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Current Challenges in Glia-Pain Biology

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A remarkable series of findings over the last decade or so has demonstrated a previously unrecognized role for CNS glia in many aspects of neuronal functioning including pain processing. In addition to their recruitment to sites of CNS damage, these cells also appear to be capable of "action at a distance," playing functional roles in areas of CNS that are quite remote from the focus of injury or disease. The implication is that the nervous system is able to initiate signals that alter the function of these glial cells, and these cells in turn release factors that regulate neuronal function. This idea has taken root, resulting in an explosion of research interest, and here we look critically at what has been reported in order to assess where knowledge is missing or uncertain.

Introduction

Pain has long been recognized to be a complex sensory experience that varies not only with the nature and extent of tissue injury, but also with a number of factors such as expectation, emotional state, attention, and history. In some unusual circumstances, there is evidence that internal regulatory systems (notably the sympathetic system) can modulate the pain experience (Day, 2008). In all of these cases, however, it has long been assumed that purely neuronal systems are involved. The pain signaling system is usually activated by activity in primary sensory neurons that leads to coordinated activation of a series of CNS areas (the so-called "pain-matrix"—Tracey, 2007), and pain modulation by physiological or psychological processes has also mostly been envisaged as being executed through neuronal control systems. It has long been recognized that a variety of nonneuronal cells might play an important role in initiating and modulating activity in primary afferent nociceptors by the release of mediators that bind to specific receptors expressed by those nociceptors. A particularly important source of such mediators is cells of the immune system that are recruited to the sites of peripheral injury and inflammation. The relative lack of efficacy of cyclooxygenase (COX) inhibitors in many chronic pain states, and encouraging clinical data on the analgesic actions of, for instance, anti-NGF in some conditions (T.J. Schnitzer, N.E. Lane, M.D. Smith, M. Brown, 2008, 12th World Cong. On Pain, abstract; Katz et al., 2009), strongly suggests that novel and important pain mediators are still to be elucidated. However, there is another form of pain modulation arising from nonneuronal cells that has been recognized and investigated only recently. This is the influence of CNS glial cells (which include some cells of the immune system, such as microglia, but other cells that are not, notably astrocytes and oligodendrocytes) on pain processing. For several decades the biology of these developmentally distinct cells has been explored, mostly from the perspective of their presumptive support role for neurons—providing metabolic homeostatis for the CNS, electrical insulation to facilitate signal transmission and integrity, and, in the case of microglia, immune surveillance cells for the CNS. Of course, disease or injury directly affecting the CNS, such as multiple sclerosis, is well recognized to sometimes lead to symptoms because of effects on these cells. But a novel action of these cells with an intriguing feature has emerged in pain processing: their effects can arise at a distance, that is, in areas of CNS that are quite remote from the focus of injury or disease. The implication is that the nervous system is able to initiate the signals that alter the function of these glial cells, and that when recruited, these glial cells in turn regulate neuronal function. This idea has taken root in the pain field and there has been an explosion of research interest in the phenomenon in the last 5 years or so. This burgeoning interest is revealing a fascinating series of neurobiological mechanisms, many of which have been recently covered in excellent reviews (Abbadie et al., 2009; Inoue and Tsuda, 2009; Milligan and Watkins, 2009; Romero-Sandoval et al., 2008b; Scholz and Woolf, 2007). Yet as this field expands and experimental data multiplies, it is perhaps worth looking critically at what has been reported and at what may still be lacking. To this end, the aim of this review is not to recap all the experimental findings to date, but to focus on a series of questions where, it seems to us, critical knowledge is missing or remains uncertain. As we will review in following sections, there is considerable evidence from preclinical studies that central glia can in some circumstances contribute critically to pain-related behavior. The important question of how much is relevant to humans, and what more could be done in the clinic, is considered at the end of this review.

What Is Glial "Activation?"

The central thrust of the argument is that CNS glial cells are, under normal conditions, bystanders in nociceptive processes but that they become "activated" following damage to peripheral tissues or nerves, and in this state now release factors that induce hyperexcitability in pain signaling pathways and thereby contribute to abnormal pain perception. Figure 1 illustrates the main features of this idea and shows two glial cell types, microglia and astrocytes, that have been studied in this context, the former much more than the latter.

Microglial cells exist in the parenchyma of the CNS and at perivascular sites. These two types have distinct properties, but both belong to the myelomonocytic lineage, which also includes monocytes and macrophages. Unlike other cells of this lineage,

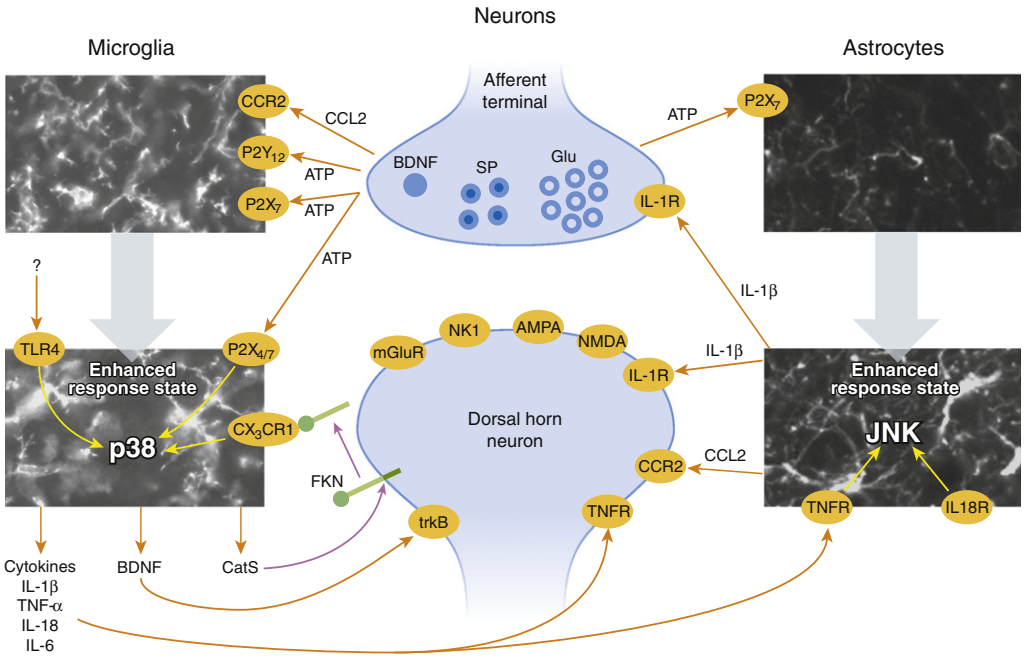

Figure 1. Pain-Related Enhanced Response States in Microglia and Astrocytes in the Dorsal Horn of the Spinal Cord
The figure illustrates the morphological changes seen in microglial cells (left) and astrocytes (right). The receptors involved in the shift from resting state to the enhanced response states are shown in yellow ellipses. In the dorsal horn of the spinal cord, primary afferent terminal fibers release glutamate, substance P, and brain-derived neurotrophic factor (BDNF), which activate their cognate receptors on postsynaptic dorsal horn neurons and transmit noxious input from the periphery to higher centers. Following peripheral nerve or tissue injury, the transmission of pain-related signals is amplified although this is not itself considered in this diagram. Alongside well-recognized neurotransmitters (glutamate, substance P, and BDNF, which activate second-order neurons), primary afferent fibers release ATP and chemokines, such as CCL2, which activate their receptors on microglia and astrocytes, thereby inducing cell transition from bystander/surveillance states to enhanced response states. Specific features of microglial and astrocytic responses are phosphorylation of MAK p38 and activation of JNK, leading to the release of cytokines, chemokines, neurotrophins, and proteases that modulate neuronal activity and contribute to nociceptive processing.

microglia mature and reside in the CNS where they represent 10%–20% of glial cells in adulthood. Microglial cells have distinct morphological and functional properties that are developed under the influence of nearby astroglia (which produce colony stimulating factors) and neurons (which can modulate microglial function via the release of neurotrophins that appear to downregulate MHC class II expression via interaction with p75 receptor) (Sievers et al., 1994).

In normal conditions, microglia perform immune surveillance of the nervous system. They exhibit ramified processes that are highly motile (Nimmerjahn et al., 2005) and express receptors for complement components, Fcγ receptor for IgG, and low levels of cell-surface immune molecules. This status changes dramatically not only following direct insults to the CNS, but also quickly after injury to peripheral nerves or peripheral tissues. Under these conditions, microglial morphology changes as cell bodies increase in size; proximal processes become thicker and distal branches appear less ramified. Microglia show increased phagocytic activity, an enhanced migratory capacity within the CNS, and increased expression of cell-surface glycoproteins including CD45 and MHC-II.

Astrocytes derive from the neuroectoderm and are intimately associated with neuronal synapses because a single astrocyte can make contact with several neurons. Astrocytes express a large number of neurotransmitter receptors and can themselves release transmitters, such as glutamate, D-serine, or ATP, upon elevation of intracellular calcium concentration. As astrocytes communicate via gap junctions, the elevation of intracellular calcium ions propagates among astrocyte networks independently from neurons. In addition, astrocytes respond to ATP via P2Y receptor activation, generation of IP3, and elevation of intracellular calcium. They can modulate synaptic neurotransmission and plasticity by exerting mGluR-mediated depression and NMDA-mediated increase in neurotransmitter release (Haydon, 2001). Furthermore, astrocytes take up the majority of extracellular glutamate via the glutamate transporter-1 (GLT1/EAAT2), which is mainly located on these cells (Tanaka et al., 1997). As with microglia, damage to peripheral nerves and peripheral tissues alters the resting state of astrocytes, most prominently near the central terminals of damaged sensory neurons (and around motoneurons). This altered state is again referred to as activation and is marked by an increase in GFAP expression.

The contribution of microglia and astrocytes to the development of abnormal pain is considered below, but one important issue for this field is to refine the simple dichotomy of "resting" and "activated" state. The case is most clear for microglia. In the normal state, microglial cells are typically highly ramified and their processes are highly motile to sample or survey the healthy CNS. In many pathophysiological conditions they change their morphology and can take on deramified forms. Associated with this morphological change are many alterations in gene expression in these cells, including the upregulation of a number

of receptors and also the production of a repertoire of cytokine and chemokine mediators and other released factors. It has sometimes been assumed that there is a single "program" of activation. But several lines of evidence suggest this is not the case. As recently reviewed (Ransohoff and Perry, 2009), different stimuli, engaging different microglial receptors, appear to lead to distinct signaling cascades within the cells and different morphological or secretory consequences. For instance, glutamate acting on microglial mGLUR2 receptors may selectively promote TNFα production while UDP acting on P2Y receptors may selectively lead to adoption of a phagocytic state. The particular repertoire of microglial responses may therefore vary with pain state and temporal course. Less is known about astrocyte activation, but it seems feasible, perhaps likely, that there will be a range of metabolic responses in astrocytes too. For these reasons, several recent reviews have made a plea to drop the term "activated glia" because it suggests a single state. We agree wholeheartedly with this sentiment and suggest the term "pain-related enhanced response states" to describe the different ways in which glial cells might respond in these conditions. An enhanced response state is evidenced both by increased expression of cell-surface receptors in these cells and by increased secretion of multiple factors. Some, but not all, of the phenotypic changes in glial cells will be relevant to pain processing, and we use the term "pain-related" to include those that are, even if the nature of what is pain-related is still an open question in many cases. A critical issue for this field is therefore to define more fully the range of response states of these cells, perhaps most productively in terms of the receptors they express and the factors they release. Comprehensive profiling of what is expressed by these cells, at both mRNA and protein levels, under different conditions is technically feasible.

What Is the Source of Glia that Show Pain-Related Enhanced Response States?

After CNS injuries, microglia respond by rapidly entering the cell cycle and undergoing local expansion (Ajami et al., 2007). This type of reactive microgliosis is a hallmark of CNS pathologies including stroke, neurodegenerative diseases, and demyelinating inflammatory diseases. Reactive astrocytes play critical roles in controlling extracellular glutamate concentrations and intercellular communication, particularly about local injury (Rossi et al., 2007). The number of microglial cells in the CNS also dramatically increases in experimental pain models, especially those involving injury to peripheral nerves. The number of astrocytes also increases although to a lesser extent than microglia and at later time points after injury (Echeverry et al., 2008). Extensive axonal degeneration appears to be a prerequisite for astroglial proliferation at the site of CNS injury. By contrast, after peripheral nerve injury, microglial cell proliferation in the spinal cord can occur in the absence of axonal degeneration (Liu et al., 2000) or neuronal death (Polgar et al., 2005). Injury to the rat facial nerve leads to mitotic divisions of microglial cells without them developing into phagocytes, as well as changes in their cytoskeletons and increased expression of complement type 3 receptors (Moran and Graeber, 2004). Injury of rodent sciatic nerve is also associated with microglial proliferation and increased immunoreactivity for microglial markers such as OX-42 and astrocytic markers (GFAP) in the somatotopically relevant areas in the spinal cord and brainstem and beyond (see below).

Division of resident cells appears to account for at least some of the increased glial cell numbers, but there is considerable controversy concerning whether blood-derived monocytes also contribute. In the case of direct CNS injury, disruption of the blood-brain barrier facilitates the inward movement of hematogenous cells, but in the case of peripherally restricted injuries, it is less clear to what extent this barrier function is compromised (Gordh et al., 2006; Brooks et al., 2006). The issue has been addressed with the use of chimeric animals in which circulating monocytes, but not resident microglia cells, in a host animal are tagged. Initial data from bone marrow of chimeric mice suggested that hematogenous monocytes infiltrate the spinal cord after peripheral nerve injury, proliferate, and differentiate into microglia (Zhang et al., 2007). This is potentially more than of academic interest because the claim that bone-marrow stem cells can penetrate the damaged brain and spinal cord has raised the hope of delivering genes to injured areas of the CNS. However, recent reports raise the possibility that inflammatory monocytes penetrate the CNS in chimeric mice because the irradiation preconditioning of the CNS (necessary in this experiment) renders the BBB more permeable, suggesting reactive microglia may not derive from the blood stream. This conclusion might not be totally correct though because nerve injury is performed 3 to 5 months after irradiation, after which time the BBB is unlikely to allow cell trafficking into the CNS. Furthermore, in a viral model associated with negligible breakdown of the BBB, inflammatory monocytes have been shown to penetrate the CNS rapidly and differentiate into microglia (Getts et al., 2008).

The issue on whether immigrant microglia intermingle with resident microglia after peripheral nerve injury remains intriguing because these two cell populations (Carson et al., 2006) might perform different roles in regulating neuronal function and contribute differentially to pain modulation.

How Important Are Glial Cells to Chronic Pain?

There is considerable evidence that glial cells can contribute to some experimental pain states, and that they indeed play an essential role in some of these. However, there remains much uncertainty as to the relative contribution of different types of glial cells, and the temporal and spatial importance of glial cells that develop enhanced response states. One concern is that we have surprisingly few good tools to address these issues. Much of the data available have been obtained by using rather general glial inhibitors. The antibiotic and immunomodulator minocycline has been extensively used as an inhibitor of glial cells. Its mechanisms of action remain somewhat uncertain, and suggestions include reduction of microglial production of proinflammatory factors such as NO and IL-1β (Lai and Todd, 2006). Fluoroacetate and its metabolite fluorocitrate are other commonly used glial inhibitors. Low doses of fluorocitrate specifically and reversibly disrupt microglial and astrocytic metabolism by blocking aconitase, which is an enzyme found in the tricarboxylic acid cycle of glia, but not that of neurons. A third general glial inhibitor is propentophylline, which decreases microglial and astrocytic responsiveness via mechanisms including increase in cAMP

through the inhibition of phosphodiesterase (PDE IV) and alteration of glutamate transporters (Tawfik et al., 2008a). L-alpha-aminoadipate is a cytotoxin specific for astrocytes and produces reduction of GFAP (Ji et al., 2006).

There are several potential problems with these inhibitors. The first is one of specificity—they may have other important biological actions that could contribute to their effectiveness. There are already suggestions that these compounds can also affect neurons, particularly in the case of high concentrations of fluorocitrate. A second problem is that the nonspecific inhibition of glial cell function may not block all (or even any) of the pronociceptive aspects of the enhanced response state of glia. As discussed above, the different possible enhanced response states are poorly defined at present and these general inhibitors may affect only some of these functions. A failure to block abnormal pain with these agents cannot be taken as definitive evidence that a particular cell type is not actively contributing to the condition. Notwithstanding these problems, there are multiple reports of the effectiveness of these treatments, particularly propentofylline, in models of chronic pain after nerve injury and cancer chemotherapy-induced neuropathy (e,g. Sweitzer et al., 2006; Tawfik et al., 2008a; Cata et al., 2008).

There are other interventions that target, at least under certain conditions, processes that are specific for a particular glial cell type. One example is cathepsin S (CatS), which is a lysosomal enzyme expressed and released by microglia in the spinal cord. CatS contributes to the maintenance of neuropathic hyperalgesia and allodynia via the liberation of the pronociceptive domain of neuronal chemokine fractalkine (FKN; also CX3CL1) (Clark et al., 2007a, 2009). Relevantly, CatS inhibitors reverse established neuropathic mechanical hyperalgesia following acute administration as well after prolonged delivery over 5 days (Irie et al., 2008), arguing for a specific role for spinal cord microglia in this process.

Another example is the expression of complement components in microglial cells that occurs rapidly after experimental injury to peripheral nerves in areas of the central terminations of the damaged nerve fibers. Complement component levels decline gradually a few weeks after injury, returning to normal levels by about 30 weeks in the spinal cord but remaining high in the gracile nucleus (Liu et al., 1995; Griffin et al., 2007). Blockade of complement 5 (C5) receptor reduces cold allodynia in neuropathic rats, indicating that complement activation converging on C5 contributes to the nocifensive response to cold associated with nerve injury (Griffin et al., 2007). The depletion of complement component C3 by intrathecal injection of cobra venom factor also reportedly alleviates established allodynia in neuropathic pain models (Levin et al., 2008), suggesting that inhibition of the complement pathway may be an effective analgesic strategy.

A putative astrocytic target is intracellular signaling protein c-*jun*-N-terminal kinase (JNK), which is activated by cytokines such as TNFα and increases the expression of the chemokine of CCL2 by astrocytes. In turn this chemokine enhances excitatory transmission in dorsal horn neurons expressing CCR2 receptors (Gao et al., 2009). Also in astrocytes, the upregulation of the metalloproteinase MMP-2 can contribute to pain mechanisms via production of IL-1β, thereby providing a non caspase-dependent source of this cytokine (Kawasaki et al., 2008). Indeed, intrathecal delivery of either JNK or MMP-2 inhibitors reverses neuropathic allodynia (Ji et al., 2006; Kawasaki et al., 2008; Gao et al., 2009).

These examples provide more confidence in the role of particular cell types in pain processing, but of course positive findings will be limited to where the correct target has been selected and where an unambiguous glial origin is demonstrable. A further complication is that different glial cell types may play distinct roles at different stages of disease progression. Indeed, there is a growing acceptance of the idea that microglia may play a critical early role, but their importance appears to wane with time, whereas astrocytes appear to play pronociceptive roles that contribute to the persistence of chronic pain. Support for these ideas comes from studies showing that pain-related enhanced responsiveness in microglia peaks soon after peripheral nerve injury and declines at later time points (Zhang and De Koninck, 2006). Pain-related enhanced responsiveness of astrocytes occurs with some delay but persists and possibly outlasts the enhanced responsiveness of microglia (Tanga et al., 2004). However, some changes in microglial expression of surface receptors have been observed as long as 50 days after nerve injury and their inhibition by fluorocitrate and propentofylline correlated with reversal of hyperalgesia (Clark et al., 2007b; Tawfik et al., 2007). Furthermore, in studies on pain after spinal cord injury, there is evidence that enhanced response states of microglia contribute to both the maintenance phase and induction (Hains and Waxman, 2006). In any case, defining the role of a particular cell type may require extensive temporal profiling. In a similar vein, the spatial distribution of contributing glia has not been extensively tested (Beggs and Salter, 2007). Most work on pain has examined spinal cord, and more specifically (and reasonably) the somatotopically appropriate areas of cord. However, it is now clear that glial activation can occur at more rostral sites in some of these peripheral nerve injury conditions, although microgliosis is not ubiquitous in the brain in these conditions, and microglial cells maintain a ramified morphology in cortical area such as the anterior cingulate and the periaqueductal gray matter (PAG) (Zhang et al., 2008). Some specific brain areas do, however, exhibit changes. Examples include altered microglial responsiveness in the gracile nucleus, the area of termination of the lesioned myelinated A fiber primary sensory neurons. Reversal of allodynia has been reported by infusion of p38 inhibitors in the gracile nucleus (Terayama et al., 2008) and in the rostral ventromedial medulla (Wei et al., 2008), and indeed, in thalamus after spinal cord injury (Zhao et al., 2007). The use of systemically administered agents of course fails to distinguish their effective site of action.

A useful starting point for investigations into glial function is to consider when and where glial cells show enhanced responsiveness in different pain states, ideally of course using a more comprehensive analysis of the nature of the altered responsiveness (for instance, quantifying some of the mediators released in this state as well as the sensitivity of the glial cells to different stimuli). However, even on a rather superficial analysis, it appears that damage associated with peripheral nerve injury is a more robust and consistent inducer of these enhanced response states compared with peripheral inflammatory

conditions. Thus, in models of neuropathic pain involving traumatic injuries of peripheral nerves, in chemotherapy-induced neuropathies, and in models of diabetic neuropathy, all workers agree that microglial and astrocytic cells in the spinal cord assume enhanced response states. Glia cells change morphology (visualized by Iba-1 and GFAP staining, for microglia and astrocytes, respectively) and microglia increase CD11b expression (revealed with OX-42 staining) with a time course of days to weeks—roughly concomitant with the presence of hyperalgesia and allodynia (Zhang and De Koninck, 2006; Peters et al., 2007 ; Wodarski et al.,2009). Consistent with the idea that glial cells contribute to neuropathic pain, glial inhibitors prevent and reverse pain response in all these models (Clark et al., 2007b; Ji et al., 2006; Tawfik et al., 2008a, 2007; Hald et al., 2008; Sweitzer et al., 2006).

In contrast, in models of peripheral inflammatory pain, evidence for microglia and astrocyte involvement in nociception is less substantial. The intraplantar injection of inflammogens such as zymosan, formalin, and carrageenan are reported to induce microglial expression of CD11b within hours, while intraplantar injection of complete Freund's adjuvant, induces it within a few days (Ledeboer et al., 2005; Clark et al., 2007b; Hua et al., 2005; Sorkin et al., 2009; Svensson et al., 2007; Raghavendra et al., 2004; Guo et al., 2007; Sun et al., 2007; Romero-Sandoval et al., 2008a). However, the degree of glial change is typically less well defined with these insults, although there is some evidence that general glial inhibitors and inhibitors of chemokine receptors (e.g., CX3CR1) can prevent some of the abnormal sensory responses associated with inflammation. Also, whether glial cells contribute to pain mechanisms in chronic inflammation is unknown. It seems likely, but is unproven, that different factors are responsible for initiating glial responses in neuropathic and inflammatory states. In neuropathic conditions, there are known to be marked changes in transmitters/modulators that are expressed and released from sensory neurons, and it seems likely that one or more of these may drive the enhanced response state of glial cells (as we discuss more fully below). In inflammatory conditions these transmitters/modulators are typically upregulated in sensory neurons, and the rapid, but weaker, glial responses that are seen appear to arise as a consequence of ongoing neuronal activity and the release of constitutively expressed factors. Indeed, because of this, it seems likely—but is again untested—that enhanced response states will be distinct in these two conditions.

Overall, there is growing confidence that microglia and astrocytes play specific roles in pain processing, though the evidence is more substantial for neuropathic pain than inflammatory pain. Furthermore, microglia are the first nonneuronal cells showing enhanced response state, while astrocytes follow with some delay. The development of selective tools for targeting microglial and astrocytic processes specifically involved in pain states would certainly help clarify the importance of glial cells.

What Factors Increase the Responsiveness of Glial Cells in Pain States and What Do These Cells Release that Affects Pain Processing?

When the CNS is directly damaged there are many ways in which signals might be generated that act on microglia and astrocytes. However, pain-related enhanced response states of glia often arise following damage to peripheral tissues or nerves, and this raises the question of what drives the altered glial responses in these circumstances. Since the precipitating event in these cases is peripheral damage, one obvious candidate is the activity in primary sensory neurons and the attendant release of neurotransmitters/modulators. These include classical transmitters such as glutamate, substance P, and brain-derived neurotrophic factor (BDNF), but also ATP, neuronal chemokines, and yet unidentified activators of Toll receptors expressed by glial cells.

Both glutamate and substance P, which are released in the dorsal horn following activation of nociceptive neurons, are capable of inducing changes in microglial cells and astrocytes. The effects are direct, via activation of NMDA receptors and NK1 receptors, on microglia (Rasley et al., 2002). Astrocytes express low levels of NK1 receptors, which are upregulated in reactive states (Palma et al., 1997; but see Guo et al., 2007) and respond to glutamate and ATP. The microglia, which of course are electrically unexcitable, show induction of the transcriptional activator NF-kB following NK1 receptor stimulation (Rasley et al., 2002). This signal is believed to regulate the synthesis of proinflammatory cytokines by the microglia. A third primary afferent transmitter, BDNF (Pezet et al., 2002), could also activate trkB receptors on spinal microglia cells, following its known release from nociceptive fiber terminals. However, following peripheral nerve injury (the best documented cause of pain-related enhanced response states in central glial), it appears that the major source of spinal BDNF becomes microglia themselves. Indeed, microglia-released BDNF has been claimed to play an important role in modulating neuronal activity by causing a depolarizing shift in the chloride reversal potential, which results in GABAergic transmission being less inhibitory, and in some spinal transmission systems, frankly excitatory (Coull et al., 2005). The evidence that substance P and glutamate actually play a role in altering the responsiveness of central glia in pain states is much weaker. *NK1* (or substance P) knockout animals do not exhibit a dramatic phenotype in neuropathic pain models. And while glutamate acting at NMDA receptors has a well-established role in promoting increases in central excitability in numerous pain states, it is difficult to determine the role of glial-expressed NMDA receptors specifically. In the spinal cord, the dorso-ventral distribution of glial cells showing these enhanced response states in these pain models is much more widespread than the termination patterns of nociceptive afferents (which are highly concentrated in the superficial laminae of the cord). So if primary afferent transmitters are important, they are unlikely to be transmitters restricted to nociceptors.

Another potential mediator of neuronal-microglial and neuronal-astrocytic communication is ATP. This can be released with activity from primary sensory neurons, although it can also be released from glial cells, and presumably from CNS neurons too, which potentially complicates interpretation of experimental data. There is considerable evidence for an important role of ATP in pain processing after nerve injury, but it is much less clear what receptors are involved, with different claims for microglial P2X4 or P2X7 receptors and the metabotropic receptor P2Y12 (Tsuda et al., 2003a; Hughes et al., 2007; Tozaki-Saitoh et al., 2008).

Much of the original emphasis was on P2X4, although not in resting microglia conditions. Following peripheral nerve injury, P2X4 receptors are upregulated in microglia in the dorsal horn (Tsuda et al., 2003b) and contribute to the development of neuropathic allodynia by inducing the release of BDNF (Coull et al., 2005; Trang et al., 2009). The pharmacological evidence for the critical role of P2X4 comes from the use of antagonists with overlapping profiles of P2X receptor antagonism supported by recent evidence in *P2X4* null mice, which do not develop mechanical hypersensitivity after peripheral nerve injury (Ulmann et al., 2008). However, because resting microglia do not express high levels of P2X4, and because microglial morphological changes evolve regardless of the absence of P2X4 receptors after nerve injury (Ulmann et al., 2008), something else is necessary to initiate and maintain the altered responsiveness of the glia. In addition, the source of ATP that does act on P2X4 receptors in pathological states has also not been established to be primary sensory neurons.

The case for P2X7 comes in part from the pharmacology of ATP receptors. High-dose ATP (mM) is needed to activate P2X7 while low doses (sub-mM) activate P2X4 and P2Y12. In vitro, ATP at 1 mM induces currents in microglia (and to a lesser extent in neurons) and induces microglial chemotaxis (Wu and Zhuo, 2008) under conditions in which glutamate and GABA (also at 1 mM) fail to produce effects on microglia (while acting on neurons). Activation of ATP receptors also causes rapid calcium response in cultured microglia (Light et al., 2006), and activation of P2X7 receptors produced release of cytokines from microglia. Moreover, and critically, blocking P2X7 receptors reportedly reverses neuropathic and inflammatory pain in some conditions. It is possible that the functional receptor on microglia is a heteromer of P2X4 and P2X7 subunits. An intriguing possibility is that the P2X4 and P2X7 receptors play different temporal roles, with the maintenance phase of microglial enhanced responsiveness dependent on P2X7 receptors and requiring high concentrations of extracellular ATP as associated with tissue stress. To further complicate matters, activation of microglial P2Y12 receptors is associated with microglial membrane ruffling, chemotaxis, movement of fine processes, and, as recently reported, pain (Tozaki-Saitoh et al., 2008). P2Y12 is not expressed by peripheral macrophages (Haynes et al., 2006).

Two further candidates for important primary afferent transmitters mediating neuronal-glial communication are the chemokines CCL2 and FKN. The chemokine CCL2 is de novo expressed in damaged sensory neurons in several neuropathic pain models, as soon as 1 day after injury. Astrocytes also upregulate this chemokine following activation with TNFα (Gao et al., 2009). CCL2 is released with activity in damaged primary afferent fibers in the dorsal horn of the spinal cord (Thacker et al., 2009). The suggestion is that this chemokine stimulates microglia via CCR2 receptors, and in support, it is reported that intrathecal neutralizing antisera to CCL2 reduces some aspects of enhanced responsiveness in glia cells and neuropathic pain behavior in these models (Thacker et al., 2009). Since all classes of damaged sensory neurons (i.e. large and small) show de novo expression of CCL2, the dorso-ventral extent of microglia showing enhanced responsiveness in the spinal cord is consistent with CCL2 mediation. *CCR2* null mice fail to develop neuropathic pain after nerve injury (Abbadie et al., 2003), although the locus of action here is unknown. However, there are some uncertainties still. One is that damaged sensory neurons also express CCR2, so actions of CCL2 on cells other than microglia are possible. A second is that in neuropathic conditions, microglia express CCR2, but it is less clear if normal glia do. Thus while the data suggest that CCL2 may be necessary for the contribution of microglia to neuropathic pain development, it is not known if this signal alone is sufficient.

The other chemokine, FKN, is a transmembrane protein that is expressed in the cell bodies of sensory neurons in the dorsal root ganglia (DRG) and intrinsic neurons in the dorsal horn, but not in CNS endothelial cells (Cardona et al., 2008). The receptor for FKN, CX3CR1, is uniquely expressed by microglia in the dorsal horn (Verge et al., 2004) and activation of CX3CR1 is pronociceptive because spinal injection of the chemokine domain of FKN is proalgesic in wild-type, but not *CX3CR1*$^{-/-}$, mice (Clark et al., 2007a). Our own recent work has revealed that the chemokine domain of spinal FKN is liberated by the cysteine protease CatS, which is expressed and released by microglial cells (Clark et al., 2009). The activation of CX3CR1 on microglia induces phosphorylation of p38 MAP kinase, which is a prerequisite for cytokine synthesis and release from microglial cells. Microglial p38 can also be activated by other chemokines, cytokines, and substance P, and lead to transcription of NF-kB and expression of IL-1, IL-6, and COX-2 enzyme. Relevantly, the inhibitors of p38 are antiallodynic in neuropathic states (Ji and Suter, 2007) and antihyperalgesic in inflammatory states (Svensson et al., 2003).

Alongside chemokine receptors, other receptors that can increase glial cell responses in pain states are the Toll receptors, which are expressed by a variety of cell types in the spinal cord. But one receptor, TLR4, is selectively expressed on microglia. TLR4 is required for the development of neuropathic allodynia, which is very much attenuated in *TLR4* null mice (Tanga et al., 2005), but it is possible that this reflects a long-term alteration in the properties of microglia in the absence of this receptor. Alternatively, an endogenous ligand for TLR4 may be released in these experimental pain models and contribute to the glial response. The identity of this ligand is not known. However, activation of spinal TLR4 by an exogenous ligand (intrathecal injection of LPS) produces hyperalgesia and activation of p38 MAPK in microglia without obvious neuronal damage (Clark et al., 2006). Application of LPS to spinal cord slices induces rapid release of IL-1β and activation of p38 with no requirement of ATP as second stimulus, as is the case in microglia in culture (Clark et al., 2006).

Together these data show that several stimuli can converge on microglia and astrocytes and together generate or sustain enhanced response states. These states appear to critically involve signaling via p38 phosphorylation and NF-kB regulation in microglia and JNK in astrocytes. What is not clear is which of these stimuli, if any, is the initial precipitating cause of microglial changes and why blocking any of so many factors prevents the microglial and astrocytic response. Different factors may play distinct roles under different conditions (e.g., different pain models) or at different times. But it also appears clear that there

are a number of feedback circuits operating in the microglial and astrocytic responses. Microglia may both release and respond to ATP and to BDNF; in neuropathic states, microglia release CatS, which can release FKN from neuronal surfaces, which in turn drives microglial responses via CX3CR1 receptors; some of the cytokines released by microglia in these pain models, such as TNFα, are able to stimulate these cells as well as astrocytes and neurons. The presence of these feedback mechanisms may explain the sensitivity of microglial cells and astrocytes to multiple signals, each of which when blocked leads to a progressive winding down of glial activity.

As reviewed above, multiple mediators can be released by glial cells in their different states of enhanced responsiveness. The inflammatory cytokines TNFα and IL-1β are frequently reported. And indeed, there is good evidence that these contribute to altered neuronal processing of painful stimuli in the spinal cord. However, there is also evidence for the release of other factors including ATP, NOS, prostanoids, BDNF, CatS, MMP-2, MMP9, and several chemokines, including CCL2. Again an issue for this field is defining the relative roles of these different mediators in the modulation of pain processing.

Do Glial Cells Contribute to Chronic Pain in Humans?

The evidence and ideas reviewed in the sections above relate to preclinical studies. While it is likely that some, perhaps much, will translate to pain states in humans, this has not been formally demonstrated, which is not surprising given the difficulty of studying spinal glial cell function in humans. And it is also worth remembering that not all findings do translate, as was unfortunately the case recently in attempts to develop a drug targeting CD-28 (see http://en.wikipedia.org/wiki/TGN1412).

The plain fact is that to date we have no direct evidence for a role of central glia in human chronic pain states. One problem is the difficulty of functionally studying these cells in humans. One possible way to circumvent this is via the use of postmortem material, and a recent study reports changes in microglia and astrocytes in the posterior horns of the spinal cord of patients with longstanding regional pain syndrome (Del Valle et al., 2009). There are, however, some potential confounding factors in this study. In this particular study the patients had been treated with morphine for their ongoing pain conditions, but treatment may have directly contributed to microglial changes. Moreover, there were signs of neuronal loss in the posterior horn and a glial response could have occurred as a result of this degeneration rather than as a primary consequence of the peripheral pathology precipitating the pain state. Nonetheless, these findings are interesting in view of the observations that the levels of proinflammatory cytokines IL-1β and IL-6 are increased in the CSF of some patients with chronic regional pain syndromes (Alexander et al., 2005). Systemic and extensive studies are of course difficult with postmortem material. However, there are some specialized resources available, such as HIV-neuropathy related tissue banks (e.g. http://www.neuro.jhmi.edu/HIV/researchers.htm) that could be exploited to this end.

There are also some in vivo opportunities, perhaps the greatest of which involves imaging techniques (Melzack, 2009). The imaging of microglial status in pain states would be desirable in both preclinical and clinical settings. The observation that the "peripheral benzodiazepine binding site" (PBD) protein complex is not normally expressed by microglia, but becomes upregulated in some pain states, may offer important opportunities, although PBD is not specific for microglia. One finding consistent with this idea is that PBD binding to microglia in the human thalamus increases many years after peripheral nerve injury (Banati et al., 2001). Therefore, PET ligands for this receptor may allow the state of microglial to be studied in vivo.

There also remains the possibility of exploiting other glial-specific receptors and pathways to evaluate the contribution of these cells in different pathologies in humans. However, nothing to date has been described that is sufficiently selective to enable unambiguous inferences to be drawn on glial function following systemic treatment with appropriate ligands.

Concluding Remarks

The neurobiology of neuronal-glial interactions is emerging as a fascinating and complex field. Such interactions are evident in many contexts, but perhaps one of the most intriguing is in the potential contribution to neuropathic pain originating from peripheral nerve injuries. This field offers the opportunity to study glial recruitment in the absence or presence of direct damage to the CNS. New facts and new mechanisms are being revealed, but as we have reviewed here, there remain numerous gaps in our knowledge. Perhaps the most significant of these relates to the clinical importance of current knowledge. Many of the experimental pain states that have been studied are not commonly seen clinically. Moreover, the preclinical studies have focused for the most part on early time points, whereas the clinical problem is usually greatly prolonged. We do not currently have many methods to study the questions in humans. Nonetheless this field offers clinical potential as well as academic interest.

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