Published in final edited form as:

Brain Res Rev. 2007 November ; 56(1): 148–169.

“Listening” and “talking” to neurons: Implications of immune activation for pain control and increasing the efficacy of opioids

Linda R. Watkins^{*}, Mark R. Hutchinson, Erin D. Milligan, and Steven F. Maier

Department of Psychology & Center for Neuroscience, University of Colorado at Boulder, Boulder, CO 80309-0345

Abstract

It is recently become clear that activated immune cells and immune-like glial cells can dramatically alter neuronal function. By increasing neuronal excitability, these non-neuronal cells are now implicated in the creation and maintenance of pathological pain, such as occurs in response to peripheral nerve injury. Such effects are exerted at multiple sites along the pain pathway, including at peripheral nerves, dorsal root ganglia, and spinal cord. In addition, activated glial cells are now recognized as disrupting the pain suppressive effects of opioid drugs and contributing to opioid tolerance and opioid dependence/withdrawal. While this review focuses on regulation of pain and opioid actions, such immune-neuronal interactions are broad in their implications. Such changes in neuronal function would be expected to occur wherever immune-derived substances come in close contact with neurons.

INTRODUCTION

Within the past 15 years, evidence has accrued that the immune system can dynamically and dramatically alter neuronal function. This review explores the dysregulation of pain by peripheral immune cells and by immune-like glial cells. The focus is on immune-neuronal interactions at three key sites: the peripheral nerve, the dorsal root ganglia, and the spinal cord. While this review uses pain modulation as the focal point, the immune-neuronal interactions that are described have far broader implications than just pain. These interactions would be expected to occur wherever immune-derived substances come in close contact with neurons. Thus these data have wide-ranging implications for alterations in neural structure and function throughout the peripheral and central nervous systems.

Regarding pain, pain serves highly adaptive, survival functions under normal circumstances. It protects the individual from harm from dangers in the environment and encourages recuperative behaviors in response to pain arising from within the body itself. However, pain can go wrong, such that pain becomes chronic and serves no adaptive, physiologically relevant function. Such pathological pain destroys lives, afflicting an estimated one-sixth of the world's population [21].

Neuropathic pain is a form of pathological pain that arises from trauma, inflammation and/or infection of peripheral nerves. Here, sensations from the affected body region are grossly

^{*}To whom correspondence should be addressed: Department of Psychology, Muenzinger Psychology Bldg, Rm D-244, Campus Box 345, University of Colorado at Boulder, Boulder, CO 80309-0345, PH: 303-492-7034, FAX: 303-492-2967, Email: Linda.watkins@colorado.edu.

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abnormal. Environmental stimuli that would never normally be perceived as pain now are, and environmental stimuli that are normally perceived as painful now elicit amplified perceptions of pain. In addition, environmental stimuli may evoke abnormal perceptions of electric tingling or shocks (paraesthesias) and/or sensations having unusually unpleasant qualities (dysesthesias). Lastly, spontaneous pain frequently occurs with varying qualities and from varying perceived body locations.

Why neuropathic pain occurs is a dilemma, as is how to successfully treat such pain. Over the past several decades, animal models of inflammatory and traumatic neuropathy have revealed a remarkable degree of plasticity in sensory nerves, sensory nerve somas, and spinal cord. For example, damaged peripheral nerve fibers may develop spontaneous activity arising not only from its peripheral nerve terminals but also from the site of axonal damage as well as from the neuronal cell bodies far from the injury site. Damaged nerves may also alter their expression of receptors along the axon, now becoming increasingly responsive to pain-inducing substances and even to substances to which sensory neurons are normally unresponsive. In addition, neurons that normally do not signal pain may alter their gene expression such that they now produce “pain” neurotransmitters. Spinal cord “pain” responsive neurons show equally remarkable plasticity along similar lines. While space does not allow more than this brief introduction to neural plasticity associated with neuropathic pain, readers are referred to several excellent reviews [17,20,21,257,274].

Based on such documented changes in neuronal function under conditions of neuropathic pain, a variety of drugs have been prescribed in hopes of controlling such pain. By-and-large these drugs fail in the great majority of patients when used to treat clinical pain, including anticonvulsants [142], antidepressants [143], opioids [104], gabapentin [202], pregabalin [48] and others. Some drugs work partially in some patients. But even when combinations of drugs are administered that target different putative causes of neuropathic pain, they fail [201]. The question naturally arises as to why current therapies fail to control neuropathic pain, given that their development and use were predicated on the results from animal models of neuropathic pain, described above. Were the results obtained from such animal models that documented neuropathy-induced changes in neuronal function simply wrong? Alternatively, might there be a critical additional mediator for the creation and maintenance of neuropathic pain?

A potentially key, recent discovery is the role of the immune system in pathological pain states, including neuropathic pain. Within the last decade, research has accrued at an ever-accelerating rate that supports the idea that immune cells in and around peripheral nerves, and immune-like glial cells in spinal cord, are key players in both the creation and maintenance of pathological pain states. Within just the past five years, this concept has taken a second leap forward with the recognition that these same immune-like glial cells compromise the efficacy of opioids for pain control.

The purpose of this review is to explore these issues, with a focus on publications within the past 5 years. We first focus on sensory nerves and then on sensory nerve somas as the targets of immune actions that enhance pain. The discussion then moves centrally to explore the role of immune-like glial cells both in pathological pain as well as in dysregulating the actions of analgesic drugs such as opioids. The basic conclusion will be that immune and glial activation can have profound effects on neuronal responses to pain and opioids, such that pain signaling is amplified and opioid efficacy is diminished as a consequence of inflammatory mediators released by these non-neuronal cells.

I. Immune activation as a driving force for pathological pain states

A. Localized immune activation associated with peripheral nerve injury and inflammation

1. Immunology of peripheral nerves—In healthy peripheral nerves, most of the ongoing immune surveillance is accomplished by immune cells that reside within the nerve itself [148,155]. Resident immunocompetent cells include Schwann cells, fibroblasts, endothelial cells, dendritic cells, macrophages and mast cells. Here, immunocompetent refers to cells that can respond to inflammation, infection and/or trauma by the production and release of inflammatory mediators classically thought of as immune-derived. In healthy nerves, these cells are “resting” in the sense that they provide active surveillance of the nerve's microenvironment but are not releasing proinflammatory mediators as they do upon activation. With the exception of circulating activated T lymphocytes [70], blood-borne immune cells have relatively limited access to peripheral nerves under normal circumstances, due to the blood-nerve barrier [170].

This scenario changes dramatically upon trauma to, and inflammation of, peripheral nerves. Upon activation by nerve trauma or inflammation, a number of these immune cells release chemoattractant cytokines called “chemokines” (e.g. macrophage inflammatory protein-2 and monocyte chemoattractant protein-1 [MCP-1]) that recruit neutrophils and macrophages from the circulation into nerve and proinflammatory cytokines, as well as nitric oxide (NO) and reactive oxygen species (ROS) that kill invading microorganisms. In addition to these useful functions, these proinflammatory chemokines and cytokines, NO, and ROS can unfortunately also directly increase nerve excitability, damage myelin, and disrupt the blood nerve barrier, thus further facilitating the movement of immune products into damaged nerve. In addition, some activated resident immune cells release degradative enzymes and acids in response to nerve trauma that exposes peripheral nerve proteins (e.g. P0, P2). Nerve proteins such as P0 and P2 are responded to as “non-self” as they are normally buried within the myelin sheath and not detected by immune cells [79]. Once released, immune derived enzymes and acids attack myelin and disrupt the blood-nerve barrier, again allowing increased access of the nerve to blood-borne immune cells [170].

Clinically relevant peripheral nerve damage can occur as a result of antibody attack, complement activation, and T-lymphocyte attack, in addition to frank trauma and inflammation (for detailed review and clinical correlations see [248]). However, given space constraints, discussion below will be limited to trauma-induced nerve damage and its associated inflammatory components. This is because these have been the focus of the most laboratory animal research to date. This focus also arises, in part, from the clinical findings that nerve biopsies from both traumatic and inflammatory neuropathies indicate that nerve biopsy levels of proinflammatory cytokines directly correlate with both the degree of axonal degeneration and neuropathic pain [119].

2. Peripheral nerve changes in response to trauma—The possibility that immune cells are involved in the development of neuropathic pain arose in the mid-1990's [50,204]. Research that followed provided convincing evidence that immune cells activated as a result of partial nerve injury importantly contribute to the resultant exaggerated pain state as well as axonal hyperexcitability and Wallerian degeneration [148,155]. Nerve injury leads to an immediate activation of calpain, a calcium activated protease in Schwann cells and associated myelin [248]. Calpain, in addition to destroying myelin, causes a rapid burst of proinflammatory cytokine and chemokine release from injured Schwann cells [236] that, in turn, leads to the recruitment of circulating neutrophils and macrophages to the injury site and further proinflammatory cytokine and chemokine release [195,207,210]. Driven by TNF and IL-1, the extracellular protease matrix metalloproteinase-9 (MMP-9) is induced in Schwann cells and macrophages, contributing to neuropathic pain, neurovascular permeability, immune

cell recruitment, demyelination and degeneration [25]. The anti-inflammatory cytokine IL-10 also rapidly falls after injury, further disrupting the balance between pro- and anti-inflammatory cytokine influences [53].

Both trauma-induced Wallerian degeneration [14] and enhanced pain [155,210] have been associated with the activity of macrophages recruited to the site of injury. Simply delaying macrophage recruitment to the site of nerve injury delays both the development of neuropathic pain and Wallerian degeneration [124,156]. Indeed, encasing a transected nerve in silastic tubing to reduce contact with recruited immune cells and their products reduces pain-like behavior [169]. Conversely, actively attracting macrophages to the injury site enhances neuropathic pain [137]. Notably, activated macrophages have been found to persist for years within human painful neuromas, suggestive that they may perseveratively influence pain from peripheral nerve damage [45].

The proinflammatory cytokines (TNF, IL-1, IL-6) appear to be the key immune-derived factors as they increase at the site of trauma in parallel with pain enhancement [35,168], being produced by macrophages as well as other resident immune and recruited immune cells. Injection of proinflammatory cytokines onto or into peripheral nerves enhances pain responsivity [209, 268]. Blockade of proinflammatory cytokine actions at the level of the sciatic nerve reduces neuropathic pain, as well as reducing immune cell recruitment and demyelination [129,195, 205,206]. Treatments, such as thalidomide which concomitantly decreases the proinflammatory cytokine TNF and increases the anti-inflammatory cytokine IL-10, prevent neuropathic pain [54]. Furthermore, neuropathic pain is prevented in IL-6 knock-out mice [185] and the magnitude of neuropathic pain correlates with both the number of activated macrophages and the number of IL-6-producing cells at the site of injury [35]. As: (a) the effects of proinflammatory cytokines synergize, and (b) each increases the production and release of the others, it is to be expected that the family of proinflammatory cytokines, rather than simply one member, is importantly involved [248].

Recent data have also provided evidence that, in addition to macrophages, mast cells, T lymphocytes, neutrophils, and Schwann cells all contribute to neuropathic pain at the site of nerve injury [148,210]. Also, products produced by immune cells beyond proinflammatory cytokines have now been implicated, including ROS, NO, growth factors, prostaglandins, activation of the complement cascade, and mast cell products [123,128,221,231]. As one example, mast cells release tryptase, which activates protease-activated receptor-2. This tryptase receptor modulates the function of transient receptor potential vanilloid-1 (TRPV1) channels, lowering their threshold for activation from 42 °C to well below body temperature [37,148]. As functional TRPV1 channels are expressed mid-axon, this provides a mechanism whereby mast cell degranulation could directly increase spontaneous activity and excitability of sensory neurons. This may have clinical relevance as mast cells remain elevated at sites of nerve trauma long after injury and mast cell degranulation/release has been proposed to contribute to neuroma pain in humans [210].

Further support for the importance of immune cells in neuropathic pain comes from the study of clonidine, an α_2 -adrenoceptor agonist. While α_2 adrenoceptors are absent from normal nerves, levels elevate in injured nerve due to expression by recruited macrophages, lymphocytes and other immune cells. Peri-sciatic clonidine at the site of nerve injury both prevents and reverses neuropathic pain [111,189]. This effect is associated with a reduction of IL-1 and TNF in sciatic nerve as well as with an elevation of the anti-inflammatory cytokine TGF- β 1 [111].

3. Peripheral nerve changes in response to inflammation—It is important to distinguish the roles of inflammation versus trauma in neuropathic pain. This is important

because: (a) nerve trauma produces both frank injury as well as inflammation, making the relative contributions of these 2 factors entangled when studying trauma models, and (b) approximately half of clinical neuropathies involve infection/inflammation in the absence of frank trauma [190]. Hence, inflammatory regulation of nerve function in the absence of traumatic injury is of both theoretical and clinical interest.

The first evidence from animal models that the manipulation of a nerve in the absence of frank trauma can produce pain enhancement came from the demonstration that simply placing immunologically activated immune cells near healthy seaslug (*Aplysia*) sensory nerves increases their excitability [30]. Later studies in rats reported that placing gut suture [137], killed bacteria [46], algae protein (carrageenan) [46], yeast cell walls (zymosan) [22] or viral components [69] near healthy sciatic nerves produces enhanced pain responses. Such changes in pain responsivity are mimicked by proinflammatory cytokines [22]. For example, TNF injected into the sciatic nerve enhances pain [209] and induces ectopic activity in single primary afferent “pain”-receptive fibers [212]. Such effects are not restricted to proinflammatory cytokines as ATP, ROS, complement activation, and phospholipase A2 similarly increase pain behaviors and peripheral nerve excitability [23,90,235,239].

As observed in studies of nerve trauma (see above), peri-sciatic clonidine also reduced pain enhancement induced by sciatic inflammation. In addition, it prevented peri-sciatic zymosan induced immune cell recruitment to the site and consequent elevations in proinflammatory cytokines [189]. Thus, both inflammatory as well as traumatic neuropathy models support that clonidine may be useful in controlling pain in such circumstances.

4. Effects of immune-derived substances on peripheral nerve—It is now clear that proinflammatory mediators can enhance peripheral nerve excitability and consequent pain behaviors. For illustrative purposes, proinflammatory cytokines will be considered here.

While proinflammatory cytokine receptors are expressed on DRG cell bodies and peripheral terminals in skin, whether these receptors are expressed along the course of the peripheral nerve fibers has, with rare exception [200], never been investigated. As other receptors, such as those responsive to ATP and capsaicin, are now recognized to be functionally expressed along axons [65,90,147], it is feasible that receptors for proinflammatory substances may be found to exist along the length of axons as well. This would be in keeping with the rapid increases in neuronal excitation observed for TNF, suggestive of receptor-mediated, direct effects on axons [212].

Support for this idea comes from the observation that TNF is retrogradely transported from the site of nerve injury to DRG somas of both injured as well as adjacent spared axons [192,200]. Intriguingly, this retrogradely transported TNF is bound to TNF receptors. This suggests that TNF internalization and transport occurred following TNF binding to its receptors expressed along the axon [200]. Thus TNF may not only directly enhance axonal excitability at the site of nerve injury but, in addition, may produce distant changes in neuronal function as well. Such receptor-mediated retrograde transport is thought to regulate gene expression and exert autocrine/paracrine actions within the DRG (see below). This provides one potential mechanism for the increased neuronal excitability of spared sensory neurons that is known to occur as a result of nearby neuroinflammation [55].

In addition to receptor-mediated effects, studies of the structure of TNF suggest that it may be able to trimerize and insert itself into lipid membranes, forming cation-permeable pores [96]. This effect is enhanced under conditions of lowered pH, as occurs at sites of inflammation [8] and is a common theme by which the immune system seeks to destroy cells under pathological conditions. In addition, TNF, as well as other proinflammatory cytokines, may

alter the functioning of endogenous sodium and calcium channels, thereby increasing membrane conductance [179,238,256].

Lastly, TNF causes demyelination of peripheral nerves, at least in part via TNF-induced release of extracellular proteases, such as MMP-9 [25]. Such demyelination of injured sensory nerve fibers induces active remodeling of the exposed axonal membrane. Remodeling leads to the insertion of sodium channels into exposed membrane, a process normally inhibited by the presence of myelin. Such *de novo* expression of sodium channels in demyelinated nerve is associated with ectopic action potentials and neuropathic pain [41].

Thus, taken together, there are multiple mechanisms by which immune products can alter neuronal function, both by receptor mediated and receptor independent events.

B. Localized immune and glial activation impacts the function of sensory neurons in dorsal root ganglia (DRG)

1. Immunology of DRGs—Under normal conditions, DRGs are comprised of sensory neuronal cell bodies and their proximal processes (including their enwrapping Schwann cells), clusters of glially derived satellite cells which closely envelop each neuronal cell body, dendritic cells, macrophages that are in close contact with neuron/satellite cell complexes, and dense networks of endothelial cells that lack a blood-brain or blood-nerve barrier [62,170]. Thus, other than neuronal cell bodies, all of the constituents of DRGs are immune or immune-like cells in that each is known to be capable of producing proinflammatory cytokines and other substances classically considered as “immune-derived” [248].

Of these cell types, perhaps the most mysterious are the satellite cells. Satellite cells have only recently begun to be investigated for their potential roles in regulating pain [62]. What is known is that satellite cells are similar to astrocytes in that they upregulate glial fibrillary acidic protein (GFAP) upon activation and that activated satellite cells express and release an array of neuroexcitatory substances, including proinflammatory cytokines [223]. The presence of satellite cells enhances the response of DRG neurons to inflammatory mediators [67] and activated satellite cells increase the neuroexcitability of DRG neurons, at least in part, via the release of IL-1 [223]. In addition, satellite cell P2X7 ATP receptors are upregulated in DRGs of humans with chronic neuropathic pain [27], suggestive that satellite cells have enhanced responsivity to ATP released by either damaged sensory neurons or by resident or recruited immune cells. Satellite cells can also regulate extracellular levels of excitatory amino acids, given their expression of the glial glutamate transporter GLAST [11]. Lastly, they appear to affect neuronal excitability via gap junctions, which form a rapid, non-synaptic means of cell-to-cell communication [62]. DRG gap junctions dramatically increase both between adjoining satellite cells and between satellite and neuronal cells, in response to peripheral inflammation or peripheral neuropathy. These changes suggest that, after peripheral inflammation, satellite cells may now have increased regulation of neuronal excitability [26,44]. Indeed, disruption of gap junction communication, using a peripherally restricted gap junction inhibitor, prevents pain enhancement from peripheral inflammation [44].

2. DRG changes in response to herniated discs—Alterations in DRG function are relevant to pathological pain arising both from peripheral nerve inflammation/trauma (see below), as well as from inflammation of the DRG itself, such as occurs in response to herniated discs. DRG changes induced by herniated discs have been a focus of study, given that back pain from herniated discs is a common source of severe pain. Acute mechanical compression of the DRG is sufficient to produce spontaneous activity in sensory afferents and to upregulate DRG mRNA and protein levels of proinflammatory cytokines (e.g., TNF, IL-1, IL-6), proinflammatory chemokines (e.g., MCP-1) as well as other proinflammatory products [98, 253,264]. In addition to the direct effects of DRG mechanical compression, herniated discs are

perceived by the immune system as “foreign” and so induce a localized inflammatory response [43]. These herniated disc-stimulated, DRG-derived proinflammatory mediators are implicated in pain enhancement [74,245]. Indeed, proinflammatory cytokines, proinflammatory chemokines, and “inflammatory soup” (bradykinin, serotonin, PGE2 and histamine) each enhance excitability of DRG neurons in normal rats, an effect that is greatly increased in neurons from rats with chronically compressed DRGs [121,127,253,270]. Correlated with this increase in neuronal excitability, TNF, IL-1 and IL-6 applied to DRG neurons *in vitro* increases the release “pain” transmitters (e.g., substance P, CGRP) [163,164,171], suggesting that proinflammatory cytokine induction in DRG may enhance pain by enhancing sensory neuron release of “pain” transmitters in spinal cord. Behaviorally, exposure of the DRG to nucleus pulposus (the shock absorbing central part of vertebral discs subject to herniation) produces pain enhancement, which is greater when combined with DRG compression [101]. Indeed, such pain enhancement directly correlates with elevations in DRG-derived proinflammatory mediators noted above [98].

In addition to DRG sources of proinflammatory mediators, there is growing evidence that immune factors derived from the herniated disc itself are involved, as well. Herniated discs are sources of a variety of neuroexcitatory substances, including NO, TNF, IL-1, IL-6, chemokines, prostaglandins, thromboxane, leukotrienes and phospholipase A2. This profile of proinflammatory products has led to the suggestion that these herniated disc-derived substances may be an additional major factor in the creation of back pain [2,19]. Indeed, enhanced pain induced by exposure of DRG to nucleus pulposus is reduced by blockade of proinflammatory factors, including TNF, thromboxane, leukotrienes, and phospholipase A2 [99,100]. Furthermore, DRG exposure to nucleus pulposus or other inflammatory mediators (e.g., IL-1, TNF) leads to spontaneous electrical activity in sensory afferents and enhanced pain responses, effects which are reduced by appropriate inhibitors [34,152,164,174,264].

3. DRG changes after peripheral nerve injury—Alterations in DRG anatomy and function occur in response to peripheral nerve injury, as well. Responses of DRG neurons are sensitized following nerve injury such that, in injured DRG, very low TNF doses produce faster, greater, and longer lasting activity in DRG neurons than observed for DRG neurons with healthy axons [193]. In addition, peripheral nerve injury distant from the DRG causes immune cells (e.g. macrophages, T-cells) to be recruited into the DRG [77,78,177] and DRG glial satellite cells to proliferate and become activated [177,210,264] (see also Section B.1, above). Such changes in DRGs are observed not only after traumatic injury to peripheral nerves, but after other pain-enhancing manipulations, as well. These include chemotherapy induced neuropathies [91], bone cancer [176], exposure of DRGs to nucleus pulposus [153,162], spinal transection [139], and ventral (motor) root transection which exposes intact sensory neurons to Wallerian degeneration of inter-mingled transected motor axons [117]. Thus, satellite cell activation and immune cell recruitment into DRG are responses common to a broad range of manipulations that enhance pain.

Invasion of the DRG by immune cells after nerve injury is thought to have functional consequences. One observation in support of this is that most macrophages recruited into DRGs after peripheral nerve injury concentrate around neurons with damaged axons [77]. Macrophages recruited to DRG after nerve injury express at least PGE2, IL-6 and CGRP [130]. These inflammatory mediators facilitate spontaneous ectopic activity in neurons and amplify their responsivity to nociceptive stimuli [130]. Similarly, depletion of circulating immune cells so to prevent their recruitment into DRG inhibits the development of hyperalgesia in response to DRG exposure to nucleus pulposus [102].

While it is clear that DRG anatomy and function are altered in response to peripheral nerve injury, it is not as yet clear why such changes occur. One hypothesis is that DRG alters its gene

expression due to signals retrogradely transported from the site of peripheral nerve injury [155] (also, see above). For example, following peripheral nerve transection, about one third of DRG neurons express IL-6 mRNA. While blocking retrograde transport by injection of colchicine into an otherwise healthy sciatic nerve did not induce IL-6 mRNA in DRG, injection of colchicine into the nerve stump after transection prevented DRG IL-6 mRNA induction [154]. This led to the conclusion that IL-6 is induced by an injury factor in the nerve stump. Immune products released at the site of nerve injury are obvious candidates. For example, mast cell degranulation (which releases a host of inflammatory mediators) in intact sciatic nerve was sufficient to stimulate IL-6 mRNA induction in DRG and, correspondingly, administration of mast cell stabilizing agents decreased IL-6 mRNA induction in DRG after sciatic injury [154]. While the identity of such retrograde signals is the subject of ongoing investigation, TNF, leukemia inhibitory factor (LIF), IL-6, and nerve growth factor (NGF) retrogradely transported from the site of peripheral nerve injury to DRG have each been posited as such signals [155].

Intriguingly, it would appear that retrograde transport occurs in intact nerves as well as damaged ones as DRG neurons with healthy axons show altered gene expression quite similar to those of damaged neurons [21]. As noted above, retrograde transport of such signals and resultant alterations in DRG function may contribute to neuropathy induced enhanced pain responses of healthy, in addition to damaged, sensory neurons [55].

An additional issue is how peripheral nerve injury leads to the: (a) activation of DRG glia (satellite cells), (b) activation of other non-neuronal DRG cells (e.g., dendritic cells, macrophages), and (c) recruitment and activation of blood-borne immune cells (e.g., macrophages, T-lymphocytes, neutrophils) [140]. Neuronally-derived NO is one candidate that has been proposed to account for such effects. DRG neuronal NO synthase is upregulated in response to both peripheral nerve injury and inflammation, and NO can activate satellite cells [44]. In addition, injury induced upregulation of proinflammatory cytokines in DRG somas has been postulated as serving an autocrine/paracrine role serving chemoattractant and activating signals for immune and glial cells [32]. Injury also upregulates proinflammatory chemokines such as MCP-1, first in neurons and later by satellite cells, which acts as a chemoattractant for immune cell influx as well as enhanced pain [140]. Thus multiple immune-derived signals in DRG may well enhance pain.

In sum, satellite glial cells, as well as resident and recruited immune cells, are well-positioned to alter the excitability of both damaged and healthy DRG neurons.

C. Peripheral nerve injury leads to glial activation in spinal cord

1. Communication from neurons to glia—Following inflammation or trauma of peripheral tissues or peripheral nerves, microglial and astrocytic activation occurs at a distance; that is, within (at least) the spinal cord. As will be discussed below, this glial activation drives pain amplification. This link between glial activation and pain enhancement was first recognized in the early-to-mid-1990's [51,52,144,251]. Studies that followed substantiated, using diverse animal models and endpoints, that spinal cord glia are key players in the creation and maintenance of enhanced pain states, including neuropathic pain [249].

However, the fact that glia within the spinal cord become activated as a consequence of inflammation/damage in the periphery raises a fundamental question. The central issue is simple: how these spinal cord glia “know” to become activated following nerve injury that has occurred far from the spinal cord? While this phenomenon was discovered ~30 years ago in a context independent of pain [109], the underlying mechanisms are only now being clarified.

The signal has to come from either neurons or humoral factors. Indeed, a number of neuron-to-glia signals that “trigger” glial activation have recently been identified. Most of this work has supported a key role of microglia as exquisite sensors of “not self” or “not normal”, leading them to be more highly responsive to CNS challenges than other cell types [108]. Microglia are also frequently the earliest glial cell type to be activated in response to peripheral inflammation and injury [115,181]. While the current literature suggests that, by-and-large, microglia are activated first and their activation, in turn, leads to the activation of astrocytes [227], it is already clear that astrocytes can also be initiators of pathological pain under certain conditions [211].

The best documented of the neuron-to-glia signals are briefly reviewed below.

2. Neuronal transmitters and modulators—Sensory neurons release an array of neurotransmitters in spinal cord upon their activation by noxious stimuli. These include substance P, excitatory amino acids, and ATP. Spinal cord astrocytes become activated by each of these transmitters via binding to NK-1 receptors, metabotropic glutamate receptors, ionotropic non-NMDA receptors (AMPA and kainite), as well as NMDA receptors [3,12]. Indeed, glia may be maximally responsive under conditions where more than one activation signal is received. For example, spinal cord astrocyte release of ATP is far greater when stimulated by glutamate plus substance P, than by either transmitter alone [252]. Receptor activation, in turn, leads to the activation of microglia and astrocytes and their consequent release of neuroexcitatory substances, including prostaglandins, IL-1, IL-6 and NO [217,218,232].

In addition, there is growing evidence that microglia can enhance pain following their activation by extracellular ATP. Microglia are exquisitely responsive to extracellular ATP, whether released by cellular damage (see below), nearby astrocytes, or neurons from either synaptic or non-synaptic regions [39,42].

Two disparate lines of evidence implicate ATP in pain enhancement, but via quite different underlying mechanisms. One line of evidence argues that microglial P2X4 receptor activation by ATP is central to enhanced pain states. These studies document that P2X4 receptors are strongly upregulated in microglia in spinal cord dorsal horn in response to peripheral nerve injury, an effect driven by fibronectin [10,87]. Treatment with P2X4 anti-sense reduces neuropathic pain, thereby implicating P2X4 signaling in such pain changes [10]. Furthermore, perispinal (intrathecal; into the cerebrospinal fluid surrounding the spinal cord) injection of microglia activated by ATP *in vitro* is sufficient to enhance pain [234] and does so through the release of brain derived neurotrophic factor (BDNF) [233]. In dorsal spinal cord, microglial BDNF release causes neuroexcitation by decreasing GABA-ergic and glycinergic inhibition [233].

Beyond P2X4, additional lines of evidence implicate microglial P2X7 and P2Y receptors in pain enhancement. Activation of these P2X7 receptors leads, in turn, to activation of p38 MAP kinase and to rapid release of IL-1, TNF, and superoxide [87,198,272]. Indeed, P2X7 activation by ATP has been argued to be the most potent stimulus for the release of IL-1 [42]. P2X7 knockout mice show suppressed inflammatory pain and neuropathic pain while retaining normal responses to pain under basal conditions [76]. In addition, rats treated with selective P2X7 inhibitors show reduced inflammatory pain and neuropathic pain, effects that were consistent across multiple animal models [76]. Such effects were evident at both behavioral and spinal cord electrophysiological levels of analysis. Regarding P2Y, this receptor has been linked to microglial activation and enhanced pain in response to peripheral inflammation [66,263].

In addition to the neurotransmitters noted above, neurons also upregulate their release of NO, prostaglandins, and dynorphin under conditions where pain facilitation occurs. Neuronal NO enhances spinal production and release of TNF, IL-1 and IL-6 [71] and PGE2 is important in the initiation of both glial activation and neuropathic pain [222]. Lastly, the pain enhancing effects of neuronally-derived spinal dynorphin have recently been linked to selective activation of microglial p38 MAP kinase which, in turn, leads to the release of PGE2 and IL-1 that enhances pain [110,217].

3. Neuronal chemokines—Chemokines are *chemoattractant cytokines*, consisting of a family of over 50 proteins. While initially thought to be solely of immune origin, it is now clear that neurons can produce and release a number of chemokines as well [216].

Fractalkine was the first chemokine discovered to be a neuron-to-glia signal. It is an unusual chemokine as it is tethered to the extracellular surface of neurons and is released upon strong neuronal activation, forming a diffusible signal [24]. Indeed, neuropathy induces a dramatic reduction in membrane bound fractalkine in DRG and spinal cord neurons, suggestive of release [273]. In spinal cord, the receptor for neuronal fractalkine is expressed only by microglia and its expression in microglia is upregulated under neuropathic pain conditions as well as by arthritis [120,196,242,273]. Intrathecal administration of fractalkine enhances pain, via activation of microglial p38 MAP kinase and the release of proinflammatory cytokines [145, 146,273]. Importantly, endogenous fractalkine also enhances pain, as neutralizing antibodies directed against the fractalkine receptor both delay and reverse the development of neuropathic pain and arthritis pain [145,146,196]. In agreement with such behavioral results, electrophysiological studies reveal that fractalkine causes hyper-responsivity of spinal neurons to brush and pinch, as well as increases in the numbers of neurons exhibiting prolonged after-discharges indicative of spontaneous pain and central sensitization [173]. Fractalkine-induced increases in neuronal excitability occurred after a delay, a result supportive of an indirect action of fractalkine on spinal neurons [273].

While fractalkine was the first chemokine implicated as a neuron-to-glia signal, it is not alone. Growing evidence implicates chemokines such as interferon-inducible protein of 10 kD (IP-10) and monocyte chemoattractant protein-1 (MCP-1) as signals that are rapidly induced in, and released from, damaged neurons [15,186]. Indeed, neurons also package and transport such chemokines to distant presynaptic terminals. This allows for remote activation of glia which express receptors for these chemokines, as occurs in spinal cord following peripheral nerve injury [269]. That such chemokines are involved in pain facilitation is supported by the observations that intrathecal injection of MCP-1 produces pain facilitation [225] and intrathecal administration of neutralizing antibodies to MCP-1 suppresses neuropathic pain [1].

4. Signaling molecules released by damaged, dying, and dead neurons—Neurons release a variety of substance that signal their damage and death. Such signals are released in spinal cord following trauma to peripheral nerves, dorsal root ganglia, or spinal cord [33]. As the clearance of myelin and other cellular debris is a remarkably protracted process continuing for many years in the human CNS compared to the periphery [241], substances released by injured neurons under such conditions may be able to provide ongoing stimulation to maintain equally protracted glial activation. In addition, there are reports of neurons in dorsal spinal cord dying secondary to peripheral nerve injury [194]. ATP is one major substance released by damaged and dying cells and has been argued to serve as an intrinsic “danger signal”, triggering the activation of nearby microglia [42]. Detection of extracellular ATP causes rapid convergence of microglial processes toward its source [39] and the release of plasminogen, a protein which enhances NMDA receptor function [88]. As ATP is also released as a neurotransmitter in response to painful stimuli, its involvement in glial activation has already been reviewed, above.

Other candidate neuron-to-glia signals include ligands for pattern recognition receptors, such as toll-like receptors (TLRs), that are expressed by glia and enables them to detect danger signals [226]. Such danger signals include substances released by cellular injury (cell membrane components such as gangliosides and lysophosphatidic acid (LPA), nuclear components such as high mobility group box 1 (HMGB1), heat shock proteins) as well as more traditionally recognized danger signals such as conserved motifs expressed by bacteria or viruses [95,175]. In response to nerve injury, TLR1, TLR2, and TLR4 have each been reported to be upregulated in CNS, linked to the production of proinflammatory cytokines (e.g. TNF, IL-1) and chemokines (e.g. MCP-1) [172]. To date, several of these damage/death signals have been examined behaviorally and found to enhance pain following intrathecal administration, including ATP, HMGB1, and LPA [89,160].

From above it is clear that there are numerous ways for neurons to trigger the activation of microglia and astrocytes in spinal cord. As will be reviewed below, this glial activation can drive pain amplification.

D. Glial activation impacts the function of pain-responsive neurons in spinal cord

1. Functions glia under basal and activated states—In normal, healthy CNS, microglia and astrocytes serve a variety of important functions. For microglia, this is primarily an active surveillance role, rapidly extending and retracting processes to constantly sample the extracellular microenvironment [184]. Astrocytes, on the other hand, are primarily involved in providing energy sources and neurotransmitter precursors to neurons, cleaning up debris, regulating extracellular levels of ions and neurotransmitters, and actively modulating synaptic transmission [64].

Under basal conditions, microglia and astrocytes do not appear to be important regulators of pain transmission. This conclusion is based on the fact that various drugs that suppress glial function or block the actions of various glial products (such as proinflammatory cytokines) do not alter responses to heat or mechanical stimuli in normal animals [63,115,144].

The roles of both microglia and astrocytes dramatically change when they are triggered to leave their basal condition and enter an activated state. In response to inflammation or damage to peripheral tissues, peripheral nerves, spinal nerves or spinal cord, both microglia and astrocytes in spinal cord become activated [141,249]. Their activation is inferred by upregulation of cell type specific activation markers, such as GFAP in astrocytes and complement receptors for microglia. Their activation is also inferred by the fact that, as discussed below, intrathecal administration of glial inhibitors resolves enhanced pain responses induced by all of the same models that induce glial activation (Table 1,2). To date, fluorocitrate and minocycline have been the drugs most commonly tested in this regard. Fluorocitrate inhibits aconitase, an enzyme in the Krebs energy cycle of astrocytes and microglia, but not neurons. Minocycline, on the other hand, inhibits microglia but not astrocytes or neurons. This difference in the mechanism of action of these drugs, along with explorations of the timecourse of expression of microglial vs. astrocytic activation markers after a manipulation that enhances pain, has allowed the relative roles of microglia and astrocytes to be explored. Typically, but not exclusively [161], microglia are observed to become activated first, followed after a delay by astrocyte activation [227]. The importance of microglia is generally thought to diminish, whereas the importance of astrocytes is generally thought to increase, over time as, quite strikingly, minocycline prevents the development of pathological pain in response to diverse manipulations yet fails to reverse enhanced pain responses once they have developed [115,181]. However, a perseverative role for microglia has recently been reported [61,230], suggestive that this issue warrants further study.

2. Mechanisms whereby glial activation affects neuronal activity—Diverse enhanced pain states are characterized by spinal cord glial activation and are suppressed by inhibitors that suppress astrocyte and/or microglial function [249]. Indeed, only one pain model to date fails to show evidence of glial involvement for as yet undefined reasons; that is, intramuscular acidic saline that induces bilateral allodynia [114] (Table 1,2). Studies aimed at identifying what glial products may modulate pain have implicated a host of neuroexcitatory substances that can be released by activated glia, including proinflammatory cytokines and chemokines, NO, ROS, ATP, prostaglandins, excitatory amino acids. Astrocytes, and recently microglia [136], have also been documented as sources of D-serine, an endogenous ligand for the glycine modulatory site on NMDA receptors. D-serine is released in spinal cord in response to peripheral inflammation, causing enhanced C-fiber mediated excitation of pain-responsive neurons [59]. In addition, glia can increase neuronal excitability and pain via downregulation of glial glutamate transporters in spinal cord as a consequence of peripheral nerve injury [16, 228].

While the mechanisms by which most glially-derived substances enhance neuronal excitability are obvious, the mechanisms underlying increased neuronal excitability by proinflammatory cytokines is only now becoming clear. What is evident is that their actions are not nearly as simple as merely binding of proinflammatory cytokines to known neuronal receptors [38,73, 166] so to induce rapid increases of neuronal excitability [167,187,191]. Several of the effects of proinflammatory cytokines on neurons have already been discussed above (see Section I.A. 4. Effects of immune-derived substances on peripheral nerves), so will not be repeated here. Beyond these, IL-1 has been shown, including in spinal dorsal horn, to enhance neuroexcitability via indirect actions; namely, by inducing the release of substance P from sensory afferents [150] and increasing calcium conductivity of neuronal NMDA receptors [191,243]. This latter effect occurs via intracellular signaling pathways leading to the phosphorylation of NMDA receptor subunits [244]. TNF increases the conductivity of glutamatergic AMPA receptors [40], increases the percentage of capsaicin-responsive cells in DRG [158], and potentiates inward currents in neuronal tetrodotoxin-resistant sodium channels [92]. TNF also increases spontaneous and evoked neurotransmitter release from presynaptic terminals [57,273]. TNF, and to a lesser extent, IL-1, upregulate the neuronal cell surface expression of both AMPA and NMDA receptors while downregulating cell surface expression of receptors for the inhibitory neurotransmitter, GABA. This pattern of changes would create an overall increase in neuronal excitatory tone [215]. Intriguingly, the AMPA receptors that are upregulated by TNF are quite unusual in that they are calcium permeable, suggesting that these AMPA receptors likely contribute to the production of neuronal NO and prostaglandins, furthering neuronal excitability [9]. Lastly, IL-1 is implicated in neuropathy-induced downregulation of G protein-coupled receptor kinase 2 (GRK2) expression in dorsal horn neurons, an action predicted to increase neuronal excitability by decreasing receptor desensitization [105].

Beyond these actions, proinflammatory cytokines lead to the release of a host of neuroexcitatory substances, including more proinflammatory cytokines, NO, ATP, prostaglandins, nerve growth factors, ROS, proinflammatory chemokines, excitatory amino acids, and BDNF [87,93,214,246]. For example, TNF stimulates the over-production and release of glutamate from microglia by upregulation of microglial glutaminase [224]. Proinflammatory cytokines can also indirectly elevate extracellular glutamate levels via downregulation of glial and neuronal glutamate transporters that serve to keep extracellular glutamate levels low under normal conditions [229]. Thus, taken together, proinflammatory cytokines exert multiple effects with the end result being neuroexcitation.

A last point worth noting here is that the effects of glial products, such as proinflammatory cytokines, on spinal cord dorsal horn neurons can depend on the presence or absence of ongoing

neuropathic pain. This is exemplified by recent work demonstrating that, in naïve rats, intrathecal TNF has no effect on spinal long-term potentiation (LTP) [126]. In contrast, in neuropathic rats, TNF receptors are upregulated in dorsal horn neurons and intrathecal TNF now induces spinal LTP. Also unlike spinal LTP in naïve rats, induction of spinal LTP by TNF in neuropathic rats involves JNK, p38 MAP kinase and NF-kappa B. Whether this alteration in the cascade reflects changes in neurons vs. glia is as yet unknown [126].

3. Beyond glia: Immunocompetent cells other than astrocytes and microglia warrant consideration—To date, virtually every spinal cord study of immune dysregulation of pain has focused on microglia and astrocytes. However, it is highly unlikely that these cells will prove to be the only non-neuronal cells involved in pain enhancement. Endothelial cells, fibroblasts, oligodendroglia, mast cells, dendritic cells, perivascular macrophages, recruited immune cells, and other cell types in both the spinal cord and overlying meninges can also produce many of the same neuroexcitatory substances as do astrocytes and microglia. It is simply an accident of evolution that astrocytes and microglia, unlike many other immunocompetent cells, have been discovered to readily upregulate cell-type specific mRNA and protein products in a graded fashion upon activation. These so-called activation markers has allowed for their activation to be easily quantified.

Recently, evidence has begun to accrue that cell types other than microglia and astrocytes are indeed responsive to conditions associated with pathological pain. For example, the meninges surrounding spinal cord have been demonstrated to increase TNF, IL-1, IL-6 and iNOS gene expression and to release TNF, IL-1 and IL-6 protein in response to immune activators such as HIV-1 gp120, administered intrathecally at a dose previously documented to enhance pain [254]. As these effects replicate *in vitro* in the absence of circulating white blood cells, these proinflammatory responses are being generated by cells intrinsic to the meninges rather than by immune cells recruited from the blood. Intriguingly, similar upregulation of proinflammatory products in the meninges occurs following peripheral nerve injury, implying that the meninges are responsive not only to inflammatory mediators within the CSF space but also to signals released by damaged sensory afferents [255].

In addition, circulating immune cells can be recruited into spinal cord after peripheral nerve injury, suggesting that immune products released by these cells may modulate pain as well. After lumbar spinal nerve lesion, marked leakage of albumin was observed by 2 weeks and persisted for over 2 months, suggestive of a defect of the blood-spinal cord barrier function localized to the spinal level innervated by the injured peripheral nerve [56]. L5 spinal nerve transection has also been reported to induce leukocyte trafficking into L5 spinal cord [219]. Here, trafficking was detected by identifying donor rat leukocytes that migrated into the spinal cord of host rats that had been previously bone-marrow irradiated, which prevents rejection of the transplanted immune cells. This approach revealed recruitment of cells with macrophage-like and T cell-like morphologies [219]. Using another approach, T-cell recruitment into the spinal cord was likewise documented after peripheral nerve injury (chronic constriction injury), but not sciatic nerve transection [77]. There was a marked concentration of these cells in the dorsal horn ipsilateral to the site of sciatic injury, co-localized with sites of microglial activation [77]. T-cell numbers remained elevated in superficial dorsal horns even 10 weeks after sciatic injury. That T-cells may contribute to neuropathic pain is supported by athymic nude rats, which lack T-lymphocytes, exhibit reduced neuropathic pain in response to chronic constriction injury [149]. Based on the observation that T-cell recruitment only occurred after sciatic nerve damage that spared sciatic axons (i.e. it occurred after chronic constriction injury but not after sciatic transection), an as yet unidentified retrograde signal has been postulated to be involved in T-cell recruitment to spinal cord [77].

From above it is clear that glial activation, and potentially recruitment and activation of other non-neuronal cells, can dramatically increase neuronal excitability and enhance pain. The effects of glially derived proinflammatory cytokines on neuronal excitability are only now becoming well understood, with diverse effects that extend well beyond simple increases in ion permeability upon cytokine binding.

4. Beyond spinal cord: Glial regulation of pain in the brain—The study of the involvement of brain glia in pain regulation is still in its infancy. Clarifying what role(s) glia play in brain processing of pain information is a very important and virtually unexplored topic. Within just the past year, it has become clear that glia in the medullary trigeminal nuclei regulate pain [60,178,265]. Beyond the first CNS synapse for sensory information, it seems highly likely that glia in the brain will be found to play important regulatory roles in pain enhancement. As will be reviewed in sections that follow, it is already clear that glia in the brain regulate many important functions; for example, responses to opioids such as morphine [80]. Furthermore, it would be anticipated that activation of brain glia would be a natural consequence of prolonged glial activation in spinal cord or trigeminal nuclei, such as occurs in response to neuropathy, spinal cord injury, multiple sclerosis, and other syndromes leading to chronic pain. This is based on prior reports that glial activation in one CNS region can lead to glial activation in projection regions [72,151]. Glial activation in brain subsequent to peripheral inflammation has been reported [183], but whether such glial activation is causal to, versus correlated with, inflammation-induced pain enhancement is as yet unknown. The possibility that peripheral inflammation-induced activation of brain glia may regulate pain has strong precedence, given the large literature on immune-to-brain communication having profound effects on brain functioning as a result of glial activation and proinflammatory cytokines, such as induction of an array of sickness responses including fever, alterations in food and water intake, activation of brain-to-spinal cord circuitry to enhance pain, induction of depressive-like behaviors, and so on [131,250]. Regarding pain, glia may be involved beyond basic sensory processing. For example, glia in the anterior cingulate cortex are now implicated in the induction of pain-related aversion via glial release of D-serine, the endogenous ligand of the glycine modulatory site on NMDA receptors [188].

II. Immune activation as a driving force for disrupting the efficacy of opioids

A. Opioid-induced glial activation undermines opioid-induced pain suppression

The mechanisms underlying neuropathic pain and morphine tolerance are strikingly similar [138]. Given this similarity, it was natural to also explore whether glia might impact the efficacy of opioids for pain control. As will be reviewed below, it is now clear from recent studies that glia do indeed compromise the ability of opioids to suppress pain [Watkins, 2005 #312; Hutchinson, 2007 #542].

Since the initial report in 2001 of a link between glia and morphine tolerance [208], evidence rapidly accumulated that chronic morphine: (a) activates both astrocytes and microglia [36, 180,208,220], (b) activates microglial p38 MAP kinase [125] and stimulates the production of spinal proinflammatory cytokines [94,180,220], both of which are associated with the development of morphine tolerance [125,182]. That glial activation was causal to, rather than simply correlated with, morphine tolerance was supported by the finding that morphine tolerance was slowed or reversed by either inhibition of spinal proinflammatory cytokines [94,180,197] or by knockout of IL-1 signaling [197]. An additional role of glia in morphine tolerance is suggested by the finding that morphine tolerance is associated with a downregulation of glial GLAST and GLT-1 glutamate transporters (the major transporters responsible for regulating extracellular levels of excitatory amino acids) in spinal cord dorsal horn, which concomitantly leads to an upregulation of extracellular excitatory amino acids [135,220]. Such data suggest that tolerance may, in part, be created by an opposing increase

in neuronal excitability due to glially induced elevations in glutamate and proinflammatory cytokines.

Importantly, a link back to neuropathic pain has also been made. What is known from clinical and laboratory animal studies is that morphine loses its efficacy in neuropathic pain patients and rats. That is, a state akin to tolerance prevails, despite the lack of prior opioid exposure, in other words “naïve opioid tolerance”. Importantly, spinal inhibition of proinflammatory cytokines abolishes morphine resistance in neuropathic animals. That is, in the presence of proinflammatory cytokine inhibition, the analgesic efficacy of even acute morphine was restored in rats with neuropathic pain [180]. This link has been strengthened by studies of “anti-analgesia”. Specifically, it has been demonstrated that prior sub-analgesic doses of morphine reduce subsequent analgesia produced by an analgesic dose of morphine. These effects are mediated by glial activation since propentofylline reinstates normal analgesia [262]. Moreover, p38 MAPK activation is also involved, again strengthening the ties to neuropathic pain. What remains unclear in this case is what glial products mediate these anti-analgesic responses.

In addition, while speculative, these data suggest a potential solution to what has come to be called “paradoxical” opioid-induced pain enhancement [103,134]. The observation in humans as well as laboratory animals is that repetitive opioid exposure leads to the development of abnormal sensitivity to pain. These effects were observed even when opioid exposure was kept constant over time in order to avoid “mini-withdrawals” between subsequent opioid doses [134,240]. This “paradoxical” opioid-induced pain enhancement was interpreted to mean that “prolonged opioid treatment not only results in a loss of opioid antinociceptive efficacy but also leads to activation of a pronociceptive system manifested as reduction of nociceptive thresholds” [134]. To date, evidence points to the involvement of NO, dynorphin, and glutamatergic NMDA receptors [134] [103]. Given: (a) the progressive activation of glia with repeated opioid exposure, (b) the evidence that dynorphin, NMDA, and NO all activate glia and induce the release of proinflammatory cytokines [71,110,125], exploring whether glial activation will solve the “paradox” of opioid-induced abnormal pain sensitivity would seem warranted for both theoretical and practical clinical reasons.

B. Opioid-induced glial activation contributes to opioid reward, dependence and withdrawal

In addition to morphine tolerance, evidence has now accrued that glia may be importantly involved in morphine dependence/withdrawal as well. In dependent populations continued exposure to opioids is required to avoid a withdrawal syndrome that would otherwise occur when opioid dosing ceases. The first indications that glia contribute to the development of morphine dependence came from the work of Raghavendra et al. [180,182] and Johnston et al. [94]. The endpoint measured in these studies was the pain enhancement that naturally occurs upon cessation of chronically administered opioids; that is, opioid withdrawal induced pain facilitation. These studies reported that withdrawal-induced pain enhancement is blocked by: (a) drugs or IL-10 (an *anti*-inflammatory cytokine) that block glial proinflammatory cytokine production, or (b) IL-1 receptor antagonist [94,180,182].

These data raised the question of whether glia may be involved in morphine phenomena mediated by the brain, in addition to spinal cord. To test for brain involvement in dependence/withdrawal, each dose of morphine in a multi-day regimen was co-administered with AV411 (ibudilast), a blood brain barrier permeable glial activation inhibitor. After the last drug dose, withdrawal was precipitated by administering an opioid antagonist. Rats that were not given the glial inhibitor exhibited robust brain mediated withdrawal signs over time, whereas rats whose glia were inhibited during morphine exposure did not [112,116]. Also, while this systemic morphine regimen upregulated astrocytic and microglial activation markers throughout the brain and spinal cord in morphine treated rats in the absence of AV411, AV411 maintained glial activation at near basal levels in morphine-treated rats [112,116]. In addition,

glia may contribute to morphine reward. Microinjection of media from activated astrocyte cultures into the nucleus accumbens or cingulate cortex increased morphine conditioned place preference an experimental measure of drug reward. In addition, *in vivo* administration of the glial modulatory drug propentofylline reduced morphine conditioned place preference [157]. Taken together, these data clearly suggest that glia, in addition to regulating pathological pain, opioid analgesia, and opioid tolerance, now should be considered as contributing to the phenomena of morphine reward and morphine dependence/withdrawal as well.

C. Opioid-induced glial activation opposes acute opioid analgesia: involvement of non-classical opioid receptors

It is also clear that, while the initial investigations reviewed above focused on the glial activating effects of chronic morphine, glia modulate the acute effects of opioid analgesia as well. Indeed, they do so in a surprising way.

Opioids (met- and leu-enkephalin) stimulate the release of IL-1 from microglial cultures [106] and blocking spinal proinflammatory cytokine increases the magnitude and duration of acute analgesia to morphine [94,197] and methadone [81]. Similarly, administration of a neutral dose of IL-1 (i.e., a low dose exerting no detectable effects on pain responsivity on its own) abolished, whereas knocking out IL-1 signaling potentiated and prolonged, morphine analgesia [197]. Intriguingly, administration of antagonists against TNF, IL-1 or IL-6 immediately upon apparent cessation of morphine analgesia rapidly “reinstates” analgesia suggesting that morphine-induced glial release of proinflammatory cytokines “masks” ongoing morphine analgesia by exerting an opposing effect [31,82,197]. Thus, IL-1 and other proinflammatory cytokines oppose the ability of acute morphine to suppress pain.

A point worth emphasizing here is that it has been *assumed* that morphine activates glia via classical opioid receptors. Based on the recent work of Hutchinson et al. which documented that opioids could alter peripheral immune function via *non*-classical receptors [83-85, 258-260], we have begun exploring the parallel issue for glia. Unlike neuronal opioid receptors, which are stereoselective, glial opioid receptors are not. Indeed, neuronally-inactive [+-]methadone upregulates mRNA for IL-1, TNF and IL-6 to at least as great a degree as does [-]-morphine and [-]-methadone upon their intrathecal administration [31]. This set of findings suggests that *different receptors* must mediate the (classical) pain suppressive effects of morphine than its (non-classical) pain enhancing effects. The clinical implications of this difference are enormous as it predicts that it should be possible to separate the neuronally mediated pain suppressive effects of opioids from their glial activating, pain enhancing effects by either (a) structurally modifying opioids to prevent their binding to the (as yet unidentified) glial non-stereoselective opioid receptor or (b) co-administering an [+-]opioid antagonist which would block glial activation while allowing opioid actions on neurons to remain unaltered. Indeed, [+-]naloxone (which is inactive at neuronal opioid receptors) potentiates morphine analgesia, delays the development of morphine tolerance, decreases [-]-naloxone-precipitated withdrawal behaviors [31] and blocks morphine induced anti-analgesia [258, 259,261,262]. Intriguingly, there is additional evidence of non-classical opioid activity of degraded endogenous opioids [240] suggesting that non-classical opioid receptors possibly mediate counter regulatory systems of pain control. Thus, early indications are that preventing opioid activation of glia may well be a clinically relevant strategy for increasing opioid analgesia while decreasing the negative consequences of repeated opioids.

III. Evidence for immune/glial regulation of pain from studies of human chronic pain patients

As the *balance* of the expression of proinflammatory cytokines relative to anti-inflammatory cytokines is of major importance in defining their impact, it is of interest to explore whether either elevated proinflammatory cytokines or suppressed anti-inflammatory cytokines may influence human pain or responsivity to opioids. To our knowledge, there have been virtually no published reports exploring whether cytokines regulate opioid analgesia, tolerance, or dependence in people. All that is known is that (a) IL-1ra polymorphisms that can alter the balance of IL-1 and IL-1ra may influence postoperative morphine consumption [13], and (b) the anti-inflammatory cytokine IL-4 induces mu- and delta-opioid receptor transcription via IL-4 responses elements in these opioid gene promoters [18,107]. In humans, a single nucleotide polymorphism within the IL-4 response element reduces its transcriptional activating potential by 50%, suggesting that a polymorphism in this region could affect human opioid receptor expression [107].

On the other hand, a few human studies have recently appeared which suggest that higher proinflammatory cytokines and/or lower anti-inflammatory cytokines may indeed be associated with enhanced pain states. Backonja and colleagues reported lower anti-inflammatory cytokine expression and elevated pro-inflammatory cytokine expression in plasma and/or lumbosacral CSF of chronic pain patients with either neuropathic pain or fibromyalgia [7]. Elevated glial activation markers, elevated proinflammatory cytokine and chemokine levels, and suppressed levels of anti-inflammatory cytokine levels in lumbosacral CSF have also been observed in complex regional pain syndrome patients, relative to controls [4,5]. Similarly, chronic widespread pain (including fibromyalgia) is associated with significantly lower gene expression and lower serum protein concentrations for both anti-inflammatory cytokines studied (IL-10 and IL-4) [237]. Elevated serum proinflammatory cytokines in serum and/or painful skin of fibromyalgia patients (reviewed in [237]) and complex regional pain syndrome patients have been reported as well (reviewed in [132]).

Another avenue for exploring the potential influence of pro- or anti-inflammatory cytokines on human chronic pain comes from the study of gene polymorphisms. There are obvious constraints on interpretation of such studies, as they do not imply whether the altered cytokine profiles impact the underlying pathogenesis versus resultant pain modulation. However, these studies may prove informative. Gene polymorphisms expected to elevate proinflammatory cytokine production have been suggested to be risk factors for low back pain [97,203], arthritis pain [165,271], discogenic pain [159], postoperative pain [13], pain of burning mouth syndrome [58], and pain of inflammatory bowel disease [267]. While little information is yet available regarding the importance of anti-inflammatory cytokine polymorphisms in human pain, a low IL-10 producing genotype has been linked to chronic pelvic pain [199] as well as rheumatoid arthritis and painful juvenile idiopathic arthritis [47].

Complementary to the data reviewed above are scattered clinical reports of the efficacy of cytokine modulating compounds on pain. For example, thalidomide and its successors (i.e., lenalidomide) can inhibit the production of proinflammatory cytokines and elevate anti-inflammatory cytokines [132]. As such, thalidomide (which readily crosses the blood brain barrier, allowing it to potentially influence glial function) and lenalidomide (whose actions are restricted to the periphery) may be intriguing compounds for clinical pain control. Clinically, there is precedence for examining TNF inhibitors as infliximab (a TNF inhibitor) reduced both proinflammatory cytokine levels and pain in a study of 2 complex regional pain syndrome (CRPS) patients [86]. Several small open label studies have supported the conclusion that thalidomide may be moderately to dramatically effective in chronic pain states, such as CRPS

[28,132]. The only study of lenalidomide in CRPS patients to date also reported a moderate to marked reduction in pain [132].

In addition to CRPS, infliximab has been reported to be efficacious in the treatment of herniated disc induced sciatica (reviewed in [97]). The pain-relieving effect occurred within 3 hr and lasted throughout the 3-mon and 1-yr follow-up. Similarly, infusion of the TNF inhibitor entanercept likewise had marked beneficial effects on lumbar radicular pain (reviewed in [97]). However, such positive results are not universally found (reviewed in [97]).

Thus, exploration of cytokine regulation of human chronic pain is in its infancy, and exploration of cytokine regulation of opioid efficacy in humans is as yet virtually non-existent. However, what little data exist to date are supportive of the conclusions drawn from animal studies reviewed above.

Given the above, however, it may off-hand appear quite puzzling that chronic pain is not simply, easily controlled by common anti-inflammatories, such as inhibitors of prostaglandins or administration of steroids [68,104]. The resolution of this seeming paradox may lie in the fact that what is classically thought of as inflammatory and damaging in the periphery (such as PGE₂) and what is classically thought of as anti-inflammatory in the periphery (such as adrenal steroids), may not be in the CNS. For example, in the CNS, PGE₂ can exert anti-inflammatory and neuroprotective effects including suppression of proinflammatory cytokines and enhancement of the production of anti-inflammatory cytokines [6,122]. In parallel, there is a growing literature supporting that steroidal “anti-inflammatories” (defined by their peripheral actions) may well not be anti-inflammatory in the CNS. For example, intrathecal steroids decrease the expression of glial glutamate transporters and increase spinal neuronal expression of NMDA receptors, effects predicted to increase the excitability of neurons [118]. Furthermore, adrenal steroids increase microglial activation including the induction of proinflammatory cytokines [49] and exert multiple proinflammatory effects in CNS [213].

CONCLUSIONS

This review has approached immune/glial regulation of pain and opioid actions at multiple levels. Regarding pain amplification, this review has described how immune and/or glial cells are a natural and inextricable part of: (a) peripheral nerves, where various types of immune cells are in intimate contact with nerve fibers and can alter axonal anatomy and function, (b) dorsal root ganglia, where the function of sensory neuronal cell bodies are modulated by ensheathing satellite glial cells and immune cells, and (c) spinal cord, where glial cells form dynamic networks that maintain and spread excitation. This review has also attempted to explore, using proinflammatory cytokines as a prime example, the multitude of ways that products released by these non-neuronal cells can enhance neuronal excitability. Finally, it extended the issue of enhanced neuronal excitability to an exploration of how activated glia dysregulate the actions of opioids. Here again, the focus was on proinflammatory cytokines, which suppress the ability of opioids to control pain and contribute to opioid tolerance and dependence/withdrawal.

There are several major points worth re-emphasizing. First and foremost is the importance of immunology as regards neuropathic pain. Classical immune cells (macrophages, T lymphocytes, mast cells, dendritic cells), immunocompetent cells that can release substances classically thought of as immune-cell products (endothelial cells, fibroblasts, keratinocytes), and immune-like glial cells (Schwann cells of peripheral nerve, satellite glial cells of DRG, microglia and astrocytes of the CNS) must all be considered as contributors to neuropathic pain. The importance of immune and glial activation to neuropathic pain has potentially profound implications both for the understanding of how such pain states occur as well as for

the development of novel therapies to treat such pain. The recognition of immune and glial involvement in such pathological pain offers hope for novel approaches to pain control.

Indeed, new treatments for neuropathic pain that target these non-neuronal cells [112,113, 133] have recently entered clinical trials sponsored by Celgene and Avigen. Celgene is testing lenalidomide, a compound that, at most, minimally crosses the blood brain barrier (so likely effective for peripheral immune cells but not CNS glia) and has greater potency than thalidomide in its ability to suppress proinflammatory cytokine production and increase IL-10 [133]. It is currently being tested in 2 large multicenter, randomized, placebo controlled studies for neuropathic pain arising from complex regional pain syndrome type I and for chronic painful radiculopathy [133]. In contrast to the Celgene compound, Avigen's AV411 (ibudilast) does cross the blood brain barrier with excellent partitioning to CNS sites. This is a compound that has been used clinically for many years in Asia for allergy and post-stroke dizziness [112]. In animal studies, AV411 suppresses proinflammatory cytokines and increases IL-10 in both peripheral immune cells and CNS glia [112]. The Avigen safety, tolerability and pharmacokinetic study enrolled 18 healthy adult volunteers (10 male, 8 female) in Adelaide, Australia. The double blind, placebo-controlled study, with single dose and two week repeat dose phases, was designed to evaluate the safety, tolerability and pharmacokinetics of AV411. In this 20-day study, volunteers were randomized to receive either 60 mg/day of AV411 or placebo in a 3:1 ratio. The standard human dose for ibudilast is 10 mg given three times per day. There were no serious adverse events in the study and most common events involved headache and nausea. A small phase IIa study in Australia involving the treatment of diabetic neuropathic pain patients, also at dose levels higher-than-approved in Asia, is currently enrolling (Avigen press release, January, 2007).

The second major point worth re-emphasizing is the importance of immunology as regards the efficacy of opioids. Although only recognized within the past ~5 years, the data are startling in their consistency across laboratories and paradigms. As such, they warrant attention. It is already clear that glia regulate morphine analgesia, tolerance, and dependence/withdrawal [80]. While it is also clear that such effects are not limited to morphine, it is as yet unknown how pervasive this glial regulation of pharmaceuticals may turn out to be. Certainly the fact that glia are also implicated in effects of drugs such as methamphetamine [157] suggests that glia may ultimately be found to exert pervasive effects on phenomena now thought of as purely neuronal.

The third major point is the pervasiveness of proinflammatory cytokines to neuropathic pain and dysregulation of opioid actions. In terms of neuropathic pain, TNF, IL-1 and IL-6 are well-established contributors to pain enhancement at the site of peripheral nerve injury and in DRG, as well as in spinal cord. While beyond the scope of this review, it may well be that these proinflammatory cytokines are important to pain amplification at supraspinal sites, as well [167,183]. It certainly is the case that glial activation at both spinal and supraspinal sites regulates the actions of opioids. While the involvement of proinflammatory cytokines in the regulation of opioids is now clearly established in spinal cord, their potential role in brain remains to be explored.

The last major point is that investigation of immune and glial regulation of neuronal function is still in its infancy. While remarkable progress has been made over the past decade, much more work needs to be done. This is, in part, because it has only recently been recognized that glia residing in various sites in the CNS are not all the same. Rather, their receptor expression and function reflect their microenvironment and, as such, must be understood in this context. Much remains to be learned about the dynamics of immune and glial regulation of neuronal functions, not only as regards to pain and opioids, but far more broadly as well.

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TABLE 1
Pain models associated with upregulation of spinal cord microglial and/or astrocyte activation markers

Complete Freund's adjuvant, subcutaneous
Formalin, subcutaneous
Phospholipase A2, subcutaneous
Zymosan, subcutaneous
Sciatic nerve injury (chronic constriction injury)
Inferior alveolar and mental nerve transection
Partial sciatic nerve ligation
Sciatic nerve inflammation with zymosan
Sciatic nerve inflammation with HIV-1 gp120
Sciatic nerve inflammation with phospholipase A2
Spinal nerve transection
Spinal nerve root injury
Spinal cord injury
Hind paw incision
Bone cancer
HIV-1 gp120, intrathecal
Lipopolysaccharide, intrathecal
Chronic opioids; opioid withdrawal-induced hyperalgesia

Modified and updated from Ledeboer et al. [114].

TABLE 2
Pain facilitation is suppressed or reversed by inhibition of spinal glial activation or proinflammatory cytokine actions

Model	Intervention
Mustard oil, topical	fluorocitrate
Carrageenan, subcutaneous	minocycline, IL-1 knockout
Complete Freund's adjuvant, subcutaneous	IL-1ra, IL-1 knockout
Formalin, subcutaneous	fluorocitrate, IL-1ra, minocycline; IL-1 knockout
Phospholipase A2, subcutaneous	fluorocitrate, IL-1ra, sTNFR
Zymosan, subcutaneous	fluorocitrate
Hind paw incision	fluorocitrate
Inferior alveolar and mental nerve transection	minocycline
Sciatic nerve injury (chronic constriction injury)	IL-1ra, IL-10; IL-1 knockout
Sciatic nerve inflammation with zymosan	fluorocitrate, minocycline
	IL-1ra, sTNFR, IL-6 antibody
Sciatic nerve inflammation with phospholipase A2	IL-1ra, anti-IL-6, IL-10
Sciatic nerve tetanic stimulation	fluorocitrate
Spinal nerve transection	propentofylline, minocycline, IL-1ra, sTNFR, anti-IL-6, methotrexate; IL-1 knockout
Spinal nerve root injury	IL-10, IL-1ra, minocycline
Spinal cord injury	fluorocitrate, IL-1ra, sTNFR, minocycline
HIV-1 gp120, intrathecal	IL-1ra
Lipopolysaccharide, intrathecal	IL-1ra, IL-10
Dynorphin, intrathecal	minocycline, IL-1ra, anti-IL-6
Fractalkine, intrathecal	

Abbreviations: IL-1ra, interleukin-1 receptor antagonist; sTNFR, soluble TNF receptor; IL-10, interleukin-10.

Modified and updated from Ledebor et al. [114]. For complete references, see [29,61,75,114,161,178,247,266]