans. Short acting antagonists have also shown to mediate decreased allodynia and central sensitization following CNS injury. Unfortunately, attempts at developing an NMDA antagonist that performs an analgesic function in alleviating NP in human subjects has been slow. One possible reason for this is the presence of NMDA receptors throughout the CNS, the non-specific activation of which can produces psychotropic effects.

GABA Receptors

The GABAergic system is a vital component of nociceptive sensory processing and modulation of its function commonly leads to the development of NP states. GABA receptors are found in both pre and post synaptic sites and function as ligand-gated chloride channels. Normal GABA receptor function is critically dependent on the activity of intracellular...
Cl⁻ concentration. Both channel types, A and B, have been implicated in NP syndromes and hyperexcitability. Loss of GABA inhibition also can produce NP. The evidence that hypofunction of GABAergic tone contributes to central NP following SCI is well documented by several experiments although the exact mechanism by which this occurs is unclear. Pharmacological treatments that enhance GABAergic function attenuates central neuropathic pain behavior and neuronal hyperexcitability following SCI. For example, intrathecal administration of GABA attenuates mechanical allodynia and hyperexcitability of spinal dorsal horn neurons following SCI. In addition, treatment with bicuculline (a GABA receptor antagonist) produces neuronal hyperexcitability and pain behavior in normal rats.

**NKCC1 and KCC2 Activity**

The maintenance of intracellular Cl⁻ concentration is essential for proper intercellular communication. The inhibitory activity of GABA receptors is critical for normal neural circuitry function and is particularly sensitive to the concentration of intracellular Cl⁻. Cation-dependent chloride transporters therefore are of vital importance in understanding NP following spinal cord injury.

Here we identify two of the major Cl⁻ regulatory proteins, the inwardly directed Na⁺-K⁺-Cl⁻ cotransporter isoform 1 (NKCC1) and the outwardly directed K⁺-Cl⁻ cotransporter isoform 2 (KCC2), both known to have a specific role in NP syndromes. Early in development, GABA acts primarily as an excitatory neurotransmitter due to high NKCC1 expression and delayed KCC2 expression, resulting in high levels of intracellular Cl⁻ and thus the maintenance of more excitable states upon activation of GABA receptors. Mature GABA neurons have a more positive equilibrium potential. It is known that following cerebral ischemia NKCC1 activation leads to astrocyte induced swelling and glutamate release, as well as neuronal Na⁺, and Cl⁻ influx during acute excitotoxicity. Inhibition of NKCC1 activity also significantly reduced infarct volume and cerebral edema following cerebral focal ischemia. Following SCI, increasing evidence has suggested that the overexpression of NKCC1 and/or downregulation of KCC2 have a significant function in the inflammatory response in the development of chronic NP. KCC2 downregulation has also been implicated in functional deficits following SCI. BDNF administration reversed these deficits. It has also been demonstrated recently that NKCC1 activity is up-regulated approximately two weeks following cSCI which precedes onset of chronic NP. Immunoblotting showed a significant transient up-regulation of NKCC1 protein in the lesion epicenter of the rat spinal cord on day 14 post-SCI. This was accompanied by a concurrent down-regulation of KCC2 protein. Moreover, inhibition of NKCC1 with its potent antagonist bumetanide (BUM) significantly reduced pain behavior in rats.

**Edemic Response**

Edema, along with the inflammation and the immune response, is an important contributor to secondary injury following both brain and SCI. Edema can be separated into vasogenic and cytogenic edema. Vasogenic edema is due to disruption of the BSCB integrity and cytogenic edema is due to increased intracellular water content. Since astrocytes outnumber neurons 20 to 1, astrocytic cytotoxic edema is believed to be a significant contributor of total cytotoxic edema due to aquaporin-4 expression. However, in order for water to have a net flux in a given direction an osmotic gradient must exist. Increasing evidence has suggested that NKCC1 may have a role in modulating this osmotic balance. In the brain, this co-transporter has been shown to play an integral part of edema formation following traumatic injury and ischemia. Another study has implicated MMP-9 in brain edema following hemorrhage. It demonstrated that hemoglobin-induced oxidative stress, resulting from the rupture of the blood-brain barrier, can trigger MMP-9 in astrocytes and consequently matrix degradation. A possible mechanism by which MMPs can increase vasogenic edema is through up-regulation of IL-1β and TNF-α. These cytokines have been directly implicated in the development of vasogenic edema in brain. Studies have also demonstrated significant correlation between increased edema and decreased neurobehavioral parameters. The extent of edema is closely related to the degree of SCI-induced motor dysfunction. However, whether the edemic response following SCI directly induces NP is still unknown.

**Immune Response**

Growing evidence is implicating a significant role of the immune response in the development of neuropathic pain, particularly through the activity of immune cells and microglia. NP has many features of a neuroimmune disorder. Consequently, therapies involving the immunosuppression and the blockade of the reciprocal signaling may produce more effective results than those aimed primarily at modulating the pain symptoms through attenuating neural activity. Several of the factors released by these immune cells, such as cytokines and neutrophic factors, are known to be involved in the development of NP. In addition to contributing to the inflammatory response, some of these signals can modulate the properties of the afferent sensory neurons.

**TNF-α**

A number of cytokines are produced following SCI. TNF-α is a prominent cytokine produced by activated immune cells and microglia. It is initially synthesized as a transmembrane protein that is then cleaved by protease to yield its active form. TNF-α interacts with a diverse family of TNF receptors and thus serves as an important intermediate in the innate immune response. While inflammation is of primary concern, TNF receptors also can induce cell proliferation, differentiation, and apoptosis. Following SCI in mice, TNF-α mRNA expression is induced as early as 15 minutes post-injury. TNF-α activity has been correlated with NP development through induction of glial and neuronal apoptosis through a NO-mediated mechanism. Evidence also exists implicating TNF-α activity in a neuroprotective role following SCI. Specifically, it is thought to protect against excitotoxic amino acids and facilitate in the maintenance of intracellular Ca++. Among the proinflammatory cytokines, IL-1β is particularly known to modulate pain sensitivity. Interleukin-1β (IL-1β), along with TNF-α, is commonly involved in the inflammatory response following CNS injury. Central administration of IL-1β was shown to produce thermal hyperalgesia and mechanical allodynia. Mice with impaired IL-1 receptor signaling exhibit no neuropathic pain, almost no self-mutilating behavior, and little ectopic neuronal activity, suggesting that IL-1β plays a critical role in the NP response. Further, rat antibody to IL-1β receptor reduced thermal hyperalgesia and mechanical allodynia in a dosage-dependent fashion in mice following nerve
injury.\textsuperscript{106} Bumetanide administration has been shown to reduce the inflammatory response through an attenuation of IL-1\(\beta\) overexpression.\textsuperscript{49} Additionally, thiazolidinediones (TZDs), potent synthetic agonists of the transcription factor peroxisome proliferator-activated receptor-gamma (PPAR gamma), were shown to induce neuroprotection after cerebral ischemia by blocking inflammation through IL-1\(\beta\) reduction.\textsuperscript{107} IL-1\(\beta\) is also known to play an important role in the upregulation of MMPs.\textsuperscript{114}

\textbf{T-lymphocytes}

Recent studies have begun to indicate a significant role of T-lymphocytes in the pathology of NP.\textsuperscript{108} It is known that these immune cells mediate inflammation following injury through the secretion of proinflammatory cytokines although it was not clear whether this produced a beneficial or detrimental outcome. Recent studies have confirmed that recruitment of T-cells produce secondary tissue damage and impair functional recovery.\textsuperscript{109} Additionally, athymic rats, which lack mature T-cells, develop a significantly reduced mechanical allodynia and thermal hyperalgesia following SCI.\textsuperscript{110} Other studies have confirmed the importance of T-cells in the onset of NP.\textsuperscript{111} T-cell infiltration and activation in the dorsal horn of the spinal cord following injury contribute to the evolution of neuropathic pain-like hypersensitivity through interferon-gamma upregulation.\textsuperscript{112} This same study also showed that IFNg signaling is required for full expression of adult neuropathic hypersensitivity.

\textbf{Neutrophils}

Neutrophils are commonly found in the blood stream and are resident in the peripheral nervous system. They are the first leukocytes to arrive at damaged tissue, and release molecules that can affect neural function.\textsuperscript{113} They are most numerous 1-3 days after SCI, are detectable for up to 10 days post injury, and heavily express MMP-9.\textsuperscript{114} Myelin basic protein, a major membrane protein component of the central nervous system, is a cleavage substrate for MMP-9, suggesting it has a supporting role in demyelination and permeability characteristics in the blood brain barrier.\textsuperscript{115} Disruption of the BSCB allows for neutrophil invasion which can contribute additional tissue damage and demyelination. Invading neutrophils will facilitate lesion expansion via necrotic mechanisms and also through the production of reactive oxygen species, pro-
essary before effective treatments can be produced.

Figure 1 is a summary of both known and probable links discussed in this review directly contributing to NP. More interactions between the discussed proteins and cells, as well as unmentioned factors, are likely, as interplay between the various contributors to NP following spinal cord injury is complex.

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