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Current Perspective

Microglial Regulation of Neuropathic Pain

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Abstract. Neuropathic pain is a highly debilitating chronic pain state that is a consequence of nerve injury or of diseases such as diabetes, cancer, infection, autoimmune disease, or trauma. Neuropathic pain is often resistant to currently available analgesics. There is a rapidly growing body of evidence indicating that signalings from spinal microglia play crucial roles in the pathogenesis of neuropathic pain. After peripheral nerve injury, microglia transform to reactive states through the expression of various genes such as cell-surface receptors (including purinergic receptors) and proinflammatory cytokines that enhance synaptic transmission in dorsal horn neurons. Inhibiting function or expression of these microglial molecules strongly suppresses pain hypersensitivity to innocuous mechanical stimuli (tactile allodynia), a hallmark symptom of neuropathic pain. A recent study also reveals that the transcription factor IRF8 (interferon regulatory factor 8) is a critical regulator of the nerve injury–induced gene expression in microglia. The present review article highlights the recent advances in our understanding of spinal microglia in neuropathic pain.

Keywords: microglia, purinergic signaling, transcription factor, neuropathic pain, spinal cord

1. Introduction

Injury to the nervous system arising from bone compression in cancer, diabetes, infection, autoimmune disease, or physical injury results in debilitating chronic pain states (so-called neuropathic pain). A troublesome symptom of neuropathic pain is tactile allodynia (pain hypersensitivity to normally innocuous stimuli), in addition to spontaneous pain and hyperalgesia (the increased pain perception of noxious stimuli). Neuropathic pain is refractory to currently available treatments such as nonsteroidal anti-inflammatory drugs and opioids (1, 2). We are now beginning to understand that neuropathic pain is not just a symptom of disease but is a consequence of disordered functioning of the nervous system (1 – 3).

From the results of studies utilizing diverse animal models of neuropathic pain, it has been indicated that neuropathic pain is a reflection of the aberrant excitability of dorsal horn neurons evoked by peripheral sensory inputs (3, 4). Emerging lines of evidence suggest that peripheral nerve injury (PNI)-induced hyperexcitability might not be a consequence merely of changes in neurons but rather of multiple alterations in glial cells. In this article, we highlight recent advances that further increase our understanding of the mechanisms underlying neuropathic pain, with a specific focus on microglia in the spinal cord.

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Invited article

2. Microglial reactivity following PNI

Microglial cells are known as resident macrophages in the central nervous system (CNS), which derive from primitive macrophages in the yolk sac (5). In the adult, microglia are ubiquitously distributed throughout CNS and have small cell body bearing branched and motile processes, which monitor the local environment in the CNS (6, 7). Microglia also show a stereotypical long-term response to a wide range of stimuli that threaten physiological homeostasis, including PNI. In response to PNI, microglia activation in the spinal cord progresses through a hypertrophic morphology, an increase in cell number, and an alteration in gene expression. It was found that spinal microglia express a receptor for the cytokine inter-

feron-γ (IFN-γR) and that stimulating IFN-γRs in naïve animals by means of intrathecal administration of IFN-γ produces a hypertrophic morphology and an increase in the microglial number. Furthermore, IFN-γR–knockout mice showed the reduction of morphological and numerical changes of microglia after PNI. Although the source of IFN-γ remains to be identified, the IFN-γ/IFN-γR system is critical in transforming resident spinal microglia into the activated state.

On the other hand, Zhang et al. have shown that bone marrow cells injected intravenously into lethally irradiated recipient mice were observed in the spinal cord parenchyma ipsilateral to PNI (8). Interestingly, bone marrow cells that had migrated into the dorsal horn underwent proliferation, expressed ionized calcium-binding adapter molecule-1 (Iba1, a marker of microglia/macrophages), and showed microglia-like morphology. However, it was also reported that the migration of bone marrow cells into the CNS might be due to experimental manipulations such as irradiation (which may influence the blood brain barrier) and injection of donor cells (9). Thus, whether, in non-irradiated mice, there is a subpopulation of spinal microglia derived from endogenous bone marrow–derived cells after PNI remains unresolved.

3. Microglial purinergic regulation of neuropathic pain

3.1. $P2X_4$ receptor ($P2X_4R$)

Purinergic P2 receptors are divided into two families, ionotropic receptors (P2X) and metabotropic receptors (P2Y). P2X receptors (of which there are seven types, $P2X_1$ – $P2X_7$) contain intrinsic pores that open upon binding of ATP (10). P2Y receptors (of which there are eight types, $P2Y_{1, 2, 4, 6, 11, 12, 13}$ and $_{14}$) are coupled to intracellular second-messenger systems through heteromeric G-proteins (11). Nucleotides are considered to be released or leaked from glial cells as well as neurons, and thus purinergic signaling plays an important role in cell-to-cell communications under physiological and pathophysiological conditions (12).

The first evidence for a causal role of microglia was the finding that PNI-induced tactile allodynia was reversed by pharmacological blockade of $P2X_4$Rs in the spinal cord (13). This is because, in the spinal cord, expression of $P2X_4$Rs was upregulated exclusively in microglia. It was indicated that PNI-induced pain hypersensitivity depends on ongoing purinergic signaling via microglial $P2X_4$Rs. A marked reduction in neuropathic pain in both mice knocked down and knocked out of $P2X_4R$ further demonstrated the necessity of $P2X_4$Rs (13 – 15). Intrathecal delivery of $P2X_4R$-stimulated microglia caused normal rats to produce allodynia, indicating the sufficiency of $P2X_4R$ (13, 16). Moreover, it was shown that activation of microglial $P2X_4$Rs stimulates the synthesis and release of brain-derived neurotrophic factor (BDNF) (14, 17) and that BDNF then causes an alteration of the transmembrane anion gradient in a subpopulation of dorsal horn lamina I neurons presumably through the downregulation of the neuronal chloride transporter KCC2, which in turn renders GABA and glycine effects depolarizing, rather than hyperpolarizing, in these neurons. Thus, $P2X_4R$-stimulated microglia release BDNF as a crucial factor to signal to lamina I neurons, causing aberrant nociceptive output that contributes to neuropathic pain (18).

From these results, upregulation of $P2X_4R$ expression in microglia would be a key process. Several studies have identified molecules that upregulate $P2X_4R$ expression in microglia in vitro (19). Among these, the extracellular matrix protein fibronectin has been well-studied. Intrathecal injection of fibronectin to naïve animals increased $P2X_4R$ expression and produced allodynia, a behavior that was not observed in $P2X_4R$-deficient mice administered fibronectin. The level of fibronectin protein was elevated in the dorsal horn after PNI (20), which was presumably due to a leakage of this protein from the blood as a consequence of a collapse of blood–spinal cord barrier functions after PNI (21). For molecular mechanisms underlying $P2X_4R$ upregulation by fibronectin, it has been proposed that fibronectin activates the Src-family kinase (SFK) member Lyn through $\alpha5\beta1$ integrins (22, 23). Activated Lyn kinase then stimulates the phosphatidylinositol 3-kinase (PI3K)-Akt and mitogen-activated protein kinase kinase (MAPK kinase, MEK)-extracellular signal-regulated kinase (ERK) signaling cascades. Signaling through the PI3K–Akt and MEK–ERK cascades has distinct roles in the upregulation of $P2X_4R$ expression in microglia at the transcriptional and post-transcriptional levels, respectively (23).

Microglia show altered $P2X_4R$ expression in response to multiple types of nervous system damage (24, 25). However, peripheral tissue inflammation caused by intraplantar injection of complete Freund's adjuvant does not increase microglial $P2X_4R$ (13). Recently, the chemokine CCL21 was shown to be a factor derived from injured DRG neurons that directly contributes to $P2X_4R$ expression in spinal microglia (26). However, the relationship between fibronectin and CCL21 remains to be elucidated.

$P2X_4$Rs are located predominantly within lysosomal compartments and are targeted there by their N- and C-terminal motifs (27). Notably, $P2X_4$Rs remain stable within lysosomes and resist degradation. When microglia are stimulated artificially by either the TLR4 agonist LPS (28, 29) or the Ca^{2+} ionophore ionomycin (27), trafficking of $P2X_4R$ protein to the cell surface occurs.

These results imply that $P2X_4R$-mediated responses in microglia might be potentially dependent on the extracellular milieu. Recently, it was found that the chemokine CCL2 increased $P2X_4R$ protein levels on the cell surface (without changing total cellular expression) via CCR2. Therefore, CCL2 might be an enhancer of $P2X_4R$ trafficking to the surfaces of microglia, and CCR2 activation may render microglial cells hyper-responsive to extracellular ATP by enhancing $P2X_4R$ expression on their cell surfaces (30).

3.2. $P2X_7$ receptor ($P2X_7R$)

Stimulation of $P2X_7Rs$ in microglia is implicated in inflammatory responses, such as release of proinflammatory cytokines [interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNFα), etc.] (31 – 33). The involvement of $P2X_7Rs$ in pain was provided by a study showing that $P2X_7R$-deficient mice exhibited reduced thermal and mechanical hypersensitivities after PNI (34). $P2X_7R$ expression was increased in the spinal cord after PNI (35, 36). The predominant type of cells expressing these receptors was microglia. Intraperitoneal and intrathecal administration of $P2X_7R$ antagonists attenuated the development of mechanical hypersensitivity (35 – 37). Activating $P2X_7Rs$ might affect neuronal excitation of dorsal horn neurons because intravenous administration of A-438079 (i.v.) reduced innocuous stimuli–evoked activity of dorsal horn neurons in neuropathic rats (38). Microglial $P2X_7R$ stimulation can also induce synthesis and release of chemokines including CCL3 and CXCL2 (39, 40). CCL3 expression in the dorsal horn was increased after PNI, and intrathecal injection of a neutralizing antibody for CCL3 reduced neuropathic pain behaviors (41). Thus, microglial $P2X_7Rs$ might participate in the neuronal hyperexcitability of dorsal horn neurons and the development of neuropathic pain through production of proinflammatory cytokines and chemokines. In addition, it has been shown that homotrimeric $P2X_7R$ is physically associated with $P2X_4R$ (28). $P2X_7$ is expressed at the cell surface, whereas the predominant localization of $P2X_4$ is in intracellular lysosome. If the trafficking of $P2X_4$ to the plasma membrane is enhanced in activated microglia (such as by CCR2 stimulation), an interaction between these two receptors might occur. Thus, it is of particular interest to investigate the interaction between $P2X_4R$ and $P2X_7R$ in neuropathic pain.

3.3. $P2Y_{12}$ receptor ($P2Y_{12}R$)

A role for $P2Y_{12}Rs$ in neuropathic pain was also suggested on the basis of reduced pain sensitivity in $P2Y_{12}R$-deficient mice, in $P2Y_{12}R$-knockdown mice, and after pharmacological blockade of the receptor (42, 43). $P2Y_{12}R$ expression levels were dramatically increased in microglial cells in the spinal cord after PNI (42, 43). Several lines of evidence have indicated that $P2Y_{12}Rs$ are implicated in the motility of microglial cell bodies and processes (6, 44 – 46). Thus, $P2Y_{12}R$ upregulation in microglia may influence the abilities of microglia to extend the tips of their branched processes toward neighboring cells. Using electron microscopy, Maeda et al. revealed that dorsal horn microglia adhere to and engulf both injured and uninjured myelinated axons, which seems to be mediated by $P2Y_{12}Rs$ (47). Thus, engulfment of myelinated axons by activated microglia via $P2Y_{12}R$ signaling in the dorsal horn may be crucial for an effective interaction between axons and microglia to induce the pathogenesis leading to abnormal pain.

4. Transcription factor IRF8: a new player for microglial activation and pain

PNI activates microglia and converts them to reactive cells. Recently, our group found that interferon regulatory factor-8 (IRF8) is a crucial player for microglial activation (48). IRF8 is a member of the IRF family (IRF1 – 9), and is expressed in immune cells such as lymphocytes and dendritic cells (49). The role of IRFs in the CNS was entirely unknown, but Masuda et al. showed that within the spinal cord, IRF8 expression was markedly upregulated in microglia, but not in neurons or astrocytes, after PNI. The microglia-specific upregulation of IRF8 started from postoperative day 1, peaked on day 3, and persisted for at least 3 weeks after PNI (48). IRF8-deficient mice showed reduction of PNI-induced tactile allodynia with no change in basal mechanical sensitivity or inflammatory pain caused by intraplantar injection of complete Freund's adjuvant. Furthermore, suppressing upregulated expression of spinal IRF8 after PNI by intrathecal administration of a small interfering RNA (siRNA) targeting IRF8, given on day 5 and 6 post-PNI to wild-type mice that had developed allodynia, caused a significant recovery of tactile allodynia. These results indicate that microglial IRF8 is necessary for the development and maintenance of tactile allodynia after PNI. In in vitro studies using cultured microglial cells overexpressing IRF8, it was shown that these cells promoted the transcription of genes associated with reactive states: those include genes involved in microglial innate responses [toll-like receptor 2 (TLR2)], chemotaxis ($P2Y_{12}R$ and the chemokine receptor CX3CR1), and inflammatory components (IL-1β, cathepsin S, BDNF, and $P2X_4R$). A mutant IRF8 lacking the DNA-binding domain failed to produce the effects observed with wild-type IRF8. Importantly, these gene expressions in the spinal cord following PNI was prevented in IRF8-deficient mice, suggesting that transcriptional activation

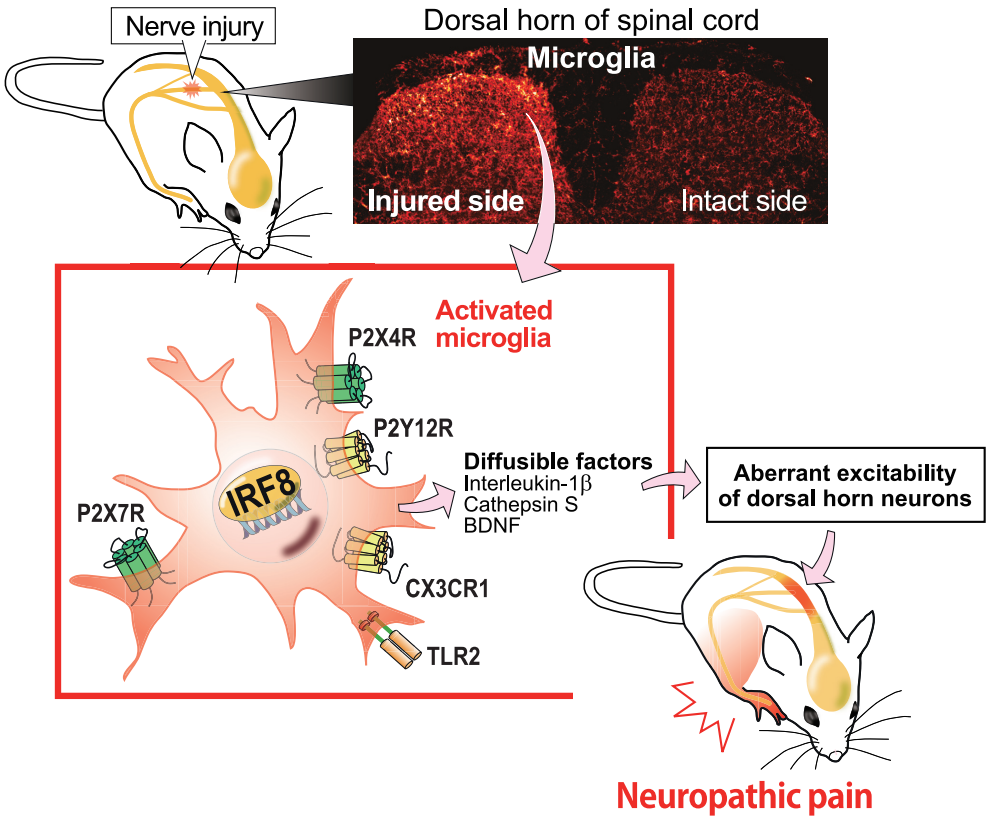

Fig. 1. Schematic illustration of the potential mechanisms by which IRF8-derived reactive microglia in the dorsal horn regulate neuropathic pain. After peripheral nerve injury, microglia become activated in the dorsal horn of the spinal cord (image: OX-42 immunofluorescence). Activated microglia show increased expression of the transcription factor IRF8 (interferon regulatory factor 8). IRF8 plays a crucial role in the upregulation of several genes in microglia caused by peripheral nerve injury. Those include purinergic receptors $P2X_4R$ and $P2Y_{12}R$, the chemokine receptor CX3CR1, toll-like receptor 2 (TLR2), interleukin-1β, cathepsin S, and brain-derived neurotrophic factor (BDNF). In the case of $P2X_7R$ expression, the role of IRF8 seems to be minor. These diffusible factors might cause aberrant excitability in the dorsal horn pain network, which might be responsible for neuropathic pain.

by IRF8, which is dependent on its ability to bind DNA, occurs in microglia in the spinal cord after PNI. However, lack of IRF8 does not result in a global defect in reactive processes of microglia because nerve injury–induced proliferation of spinal microglia is not affected by IRF8 deficiency. Furthermore, the impact of microglial IRF8 in modulating pain was demonstrated from the data showing that transferring IRF8-overexpressing microglia spinally to normal mice produced pain. The behavioral alteration was not observed in mice that were spinally transferred the mutant IRF8–transduced microglia. Also, pretreating IRF8-transduced microglia with a cocktail containing a function-blocking antibody for IL-1β and an inhibitor of cathepsin S attenuated tactile allodynia by IRF8-transduced microglia. Thus, these results imply that inflammatory signals derived from microglia overexpressing IRF8 can produce allodynia. Collectively, it was suggested that IRF8 activates a program of gene expression that transforms microglia into a reactive phenotype driving neuropathic pain, and these results provide a new mechanism for microglial activation (Fig. 1). This picture also raises several questions. One is how IRF8 expression is upregulated in microglia after nerve injury. It has been reported that IFN-γ increases IRF8 expression in vitro (50), the deficiency of IFN-γ receptor fails to prevent PNI-induced IRF8 expression in the spinal cord (48). Thus, it is proposed that an IFN-γ–independent mechanism is involved. Considering the fact that IRF8 upregulation occurs in response to nerve injury but not to peripheral tissue inflammation, it is thus possible that the upregulation of IRF8 would be induced by molecules that are associated with nerve injury. Indeed, IRF8 upregulation is also induced in brain microglia by hypoglossal nerve axotomy and kainic acid–induced neuronal injury (48). However, nerve injury–related molecules that induced IRF8 upregulation remain to be identified. Also, whether IRF8 directly binds to promoter regions of these genes and promotes their expression remains to be determined. Answers to these issues will provide new insights into the molecular mechanisms underlying microglial activation and neuropathic pain.

5. Conclusion

We have primarily focused on the roles of spinal microglia in neuropathic pain. Pharmacological, molecular and genetic manipulations of the function or expression of these microglial molecules have been shown to influence nerve injury–induced pain behaviors and hyperexcitability of the dorsal horn pain pathway. Therefore, spinal microglia activity makes a critical

contribution to pathologically enhanced pain processing in the dorsal horn, and microglia might be promising targets for treating neuropathic pain. It is expected that an increased understanding of the functions of microglia will provide us with exciting insights into pain mechanisms and clues to aid the development of new therapeutic agents for the management of neuropathic pain.

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