Microglia-Mediated Regulation of Neuropathic Pain: Molecular and Cellular Mechanisms

Makoto Tsuda

Department of Life Innovation, Graduate School of Pharmaceutical Sciences, Kyushu University; 3–1–1 Maidashi, Higashi-ku, Fukuoka 812–8582, Japan.

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Pain is a defense system that responds rapidly to harmful internal and external stimuli through the somatosensory neuronal pathway. However, damage to the nervous system through cancer, diabetes, infection, autoimmune disease, chemotherapy or trauma often leads to neuropathic pain, a debilitating chronic pain condition. Neuropathic pain is not simply a temporal continuum of acute nociceptive signals from the periphery, but rather due to pathologically altered functions in the nervous system, which shift the net neuronal excitatory balance toward excitation. Although alterations were long thought to be a result of changes in neurons, but an increasing body of evidence over the past decades indicates the necessity and sufficiency of microglia, the tissue-resident macrophages of the spinal cord and brain, for nerve injury-induced malfunction of the nervous system. In this review article, I describe our current understanding of the molecular and cellular mechanisms underlying the role of microglia in the pathogenesis of neuropathic pain and discuss the therapeutic potential of microglia from recent advances in the development of new drugs targeting microglia.

Key words microglia; neuropathic pain; spinal cord

1. INTRODUCTION

Human and other organisms acquire nociceptive pain as a defense system that rapidly responds to a wide range of harmful internal and external stimuli that threaten physiological homeostasis. Noxious stimuli such as heat and mechanical and chemical irritants to the skin excite nociceptive primary afferent sensory neurons, called nociceptors (thin, myelinated Aδ and unmyelinated C fibers). The excitatory information is then conveyed to the spinal dorsal horn (SDH) and activates brain-projecting nociceptive pain transmission neurons in lamina I1–4 via complex neuronal circuits. The information then goes up to the brainstem and higher brain regions related to sensory and affective components of pain.1,5–7 In contrast, somatosensory signals evoked by innocuous stimuli such as touch to the skin are also conveyed to the SDH through low threshold mechanoreceptors (LTMRs) including thick, myelinated Aβ fibers, which activate distinct SDH neuronal circuits. These Aβ fibers anatomically connect to nociceptive lamina I neurons via excitatory and inhibitory interneurons, but the signaling flow from Aβ fibers is normally interrupted by inhibitory synaptic inputs from γ-aminobutyric acid (GABA)- or glycine-containing interneurons. Thus, under normal healthy conditions, Aβ fibers do not activate nociceptive projection neurons and do not cause pain. As such, the accurate perception of pain, as well as other sensations, requires properly wired neuronal circuits and their correct function.

However, following damage to the nervous system such as by cancer, diabetes, infection, autoimmune disease, chemotherapy and trauma, this system can malfunction. These conditions often cause a debilitating chronic pain syndrome (termed neuropathic pain). A cardinal symptom of neuropathic pain is mechanical allodynia (touch-evoked pain). Neuropathic pain does not resolve even after the healing of tissue damage, and can persist for long periods of time. Thus, it is considered that the pain is not simply a temporal continuum of acute nociceptive signals from the periphery, but rather due to pathologically altered functions in the nervous system.2,4,8,9 Pathological changes and their molecular and cellular mechanisms have been studied using models of neuropathic pain, for example rodent models established by peripheral nerve injury (PNI). A growing body of evidence indicates that PNI causes anatomical and functional modifications in primary afferent sensory neurons, SDH neurons and the brain. These modifications shift the balance between synaptic excitation and inhibition toward excitation, which may account for development and maintenance of chronic pain.5,4,8,9 These alterations were long thought to be a consequence simply of changes in neurons, but accumulating evidence during the last 15 years indicates the important role of non-neuronal cells of the nervous system, including monocytes, macrophages, T cells, and glial cells.10,11 In this review article, I describe the molecular and cellular mechanisms underlying the role of microglia in the pathogenesis of neuropathic pain and also discuss the therapeutic potential of microglia from recent advances in the development of new drugs targeting microglia.

2. MICROGLIA

Microglial cells are known as the tissue-resident macrophages of the central nervous system (CNS) and constitute 5–10% of total cells in the adult CNS. Microglia were originally described by Pio del Rio-Hortega in 191912 and proposed to have a mesodermal origin.13 By fate mapping analyses, erythromyeloid progenitors generated in the embryonic yolk sac were identified as the origin of microglia.14 The progeni-
tors develop into microglia progenitors via an immature and more mature stage, then leave the yolk sac and migrate to the brain through blood vessels, and, terminally differentiate into microglia. In the healthy CNS, microglia remain throughout life and are maintained by self-renewal. As microglia have a unique molecular signature compared with other myeloid and immune cells such as circulating monocytes or macrophages, a distinct gene expression program is required for the development of microglia. It has been shown that interleukin-34 (IL-34) is crucial for their development and transforming growth factor-β (TGF-β) is required for promoting terminal differentiation and acquiring adult microglia properties. For maintaining microglia in adults, colony stimulating factor 1 receptor (CSF1R) signaling might have an ongoing role. Microglia in the adult CNS represent a unique morphology, which has a small soma with thin and branched processes whose motility is highly dynamic. Furthermore, microglia directly contact presynaptic terminals and dendritic spines and, in response to neuronal excitation, extend their processes toward highly active synapses. Now, microglia in the CNS are received much attention as being crucial for forming and refining neuronal circuitry and network connectivity, and contributing to neuronal plasticity.

3. MICROGLOSIS IN THE SPINAL DORSAL HORN AFTER PNI

Injury to the peripheral nerves activates microglia in the SDH (Fig. 1). In the late 1970s, it was found that non-neuronal cells (which were later identified as microglia) are increased in the SDH after PNI. Two possibilities have been considered as the mechanisms for the PNI-induced SDH microgliosis. First is proliferation of resident microglia because after PNI, SDH microglia was found to be immunohistochemically labeled by proliferation markers. A recent study examining the detailed temporal kinetics of microgliosis by a short-pulse labeling of proliferating cells showed a narrow time window for rapidly inducing a proliferation burst of SDH microglia after PNI. One potential candidate factor recently identified is CSF1. Consistent with the kinetics of microglial proliferation after PNI, CSF1 expression is rapidly induced in injured dorsal root ganglion (DRG) neurons. This expression seems to involve IL-1β from surrounding satellite glia. Conditional knockout of CSF1 in DRG neurons prevented the PNI-induced microglial proliferation, and, conversely, intrathecal administration of CSF1 to normal mice induced proliferation, indicating that CSF1 in injured DRG neurons is necessary and sufficient to induce microglial proliferation after PNI. In contrast, it should be noted that the upregulation of CSF1 is not transient but persists until a few weeks after PNI when microglial proliferation has already terminated, suggesting a distinct role for CSF1–CSF1R signaling at this later phase, such as the control of the expression of microglial genes. The second possible mechanism could be infiltration of bone marrow (BM)-derived circulating monocytes into the parenchyma of the SDH, which then differentiate into microglia-like cells. This infiltration of BM cells was found in BM chimeric mice that had received lethal irradiation with BM transplantation. The dose of irradiation used was high, which can produce toxic side effects including an induction of chemoattractants and a disruption of the blood brain/spinal cord barrier. On the other hand, in BM chimeric mice generated by a lower irradiation dose, such infiltration of BM cells was not observed. In addition, several studies also showed no contribution of circulating monocytes to the PNI-induced microgliosis using parabiosis mice (a model in which two mice are surgically joined and share circulating blood) and transgenic mice enabling distinct visualization

Biography
Makoto Tsuda received his Ph.D. from Hoshi University (Tokyo, Japan) in 1998. He then worked as a postdoctoral fellow in the laboratories of Dr. Kazuhide Inoue at National Institute of Health Science (NIHS; Tokyo, Japan; 1998–2002) and of Dr. Michael Salter at Hospital for Sick Children (Toronto, Canada; 2002–2004). He then returned to the laboratory of Dr. Inoue at NIHS as Research Associate (2004–2005). He was appointed as Assistant Professor at Graduate School of Pharmaceutical Sciences, Kyushu University (Fukuoka, Japan) in 2005, as Associate Professor in 2006 and as Professor (2014). He received The Award for Young Investigator of JPS (2007), The Award of Young Scientist from MEXT of Japan (2007), The Award for Distinguished Young Investigator of JSN (2007), and The Pharmaceutical Society of Japan Award for Divisional Scientific Promotion (2019). Work in his laboratory is primarily directed at elucidating glia–neuron interactions in the spinal cord and brain, to understanding the cellular and molecular mechanisms of pain and itch, and to devising strategies for new types of pain and itch relieving medications.
of resident microglia and circulating monocytes. Together, it is now appreciated that local expansion of resident microglia by proliferation is the primary cellular mechanism for SDH microgliosis after PNI. Nevertheless, it should be noted that infiltration of circulating monocytes might occur in the SDH of other models of neuropathic pain. For example, in experimental autoimmune encephalomyelitis (EAE; a model of multiple sclerosis, with chronic pain being a common symptom), monocytes massively infiltrated into the spinal cord with demyelinating lesions, although these monocytes did not permanently contribute to the resident microglia pool.

4. CAUSAL LINK BETWEEN MICROGLIA AND NEUROPATHIC PAIN

Following initial reports of PNI-induced microgliosis in the SDH, the role of microglia in neuropathic pain remained unclear for about thirty years. In 2003, a causal link was first uncovered by studies investigating the role of the ionotropic purinergic receptor subtype P2X4 receptor (P2X4R) and p38 mitogen-activated protein kinase (p38MAPK). P2X4R is a subtype of the P2X family consisting of ATP-gated ionotropic receptors, which are formed by three subunits, permeable to Na+, K+, and Ca2+. It was found that expression of P2X4R in the SDH is upregulated exclusively in microglia after PNI, and that pharmacological blockade of P2X4R suppress the PNI-induced mechanical allodynia, indicating the necessity of microglial P2X4R in neuropathic pain. Furthermore, intrathecal administration of P2X4R-stimulated cultured microglia to normal rats induced allodynia, indicating its sufficiency. The EAE model of multiple sclerosis—with its common symptom of chronic pain—showed microglial activation and P2X4R upregulation in the SDH, and mechanical allodynia. An increase in expression of P2X4R was also found in SDH microglia of a model of acute inflammatory demyelinating polyradiculoneuropathy, the most common subtype of Guillain-Barre syndrome. In a model of herpetic pain, microglia-selective upregulation of P2X4R in the SDH was also found, and its time course corresponded with mechanical allodynia. The herpes-induced mechanical allodynia was suppressed by pharmacological blockade of P2X4Rs. Thus, microglia activation and their expression of P2X4R are commonly observed phenomena in diverse models of neuropathic pain. How- ever, the contribution of microglial P2X4R in neuropathic pain may be different between males and females. It was reported that PNI did not upregulate P2X4R expression and that pharmacological inhibition of P2X4R had no effect on mechanical allodynia in female mice. A sex difference in the role of microglial molecules in neuropathic pain was also reported in Toll-like receptor 4 (TLR4) and p38MAPK, both of which have a crucial role in spinal microglia in males. Nevertheless, there are also reports showing no obvious sexual dimorphism in the suppressive effect on PNI-induced pain by genetic knockout of microglia-selective genes including the G-protein-coupled P2 receptor P2Y12R and C-X3-C motif chemokine receptor 1 (CX3CR1). In addition, P2X4R upregulation in spinal microglia of females has been observed in rodent models of bone cancer pain and herpetic pain. In the latter model, microglial P2X4R activates the brain-derived neurotrophic factor (BDNF)-tropomyosin-related kinase B (TRKB) signaling pathway. Thus, further work needs to be performed to clarify the sex-dependent contribution of SDH microglia in other models of neuropathic pain.

5. MOLECULAR MECHANISMS FOR EXPRESSION OF MICROGLIAL P2X4R

PNI-induced microglial activation dramatically changes expression of genes. Besides P2X4R, numerous microglia-selective molecules (approximately 40) are currently identified as being implicated in PNI-induced pain. One of the key regulators which govern a microglia-selective transcription in the context of PNI is interferon regulatory factor 8 (IRF8), a member of the IRF family. It was demonstrated that after PNI, expression of IRF8 in the SDH is upregulated exclusively in microglia and IRF8 regulated expression of P2X4R and other microglial genes including P2Y12R, TLR2, and CX3CR1 and diffusible factors (IL-1β, cathepsin S (CatS) and BDNF). IRF8 directly regulates other members of the IRF family such as IRF1 and IRF5. IRF5 was shown to bind to the putative promoter region of P2X4R and enhance its expression. Indeed, a deficiency of IRF5 suppressed the PNI-induced spinal P2X4R upregulation and neuropathic allodynia. Therefore, the IRF8–IRF5 transcription axis would be an important mechanism for producing P2X4R-expressing microglia after PNI and neuropathic pain (Fig. 2). A recent study also showed that v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB), which is a basic leucine zipper transcription factor, is also involved in microglial P2X4R upregulation after PNI. Microglia-selective knockout of MAFB in adult mice suppresses the PNI-induced mechanical allodynia and a knockdown of MAFB reduces P2X4R. However, spinal MAFB knockdown had no effect on established mechanical allodynia, suggesting that MAFB plays an important role in initiation of P2X4R-expressing microglia and neuropathic pain after PNI.

What extracellular factor(s) triggers P2X4R expression in microglia? Because P2X4R expression in SDH microglia was found unaltered in a chronic pain model associated with peripheral tissue inflammation, microglial P2X4R upregulation might involve factors released from damaged DRG neurons. Possible candidates include CSF1 and cysteine-cysteine chemokine ligand 21 (CCL21), whose expressions were found to be increased in injured DRG neurons. CSF1 also increased expression of IRF8 in spinal microglia. In addition, other extracellular factors whose source remains to be determined could also be involved in the P2X4R expression in microglia.

6. CELLS RELEASING ATP TO ACTIVATE MICROGLIA

Extracellular ATP must be needed to activate microglial P2X4Rs. ATP has been reported to be released from primary afferents, SDH neurons and glial cells. However, the cell type responsible for releasing ATP within the SDH in the context of PNI was unknown. We recently demonstrated that SDH neurons that express vesicular nucleotide transporter (VNUT, also known as SLC17A9, a secretory vesicle protein responsible for storage and release of ATP) are a crucial source of the ATP that causes pain hypersensitivity. It was found that PNI increases expression of VNUT and extracel-
lular ATP content within the spinal cord. A deficiency of VNUT attenuated the PNI-induced mechanical allodynia. This was phenocopied by a specific knockout of VNUT in SDH neurons, but not in primary sensory neurons, microglia or astrocytes. Thus, VNUT in SDH neurons is necessary for ATP release that contributes to neuropathic pain.

7. MICROGLIA-DERIVED FACTORS CRUCIAL FOR NEUROPATHIC PAIN

Activated microglia also express a variety of humoral factors such as proinflammatory cytokines, chemokines and trophic factors. After stimulation of P2X4R in microglia, BDNF was found to be released.82,83) BDNF was then shown to act on TrkB in lamina I neurons and to induce an altered transmembrane anion gradient by downregulating K⁺–Cl⁻ co-transporter 2 (KCC2), which causes changes in GABA- and glycine-evoked responses from inhibitory to excitatory and mechanical hypersensitivity82) (Fig. 2). This abnormal excitation results in potentiating glutamatergic excitation via glutamate receptors. The resulting hyperexcitability of pain transmission in neurons contributes to neuropathic pain.

Fig. 2. P2X4R-Expressing Spinal Microglia in Neuropathic Pain

After PNI, microglia in the spinal dorsal horn (SDH) become activated and undergo robust proliferation (microgliosis) after nerve injury. These microglia upregulate P2X4R expression through an IRF8–IRF5 transcriptional axis. IRF8 induces IRF5 expression, and then IRF5 directly binds to the promoter region of the P2xr4 gene and induces expression of P2X4R mRNA. Microglial P2X4R is activated by extracellular ATP released from SDH neurons and, in turn, releases bioactive diffusible factors, such as BDNF. BDNF then downregulates KCC2 in SDH pain transmission neurons, via TrkB, which causes an increase in intracellular Cl⁻ and leads to a depolarizing shift in the anion reversal potential. Under these conditions, GABA or glycine released as a result of innocuous stimulation induces neuronal depolarization. The depolarization and TrkB signaling also result in potentiating glutamatergic excitation via glutamate receptors. The resulting hyperexcitability of pain transmission in neurons contributes to neuropathic pain.

In the SDH, P2X7R mediates ATP-induced IL-1β release from TLR4-primed microglia.80) PNI-induced IL-1β transcription in the spinal cord involves TLR287) and TLR4.80) At a post-transcription level, the nod-like receptor family, pyrin domain containing-3 protein (NLRP3) inflammasomes activate procaspase-1, which promotes pro-IL-1β processing and secretion of mature IL-1β.89) IL-1β has been shown to phosphorylate NMDARs90) and to enhance excitatory synaptic transmission.91–93) IL-1β also decreases GABA- and glycine-mediated synaptic inhibition.92) In addition, microglial IL-1β acts on astrocytes, which contributes to neuropathic pain.94) SDH astrocytes also become activated after PNI and contribute to maintenance of pain hypersensitivity,95–97) suggesting a crucial role of microglia–astrocyte signaling in pain chronicity in the context of PNI. As for TNFα, its expression in the SDH was found to be increased in microglia after PNI via p38MAPK.98) In SDH neurons, TNFα rapidly increases excitatory responses evoked by activation of NMDARs and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA receptors).92,99) In microglia, activation of TNF receptors increases expression of BDNF, which was shown to increase dendritic structural remodeling and synaptic connectivity strength in lamina I SDH neurons.100) Collectively, TNFα modulates synaptic structure and strength in SDH neurons by multiple mechanisms involving direct and indirect effects.

CatS is a lysosomal cysteine protease that was found to be released from microglia.101) It was shown that microglial CatS released in response to P2X7R activation via p38MAPK cleaves membrane-bound fractalkine expressed on SDH neurons and astrocytes.102) The cleaved fractalkine is considered to act on microglia again because the fractalkine receptor
CX3CR1 is expressed exclusively in microglia. Activation of the P2X7R–p38MAPK–CatS–fractalkine–CX3CR1 pathway leads to IL-1β secretion from microglia, which in turn modulates synaptic excitation and inhibition, as described above.

8. DEVELOPMENT OF MICROGLIA-TARGETING DRUGS

The preclinical studies described indicate great interest in microglia as a promising target for treating neuropathic pain. To date there are no clinically approved drugs that selectively target microglia and their molecules, but drug discovery efforts are currently in progress. As for P2X4Rs, some selective antagonists were developed. These include the benzodiazepine derivative 5-BDBD, the N-substituted phenoxazines PSB-12054 and PSB-12062, and the phenylurea BX430. The compound NP-1815-PX which was more recently identified as a novel P2X4R antagonist was easily dissolved in water and inhibited rodent and human P2X4Rs with a high potency. Among the P2XRs tested, this compound was selective for P2X4R. Most importantly, NP-1815-PX had an anti-allodynic effect in pathological chronic pain models without any alterations in acute physiological pain responses or motor coordination, which are predicted therapeutic benefits of this antagonist. Unfortunately, NP-1815-PX had poor penetration to the CNS tissues, but the pharmaceutical company Nippon Chemiphar successfully developed a more potent and specific P2X4R antagonist with CNS-penetrating properties (NC-2600), which are now under clinical trials in Japan. Furthermore, recent studies developed monoclonal antibodies for extracellular head domains of P2X4Rs, a region that is an important site for modulation of this receptor. Among the developed antibodies, immunoglobulin G (IgG) isolated from adult mice. This is an important issue that should be clarified by further investigations. In addition, a chemical screening of a library identified the antidepressant duloxetine as a clinically approved drug that has an inhibitory effect on rodent and human P2X4Rs and a reversal effect on the PNI-induced mechanical allodynia. For drugs controlling ATP release, the first-generation bisphosphonate clodronate was identified as a potent and selective allosteric inhibitor for VNUT. Clodronate impaired vesicular ATP release from neurons and attenuated neuropathic pain. For P2X7R–CatS–fractalkine–CX3CR1–p38MAPK–IL-1β pathway, much effort has been made to develop selective P2X7R antagonists. These include a-740003 dose-dependently reduced nociception in rodent models of neuropathic and inflammatory pain, which was lost in IL-1β-knockout mice. While several compounds had problems—especially a poor CNS penetration—JNJ-47965567 was identified as a brain-penetrant P2X7R antagonist and had analgesic effects on neuropathic hypersensitivity. JNJ-42253432 had a higher CNS penetrating property but, surprisingly, no analgesic effect. While mechanical hypersensitivity in a collagen-induced arthritis model is attenuated by intrathecal P2X7R antagonists, clinical trials of P2X7 antagonists in rheumatoid arthritis have failed. The role of P2X7R is thus still controversial. A selective inhibitor for CatS was also reported to attenuate neuropathic allodynia and to potentiate an anti-allodynic efficacy of pregabalin without influencing its side effects. For the complement receptor, complement component fragment 5a receptor (C5aR), which has been implicated in PNI-induced pain, a potent and orally active C5aR non-competitive allosteric inhibitor, DF2593A, suppressed pain behaviors in various pain models including neuropathic pain.

Discovery of a tool to monitor microglia activity or state in the spinal cord and brain would provide an extremely important step forward to understanding the alteration in microglial functions in neuropathic pain patients. Recently, a technique for developing induced microglia-like (iMG) cells from human blood monocytes was reported. The origin of circulating monocytes in adults could be different from brain microglia in humans, but a similar pattern of gene expression was observed between iMG and primary microglia obtained from fetal brain tissue. Of particular interest, iMG cells of patients with fibromyalgia were demonstrated to show a TNFα-releasing inflammatory phenotype that correlated with pain severity. Although further studies are needed to elucidate the role of iMG cells in chronic pain and their function in patients with other chronic pain conditions, iMG cells may be used to study the mechanisms of neuropathic pain and also as biomarkers for diagnosis and therapeutics.

9. MICROGLIA AND OPIOID TOLERANCE/DEPENDENCE

An alternative benefit of microglia-targeting drugs in pain therapy would be to increase the usefulness of opioids, especially tolerance to their analgesic effect and dependence. Chronic treatment with morphine activates microglia in the SDH and some brain regions and depleting spinal microglia or inhibiting microglial molecules suppresses analgesic tolerance to opioids. However, spinal microglia had little role in already established tolerance, suggesting a contribution to development but not maintenance of analgesic tolerance to opioids. Furthermore, it was also found that spinal microglia depletion attenuates the behavioral sequelae of withdrawal from chronic morphine. Mechanistically, microglia activated by chronic morphine treatment release ATP via P2X7R, which in turn leads to long-term synaptic facilitation in the SDH and the resultant withdrawal signs. From these results, it is suggested that targeting spinal microglia might selectively prevent the undesirable side effects related to chronic use of opioids without reducing their analgesic effect. However, how opioids activate microglia remains controversial. While some studies report that opioids increase expression of microglial molecules (such as P2X4R, P2X7R, and pannexin 1) in primary cultured microglia via ß-opioid receptors (MOR), another study shows that MOR is not expressed in spinal cord microglia isolated from adult mice. This is an important issue that should be clarified by further investigations.

10. CONCLUSION

Accumulating evidence from basic pain research has not only demonstrated the necessity and sufficiency of spinal cord microglia in the pathogenesis of neuropathic pain, but also greatly advanced our understanding of the mechanisms of this contribution at the molecular and cellular levels. The
transcriptome analysis of microglia-selective genes and microglia heterogeneity will accelerate investigations. Furthermore, recent work has also showed a crucial role for brain microglia in somatosensory and/or emotional aspects of neuropathic pain. Because pharmacological, molecular, and genetic operations of the function or expression of microglia-expressing molecules strongly influence neuropathic pain behaviors and have no effect on acute pain under normal conditions, microglia and their molecules might be potential targets for treating neuropathic pain. Structure-based drug discovery and technological advances in establishing iMG from patients’ monocytes will allow us to establish a strategy to strongly suppress activated microglia and to diagnose neuropathic pain.

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Conflict of Interest The author declares no conflict of interest.

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