Neuronal and microglial mechanisms for neuropathic pain in the spinal dorsal horn and anterior cingulate cortex

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Abstract
Neuropathic pain is a debilitating chronic pain condition occurring after damage in the nervous system and is refractory to the currently available treatments. Major challenges include elucidating its mechanisms and developing new medications to treat it. Nerve injury-induced pain hypersensitivity involves aberrant excitability in spinal dorsal horn (SDH) neurons as a consequence of dysfunction of inhibitory interneurons and of hyperactivity of glial cells, especially microglia, the immune cells of the central nervous system. Evidence of this is found using animal models to investigate the molecular and cellular mechanisms of neuropathic pain. The pathologically altered somatosensory signals in the SDH then convey to the brain regions, including the anterior cingulate cortex (ACC). In these regions, nerve injury produces pre- and postsynaptic long-term plasticity, which contributes to negative emotions and anxiety associated with chronic pain conditions. Furthermore, recent evidence also indicates that the descending projection pathways from the ACC directly and indirectly to the SDH (the top-down corticospinal network) regulate nociceptive sensory transmission in the SDH. Thus, understanding a possible connection between the SDH and ACC, including a neuron-microglia interaction, may provide us with insights into the mechanisms used to amplify pain signals related to neuropathic pain and clues to aid the development of new therapeutic agents for the management of chronic pain.

Keywords: anterior cingulate cortex, microglia, neuropathic pain, spinal dorsal horn neurons, synaptic plasticity, top-down modulation.

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Abbreviations used: 5-BDBD, 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one; AC1, adenylyl cyclase type 1; ACC, anterior cingulate cortex; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; BDNF, brain-derived neurotrophic factor; BM, bone marrow; BX430, 1-(2,6-dibromo-4-isopropyl-phenyl)-3-(3-pyridyl)urea; CFA, complete Freund’s adjuvant; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CNS, central nervous system; CSF1, colony stimulating factor 1; DPRS, descending pain regulation system; DRG, dorsal root ganglion; EPM, elevated plus maze; FG, fluoro-gold; GFAP, glial fibrillary acidic protein; HCN, hyperpolarization cyclic nucleotide; HRP, horseradish peroxidase; IFN-γ, interferon γ; IRF8, interferon-regulatory factor 8; KCC2, potassium chloride cotransporter 2; LTP, long-term potentiation; NP-1815-PX, 5-[3-(5-thioxo-4H-[1,2,4]oxadiazol-3-yl)phenyl]-1H-naphthol[1,2-b][1,4]diazepine-2,4(3H,5H)-dione; P2X4R, P2X4 receptor; PAG, periaqueductal gray; PBN, parabrachial nucleus; PKA, protein kinase A; PKMε, protein kinase Mε; PNI, peripheral nerve injury; Post-LTP, postsynaptic LTP; Pre-LTP, presynaptic LTP; PSB-12054, N-(benzoyloxy)carbonylphenoxazine; PSB-12062, N-(p-methylphenyl)sulfonylphenoxazine; RVM, rostro-medial ventral medulla; SDH, spinal dorsal horn; TBS, theta burst stimulation; VNUT, vesicular nucleotide transporter; WDR, wide dynamic range.
Acute physiological pain, such as pain evoked by noxious heat or mechanical stimuli, plays an essential role as an early warning system, alerting us to the presence of damaging stimuli. If one has damage in peripheral tissues which leads to enhanced sensitivity to noxious stimuli, such pain might lead to behaviors that minimize physical contact with the damaged area and, consequently, assist in wound repair. In contrast, pathological pain is not dependent on the presence of tissue-damaging stimuli. Neuropathic pain, a debilitating chronic pain condition, is often a consequence of nerve injury or of diseases such as cancer, diabetes mellitus, infection, autoimmune disease, and trauma. Symptoms of neuropathic pain are spontaneous pain, hyperalgesia (the increased pain perception of noxious stimuli), and mechanical allodynia (pain hypersensitivity to normally innocuous stimuli). Neuropathic pain, especially allodynia, is often resistant to currently available drug treatments such as non-steroidal anti-inflammatory drugs and even opioids, but only when administered at doses that do not produce significant side effects. We are now beginning to understand that neuropathic pain is not just a symptom of disease, but is an expression of pathological operations of the nervous system. Thus, elucidating mechanisms for neuropathic pain and developing new therapeutic agents for its treatment are major challenges.

Somatosensory information from the periphery is conveyed to the spinal dorsal horn (SDH) via primary afferent sensory neurons, properly processed by neural circuits in the SDH, and in case of noxious stimulus, transmitted to nociceptive projection neurons in lamina I neurons (Braz et al. 2014; Prescott et al. 2014; Peirs and Seal 2016). Primary afferents are broadly divided into two classes: nociceptive and non-nociceptive, which respond to noxious and innocuous stimuli, respectively (Todd 2010; Braz et al. 2014). The nociceptor classification refers to mainly thin, myelinated Aδ fibers and unmyelinated C fibers. These fibers detect noxious stimuli (i.e., heat, mechanical and chemical irritants). The latter low-threshold mechanoreceptors, such as large diameter, thick, myelinated Aβ fibers, detect innocuous mechanical stimuli (i.e., light touch). The terminals of C and Aδ fibers in the SDH are concentrated in the superficial lamina, and these fibers activate projection neurons and excitatory interneurons (Fig. 1). In contrast, the terminals of Aβ fibers are concentrated in the deeper lamina, and mainly target excitatory and inhibitory interneurons and projection neurons located in the deeper SDH (not shown). Although Aβ fibers polysynaptically connect to lamina I projection neurons, the functional link is considered to be normally strongly repressed by inhibitory interneurons (Fig. 1). Thus, under normal healthy conditions, Aβ fibers do not activate nociceptive projection neurons and do not cause pain. However, the neuronal networks in the SDH are modulated and modified under pathological conditions, such as peripheral tissue inflammation and peripheral nerve injury (PNI), and behaviorally, noxious stimuli can produce enhanced pain (hyperalgesia), and innocuous mechanical stimuli can produce pain (mechanical allodynia). Furthermore, the majority of nociceptive projection neurons in lamina I SDH neurons provide ascending signals to several brain regions, such as the parabrachial nucleus (PBN) and thalamus (Todd 2010; Bliss et al. 2016). These regions further neuronally connect with the somatosensory cortex, amygdala, prefrontal cortex, insular cortex, and anterior cingulate cortex (ACC), areas where human brain imaging studies have shown to be core brain regions related to sensory and negative emotional information of the pain experience and chronic pain (Apkarian et al. 2005; Bushnell et al. 2013).

Molecular and cellular mechanisms for the development and maintenance of neuropathic pain have been investigated using various animal models. The underlying mechanisms are not fully understood, but approaches including recent genetic technologies to enable ablation or silence a specific cell population in the pain pathway (Duan et al. 2014; Prescott et al. 2014; Bourane et al. 2015; Foster et al. 2015; Han et al. 2015; Petitjean et al. 2015; Bliss et al. 2016; Peirs and Seal 2016) have provided advances in the understanding of neuronal circuits in the SDH and brain crucial for neuropathic pain. Furthermore, a growing body of evidence has shown that PNI-induced synaptic hyperexcitability might not be a consequence simply of changes in neurons, but rather of multiple alterations in glial cells, such as microglia, the immune cells of the central nervous system (CNS) (Tsuda et al. 2005; Beggs et al. 2012; Salter and Beggs 2014; Ji et al. 2016). In this review, we highlight recent advances in our understanding of the mechanisms that underlie neuropathic pain with a focus on the role of neuron–microglia communications in the SDH and synaptic plasticity in the anterior cingulate cortex (ACC), based largely on findings from animal studies.

**Spinal mechanisms for neuropathic pain**

**SDH neurons**

SDH is the first gate of the CNS where periphery somatosensory signals input. The SDH is characterized by a distinct laminar structure, and a number of excitatory and inhibitory interneurons that form a complex network through which modality-specific somatosensory information is properly processed (Todd 2010; Braz et al. 2014; Peirs and Seal 2016). However, under chronic pain conditions, this system seems to collapse, especially in case of mechanical allodynia (pain caused by normally innocuous mechanical stimuli). The underlying mechanisms for why innocuous mechanical stimuli can produce pain are not yet fully understood, but it has been proposed that a pathologically altered connection of Aβ fibers to nociceptive lamina I SDH neurons might be established after PNI. In animals with PNI, expression of c-Fos and phosphorylation of ERK was observed in superficial SDH neurons by...
Fig. 1 Neuronal circuit in the spinal dorsal horn (SDH), microglial modulation of SDH neurons, and synaptic plasticity in the anterior cingulate cortex (ACC). Lower panel: Primary afferents convey somatosensory information from the periphery to the SDH. Nociceptive information is mainly mediated by Aδ and C fibers, and innocuous mechanical information is mediated by Aβ fibers. C and Aδ fibers terminate in the superficial SDH and activate projection neurons and excitatory interneurons. The terminals of Aβ fibers are concentrated in the deeper SDH, and connect to excitatory and inhibitory interneurons. Aβ fibers polysynaptically connect to lamina I projection neurons via SDH interneurons including PKCγ-expressing neurons, but the functional link is normally strongly repressed by inhibitory interneurons positive to GABA, glycine (Gly), dynorphin (Dyn), and parvalbumin (PV). After peripheral nerve injury, SDH microglia become activated and up-regulate P2X4 receptor (P2X4R) expression through an interferon-regulatory factor 8 (IRF8)–IRF5 transcriptional axis. IRF8 induces IRF5 expression, and then IRF5 directly binds to the promoter region of the P2rx4 gene and induces expression of P2X4R mRNA. P2X4R is activated by extracellular ATP released from SDH neurons and, in turn, releases bioactive diffusible factors, such as BDNF. BDNF down-regulates potassium chloride cotransporter 2 (KCC2), which causes depolarization of these neurons following stimulation by GABA and glycine. The resultant hyperexcitability in the SDH pain network induced by microglial factors may be responsible for neuropathic pain. Upper panel: Postsynaptic (post) and presynaptic (pre) long-term potentiations (LTP) in the ACC. Post-LTP induction requires the activation of NMDA receptors (NMDAR), Ca^{2+} via NMDAR and voltage-gated Ca^{2+} channels (VGCC) and calmodulin stimulate adenylyl cyclase type 1 (AC1), and protein kinase A (PKA) phosphorylates AMPAR GluA1 subunit. PKA also activates the transcription factor cAMP response element-binding protein (CREB). PKMζ up-regulates GluA1–GluA2 heteromers. For pre-LTP, presynaptic kainate receptor GluK1 and AC1 activation are necessary for its induction. cAMP binds to the hyperpolarization cyclic nucleotide (HCN) channel to increase its sensitivity, and PKA enhances vesicle fusion. The SDH and ACC functionally connect via the ascending parabrachial nucleus (PBN)–amygdala pathway, and the descending direct and indirect periaqueductal gray (PAG)–rostromedial ventral medulla (RVM) pathways, respectively.

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light touch and Aβ fiber stimulation (Zhang et al. 2007a; Matsumoto et al. 2008). Several lines of evidence indicate a crucial role of a subpopulation of SDH neurons in neuropathic pain. It was shown that mice lacking protein kinase Cy that was expressed specifically in the SDH neurons located in the inner area of lamina II failed to develop the PNI-induced pain hypersensitivity (Malmberg et al. 1997). Protein kinase Cy+ SDH neurons receive not only Aβ fiber input (Neumann et al. 2008; Lu et al. 2013), but also inhibitory glycinergic inputs activated by Aβ fibers that form a feed-forward inhibitory circuit, thereby preventing Aβ input from activating the nociceptive pathway (Lu et al. 2013). Disruption of this glycinergic inhibitory control after PNI has been considered to be involved in mechanical allodynia. Furthermore, recent genetic technologies have demonstrated that ablation or silence of subpopulations of SDH interneurons that are positive to dynorphin (Duan et al. 2014), somatostatin (Duan et al. 2014), parvalbumin (Petitjean et al. 2015), calretinin (Peirs et al. 2015), vesicular glutamate transporter (Peirs et al. 2015), and glycine transporter 2 (Foster et al. 2015) produces mechanical allodynia. Coull et al. (2003) showed that PNI down-regulates potassium-chloride cotransporter 2 (KCC2), and causes an increase in intracellular Cl− levels, which leads to the collapse of the transmembrane anion gradient in SDH neurons. This alteration, in turn, induces depolarization of these neurons after stimulation by γ-aminobutyric acid (GABA) and glycine (Coull et al. 2003). From these findings, it has been proposed that the PNI-induced hyperexcitability of SDH neurons and mechanical allodynia might involve loss of function of inhibitory interneurons (cf. diminished activity of these neurons, and reduced or inverted effectiveness of GABA and glycine) (Braz et al. 2014; Prescott et al. 2014; Peirs and Seal 2016).

**Microglia**

A rapidly growing body of evidence indicates that spinal glial cells, in particular microglia, play a critical role in the pathogenesis of neuropathic pain (Tsuda et al. 2005; Beggs et al. 2012; Salter and Beggs 2014; Ji et al. 2016). Microglial cells are known as tissue-resident macrophages in the CNS and constitute 5–10% of total cells in the adult CNS. Recent fate mapping studies revealed that microglia arise from early yolk sac-derived precursors that leave the yolk sac on E8.5–E9.0 and migrate to the neuroectoderm via the primitive blood-stream (Ginhoux et al. 2010; Kierdorf et al. 2013a). The yolk sac-derived microglia are thought to remain throughout life and to be maintained by self-renewal in the healthy CNS, with little contribution from bone marrow-derived monocytes/macrophages (Ransohoff and Cardona 2010; Prinz and Priller 2014; Crotti and Ransohoff 2016).

Microglia in the SDH rapidly respond to PNI: cell body hypertrophy with thickened and retracted processes, increased cell numbers, and increased staining of microglial markers, such as CD11b and ionized calcium-binding adapter molecule-1 (Tsuda et al. 2005). Signaling molecules derived from damaged primary afferents might be important for microglial activation. Studies have suggested metallo-proteinase-9 in injured dorsal root ganglion (DRG) neurons as candidates (Kawasaki et al. 2008a). The number of microglia in the SDH is markedly increased after PNI (Tsuda et al. 2013), which might be associated with proliferation of resident microglia (Gehrmann and Banati 1995; Gu et al. 2016b; Tashima et al. 2016). It was found that within the SDH, microglia expressed interferon-γ (IFN-γ) receptors in a cell type-specific manner (Tsuda et al. 2009b) and that IFN-γ receptor knockout mice displayed a suppression of the PNI-induced morphological changes and proliferation of microglia. Recent studies reported that expression of colony stimulating factor 1 (CSF1) is induced in injured DRG neurons at an early phase and that conditional deficiency of CSF1 in DRG neurons reduced proliferation of spinal microglia after PNI (Guan et al. 2016; Okubo et al. 2016). Alternatively, it was also reported that in bone marrow (BM) chimeric mice, circulating BM-derived monocytes/macrophages infiltrate the SDH parenchyma after PNI and differentiate into microglia-like cells (Zhang et al. 2007b; Echeverry et al. 2011; Padi et al. 2012; Isami et al. 2013). However, these chimeric mice received a high dose of whole-body irradiation which produces toxic effects, such as chemotactarctant induction and blood–brain barrier disruption (Hwang et al. 2006; Mildner et al. 2011; Kierdorf et al. 2013b; Larochelle et al. 2015). This, therefore, could allow the infiltration of circulating blood cells into the SDH. In a recent study which evaluated this issue by irradiation with various doses and also by parabiosis, a model without irradiation and BM transplantation, it was shown that BM-derived cells do not contribute to the population of SDH microglia after PNI (Tashima et al. 2016). Therefore, the PNI-induced spinal microgliosis may not be a consequence of recruitment of BM-derived cells, but may rather be associated with a local expansion of resident microglia because of their proliferation activity in response to PNI (Tashima et al. 2016). This is supported by a study using genetic mouse models in which microglia and peripheral monocytes/macrophages are differentially labeled (Gu et al. 2016b).

The first evidence for a causal role of microglia in neuropathic pain was revealed by studies on the purinergic P2 receptors, especially the ionotropic P2 subtype P2X4 receptors (P2X4R) (McCleskey 2003; Tsuda et al. 2003). P2X4R are cation-selective channels with almost equal permeability to Na+ and K+ and significant permeability to Ca2+ (Ralevic and Burnstock 1998; Khakh et al. 2001; North 2002), with three subunits forming one ion-channel (Kawate et al. 2009; Shinozaki et al. 2009). It was found that expression of P2X4R increased exclusively in spinal microglia after PNI and pharmacological blockade and genetic knockout of the receptor suppressed the PNI-induced

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mechanical allodynia (Tsuda et al. 2003, 2009a; Ulmann et al. 2008). From the findings that intrathecal P2X4R-stimulated microglia to normal rats induced allodynia, it was indicated that P2X4R-expressing microglia are not only necessary but sufficient to produce mechanical allodynia (Tsuda et al. 2003, 2005). In other models (experimental autoimmune neuritis and herpetic pain), up-regulation of P2X4R in SDH microglia was also observed, the time course of which corresponded to mechanical allodynia (Zhang et al. 2008b; Matsumura et al. 2016). A recent study raised the possibility that microglial pain processing involving P2X4R after PNI may only be essential in male mice. In female mice, there was no up-regulation of spinal P2X4R expression after PNI and no effect of pharmacological blockade of spinal P2X4Rs on mechanical allodynia (Sorge et al. 2015). However, it was also recently shown the up-regulation of P2X4R in SDH microglia in female mice of a herpetic pain model (Matsumura et al. 2016). Thus, further work needs to be performed to clarify the sex differences in other models of neuropathic pain.

For up-regulation of P2X4R expression selectively in microglia, several studies revealed molecular mechanisms (Tsuda et al. 2008a,b, 2009c). One of the key factors is interferon-regulatory factor 8 (IRF8), a member of the IRF family (IRF1–9) which is expressed in immune cells, such as lymphocytes and dendritic cells (Tamura et al. 2008). It was found that within the SDH, expression of IRF8 was up-regulated exclusively in microglia after PNI (Masuda et al. 2012). In primary cultured microglial cells, IRF8 promoted expression of P2X4R as well as other genes associated with reactive states. In IRF8 knockout mice, the PNI-induced P2X4R up-regulation and mechanical allodynia were suppressed. Furthermore, it was found that IRF5 expression was also induced selectively in spinal microglia after PNI (Masuda et al. 2014), which required IRF8 (Kurotaki et al. 2013; Masuda et al. 2014). Interestingly, IRF5 was found to bind directly to the promoter region of the P2rx4 gene and to induce P2X4R expression in microglial cells. IRF5 knockout mice indeed showed a reduction in spinal P2X4R up-regulation and allodynia after PNI (Masuda et al. 2014). Therefore, an activation of the IRF8–IRF5 transcriptional axis after PNI plays a pivotal role in producing P2X4R-expressing microglia in the SDH and neuropathic pain (Masuda et al. 2014) (Fig. 1).

The findings described above suggest that such pain behaviors require extracellular ATP in the spinal cord to activate P2X4R and other P2 receptors. It was recently found that mice lacking vesicular nucleotide transporter (VNUT; also known as Slc17a9), a secretory vesicle protein responsible for the storage and release of ATP (Sawada et al. 2008), displayed a reduction in the PNI-induced allodynia (Masuda et al. 2016). A similar phenotype was also observed in spinal VNUT knockout mice, indicating that VNUT-expressing cells residing in the spinal cord may be crucial for mechanical allodynia. By using mice whose VNUT was conditionally disrupted by the Cre-loxP system, it was revealed that VNUT expressed in SDH neurons, but not in astrocytes, microglia, or primary afferents, contributes to the PNI-induced allodynia. Furthermore, ATP release from the spinal cord slices was also suppressed in SDH neuron-specific VNUT knockout mice. These results strongly suggest that VNUT-dependent exocytic ATP release from SDH neurons is a crucial mechanism for neuropathic pain.

For drug discovery, some P2X4R antagonists were developed (Jacobson and Muller 2016). Those include the benzodiazepine derivative 5-(3-bromophenyl)-1,3-dihydro-2H-benzo[d][3,2-e]-1,4-diazepin-2-one (5-BBDDB) (Donnelly-Roberts et al. 2008; Balazs et al. 2013), the N-substituted phenoazines – N-(benzoxycarbonyl)phenozone (PSB-12054) and N-(p-methylphenyl)sulfonylphenozone (PSB-12062) – and the phenylurea – 1-(2,6-dibromo-4-isopropyl-phenyl)-3-(3-pyridyl)urea (BX430) (Ase et al. 2015). However, there were drawbacks in potency, selectivity, and water solubility. The compound 5-[3-(5-thioxo-4H-[1,2,4]oxadiazol-3-yl)phenyl]-1H-naphtho[1,2-b][1,4]diazepine-2,4(3H,5H)-dione (NP-1815-PX) was recently identified as a novel P2X4R antagonist that resolved these problems (Matsumura et al. 2016). NP-1815-PX 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 was the first P2X4R antagonist to produce an anti-allodynic effect in pathological chronic pain models without any alterations in acute physiological pain responses or motor coordination, which suggests a predicted therapeutic benefit of this antagonist.

**Microglia–neuron interaction in the SDH**

One of the mechanisms by which activated microglia alter the pain processing network within the SDH might involve microglia-derived signaling molecules. It was demonstrated that activation of microglial P2X4R leads to a release of brain-derived neurotrophic factor (BDNF) (Ullmann et al. 2008; Trang et al. 2009). BDNF subsequently induced an altered transmembrane anion gradient in a subpopulation of lamina I neurons in the SDH by down-regulating KCC2 (Coull et al. 2005), which caused changes in GABA- and glycine-evoked responses from inhibitory to excitatory and mechanical allodynia (Coull et al. 2005) (Fig. 1). Microglia-derived proinflammatory cytokines were also found to modify excitatory or inhibitory synaptic transmission and neuronal activity in the superficial SDH neurons (Ikeda et al. 2007; Kawasaki et al. 2008b). Exogenous application of IL-1β enhances NMDA receptor-mediated Ca2+ response via activating the tyrosine protein kinase Src (Viviani et al. 2003) which is known to enhance NMDA receptor activity in SDH neurons (Yu et al. 1997; Woolf and Salter 2000) and neuropathic pain (Liu et al. 2008). IL-1β also decreases

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GABA<sub>A</sub> receptor-mediated currents (Wang et al. 2000). Furthermore, local spinal administration of ATP-stimulated microglia alters the phenotype of in vivo nociceptive lamina I projection neurons, such that they relay innocuous mechanical input (Keller et al. 2007). In this study, the recorded lamina I neurons were confirmed to project to the PBN (Keller et al. 2007), and it seems that the lamina I neurons might output signals to the PBN. Considering the fact that the PBN neuronally connects with the amygdala and ACC (Todd 2010; Bliss et al. 2016), it leads to a hypothesis that microglially altered somatosensory signals in the SDH may contribute to synaptic alterations in these regions and also to negative emotions associated with chronic pain conditions.

Mechanisms for neuropathic pain in the ACC
Persistent nociceptive information produces negative emotions such as anxiety and depression by engaging mood-related regions in the brain. According to the IASP definition, one cannot experience pain without the CNS. In the CNS, the amygdala, prefrontal cortex, insular cortex, and ACC play crucial roles for chronic pain (Apkarian et al. 2005; Nakao et al. 2012; Bushnell et al. 2013; Neugebauer 2015; Zhuo 2016). The ACC is a major cortical area involved in various higher brain functions, such as nociception, chronic pain, cognition, and emotions. This area is also important in the emotional/aversive component of pain, as stimulation of the ACC reverses avoidance behavior in the place escape/avoidance paradigm in a model of neuropathic pain (LaBuda and Fuchs 2005).

Human imaging studies have clearly shown the importance of the ACC in chronic pain (Apkarian et al. 2005; Shackman et al. 2011), which have been corroborated by animal studies that have demonstrated a critical involvement of the ACC in nociception and chronic pain. In particular, in vivo recordings of neural activity have provided evidence that the ACC plays a critical role in nociception and injury (Wu et al. 2005b). In vivo electrophysiological studies demonstrate that digit amputation causes synaptic potentiation in rats (Wei and Zhuo 2001; Wu et al. 2005b), and in vivo field recording have shown amputation-mediated long-lasting potentiation of ACC activity (Wei and Zhuo 2001).

Anatomy and basic transmission: chronic pain models produce synaptic plasticity in the ACC
The ACC is composed of layers I, II, III, V and VI, and displays excitatory and inhibitory neurons within all layers. So far, the mechanisms of excitatory synaptic transmissions in the ACC have mainly been studied using models of chronic pain (Zhuo 2008, 2014, 2016). Animal models of chronic pain have been shown to cause excitatory synaptic plasticity at both presynaptic and postsynaptic sites within the ACC (Fig. 1). Postsynaptic mechanisms include Ca<sup>2+</sup> influx enhancement via activation of NMDA receptors. Protein kinase A (PKA) signaling plays important roles via adenylyl cyclase type 1 (AC1) and phosphorylation of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunit GluA1 at the Ser845 site (Fig. 1). In addition, PKA signaling is also critical for postsynaptic changes. It is activated downstream of Ca<sup>2+</sup>-calmodulin-stimulated AC1 and phosphorylates AMPA receptors containing GluA1 subunits at the Ser845 site (Wu et al. 2005a; Zhao et al. 2006; Xu et al. 2008; Toyoda et al. 2009). Multiple chronic pain models have documented effects on presynaptic mechanisms within the ACC, including nerve injury models, chronic inflammatory pain, and visceral pain models, which all facilitate glutamatergic transmitter release in layer II/III pyramidal neurons. Furthermore, paired pulse ratio, which is a measurement of presynaptic changes, is also altered in these major models (Zhao et al. 2006; Xu et al. 2008; Toyoda et al. 2009; Li et al. 2010; Liu et al. 2015). Cumulative alterations of excitatory presynaptic transmission suggest that presynaptic plasticity may play a functional role in chronic pain.

Long-term potentiation in the ACC and chronic pain
Long-term potentiation (LTP) is a key cellular mechanism for learning and memory, and chronic pain (Bliss and Collingridge 1993; Zhuo 2014; Bliss et al. 2016). In the ACC, at least two types of LTP have been documented (Zhao et al. 2005; Zhuo 2008, 2016; Koga et al. 2015a). One is a postsynaptic form of LTP (post-LTP) which is driven by a glutamatergic NMDA-dependent mechanism (Fig. 1). Another one is a presynaptic form of LTP (pre-LTP) that is an NMDA-independent form of long-term plasticity. Pairing protocol and theta burst stimulation (TBS) can produce post-LTP in whole-cell patch clamp or field recording methods. Sixty-four multi-unit field recordings have also captured postsynaptic LTP, as application of (TBS) can increase field potentials and enhance the numbers of active channels showing LTP (Chen et al. 2014b).

Pharmacological and genetic manipulation studies revealed that post-LTP mechanisms within the ACC require activation of AC1, GluA1 phosphorylation, activation of protein kinase M<sub>C</sub> (PKM<sub>C</sub>), in addition to activation of NMDA receptors containing GluN2B subunits (Liauw et al. 2005; Li et al. 2010). Studies indicated that the postsynaptic form of ACC-LTP contributes to PNI-induced enhancements in nociception (Li et al. 2010). Importantly, microinjections of selective inhibitors of AC1, GluN2B, or PKM<sub>C</sub> reduce nociceptive behaviors in murine models of chronic pain (Wu et al. 2005a; Li et al. 2010; Wang et al. 2011; King et al. 2012).

Pre-LTP in the ACC for chronic pain-related anxiety
In addition to nociception and chronic pain, the ACC is also involved in negative emotions such as anxiety and fear. For example, inactivation of the ACC through microinjection of...
a GABA agonist, muscimol, reduced anxiety-like behaviors in adult mice (Kim et al. 2011). Moreover, multiple studies have shown that chronic pain or visceral pain models, which alter ACC function, cause enhancements in anxiety-like behaviors in the elevated plus maze, open field, or light–dark tests (Narita et al. 2006a; Matsuzawa-Yanagida et al. 2008; Zhang et al. 2014). Importantly, microinjection of the selective serotonin reuptake inhibitor paroxetine into the ACC can prevent anxiety-like behaviors under a chronic pain condition, suggesting that the ACC plays a critical role in pain and anxiety in animals (Matsuzawa-Yanagida et al. 2008).

Although multiple reports have investigated the mechanisms involved in pre-LTP in the hippocampus and amygdala (Nicoll and Schmitz 2005; Shin et al. 2010; Bliss and Collingridge 2013), the mechanisms of pre-LTP within the ACC have yet to be fully elucidated. Although chronic pain models and PNI had been shown to cause presynaptic plasticity, the physiological role was unclear. Recently, we showed the mechanisms of pre-LTP in the ACC and investigated that the cingulate pre-LTP contributes to anxiety-like behavior and is involved in PNI-induced anxiety-like behaviors (Koga et al. 2015a,b). Low-frequency stimulation can produce LTP in the ACC and amygdala that can last for at least 1 h (Shin et al. 2010; Koga et al. 2015a, b). It can also alter the paired pulse ratio, indicating that the potentiation induced in the ACC by this low-frequency stimulation is of presynaptic origin. Furthermore, within the ACC, several critical mechanisms for pre-LTP have been studied. Using pharmacological drugs and transgenic mice, we found that GluK1-containing kainate receptors, L-type voltage-gated Ca\(^{2+}\) channels, PKA pathway via AC1, and hyperpolarization cyclic nucleotide-gated (HCN) channels are necessary for the expression of cingulate pre-LTP (Fig. 1). In particular, HCN channels were found to play an important role in both the induction and maintenance of pre-LTP, without affecting basal synaptic transmission.

Interestingly, chronic inflammatory and neuropathic pain models reduce pre-LTP in the ACC. On the other hand, mice exposed to acute noxious thermal stimulation or innocuous warm stimulations show normal pre-LTP (Koga et al. 2015a). These results suggest that cortical pre-LTP is selectively occluded in chronic pain models. Fear and anxiety are two major emotions which are involved in the functions of the ACC (Vogt 2005; Zhao et al. 2005; Bliss et al. 2016). The ACC of mice trained in the fear-conditioning paradigm, either immediately after or 2–3 days after conditioning, produced normal pre-LTP, indicating that pre-LTP is not affected by fear conditioning (Koga et al. 2015a). Next, it was tested whether pre-LTP may be involved in anxiety-like behaviors. The mice were placed in a standard elevated plus maze (EPM) for 5 min and brain slices were prepared immediately after the exposure. The pre-LTP was partially reduced in the mice exposed to the EPM. The mice exposed to a raised open platform, which was a modified EPM in which the open arms were blocked (to increase the anxiety inducing component of the paradigm). After exposure for 5 min in the raised open platform, pre-LTP was absent. As a control, mice exposed to closed arms for 5 min showed normal pre-LTP. To establish whether the effect of anxiety is selective for pre-LTP, post-LTP was examined in the mice that were exposed to the open platform. A pairing protocol could lead to normal post-LTP. These results suggest that anxiety-like behavior occluded pre-LTP. Taken together, chronic pain and anxiety both result in selective occlusion of pre-LTP.

As mentioned earlier, PNI models increase anxiety-like behaviors in mice. Fear and anxiety are two major emotions which are involved in the functions of the ACC. We recently showed that bilateral microinjections of an HCN channels inhibitor, ZD7288, into the ACC of mice with PNI, reduce the PNI-induced anxiety-like behaviors. These local injections into the ACC also robustly alleviated PNI-induced pain hypersensitivity (Koga et al. 2015a).

**Role of glial cells in synaptic plasticity in the ACC**

The role of astrocytes and microglia activation in the ACC in chronic pain remains to be fully understood. It is likely that the activation of glial cells in the ACC may be time dependent and/or types of chronic pain model-dependent manners. At 3 and 14 days after an injection of complete Freund’s adjuvant (CFA) into the hindpaw, a model of peripheral inflammation, both mRNA and protein of glial fibrillary acidic protein (GFAP), an astrocytic marker, were significantly increased in the bilateral ACC. However, 4 h after the CFA injection, the mRNA level of GFAP in the ACC did not change. Twelve hours after, the protein level of GFAP in the ACC did not change in the CFA-treated group (Chen et al. 2012). Interestingly, optical imaging with voltage- and Ca\(^{2+}\)-sensitive dye imaging showed that astrocytes are activated for 3 days after CFA injection in the ACC (Ikeda et al. 2013). Long-term facilitation of neuronal excitation in the ACC in this model requires the activation of astrocytes (Ikeda et al. 2013). Sciatic nerve ligation also caused a dramatic increase in GFAP-like immunoreactivity, which is located in the dendritic astrocytes, with its expanding distribution in the ACC (Narita et al. 2006b; Kuzumaki et al. 2007). Using a formalin-induced conditioned place avoidance model, which reflects the pain-related negative affective state induced by nociceptive stimuli, GFAP protein was also enhanced in the ACC (Lu et al. 2011). On the other hand, common peroneal nerve ligation in adult mice selectively activated microglia in the spinal cord, but not higher cortical regions (Zhang et al. 2008a). Furthermore, in the ACC, long-term synaptic plasticity, such as post-LTP and pre-LTP, was insensitive to minocycline, an inhibitor of microglial activation, suggesting that the activation of microglia may not be involved in long-term synaptic plasticity (Song et al. 2015). Three days after unilateral spared nerve
injury, the quantification of immunoreactivity did not reveal a significant increase in GFAP immunoreactivity in the mice ACC (Ikeda et al. 2013).

**Top-down modulation of pain: ACC–brainstem–SDH descending pathways**

Although various studies demonstrated that the ACC plays an important role in the process of pain and pain-related emotional changes, little is known about its output modulatory pathway. It is well established that the density of nociceptive input can be regulated by the periaqueductal gray (PAG)–rostromedial ventral medulla (RVM)–SDH pathway (Basbaum and Fields 1984; Gebhart 2004; Ossipov et al. 2014), the so-called descending pain regulation system (DPRS). Whether ACC’s effect is mediated through the DPRS is thus interesting to explore.

Anatomic evidence showed that efferent fibers from the ACC innervate the PAG (An et al. 1998; Floyd et al. 2000). Human imaging works revealed that placebo analgesia is related to the enhanced connectivity between ACC and PAG (Eippert et al. 2009). This analgesia effect may be µ-opioid receptor dependent, for systemic injection of naloxone blocked the blood oxygenation level-dependent responses in both ACC and PAG (Petrovic et al. 2002; Zubieta et al. 2005; Eippert et al. 2009). Since the output neurons in the ACC are mainly glutamatergic pyramidal cells and optical stimulation of the channelrhodopsin 2-expressing ACC pyramidal cells induced behavioral hyperalgesia (Kang et al. 2015), it is possible that µ-opioid receptor activation in the ACC directly inhibits the ACC output neurons, which might sequentially decrease the release of presynaptic glutamate to PAG. In the PAG, especially on its ventral part, the mechanism of opioid receptor-induced analgesia has been well studied. µ-Opioid peptides inhibit the activity of GABAergic interneurons and disinhibit the RVM-projecting neurons (Vaughan et al. 1997; Morgan et al. 2008; Lau and Vaughan 2014; Chen et al. 2016). Although the postsynaptic targets of the ACC–PAG projecting fibers are not clear yet, it is less possible that the ACC–PAG projecting terminals directly connect with RVM-projecting neurons. They may potentiate the activity of GABAergic interneurons and enhance the tonic GABAergic inhibition on the RVM projecting neurons, so that the ACC activation causes a hyperalgesic effect. However, the possibility that ACC directly sends projections to the RVM projecting neurons cannot be excluded. In this case, the ACC–PAG pathway may play a homeostasis effect to balance the ACC stimulation-induced hyperalgesia.

Unlike with PAG, the connection from the ACC to the RVM has not been proved. After injection of retrograde tracer horseradish peroxidase (HRP) into the rat nucleus raphe magnus, retrogradely labeled cells were not observed in the ACC (Carlton et al. 1983), suggesting that there is no direct projection from the ACC to the RVM. Although it has been reported that microinjection of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and lidocaine into the RVM blocked ACC stimulation induced pain facilitation (Calejesan et al. 2000), this effect may not be via direct ACC–RVM projection, but by some unknown mechanisms. One explanation is that ACC stimulation simultaneously activates the ACC–PAG–RVM or ACC–dorsal reticular nucleus–RVM pathways (Zhang et al. 2005). However, work to determine the anatomical and functional connection between ACC and RVM is definitely needed for the future.

Whether the ACC sends projections to the spinal cord remained obscure for many years. Although Galea and Darian-Smith (1994) and Gabbott et al. (2005) showed that some parts of the dorsal ACC project to the cervical SDH in rat and macaque monkey, the systematic investigation of the ACC–SDH pathway has been carried out in recent years (Chen et al. 2014a,c). After injection of retrograde tracer fluoro-gold or HRP into the cervical SDH of mouse, fluoro-gold- or HRP-labeled neurons are found in the layer V of rostral ACC. These retrogradely labeled neurons were distributed on both sides of the ACC, with a significant contralateral preference to the injection site. Further injection of the retrograde tracer phaseolus vulgaris leucoagglutinin and the gene-edited rabies virus confirms the ACC–SDH pathway: the projecting fibers are observed to distribute mainly in the lamina I–III of the cervical and lumbar SDH (Chen et al. 2014a). The ACC–SDH pathway possibly plays a role in pain modulation, since c-Fos proteins are expressed in the ACC–SDH projecting neurons in mice with PNI. More importantly, in mice with PNI, increased expression of GluA1 and potentiated postsynaptic responses exclusively happen on the ACC–SDH projecting neurons. Inhibition of the increased expression of GluA1 by microinjection of the GluA1 antagonist NASPM into the ACC reversed the postsynaptic LTP and behavioral mechanical allodynia, indicating the ACC–SDH pathway is important for the maintenance of neuropathic pain (Chen et al. 2014a,c). Our more recent in vivo works showed that high frequency of electrical stimulation in the ACC significantly increased the frequency and amplitude of the spontaneous excitatory postsynaptic currents in SDH lamina I/II neurons (unpublished data), providing a direct evidence that ACC potentiates the SDH synaptic transmission. However, Senapati et al. (2005) and Ma et al. (2011) also showed that high-frequency electrical stimulation of ACC inhibits the spikes of rat spinal wide dynamic range (WDR) neurons. However, since ACC projecting fibers and terminals only distribute in the lamina I–III (Chen et al. 2014a), this inhibitory effect on the lamina IV–V WDR neurons may not come from the direct synaptic transmission from the ACC. As discussed by Senapati et al. (2005), the inhibitory responses may be mediated by the ACC–PAG–RVM pathway. However, how the ACC-projecting fibers affect SDH neurons and whether RVM is
necessary for the ACC–SDH pathway remains largely obscure. The function of ACC–SDH pathway in pain regulation also needs further investigation in the future.

Summary and future direction

In this review, we highlighted recent advances in our understanding of the mechanisms that underlie neuropathic pain, with a focus on the role of neuron–microglia communications in the SDH and synaptic plasticity in the ACC (Fig. 1). For the former, microglial responses to PNI underlie the pathological alteration in the processing of somatosensory information required to explain the principal components and cardinal symptoms of neuropathic pain. The roles of spinal microglia-expressing P2X4R was primarily described, but other purinergic receptors P2X7R and P2Y12R have been shown to contribute to neuropathic pain (Chessell et al. 2005; Honore et al. 2006; Kobayashi et al. 2008, 2011; Tozaki-Saitoh et al. 2008; Ochi-Ishi et al. 2014; Gu et al. 2016a). It is known that microglia have several functions including microgliosis, migration, phagocytosis, and synaptic pruning (Kettenmann et al. 2011). Whether these diverse responses are causally involved in the pathogenesis of neuropathic pain remains unclear. Recent studies using genetic technologies identified a subpopulation of SDH neurons crucial for neuropathic pain (Peirs and Seal 2016). It will be therefore interesting to investigate in the future how each of the diverse functions of microglia regulates activity of subpopulations of SDH neurons and modulate its circuits. In addition, whether spinally altered somatosensory signals are involved in synaptic plasticity in the brain should also be addressed in the future.

For the latter, we described that pre- and postsynaptic LTP in the ACC contributes to the mediation of an anxiety signal under chronic pain conditions (Fig. 1). While findings about the synaptic mechanisms have mainly focused on excitatory synaptic transmission and plasticity (Zhuo 2008, 2014), the role of inhibitory mechanisms is not yet fully understood. Recently, Kang et al. (2015) reported that selective activation or inhibition of ACC neurons by optogenetic techniques can regulate nociceptive behaviors. In addition, the selective activation of pyramidal neurons can rapidly and acutely decrease nociceptive behaviors. On the other hand, the inhibition of pyramidal neurons in the ACC can reduce hypersensitivity in an inflammatory pain model. A similar effect is obtained by activation of parvalbumin-expressing, but not somatostatin-expressing, interneurons. Therefore, future investigations must next target inhibitory interneurons to understand the functions of selective projections, spreading of neural networks, or microcircuits in the ACC for producing chronic pain and anxiety. For the descending output of the ACC for the top-down facilitation of pain, there are two pathways: the ACC–SDH and ACC–brainstem–SDH pathways. These two pathways may work in parallel and offer complementary effects to regulate nociceptive sensory transmission in the spinal level. The ACC–SDH pathway should offer a rapid enhancement, since glutamate released from ACC projecting fibers may acutely affect spinal transmission, through different types of glutamate receptors, such as NMDA, AMPA, kainite, and metabotropic glutamate receptors, on SDH neurons (Li and Zhuo 1998; Li et al. 1999; Kerchner et al. 2001). ACC–brainstem–SDH pathway should provide a prolonged facilitatory regulation (inhibitory regulation may also exist for homeostasis) since serotonin and noradrenaline released from brain DPRS are the major mediators for long-term modulatory effects in spinal transmission (Zhuo and Gebhart 1991; Fields 2004; Gebhart 2004). In this pathway, not only shallow layers (lamina I–III) but also deep layers (lamina IV–V) of SDH neurons should be influenced.

Considering that ACC converges emotional and sensory information, it is also possible that the top-down pathways contribute not only to the regulation of pain intensity, but also the interaction between pain and emotional changes. A potential example seems to be phantom limb pain: emotional changes may trigger enhanced spikes of ACC output neurons and sequentially inhibit the brainstem DPRS and potentiate the activity of SDH neurons. Both the potentiated spontaneous and innocuous stimulation-induced activity in SDH neurons, which may involve activated microglia, will input noxious information to the brain and in turn cause pseudo ‘pain’-like sensation without further noxious peripheral inputs. It thus appears that elucidating the possible amplification mechanism between SDH and ACC may provide us with exciting insights into the mechanisms of neuropathic pain and clues to aid the development of new therapeutic agents for the management of chronic pain.

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