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Research Overview

Neural-Glial Interaction in the Spinal Cord for the Development and Maintenance of Nerve Injury-Induced Neuropathic Pain

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Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT Damage to the nervous system often results in neuropathic pain. Current treatment for this disabling state is unsuccessful due to our incomplete understanding of cellular mechanisms causing this pain. Although glial cells were largely ignored in most textbooks of pain, accumulating evidence over the last decade indicates an important role of glial cells in the pathogenesis of pain. Both microglia and astroglia are activated in the spinal cord after peripheral nerve injury. Importantly, activated microglia and astroglia produce multiple inflammatory mediators and neuromodulators, acting on primary afferents or dorsal horn neurons and leading to an enhancement and maintenance of dorsal horn neuron sensitization and subsequent pain sensitization. This neural-glial interaction after peripheral nerve injury is likely to be triggered by signaling molecules released in the spinal cord from central terminals of damaged sensory neurons, stimulating surrounding glial cells. In addition, there is a microglial-astroglial interaction; microglia activation occurs before astroglial activation and is known to cause astroglial activation. Glial activation is further enhanced by microglial-microglial interaction and astroglial-astroglial interaction. Many signaling molecules (e.g., MAP kinases, ATP receptors, chemokine receptors) are exclusively activated in spinal microglia or astroglia after nerve injury, and an inhibition of these molecules can attenuate neuropathic pain. Since traditional pain-killers are designed against neuronal targets and are only partially effective to treat neuropathic pain, searching for signaling molecules that are induced in spinal glia in neuropathic pain conditions will identify novel targets for the management of this debilitating chronic pain. Drug Dev. Res. 67:331–338, 2006.

Key words: microglia; astrocytes; spinal cord; MAP kinase; neuropathic pain

INTRODUCTION

Damage to the nervous system often results in neuropathic pain. Many patients in the pain clinic suffer from neuropathic pain due to injury to the peripheral nervous system (e.g., peripheral nerves, dorsal root ganglia [DRG], and dorsal roots) or the CNS (e.g., spinal cord and thalamus). These injuries may result from major surgeries, diabetic neuropathy, amputation, viral infection, trauma, and stroke [Ji and Strichartz, 2004; Woolf and Mannion, 1999]. Neuropathic pain in animal models is mainly produced by peripheral nerve injuries and is highly reproducible. Most studies on mechanisms of neuropathic pain are based on peripheral nerve injury model. Therefore,

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in this review, we will focus on nerve injury–induced neuropathic pain. Mechanical allodynia (painful responses to normally innocuous tactile stimuli) is the most distinct symptom of neuropathic pain, despite the fact that neuropathic pain is also characterized by cold allodynia and hyperalgesia (increased responsiveness to noxious heat and mechanical stimuli), as well as spontaneous pain described as shooting, lancinating, or burning pain [Ji and Strichartz, 2004; Woolf and Mannion, 1999]. Our incomplete understanding of molecular and cellular mechanisms underlying the development and maintenance of neuropathic pain results in limited success in treating this pain.

NEURAL MECHANISMS OF NEUROPATHIC PAIN

Nerve injury–induced pain hypersensitivity can be caused by both peripheral and central mechanisms. Neuropathic pain was originally thought to arise from injury discharge from the site of axonal injury and from the development of ectopic activity in the injured DRG neurons [Devor and Seltzer, 2000]. Spontaneous discharge also develops in non-injured neurons and increased pain sensitivity is found in intact nerve territories [Decosterd and Woolf, 2000; Kim and Chung, 1992; Ma et al., 2003; Wu et al., 2001]. Inflammatory mediators released from damaged axons or infiltrating immune cells play a critical role in the generation of spontaneous activity and genesis of neuropathic pain. Peripheral nerve injury also induces marked phenotypic changes in DRG neurons and both upregulation and downregulation of numerous genes could contribute to the maintenance of injured-induced hypersensitivity of sensory neurons [Ji and Strichartz, 2004].

Central sensitization is an activity-dependent plasticity of nociceptive neurons in the CNS, particularly in the dorsal horn of the spinal cord [Ji et al., 2003; Mantyh et al., 2002; Woolf and Salter, 2000]. Spontaneous activity from primary afferents is believed to drive central sensitization. While peripheral sensitization (a counterpart of central sensitization) appears to trigger nerve injury–induced neuropathic pain, central sensitization may directly cause neuropathic pain after spinal cord injury, termed as central neuropathic pain. Central sensitization is induced by enhanced excitatory synaptic transmission (e.g., potentiated AMPA and NMDA currents due to increased activity of the glutamate AMPA and NMDA receptors), a mechanism that is also implicated in long-term potentiation of hippocampal neurons that underlies learning and memory [Ji et al., 2003]. Decreased inhibitory synaptic transmission also plays an important role in central sensitization after nerve injury. In addition, altered synaptic connectivity, increases in descending facilitatory influences, direct projection of large diameter myelinated fibers to dorsal column nucleus, and aberrant sympathetic influence could also contribute to the development and/or maintenance of neuropathic pain [Ji and Strichartz, 2004; Ossipov et al., 2002; Porreca et al., 2002; Woolf and Mannion, 1999]. Like long-term memory, maintenance of central sensitization and chronic pain requires gene transcription, and the transcription factor CREB (cAMP response element binding protein) appears to be important for regulating the transcription of many pronociceptive genes. Unlike long-term memory that is predominantly contributed to by neuronal mechanisms, neural-immuno or neural-glial interaction after nerve injury plays a critical role in enhancing and maintaining central sensitization [Ji et al., 2003].

SPINAL GLIA AND PAIN SENSITIZATION

Although pain hypersensitivity was originally thought to result exclusively from the altered activity of neurons, glial cells also express various receptors for neurotransmitters and neuromodulators [Ji and Strichartz, 2004; Steinhauser and Gallo, 1996]. The neuron-centered view of pain regulation is changing. Early evidence implying a role of spinal glia in pain sensitization came from the following studies: (1) Peripheral nerve injury induces activation of microglia and astrocytes in the spinal cord [DeLeo and Yezierski, 2001; Garrison et al., 1991; Watkins et al., 2001]; (2) Spinal injection of a glial toxin fluorocitrate can reduce inflammatory pain [Meller et al., 1994; Watkins et al., 1997]; (3) Propentofylline, a glial modulating agent, exhibits anti-allodynia properties in neuropathic pain in rats [Sweitzer et al., 2001b]; (4) Intrathecal injection of an HIV envelope glyprotien gp-120 activates spinal glia and induces hyperalgesia [Milligan et al., 2001]; and (5) Several proinflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α are primarily synthesized in glial cells and have been implicated in pain hypersensitivity [Sweitzer et al., 2001a; Watkins et al., 2001; Watkins and Maier, 2003]. However, these proinflammatory cytokines can also be synthesized in neurons after nerve injury, and fluorocitrate and propentofylline do not differentiate different subtypes of glial cells. Several recent studies have provided further evidence for spinal glial regulation of neuropathic pain.

MICROGLIA AND NEUROPATHIC PAIN

Microglia are regarded as resident macrophages in the CNS. In resting status, microglia are ramified with thin branches. Once activated, microglia become amoeboid with thick and short branches (Fig. 1). They show the quickest responses to nerve injury among

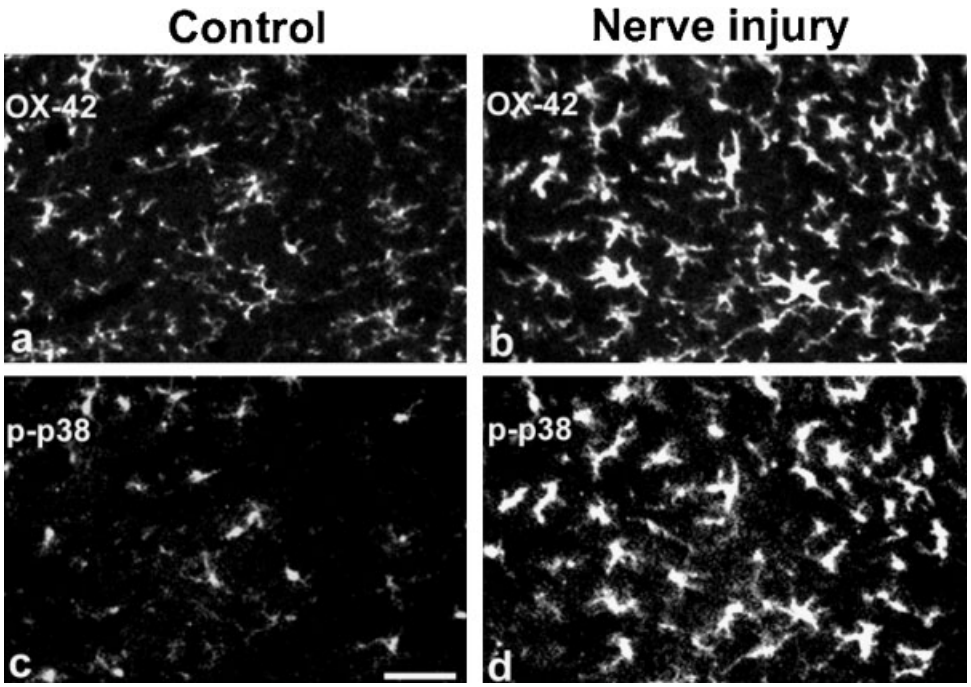

Fig. 1. **a,b:** Peripheral nerve injury by spinal nerve ligation induces a profound activation of microglia in the spinal cord dorsal horn, as indicated by increased expression of OX-42 (an antibody recognizing the microglial marker complement receptor-3 (CD-11b, or Mac-1) as well as by a distinct morphological change from a resting ramified shape in control non-injured spinal cord (a) to an active amoeboid shape 3 days after nerve ligation (b). **c,d:** Spinal nerve ligation also induces a drastic activation of p38 MAP kinase, as shown by increased phosphorylation (p-p38). a,c and b,d are the same spinal sections that are double stained for OX-42 and p-p38. p-p38 immunoreactivity is completely overlapped with that of OX-42, suggesting that p38 is only activated in spinal microglia. Scale bar = 20 μm.

all the glial subtypes [Kreutzberg, 1996]. Nerve injury induces the expression of microglial markers (e.g., Mac-1, TLR4, CD 14) within 4 hr [DeLeo et al., 2004]. In particular, nerve injury induces a rapid activation (phosphorylation) of p38 MAPK (mitogen-activated protein kinase) in the injured side of the spinal cord. p38 is not activated in spinal cord neurons in either control or injury conditions. Instead, p38 is exclusively activated in OX-42 (an antibody for complement receptor 3/Mac-1)-positive microglial cells in the spinal cord by nerve injury [Jin et al., 2003; Tsuda et al., 2005]. Intrathecal administration of a p38 inhibitor SB203580 can prevent spinal nerve ligation-induced mechanical allodynia up to 2 weeks. Moreover, SB203580 can further reverse established mechanical allodynia, when administrated 10 days after nerve injury, although this reversal is only partial [Jin et al., 2003; Tsuda et al., 2004; but see also Schafers et al., 2003]. In a sciatic inflammatory neuropathy (SIN) model, neuropathic pain can both be prevented and reversed by intrathecal injection of p38 inhibitor CNI-1493 [Milligan et al., 2003]. A recent study shows that β-isoform of p38 is expressed in spinal microglia and knock down of this isoform prevents spinal pain sensitization [Svensson et al., 2005].

In addition to p38, we have recently shown that another MAPK family member ERK (extracellular signal-regulated kinase) is also induced by spinal nerve ligation in microglia in the first several days after injury. Blockade of this activation with a MEK inhibitor attenuates nerve ligation-induced mechanical allodynia [Zhuang et al., 2005]. It remains to be investigated how activation of p38 and ERK in spinal microglia can facilitate pain, although regulation of the synthesis of inflammatory mediators/neuromodulators is a highly implicated mechanism. It is also unclear whether p38 and ERK are required for microglial activation after nerve injury.

Interestingly, nerve injury–induced p38 activation in spinal microglia is reduced in mice lacking chemokine receptor CCR2. CCR2 is also induced in spinal microglia. Importantly, neuropthatic pain after nerve injury is diminished in these mice [Abbadie et al., 2003]. In addition, chemokine receptor CX3CR1 is induced in spinal microglia by nerve injury [Verge et al., 2004] and is also required for neuropathic pain sensitization [Milligan et al., 2004]. Fractaline, the only ligand for CX3CR1, appears to induce p38 activation in spinal microglia (Zhuang and Ji, unpublished observation). Interestingly, ATP receptor P2X4 is specifically

induced in spinal microglia after nerve injury, and mechanical allodynia is reduced by knocking down this receptor. Importantly, injection of ATP-activated microglia into the spinal cord induces mechanical allodynia, indicating that microglial activation is sufficient to induce pain sensitization [Tsuda et al., 2003]. Like CCR2, activation of P2X4 receptor by ATP in spinal microglia is likely to activate p38 or ERK to regulate pain sensitization. Recently, Tanga et al. [2005] showed that Toll-like receptor 4 (TLR4) is induced in spinal microglia by nerve injury. This receptor is required for microglial activation and development of neuropathic pain [Tanga et al., 2005].

Is microglial activation required for the development or maintenance of neuropathic pain? A microglial inhibitor minocycline is shown to prevent or delay neuropathic pain. However, this inhibitor can not reverse the established neuropathic pain when given several days after nerve injury, indicating a role of spinal microglia in the early development of neuropathic pain [Ledeboer et al., 2005; Raghavendra et al., 2003]. One argument could be that minocycline may not block all the signals in microglia, and that p38 activation and P2X4 induction in spinal microglia are still maintained even at 2 weeks after nerve injury. Nevertheless, microglia are more important for the early development of neuropathic pain.

ASTROGLIA AND NEUROPATHIC PAIN

In contrast to increasing evidence for microglial regulation of neuropathic pain, little is known about the role of spinal astrocytes in pain regulation. Nerve injury also induces a marked induction of GFAP (glial fibrillary acidic protein), the most used marker for astrocytes, in the spinal cord (Fig. 2). Also, astrocytes have the greatest number in the CNS and exhibit strong structural interrelationship with neurons in all regions of the CNS, enwrapping synaptic terminals. Astrocytes are positioned in such a way that they have the potential to be a pathway for signaling between glia and neurons. On the one hand, astrocytes can "listen" to neurons. Astrocytic processes form part of synapses (Tripartite synapses) [Araque et al., 1999]. Neurotransmitter (glutamate) released from presynaptic terminal not only acts on postsynaptic receptors, but can also spill over to bind glutamate receptors on astrocytic processes. Activation of glutamate receptors on astrocytes leads to an increase in intracellular Ca^{2+} via release from intracellular Ca^{2+} store. On the other hand, astrocytes can also "talk" to neurons. An increase in astroglial Ca^{2+} levels results in glutamate release from astrocytes next to the synapse, activating synaptic receptors and altering synaptic transmission [Haydon, 2001].

Do microglia and astrocytes play distinct roles in pain regulation? Astroglial activation is typically preceded by microglial responses [Kreutzberg, 1996]. For example, nerve injury induces the expression of microglial markers (e.g., Mac-1) within a few hours, but astroglial maker GFAP is induced after a few days [DeLeo et al., 2004] (Fig. 2). Interestingly, we found a sequential activation of ERK in the spinal cord after nerve ligation, first in microglia (first several days), then in astrocytes (after day 10). At a late time point (day

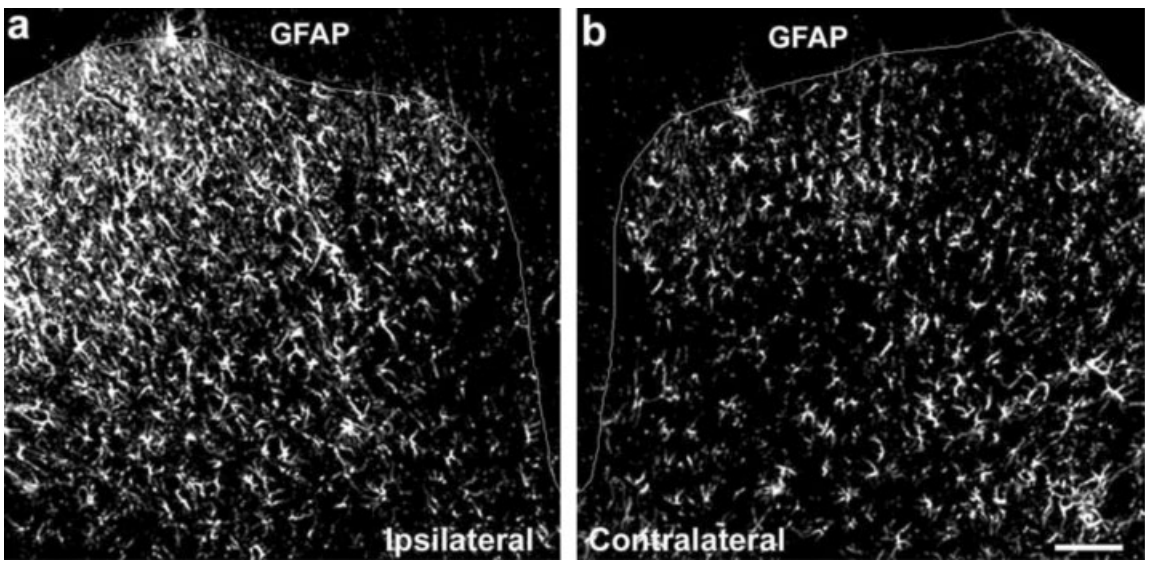

Fig. 2. a,b: Spinal nerve ligation produces a marked activation of astrocytes in the medial spinal cord dorsal horn, as indicated by increased expression of GFAP, an astroglial marker, as well as by distinct morphological changes of astroctytic processes from thin ones in non-injured contralateral spinal cord to thick ones (sign of hypertrophy) in injured ipsilateral spinal cord one week after nerve injury. Scale bar = 50 μm.

21), ERK is exclusively activated in spinal astrocytes [Zhuang et al., 2005]. These results support a distinct role of microglia vs. astroglia in the early vs. late development of neuropathic pain. Importantly, intrathecal inhibition of ERK at this late time point reversed nerve injury–induced mechanical allodynia [Zhuang et al., 2005]. A recent study also supported a role of spinal astrocytes in pain sensitization. Implantation of neural stem cells into the injured spinal cord produced a severe side effect, mechanical allodynia. Since most implanted stem cells become astrocytes in the spinal cord, stem cell–induced allodynia is likely to be attributed to the action of astroglia [Hofstetter et al., 2005].

Furthermore, astrocytes are characterized by forming gap junctions between them. Gap junctions exist at apposing plasma membranes of many cell types contributing to local metabolic hemostats and synchronization of cellular activities by allowing direct intercellular movements of ions, metabolites, and second messenger molecules up to 1,000 Daltons. These junctions are composed of hemi channels called connexons. In the spinal cord, gap junctions are predominantly formed between astrocytes. Connexin43 is regarded as the main functional protein for gap junctions in astrocytes [Nagy et al., 2004]. Studies in cell cultures and later in brain slices showed that transmitters that are released from neurons induce transient elevation of internal Ca^{2+} levels in astrocytes. Furthermore, glutamate-stimulated Ca^{2+} elevations spread from one astrocyte to another, indicating that Ca^{2+} signaling between astrocytes might form the basis of a long-range signaling pathway for cell-cell communication that depends on gap junctions [Haydon, 2001]. Therefore, gap junctions are implicated in the propagation of damage from the core to adjacent zones after brain ischemia.

Interestingly, gap junction blockade has been shown to influence the spread of pain. In a nerve inflammation model, high concentration of zymosan delivered to the sciatic nerve can produce a "mirror pain" in the contralateral paw. This mirror pain is suppressed by intrathecal injection of a gap junction blockade [Spataro et al., 2004].

NEURAL-GLIAL INTERACTION FOR NEUROPATHIC PAIN

From Neurons to Glia

The first question is: how does the neural signal get to the glia and induce glial activation? As described in Figure 3, the whole cascade should be initiated by

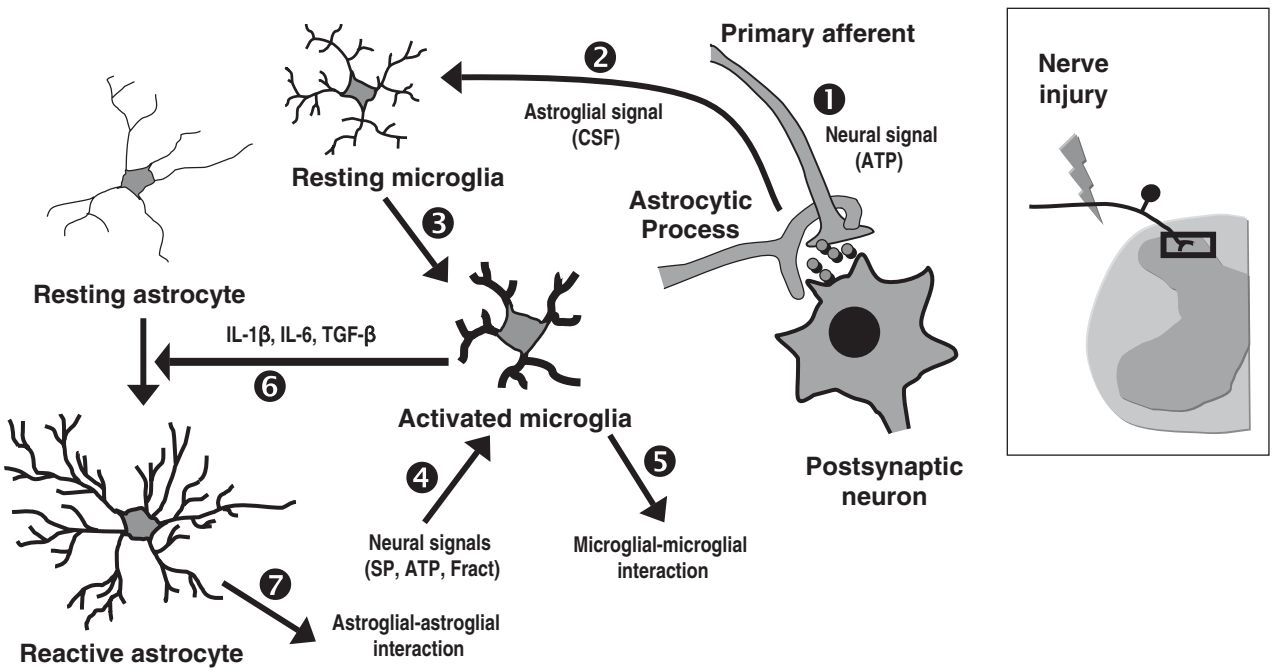

Fig. 3. Putative signals from neurons to glia. Damage to the peripheral axons of primary sensory neurons will cause a change in the central terminals of these neurons in the spinal cord (right). The affected terminals will release some signal molecule (e.g., ATP) to stimulate astrocytes next to synapses (Step-1). Astrocytes could subsequently release some trophic factor (e.g., CSF) to stimulate microglia (Step-2), casing microglial activation (Step-3). Microglia are also activated by molecules released from neurons (e.g., substance P, fractalkine, ATP, Step-4). Activated microglia can trigger additional microglial activation (Step-5). Microglia further activate astrocytes by releasing several stimulating factors (e.g., IL-1β, IL-6, TGF-β, Step-6). There is also a mutual activation between astrocytes (Step 7).

signal molecules released from spinal central terminals of damaged sensory neurons after peripheral nerve lesion, acting on nearby glial cells. Since synapses make intimate structural relationships and functional interaction with astrocytic processes, it is reasonable to assume that astrocytes would be the first glial cells to sense the abnormality or homeostasis disturbance of neurons. In response to a neuronal signal (e.g., ATP), astrocytes may synthesize and release some factor, such as CSF (colony-stimulating factor) or chemokine (e.g., CCR2 ligand), that can induce microglial activation. It is also possible that neurons, probably in response to astroglial signal, could release several diffusible factors such as the neuropeptide substance P and the chemokine fractalkine, and ATP to induce microglial activation (Fig. 3). However, how effective these diffusible factors are to cause microglial activation in vivo remains to be tested. Upon activation, microglia can further activate adjacent microglia by autocrine mechanisms, which can not only maintain microglial activation, but also spread microglial activation beyond the region terminated by primary afferents.

There is ample evidence showing that microglial activation leads to astroglial activation. Elimination of proliferating microglia suppresses the upregulation of GFAP in the spinal cord following nerve injury and microglial inhibition prevents nerve injury–induced GFAP upregulation [Aldskogius and Kozlova, 1998; Raghavendra et al., 2003]. Microglia can synthesize and release IL-1β, IL-6, and TGF-β (transforming growth factor-β), and each has been shown to induce astroglial activation (Fig. 3). Other factors such as bFGF (basic fibroblast growth factor), CNTF (ciliary neurotrophic factor), and endothelin, which can be released from different cell types, will also cause astroglial activation [Aldskogius and Kozlova, 1998]. Like microglia activation, activated astroglia can also activate other astrocytes via autocrine mechanisms (Fig. 3). Furthermore, astrocytes are heavily connected by gap junctions, and gap junction communication could be enhanced after

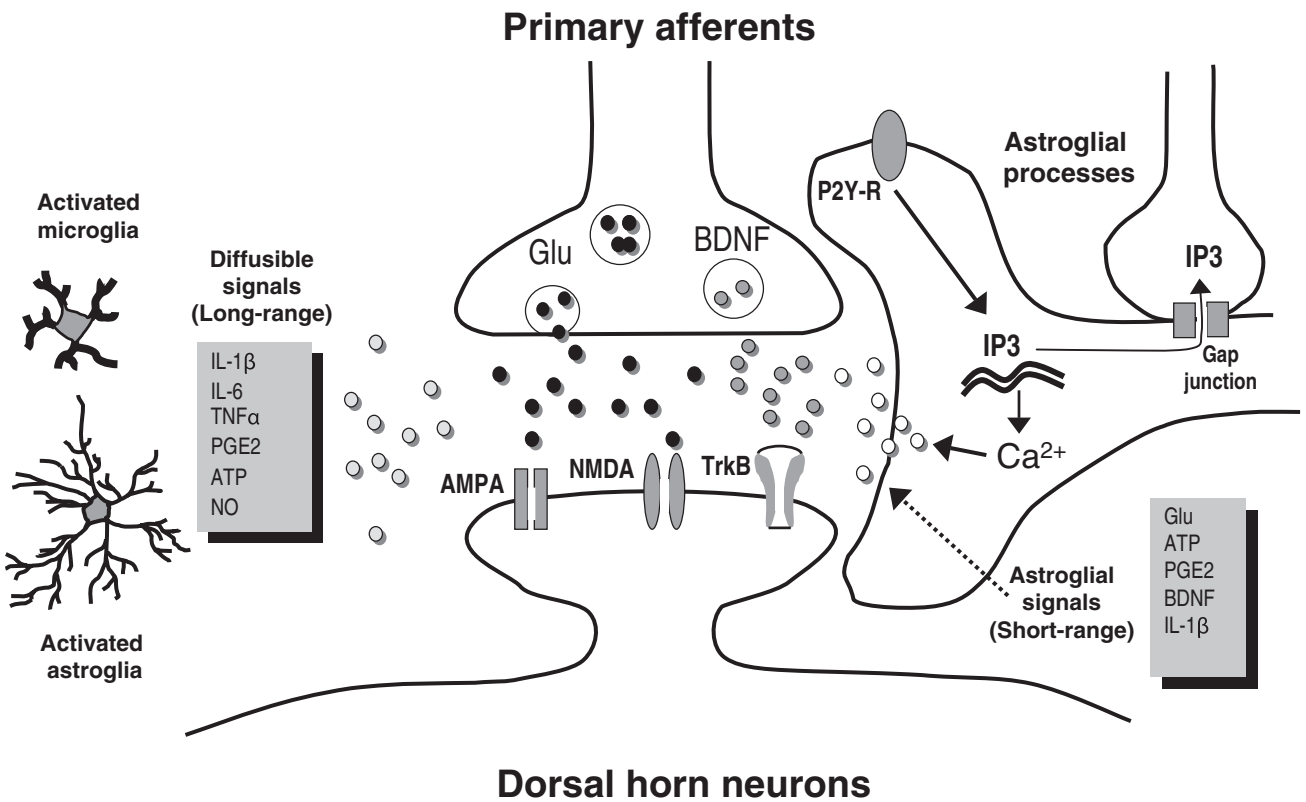

Fig. 4. Putative signals from glia to neurons. Astrocytic processes make very close contact with synapses. After nerve injury, astrocytes not only release neurotransmitters (e.g., glutamate, ATP), but may also release neuromodulators (e.g., BDNF, PGE_2, IL-1β, IL-6) in a short range to enhance synaptic transmission. In addition, diffusible factors (such as IL-1β, IL-6, NO), in a long range, may also affect presynaptic as well as postsynaptic sites to increase synaptic strength. At postsynaptic site, receptors for glutamate (AMPA and NMDA) and BDNF (TrkB) are required for neuropathic pain sensitization. Moreover, the activity of AMPA, NMDA, and TrkB receptors can be regulated by glial factors in a way that excitatory synaptic transmission is potentiated, leading to neuropathic pain hypersensitivity.

astroglial activation, leading to long-range cell-cell signaling.

From Glia to Neurons

The next question is: how can glial activation lead to the sensitization of pain transmission neurons and subsequent pain facilitation? As mentioned above, astrocytes not only receive signals from synapses, but also talk to synapses by releasing the major excitatory neurotransmitter glutamate in a Ca^{2+} -dependent way (Fig. 4). Activated astrocytes may also synthesize and release the neurotrophin BDNF (brain-derived neurotrophic factor), and PGE_2 (prostglandin E_2) via upregulation of its upstream enzyme Cox-2 (cyclooxygenase-2). PGE_2 can sensitize dorsal horn neurons not only by enhancing the release of neurotransmitters from presynaptic terminals, but also by suppressing inhibitory GABA current at postsynaptic sites. BDNF could sensitize postsynaptic dorsal horn neurons via regulating the activity of NMDA receptors [Scholz and Woolf, 2002].

Synaptic activity of dorsal horn neurons might also be affected by diffusible messengers released from activated microglia and astroglia (Fig. 4). These messengers, such as IL-1β, IL-6, and NO (nitric oxide), have been shown to act on presynaptic site-enhancing neurotransmitter release, although a direct postsynaptic effect may also exist [Ji and Strichartz, 2004; Samad et al., 2001; Watkins et al., 2001]. All the discussed molecules that are released from glial cells play a role in pain sensitization, presumably via a spinal mechanism. These glial factors could further maintain pain hypersensitivity by inducing gene expression.

CONCLUSIONS

Neural-immuno or neural-glial interaction has been receiving more and more attention in recent years. Accumulating evidence supports an important role of spinal glia for the development and maintenance of nerve injury–induced neuropathic pain. However, it is still far from clear how neurons and glia interact in vivo. Microglia and astroglia can both be activated in the spinal cord after nerve injury and may release similar diffusible factors to affect neuronal sensitivity, therefore exhibiting overlapping roles in neuropathic pain sensitization. However, the fact that astrocytes have very close contact to synapses and can be more persistently activated after nerve injury may support a more unique role of this subtype of glial cells for maintaining pain facilitation. The role of another type of glial cells, oligodendrocytes, in neuropathic pain still remains unclear. There is an increasing list of signaling molecules that are exclusively induced in spinal microglia or astroglia. Identification of these molecules will provide new targets for the management of neuropathic pain, a disabling pain condition that affects millions of Americans and that is not very successfully treated by current drugs aimed at targeting neuronal cells.

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