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Activity-triggered tetrapartite neuron-glial interactions following peripheral injury

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Abstract

Recent studies continue to support the proposition that non-neuronal components of the nervous system, mainly glial cells and associated chemical mediators, contribute to the development of neuronal hyperexcitability that underlies persistent pain conditions. In the event of peripheral injury, enhanced or abnormal nerve input is likely the most efficient way to activate simultaneously central neurons and glia. Injury induces phenotypic changes in glia and triggers signaling cascades that engage reciprocal interactions between presynaptic terminals, postsynaptic neurons, microglia and astrocytes. While some responses to peripheral injury may help the nervous system to adapt positively to counter the disastrous effect of injury, the net effect often leads to long-lasting sensitization of pain transmission pathways and chronic pain.

Introduction

Most recent studies continue to support the proposition that non-neuronal components of the nervous system, mainly glial cells and associated chemical mediators, contribute to the development of neuronal hyperexcitability that underlies persistent pain conditions. As microglia are considered innate immune cells of the so-called immune-privileged brain, their responses to peripheral injury with collaborative involvement of astroglia and cytokines are considered a type of neuroinflammation [1,2]. This type of neuroinflammation, however, is remote from the site of injury and characteristically distinct from the conventional meaning of “inflammation”. It is generally devoid of cardinal signs of inflammation in the brain and spinal cord, not necessarily deleterious to neurotransmission as seen in other degenerative neurological diseases, and most importantly, it depends upon enhanced afferent neuronal activity after peripheral injury. Thus, the central “neuroinflammation” induced by peripheral injury is deemed “neurogenic” [2-4], which consists of a plethora of mutual signaling between neurons and glia via chemical mediators and their receptors. While some of these

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responses to peripheral injury may help the central nervous system (CNS) to adapt positively to counter the disastrous effect of injury, the net effect often leads to long-lasting sensitization of pain transmission pathways and chronic pain. We will briefly discuss some recent literature on injury-induced central neuron-glial interactions and their significance in persistent pain.

Glial response to peripheral injury in humans

Despite ample evidence from animal studies, it has been a challenge to directly demonstrate the involvement of glia in human chronic pain conditions [see 5]. Indirect evidence suggests that humans also exhibit glial response to injury. Increased levels of astroglial marker glial fibrillary acidic protein (GFAP) and S100β were observed in postmortem spinal cord dorsal horn tissues from Human Immunodeficiency Virus (HIV) patients with chronic pain [6]. Increased CNS inflammatory cytokine levels in chronic pain patients have been observed [see 7].

Utilizing an improved *in vivo* marker of glial activation with integrated positron emission tomography (PET)-magnetic resonance imaging (MRI), Loggia et al [8**] have recently provided the first observation suggesting brain glial activation in chronic pain patients. They imaged the translocator protein (18kDa) (TSPO) through its specific binding to a newly developed PET radio ligand ^{11}C-PBR28 in patients suffering from chronic low back pain. The TSPO was first described as the peripheral-type benzodiazepine receptor or recognition site [9]. It was found later that TSPO was also expressed in microglia, astrocytes and neurons [see 10, 11]. Interestingly, TSPO has been selectively upregulated in spinal astrocytes and microglia, but not in neurons, following L5 spinal nerve injury in rats [11] and has been used as a marker of increased glial activity after CNS injury in imaging studies [see 8**]. Peripheral immune challenge with lipopolysaccharide (LPS) induced an increased TSPO binding in the mouse brain with another TSPO PET ligand ^{18}F-PBR06 [12].

In comparison between matched pairs controlling TSPO polymorphism, age and sex, the levels of TSPO, assessed by standardized uptake values for ^{11}C-PBR28, was significantly increased in the thalamus and other regions including the insula, middle and posterior cingulate cortex, and ventromedial prefrontal cortex in patients with low back pain. These observations are consistent with previous report that limb denervation injury leads to long-lasting increase in binding of ^{11}C(R)-PK11195, the earlier generation of the TSPO ligand, in the human thalamus [13]. The increased ^{11}C-PBR28 tracer binding appeared somatotopically relevant in the somatosensory and motor cortices receiving input related to the pain in the lower back and leg, indicating correlation of increased glial activity with pain-related neuronal input [8**].

Activity-dependent microglial activity

Microglia are sensitive to their environment. In their resting state, microglia constantly scan and monitor their surroundings and respond to changes in homeostasis [see 14-16]. In a broader sense, changes in the extracellular milieu can also be induced by injury distant from the CNS, resulting from influx of chemical mediators and neurotransmitters released from afferent terminals activated by enhanced or abnormal input activity after injury. Studies have

shown the contribution of primary afferent input to increased microglial activity after injury [17] and the development of pain-related behavior is delayed after peripheral nerve block [18]. In fact, peripheral nerve injury leads to increased activity of microglia in brain regions directly related to pain, mood and affect, as well as reward circuitry [19].

Microglia maintain a relationship with neurons, which positions microglia to perceive changes in neuronal activity. In the cortex, Baalman et al. [20] show that a subset of perineuronal microglia are specifically associated with the axon initial segment through a single process. Cilium-like protrusions of microglial processes closely appose or wrap around dendritic spines, presynaptic terminals, synaptic cleft and even perisynaptic astrocytic processes (Fig. 1A,B) [21]. It is interesting to note that microglia adjust their morphology and location in a dynamic fashion to sensory input. Tremblay et al. [21] demonstrate that altering light exposure induces changes in microglia processes in the visual cortex of juvenile mice, involving microglial contact/apposition with synaptic structures and motility of microglial processes. Visual deprivation reduced microglial process motility, which was reversed after light-reexposure. Evo et al. [22*] observed that microglial processes converge at dendritic spines in mouse brain slices in response to reduced extracellular calcium. They describe this phenomenon as “microglial process convergence”, which is apparently mediated by purinergic signaling. Conceivably, a reduction in extracellular calcium might mimic the effect of intense synaptic activity. Even in the resting state, the transient contacts of microglial processes with synaptic neural elements are modulated by neuronal activity [23]. Thus, microglia behavior is clearly regulated by sensory-driven neural activity.

The microglial cell membrane expresses a diversity set of receptors. What chemical signals do microglia pick up as a sign of injury? Among a handful of candidates, purines, as neurotransmitters, appear on the top of the list [15, 24-28*], although ATP could also be derived from other sources such as astrocytes [29]. Microglia-neuronal contact alters with changes in neuronal input. In P2Y12 receptor (P2Y12R) knock-out mice, microglial process convergence towards neuronal dendrites was eliminated [22*]. Tatsumi et al [30] recently show that microglial P2Y12R signaling following nerve injury involves downstream Rho-associated protein kinase (ROCK) that acts at actin. The ROCK inhibitor H1152 attenuated neuropathic pain behavior and reversed nerve injury and 2Me-SADP-induced retraction of microglial processes, implicating an effect on pain-related microglial motility.

The other top candidate that mediates neuronal input to microglia is the chemokine CX3CL1 [chemokine (C-X3-C motif) ligand 1], or fractalkine [31-35]. CX3CL1 is expressed in spinal cord dorsal horn and dorsal root ganglion neurons but not glia and its receptor CX3C chemokine receptor 1 (CX3CR1) is expressed exclusively in microglia [36]. Mice lacking CX3CR1 exhibited a delay in the development of allodynia following administration of the chemotherapeutic agent vincristine [37]. Direct application of CX3CL1 to spinal slices strengthens synaptic transmission between primary afferent C-fibers and dorsal horn lamina I neurons via microglial activity [38*]. Interestingly, this synaptic facilitatory effect of CX3CL1 does not depend on neural activity, which is consistent with a cascade related to CX3CL1/CX3CR1 signaling after peripheral injury. The functional chemokine domain of CX3CL1 is normally tethered to the membrane and released after cleaving by proteases. The

cleavage of CX3CL1 is achieved by the cysteine protease cathepsin S (CatS) from microglia. Nerve injury induces CatS in spinal microglia in the region receiving damaged primary afferent terminals, followed by CX3CL1 cleavage [see 34]. Thus, direct application of CX3CL1 may bypass the cleavage step of the membrane-tethered CX3CL1 and produces an effect that is downstream to initial injury-related neuronal input.

There is still much to be learned on the nature of inputs that switch microglia from a surveying phenotype to an active state; and it is not fully understood how glial cells decode information encoded by multiple integrated chemical mediators. It is interesting to note that application of ATP alone promoted motility of microglia, while application of a list of neurotransmitters [glutamate, substance P, gamma-Aminobutyric acid (GABA), serotonin (5-HT) etc.], neurotrophic factors (nerve growth factor, brain-derived neurotrophic factor), and chemokines [chemokine (C-C motif) ligand 2 (CCL2), CX3CL1 etc.], even direct nerve stimulation, had no effect [22*,39]. To induce release of CatS in microglia, ATP alone was insufficient. Only with priming by LPS, ATP activates P2X7R on microglia and leads to release of CatS. It seems that to reproduce a full spectrum of ‘activated’ microglia phenotype after injury, a combination of signals that more closely mimic *in vivo* conditions are necessary. The factors that help to maintain microglia in the resting state are also worth attention. CD200R is expressed in microglia and interacts with neuronal CD200, the OX-2 membrane glycoprotein. Loss of CD200-CD200R signaling facilitates microglial activation in CNS after peripheral injury [see 32]. Down-regulation of CD200/CD200R1 in the knee synovium is associated with a painful condition [40].

Astroglia and neuronal activity after injury

Unlike microglia developed from macrophages of mesodermal hematopoietic cells, astrocytes are derived from the ectoderm and are involved in synaptic activity through their intimate anatomical relationship with neurons originating from the same germ layer. Ample evidence supports a role of astrocytes in the development of persistent pain hypersensitivity after injury [41-43]. Inhibition of astrocyte activity has been associated with the antihyperalgesic effect of pioglitazone, a peroxisome proliferator-activated receptor gamma agonist [44]. Direct nerve stimulation induced astroglial activity and cytokine release [45] and prolonged local anesthesia block of afferent neurons delayed the onset of pain-related behavior and reduced nerve injury-induced astrocytic responses [46], indicating that astrocytic response is regulated by neural input. Studies have shown that activation of microglia usually preceded reactive astrocytosis after injury [47,48*]. However, this phenomenon is not universal. In chemotherapy-induced peripheral neuropathic pain, glial activation is only seen for astrocytes, but not microglia [49, also see 50]. In a female bone cancer pain model, reactive astrocytosis occurred earlier than microglial activation and microglia did not appear to be involved in the initiation, but was critical in the maintenance, of persistent pain hypersensitivity [28*].

Astrocytes release gliotransmitters that contribute to synaptic activity. D-serine as a coagonist of the NMDA (N-methyl-D-aspartate, GluN) receptor is released from astrocytes. From an activity-dependent point of view, a recent work shows that release of D-serine from astrocytes in culture can be induced by ATP through astrocytic P2X7 receptors [51]. This

release is not calcium-dependent, consistent with growing awareness that astrocytic Ca^{2+} dynamics is not necessarily linked to synaptic activity [see 52]. The ATP-stimulated D-serine release from astrocytes, however, requires functional pannexin-1 channels [51]. Pannexin-1 is homologous to the invertebrate gap junction protein innexin, but does not structurally bridge neighboring cells [53]. The major function of pannexins is to facilitate communication between the extra- and intra-cellular compartments. Apparently, pannexin plays a role in neuron-astrocytic signaling after injury. Blocking pannexin-1 attenuated neuropathic pain behavior in rats [54].

A unique contribution of astrocytes to excitatory synaptic activity is removal of glutamate from the extracellular fluid through glutamate transporter-1 (GLT-1, EAAT2), which is a major determinant of glutamate receptor activation and related neuronal excitability. Peripheral nerve injury down-regulates GLT-1 in the spinal cord dorsal horn [55], which may lead to accumulation of glutamate in the synaptic cleft. Restoring GLT-1 with ceftriaxone relieved hyperalgesia [56]. The down-regulation of GLT-1 results in an enhanced extrasynaptic NMDA receptor activation, likely involving glutamate spillover to the extrasynaptic site [57].

It is important to appreciate that the activity of GLT-1 is not limited to glutamate recycling. Sodium is cotransported with glutamate into astrocytes so that neuronal sodium load can be reduced. Sodium imaging in brain slices shows that neuronal activity triggers a transient increase in Na^+ in astrocytes [58, also see 59]. Interruption of glutamate transport and associated uptake of extracellular K^+ via activity of astrocytic Na^+-K^+ ATPase led to elevated neuronal sodium and prolonged epileptiform burst activity in mouse hippocampal slices [58]. Thus, astrocytes are crucially important for neuronal sodium homeostasis particularly in the phase of intense activity.

Astrocytes form functional syncytium through gap junction proteins such as connexin43 (Cx43, GJ1). Six connexins assemble into a connexon and two connexons from adjacent cells face each other to form a gap junction channel. Gap junctions allow flow of ions and chemical mediators thus facilitating intercellular communication. Connexons that are not opposing a counterpart from other cells form hemichannels that provide an additional form of intra- and extra-cellular communication. Administration of carbenoxolone, a non-selective gap junction decoupler, has been shown to attenuate behavioral pain hypersensitivity in animal models [48*, 60-63], suggesting that astrocytic gap junctions are involved in persistent pain. Potential involvement of gap junctions in persistent pain gives a hint on the mechanisms underlying spread of excitation to a non-injured territory, possibly mirroring pain that occurs contralateral to the site of injury [60].

The proposed contribution of astrocytic gap junctions to persistent pain, however, requires further attention. In an *in vitro* astrocyte scratch injury model, the spread of injury involves an increase in Cx43 hemichannel, but not gap junction channel activity [64]. In mouse hippocampal slices, LPS induces astroglial Cx43 hemichannel opening without an effect on gap junction activity [65]. Chen et al. [48*] show that tumor necrosis factor (TNF)-activated astroctytes induce mechanical allodynia involving Cx43. However, the effect of TNF was on Cx43 hemichannel activity in astrocytes as shown by ethidium bromide uptake in cultures,

but not on gap junction channels [48*]. While an involvement of astrocytic gap junctions cannot be ruled out, further studies should distinguish the role of Cx hemichannels in injury-induced pain hypersensitivity.

Integrated neuron-glial signaling in persistent pain

Studies on neuron-immune interactions in pain have led to a concept that the basic functional unit for spinal/trigeminal pain processing is tetrapartite, likely consisting of four components, presynaptic terminals (primary afferents), postsynaptic neurons/dendrites, and astrocyte and microglial processes [66-68]. Although there is no direct evidence at the spinal level, this concept is supported by functional anatomy data from brain that demonstrate mutual contacts between the four components in the brain and their dynamic sensitivity to sensory input and signaling molecules [20-22*] (Fig. 1).

Ample evidence has shown comprehensive reciprocal neuron-glial and glia-glial signaling following peripheral nerve or tissue injury, involving neurotransmitters/modulators, cytokines/chemokines, growth factors, proteases, and their receptors/substrates [69,70]. For example (Fig. 1C), ATP released from primary afferent central terminals can engage P2X3R on neurons [see 26], P2X4R/P2Y12R on microglia [see 24,30], and P2X7R on astrocytes [51]. Microglia release CatS that cleaves CX3CL1 tethered to spinal neurons and TNF/interleukin (IL)-18 that acts on astrocytes [48*, 71**]. Astrocytes can interact with C-C chemokine receptor type 2 (CCR2) on microglia through release of Chemokine (C-C motif) ligand 7 (CCL7) [72], and with chemokine (C-X-C motif) receptor 2 (CXCR2) on neurons through CXCL1 [48*,62]. Astrocytic IL-17 facilitates NMDA receptor phosphorylation through IL-17R on neurons [73]. Both microglia and astrocytes can release IL-1β that activates neuronal IL-1R [74,75]. Activation of IL-1R facilitates NMDA receptor phosphorylation in neurons [74], enhances glutamate release from primary afferents in spinal dorsal horn [76], increases endocytosis of GLT [77], and inhibits GABAergic neurotransmission (Fig. 2A) [78**]. All these signaling events can be triggered by peripheral injury or induced by inflammatory agents, and are mediated by cellular pathways involving multiple protein kinases and auxiliary factors, and importantly, linked to behavioral hyperalgesia.

The tetrapartite model of the spinal pain-processing unit should be understood in the context of different synaptic circuitry. In a GABAergic “inhibitory” synapse, decreased synaptic activity can be induced following LPS-induced IL-1β release from microglia, suppressed astrocytic GLT activity, and reduced GABA synthesis in presynaptic neurons (Fig.2A) [77,78**]. Spinal pain processing is subject to descending modulation. Descending serotonin produces facilitation by activating 5-HT_3 receptors on spinal neurons, followed by upregulation of glial markers and behavioral hyperalgesia in rats [71**]. These effects of 5-HT are mediated by a signaling cascade that involves neuronal CX3CL1, microglial IL-18, astrocytic IL-1β and their respective receptors CX3CR1 (microglia), IL18R (astrocytes) and IL-1R (neurons) (Fig. 2B). Thus, pain-related neuron-glial signaling in the spinal cord can also be triggered or modulated by brainstem centrifugal input.

Neuroprotection, antinociception and glial activity

Further analysis of PET/MRI data indicated that the increased TSPO in the thalamus in human chronic pain patients is negatively associated with levels of pain and proinflammatory cytokine IL-1β [8**], suggesting a protective role of glial activation. Neuroprotection is in fact a major function of glia, although the net effect of increased glial activity after injury is often pronociceptive [3,79,80]. The TSPO agonist has been shown to attenuate persistent pain in animal models [10,11]. This raises the possibility of targeting TSPO for pain relief. Glia secrete anti-inflammatory cytokine IL-10 that enhances analgesia [81,82]. The A3 adenosine receptor agonist IB-MECA attenuated paclitaxel-induced pain-related behavior by reducing proinflammatory TNF and IL-1β and increasing anti-inflammatory IL-10. IB-MECA also attenuated nitration of astrocytic GLT-1 and glutamine synthetase [83], suggesting recovery of function of these proteins in synaptic glutamate homeostasis. Thus, in manipulating glia for pain relief, it is intuitive to selectively engage their protective mechanisms, not simply suppress glial activity.

Concluding remarks and additional comments

Neuron-glial interactions continue to attract major interest in searching for mechanisms and treatment of chronic pain. In the event of peripheral injury, the enhanced or abnormal nerve input is likely the most convenient and efficient way to alert simultaneously central neurons and glia, although infiltration of peripheral immune cells and immune mediators through a compromised blood-brain barrier/blood-spinal cord barrier may also play a role [50]. Injury-triggered signaling cascade engage reciprocal activation between presynaptic terminals, postsynaptic neurons, microglia and astrocytes, leading to long-lasting persistent pain.

It is unclear whether oligodentrocytes, the third major glia type in the CNS, interact similarly with neurons and other glial cells in response to peripheral injury. Gritsch et al. [84**] report that genetic ablation of oligodendrocytes induced cold and mechanical pain hypersensitivity in mice. It was found that the occurrence of pain hypersensitivity after diphtheria toxin-induced oligodendrocyte loss correlated with axon degeneration in the spinothalamic tract and upregulation of amyloid precursor proteins, a marker of axonal pathology. However, the microglial and astrocytic responses occurred about 3 weeks later except an early upregulation of one microglial marker, Iba-1 (ionized calcium-binding adapter molecule 1). It seems that interruption of interaction between oligodendrocytes and axons is sufficient to induce hyperalgesia and microglia/astrocytes do not contribute to the initiation of pain hypersensitivity in these mice. The dissociation of persistent pain hypersensitivity and glial responses has also been noticed in other models [85,86]. Further studies are expected to delineate a distinct role of neuron-glial interactions under specific pain conditions.

There has been a continuing effort in translating preclinical findings on neuron-glial interactions in pain. However, the clinical trials of agents targeting glia and pain-related chemokines/receptors have not been successful [see 1,8**,87 for further details]. For example, it is disappointing that a well-studied glial modulator propentofylline did not produce pain relief in post-herpetic neuralgia patients [88]. Minocycline, a microglial inhibitor, did not produce clinically meaningful pain relief [89,90]. AZD2423, a CCR2

antagonist, is ineffective against posttraumatic neuralgia [91]. To search for answers to negative clinical findings, Landry et al. [88] have provided some clues regarding potential relevant differences in function of microglia and macrophages between humans and rodents, which might explain observed failure in clinical trials. Finding explanations of failed trials is no easy task and there should be controlled direct comparisons between clinical and pre-clinical conditions [see 92]. Besides potential differences in biology and trial design issues, we need to rethink whether we have sufficient understanding of the mechanisms and to pursue coordinated preclinical and clinical studies in a disease-specific fashion.

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Highlights

-Brain glial activation is observed in patients suffering from chronic low back pain.

-Microglia maintain a dynamic relationship with neurons.

-Astrocytic connexin hemichannels play a role in injury-induced pain hypersensitivity.

-Neuron-glial signaling in the spinal cord can be triggered by descending input.

-Enhanced glial activity also offers neuroprotection as suggested by human studies.

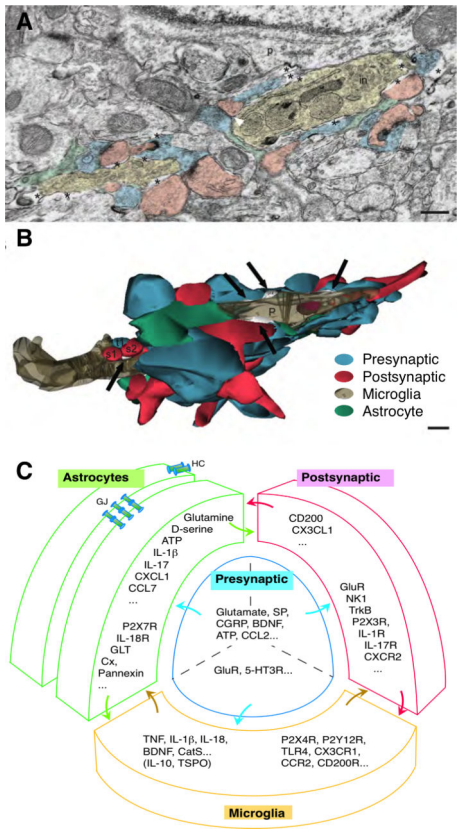

Fig. 1.

The tetrapartite model of the neuron-glial interactions. **A**. Electron microscope image showing oppositions between the presynaptic axon terminals (blue), postsynaptic dendritic spines (pink), microglia (beige) and perisynaptic astrocytes (green). Note a microglia contiguous to a neuronal perikaryon (p) with its associated extracellular space (asterisks) and contacted synapse-associated elements. in, cellular inclusion. Scale = 250 nm. (Adapted from [21] Tremblay et al. PLoS Biology, 2010, 8(11):e1000527, Fig. 2A.) **B**. Partial 3-D reconstruction of the microglial proximal process (P) cut in transverse. Note that microglial processes directly contact multiple presynaptic axon terminals (blue), postsynaptic dendritic spines (red), and perisynaptic astrocytic processes (green). Similar cellular relationship exists for axon terminals, dendrites and astrocytes. Black arrows indicate extracellular space pockets of various size and shape (white). s1, s2, two dendritic spines; t1, one axon terminal. Scale = 250 nm. (Adapted from [21] Tremblay et al. PLoS Biology, 2010, 8(11):e1000527, Fig. 2B.) **C**. The tetrapartite model of the neuron-glial interactions in spinal nociceptive processing. Note mutual contacts between all four components of the model. Example lists of neurotransmitters/modulators, cytokines/chemokines, growth factors, proteases, and their receptors/substrates involved in nociceptive processing are shown in respective compartments. Two astrocytes are shown connected by astrocytic gap junctions. Abbreviations: **5-HT**, serotonin; **BDNF**, brain-derived neurotrophic factor; **CatS**, cysteine protease cathepsin S ; **CCL2**, chemokine (C-C motif) ligand 2; **CCL7**, Chemokine (C-C motif) ligand 7; **CCR2**, C-C chemokine receptor type 2; **CGRP**, calcitonin gene-related peptide; **CX3CL1**, chemokine (C-X3-C motif) ligand 1; **CX3CR1**, CX3C chemokine

receptor 1; **CXCL1**, chemokine (C-X-C motif) ligand 1; **Cx**, connexon; **CXCR2**, CXC chemokine receptor 2; **HC**, connexin hemichannel; **IL**, interleukin; **GJ**, gap junctions; **GLT**, glutamate transporter; **GluR**, glutamate receptors; **NK1**, neurokinin 1; **R**, receptor; **SP**, substance P; **TNF**, tumor necrosis factor; **TrkB**, Tropomyosin receptor kinase B; **TSPO**, translocator protein; **TLR4**, Toll-like receptor 4;

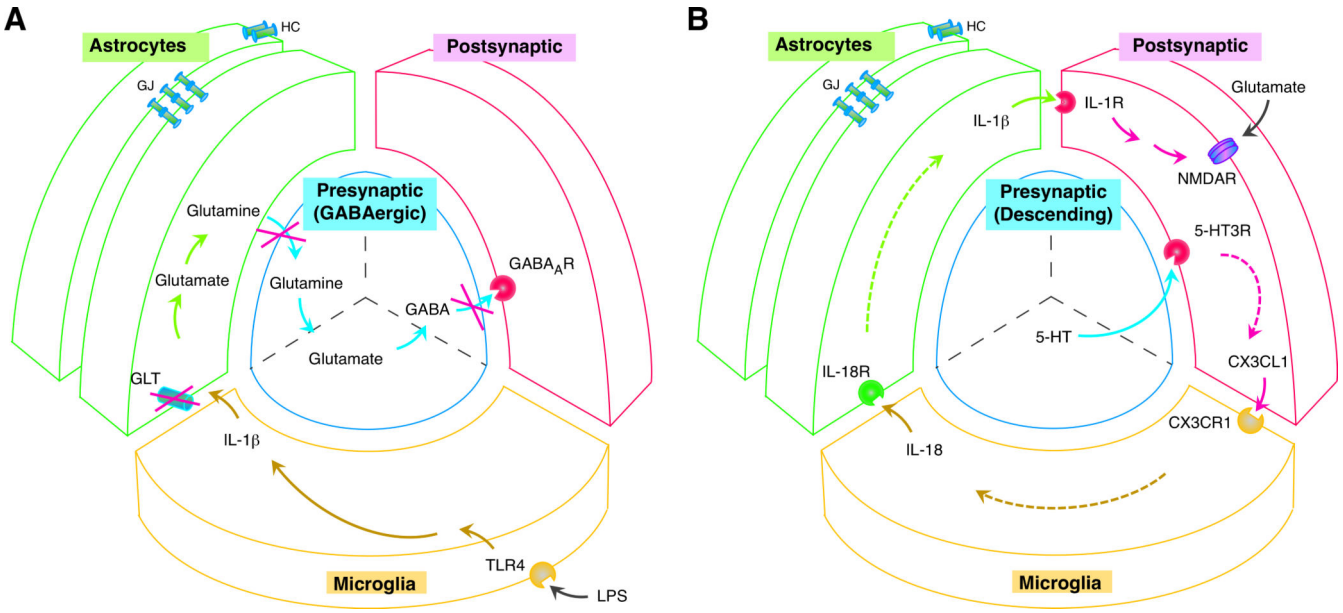

Fig. 2.

Four-way neuron-glial interactions in spinal nociceptive processing. **A**. Decreased GABAergic inhibition in the spinal dorsal horn induced by lipopolysaccharide (LPS) activation of microglia through TLR4, IL-1β release from microglia, suppressed astrocytic glutamate transporter activity after IL-1β-induced GLT endocytosis, and reduced glutamate-glutamine cycle-dependent GABA synthesis in presynaptic neurons [Adapted from 77,78**]. Reduced GABAergic inhibition unleashes postsynaptic excitatory neurons and contributes to pain hypersensitivity. **B**. Descending 5-HT-induced neuron-glial interactions and related signaling cascade that underlies pain hypersensitivity. Descending serotonin (5-HT) activates 5-HT3 receptors on spinal neurons, followed by a signaling cascade that involves release of neuronal CX3CL1, microglial IL-18, and astrocytic IL-1β and activation of their respective receptors CX3CR1 (microglia), IL18R (astrocytes) and IL-1R (neurons) [Adapted from 71]. IL-1R facilitates NMDA receptor activity that leads to neuronal hyperexcitability and behavioral hyperalgesia. Abbreviations: GABA, gamma-Aminobutyric acid; LPS, lipopolysaccharide; NMDAR, N-methyl-D-aspartate receptor; see Fig. 1 caption for other abbreviations.