Chapter 7
Microglia in the CNS and Neuropathic Pain

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Abstract Neuropathic pain occurring after peripheral nerve injury is not simply a consequence of temporal continuity of acute nociceptive signals, but rather of maladaptive nervous system function. Over the past decades, a body of literature has provided evidence for the necessity and sufficiency of microglia, the tissue-resident macrophages of the central nervous system, for nerve injury-induced alterations in synaptic function. Recent studies have also revealed active roles for microglia in brain regions important for emotion and memory. In this chapter, I highlight recent advances in our understanding of the mechanisms that underlie the role of spinal and brain microglia in neuropathic pain, with a focus on how microglia are activated and alter synaptic function. I also discuss the therapeutic potential of microglia from recent advances in the development of new drugs targeting microglia, which may facilitate translation from the bench to bedside.

Keywords Microglia · Neuropathic pain · Spinal cord · Brain

7.1 Introduction

Injury to the nervous system as a consequence of cancer, diabetes, infection, autoimmune disease, chemotherapy, and trauma often causes debilitating chronic pain syndrome (neuropathic pain). Its symptoms include spontaneous pain, hyperalgesia (increased pain by a stimulus that normally provokes pain), and allodynia (pain due to a stimulus that does not normally provoke pain). Neuropathic pain does not resolve even after the overt tissue damage has already healed and can persist for long periods of time, indicating that the pain is not simply a temporal continuum of acute nociceptive pain, but rather due to pathologically altered nervous system function [5, 58, 79, 105]. Such pathological alterations have been extensively studied

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using rodent models of neuropathic pain, for example, models developed by peripheral nerve injury (PNI). Accumulating evidence indicates that PNI causes a variety of plastic modifications in neuronal synapses, connections, and networks at the molecular and cellular levels. These modifications shift the balance between synaptic excitation and inhibition in lamina I projection neurons toward excitation, which may account for development and maintenance of pain hypersensitivity [5, 58, 79, 105]. These alterations were long thought to be a consequence simply of changes in neurons, but mounting evidence indicates the important role of non-neuronal cells of the nervous system, including monocytes, macrophages, T cells, and glial cells [43, 45]. Microglial cells, which are known as the tissue-resident macrophages of the central nervous system (CNS) and constitute 5–10% of total cells in the adult CNS, have received much attention. In the late 1970s, it was found that non-neuronal cells (which were later identified as microglia) are increased in the spinal dorsal horn (SDH) after PNI [27, 28]. About 30 years later, a causal role of spinal microglia in neuropathic pain was first reported [46, 97]. Currently, numerous microglia-selective molecules (approximately 40) implicated in PNI-induced pain have been identified, providing compelling evidence that microglia are the key cell type for pathogenesis of neuropathic pain. In this chapter, we highlight recent advances in understanding of the role of CNS microglia in neuropathic pain.

7.2 Microglia

Microglia were originally described by Pio del Rio-Hortega in 1919 [19] and proposed to have a mesodermal origin [51]. In fate mapping studies enabling cell marking and gene regulation at the developmental stage, prenatal hematopoietic precursor cells were identified as the origin of microglia [29, 30, 52]. Microglia arise from yolk sac precursors genetically labelled as runt-related transcription factor 1 (Runx1)-expressing cells. Erythromyeloid progenitors in the yolk sac develop into microglia progenitors via an immature and more mature stage. The progenitors then leave the yolk sac, migrate to the brain through blood vessels, appear in the neuroepithelium with an amoeboid morphology, and finally take on a ramified. The development of microglia is independent of transcription factors required for development of other myeloid cell populations [52, 83]. As microglia have a unique molecular signature compared with other myeloid and immune cells [7, 25, 31, 37], this indicates a distinct developmental program of microglia from other myeloid cell types. The microglial development program is regulated by interleukin-34 (IL-34) signaling via CSF1R [29, 103]. Promoting terminal differentiation and acquiring adult microglia properties require TGF-β1 as a key factor [7]. In the healthy adult CNS, microglia remain throughout life and are maintained by self-renewal [88] with little contribution from bone marrow-derived circulating monocytes [2]. For maintaining microglia in adults, CSF1R signaling might have an ongoing role since pharmacological inhibition of CSF1R eliminates microglia in the adult brain [21]. In adults, microglia represent a morphologically unique type of cell, which, under normal conditions, has a small

soma bearing thin and branched processes. Two photon in vivo imaging studies have revealed that microglia processes are highly dynamic [17, 18, 73]. The processes of microglia rapidly move toward the site of injury [18, 34]. Furthermore, microglia directly appose synaptic regions (presynaptic terminals and dendritic spines) and, in response to neuronal activity, steer their processes toward active synapses, which facilitates contact with highly active neurons [102]. Now, microglia in the CNS are increasingly recognized as being crucial for sculpting the structure of the CNS, refining neuronal circuitry and network connectivity, and contributing to plasticity.

7.3 Microgliosis After PNI

As seen in the initial reports in the late 1970s [27, 28], PNI increases the number of microglia in the SDH. Such microgliosis is considered to occur through two mechanisms. First is proliferation of resident microglia because SDH microglia are immunohistochemically labelled by proliferation markers [26, 42]. Second is infiltration of bone marrow-derived circulating monocytes into SDH, which differentiate into microglia-like cells [106]. However, the latter was only observed in bone marrow chimeric mice receiving a high dose of irradiation [87], a treatment that can produce toxic effects including disruption of the blood-brain/spinal cord barrier [59]. Recent studies demonstrated no contribution of circulating monocytes to the PNI-induced microgliosis in the SDH, using parabiosis mice (a model in which two mice are surgically joined and share circulating blood in order to generate a chimera without irradiation and transplantation) [87] and transgenic mice enabling distinct visualization of resident microglia and circulating monocytes [32]. Therefore, local expansion of resident microglia by proliferation is the primary cellular mechanism for SDH microgliosis after PNI [32, 87]. Nonetheless, it should be noted that monocyte infiltration might be dependent on the neuropathic pain model. For example, in experimental autoimmune encephalomyelitis (a model of multiple sclerosis, with chronic pain being a common symptom), massive monocyte infiltration is observed in the spinal cord with demyelinating lesions [1]. However, these monocytes do not permanently contribute to the resident microglia pool.

SDH microgliosis seems to be a crucial step in neuropathic pain because interrupting this process suppresses PNI-induced pain hypersensitivity [32]. What triggers microgliosis? There are currently many reports showing that gene knockout reduces PNI-induced microgliosis [43]. Among them, neuregulin-1 might be one candidate. This is expressed in dorsal root ganglion (DRG) neurons, and its receptor ErbB2 is activated in spinal microglia after PNI [8]. Inhibition of neuregulin-1/ErbB2 signaling suppresses the PNI-induced microgliosis. Another potential candidate factor recently identified is colony-stimulating factor 1 (CSF1). CSF1 is rapidly induced in injured DRG neurons [33, 77] presumably by IL-1β signaling from surrounding satellite glia [61]. By contrast, IL-34 expression was not changed in DRG neurons [77]. The PNI-induced microglial proliferation and mechanical hypersensitivity were reduced by conditional knockout of CSF1 in DRG neurons

[33] and intrathecal administration of a CSF1R inhibitor [77]. Conversely, intrathecal CSF1 administration to normal mice induced proliferation and pain [33]. These findings suggest that CSF1 in injured DRG neurons activates CSF1R in microglia and induces proliferation. DNAX-activation protein 12 (DAP12) is a putative molecule downstream of CSF1R signaling, but the PNI-induced microglial proliferation might underlie a DAP12-independent mechanism because DAP12-deficient mice had no effect on the proliferation [33]. However, DAP12-deficient mice do not show PNI-induced pain [33, 55] or increased microglial number [55]. Thus, it is conceivable that DAP12-dependent signaling might presumably be involved in microglial migration from surrounding areas or changes in survival [55]. In addition, it should be noted that the upregulation of CSF1 and CSF1R persists until a few weeks after PNI [33, 77], when microglial proliferation has already terminated [32], suggesting a distinct role for CSF1-CSF1R signaling at this later phase, such as the control of the expression of microglial genes.

7.4 Molecularly Activated Microglia After PNI

SDH microglia are in an activated state following PNI through a change in their gene expression. For this process, one of the key regulators is interferon regulatory factor 8 (IRF8), a member of the IRF family [85]. Within the SDH, IRF8 is upregulated exclusively in microglia after PNI [66]. IRF8 regulates microglial genes including cell surface responses such as purinergic P2 receptors (P2X4R and P2Y12R), toll-like receptor 2 (TLR2), and C-X3-C motif chemokine receptor 1 (CX3CR1) and diffusible factors (IL-1β, cathepsin S (CatS), and brain-derived neurotrophic factor (BDNF)). The mechanism underlying IRF8 expression remains to be determined, but microglial IRF8 in the SDH has been shown to be upregulated by intrathecal administration of CSF1 or an activator of triggering receptor expressed on myeloid cells 2 (Trem2) [33, 55]. IRF8 also directly regulates transcription of IRF1 and IRF5 [63, 64]. It was found that IRF5 binds to the P2X4R promoter and induces its expression [64]. Loss of IRF5 suppresses the PNI-induced spinal P2X4R upregulation and pain hypersensitivity. Thus, the IRF8–IRF5 transcription cascade would be a core mechanism for producing P2X4R-expressing microglia after PNI and neuropathic pain. Microglial P2X4R upregulation also involves factors released from damaged DRG neurons such as CSF1 [33] and cysteine-cysteine chemokine ligand 21 (CCL21) [3] and by other extra- and intracellular factors [95, 96, 98, 99]. Pharmacological blockade and genetic knockout of P2X4R suppress the PNI-induced mechanical hypersensitivity [94, 97, 100]. Intrathecal administration of P2X4R-stimulated cultured microglia to normal rats induces allodynia, indicating that P2X4R-expressing microglia are not only necessary but sufficient to produce pain hypersensitivity [92, 97]. For activating P2X4Rs, extracellular ATP is required. ATP is known to be released from primary afferents [71], SDH neurons [47], and glia [6, 22, 41], but it was recently found that SDH neurons that express vesicular nucleotide transporter (VNUT [82], also known as SLC17A9; a secretory vesicle

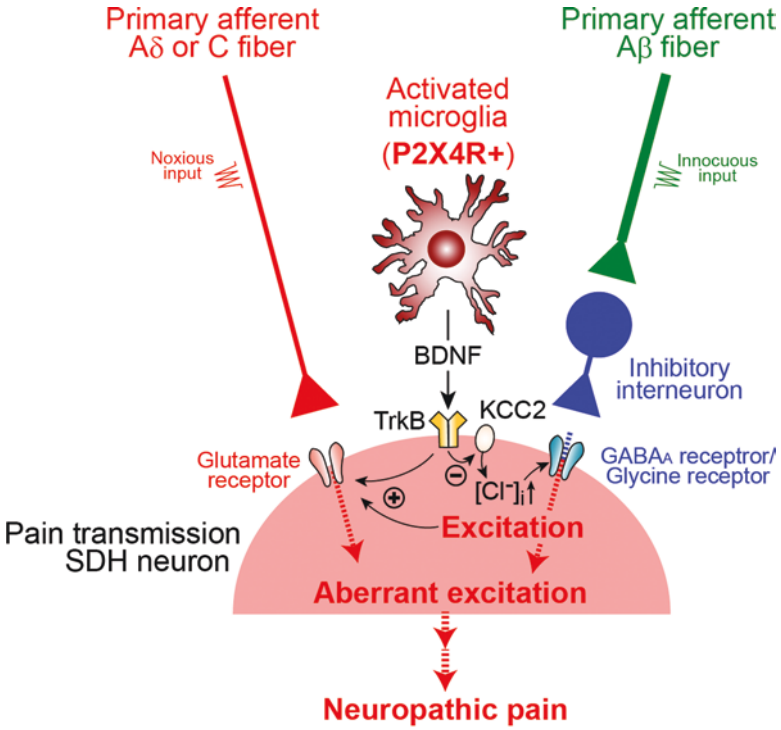

Fig. 7.1 Role of P2X4R-expressing spinal microglia in neuropathic pain. After PNI, microglia in the SDH become activated. The activated microglia upregulate P2X4R expression. P2X4R-stimulated microglia releases the signaling molecules BDNF. BDNF downregulates KCC2 in SDH pain transmission neurons, via 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. TrkB signaling also potentiates glutamatergic excitation via glutamate receptors. The resulting hyperexcitability of pain transmission in neurons contributes to neuropathic pain

protein responsible for storage and release of ATP) are a crucial source of the ATP that causes pain hypersensitivity [65]. Following stimulation of P2X4R, microglia release BDNF [16, 91]. BDNF activates tyrosine receptor kinase B (TrkB), in lamina I neurons, and induces an altered transmembrane anion gradient by downregulating KCC2, which caused changes in GABA- and glycine-evoked responses from inhibitory to excitatory and mechanical hypersensitivity [16] (Fig. 7.1). This change also potentiates their glutamatergic excitation via N-methyl-D-aspartate receptors (NMDAR) [38]. The crucial role of microglial BDNF was demonstrated by the finding that microglia-selective BDNF deficiency reduces PNI-induced pain [84]. By contrast, the conditional knockout of BDNF in primary afferent neurons has no effect [107]. These studies identifying the microglial P2X4–BDNF–KCC2 pathway provide evidence for the causal role of microglia-to-SDH neuronal signaling in neuropathic pain (Fig. 7.1).

Another microglial signaling to SDH neurons for neuropathic pain involves inflammatory factors. In particular, IL-1β and tumor necrosis factor-α (TNFα) have been extensively studied [43]. Important microglial receptors for producing and releasing these proinflammatory cytokines might be P2X7R and TLRs [10, 53, 54, 86]. In the SDH, P2X7R is required for ATP-induced IL-1β release from TLR4-primed microglia [13]. PNI-induced IL-1β transcription in the spinal cord involves TLR2 [53] and TLR4 [86]. At a posttranscription level, the Nod-like receptor family, pyrin domain containing-3 protein (NLRP3) inflammasomes activate pro-caspase-1, which promotes pro-IL-1β processing and secretion of mature IL-1β [35]. P2X7R is one of the most potent activators of the NLRP3 inflammasome [20]. IL-1β has been shown to phosphorylate NMDARs [101] and to enhance excitatory synaptic transmission [11, 50, 80]. IL-1β also decreases GABA- and glycine-mediated synaptic inhibition [50]. In addition, microglial IL-18, which can also be produced via NLRP3 inflammasomes, signals to astrocytes and contributes to neuropathic pain [69]. SDH astrocytes also become activated after PNI and contribute to maintenance of pain hypersensitivity [56, 93, 110], suggesting a crucial role of microglia-astrocyte signaling in chronicity of neuropathic pain.

TNFα is also a potent neuromodulator contributing to neuropathic pain. Expression of this cytokine in the SDH is exclusively increased in microglia after PNI via p38 mitogen-activated protein kinase (p38MAPK) [48]. TNF receptors (TNFR) in the SDH are found in multiple cell types [48]. In SDH neurons, TNFα rapidly increases excitatory responses evoked by activation of NMDARs and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) in SDH neurons [50]. TNFα has recently been shown to contribute to a form of synaptic plasticity for pain amplification in the SDH [57]. TNFR expressed at presynaptic terminals of primary afferents modulates glutamate release [78]. Furthermore, microglia, astrocytes, and endothelial cells in the SDH also express TNFR [48]. Microglial TNFR activation increases expression of BDNF, which leads to an increase in dendritic structural remodeling and synaptic connectivity strength in lamina I SDH neurons [62]. TNFα acts on astrocytes and enhances expression of chemokines, which rapidly increase excitatory synaptic transmission [9, 24]. In endothelial cells, TNFR upregulates cyclooxygenease-2 (COX-2) and prostaglandin I2 synthase (PGIS) [48]. Pharmacological inhibition of COX-2 and prostaglandin I2 (IP) receptors reduces pain hypersensitivity. Since IP receptors are localized in SDH neurons [48] and primary afferents [76], microglial TNFα can activate neurovascular communication and produce pain [48]. 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 is also a crucial microglial molecule for a communication to SDH neurons and for neuropathic pain [14]. CatS expression is upregulated in microglia in the SDH after PNI. Microglial CatS is released in response to P2X7R activation via p38MAPK and then cleaves membrane-bound fractalkine expressed on SDH neurons and astrocytes [12]. The cleaved fractalkine is considered to act on microglia again because the fractalkine receptor CX3CR1 is found exclusively in microglia [32, 109]. Activation of the P2X7R–p38MAPK–

CatS–fractalkine–CX3CR1 pathway leads to IL-1β secretion from microglia [11], which in turn modulates synaptic excitation and inhibition, as described above.

7.5 Brain Microglia and Neuropathic Pain

Recent studies have shown that PNI also activates microglia in several brain regions. These include the thalamus, amygdala, ventral tegmental area (VTA), nucleus accumbens (NAc), ACC, bed nucleus of stria terminalis, hippocampus, and periaqueductal gray [62, 68, 72, 89, 90]. Although the mechanism underlying microglia activation in the brain after PNI remains unknown, the role of brain microglia in neuropathic pain has recently been shown. It was found that inhibition of VTA microglia activation suppresses the PNI-induced reduction of dopamine release in the NAc and altered reward behavior [89], suggesting that activated microglia contribute to impairment of the VTA–NAc mesolimbic dopamine system after PNI. In the hippocampal CA1 region, dendritic structural complexity (including spine density), functional synaptic connectivity and BDNF levels were all reduced in PNI mice [62]. Microglial ablation and TNFR deficiency also prevented pain hypersensitivity and memory deficits after PNI. These findings provide evidence indicating that PNI activates brain microglia, which contributes to structural and functional synaptic alterations and pain hypersensitivity, as well as reward and memory deficits of PNI. It was also found that PNI also causes infiltration of circulating monocytes selectively in the central nucleus of the amygdala about 1 month later [81]. The infiltrated cells expressed IL-1β, and blocking the IL-1β signal reversed anxiety but not mechanical hypersensitivity. Because information about the aversive nature of the pain experience is thought to be processed in the central nucleus of the amygdala [4], ongoing signaling derived from infiltrated monocytes might also be crucial for the emotional component of neuropathic pain.

7.6 Therapeutic Implications

The mounting findings from studies using preclinical models described above provide much interest in microglia as a promising target for treating neuropathic pain. There are so far no clinically approved drugs that selectively target microglial molecules, but drug discovery efforts are currently in progress. A recent study identified NP-1815-PX as a novel P2X4R antagonist with a potent inhibition to rodent and human P2X4Rs [67]. Intrathecal administration of this compound to pathological pain models produces an anti-allodynic effect. Unfortunately, NP-1815-PX had poor CNS penetration, but the pharmaceutical company Nippon Chemiphar successfully developed a more potent and specific P2X4R antagonist with CNS-penetrating properties (NC-2600), which has been tested in phase I trials in Japan. Furthermore, the first-generation bisphosphonate clodronate was identified as a

potent and selective allosteric inhibitor for VNUT. Clodronate has shown to impair vesicular ATP release from neurons and to attenuate neuropathic pain [49]. Thus, these compounds can inhibit the activation of the P2X4R–BDNF–TrkB–KCC2 signaling pathway. P2X7R antagonists [44] and CatS inhibitor [36] could target the P2X7–CatS–fractalkine–CX3CR1–p38 MAPK–IL-1β pathway.

An alternative therapeutic potential of microglia for treating pain might be to increase the usefulness of opioids. Recent studies have revealed a crucial role of spinal and brain microglia in these side effects of opioids. Chronic morphine treatment activates microglia in the SDH and some brain regions [40]. Analgesic tolerance to opioids is suppressed by depleting spinal microglia [60] and by inhibiting microglial molecules [39, 60, 104, 108]. However, spinal microglia have little role in already established tolerance [23], suggesting that spinal microglia contribute to the development, but not maintenance, of morphine analgesic tolerance. Furthermore, morphine is known to produce a paradoxical increase in pain sensitivity. This side effect seems to be dependent on microglial P2X4R signaling in the SDH [23]. Moreover, it was also recently found that spinal microglia depletion also attenuates the behavioral sequela of withdrawal from chronic morphine [6]. Microglia activated by chronic morphine treatment release ATP via pannexin 1 that has interacted with P2X7R, and inhibition of microglial ATP release attenuates withdrawal behavior and long-term synaptic facilitation [6]. These findings suggest that targeting spinal microglia might selectively prevent the undesirable side effects caused by chronic opioid use without reducing their pain-relieving effect. However, whether opioids act directly on μ-opioid receptors (MOR) expressed by microglia remains controversial. Some studies showed that opioids upregulate microglial molecules (like P2X4R, P2X7R, and pannexin 1) in cultured microglial cells in vitro via microglial MOR, but a recent study reported that MOR is undetectable in spinal microglia isolated from adult mice. The latter study also showed that a conditional loss of MOR in primary afferent nociceptors eliminates morphine-induced tolerance and hyperalgesia without suppressing activation of spinal microglia [15]. Further investigation is needed to clarify this issue.

Several studies have recently established methods for generating human microglia through the differentiation of induced pluripotent stem (iPS) cells to erythromyeloid progenitor-like cells [70], which may provide a major step forward to understanding an alteration in microglial functions in neuropathic pain patients. If circulating monocytes recruited to the brain also contribute to neuropathic pain [81], a technique for developing induced microglia-like (iMG) cells from human blood monocytes [75] would be useful. It was recently found that iMG cells of fibromyalgia patients display a TNFα-releasing inflammatory phenotype, and interestingly the ability of iMG cells to release this cytokine correlates with the pain severity of patients [74]. Thus, it is possible that iMG cells may be used to study the mechanisms of neuropathic pain and also as biomarkers for diagnosis and therapeutics. However, it should be noted that there are dramatic differences between cultured microglia and microglia in vivo [7], and thus further studies are needed to examine whether human microglia derived from iPS cells and human iMG derived from monocytes are indeed useful for translation.

7.7 Conclusions

An accumulating body of literature has not only provided compelling evidence for the necessity and sufficiency of microglia in neuropathic pain but also greatly advanced our understanding of the molecular and cellular mechanisms of this contribution. The recent identification of microglia-selective genes [7, 25, 31, 37] will accelerate investigations. Furthermore, recent work has revealed a crucial role for brain microglia in sensory and/or emotional aspects of neuropathic pain, although the underlying mechanism(s) remain unknown. Because pharmacological, molecular, and genetic manipulations of the function or expression of microglial molecules substantially influence chronic pain behaviors and have no effect on acute physiological pain under normal conditions, glial cells and their expressing molecules might be good targets for treating chronic pain. Indeed, potent and selective antagonists and/or inhibitors targeting microglial molecules have been developed and exhibit therapeutic effects on neuropathic pain hypersensitivity in preclinical models. Structure-based drug discovery together with technological advances in establishing human microglia from iPS cells and iMG from circulating monocytes from patients will help us to establish a strategy to effectively suppress activated microglia and to diagnose neuropathic pain.

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