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Mini review

Emerging role of astroglia in pain hypersensitivity

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Summary Recent studies suggest that astroglia, a major non-neuronal cell type in the central nervous system, actively participate in synaptic activity and potentially contribute to neurological disorders including chronic pain. Astroglia exhibit a hyperactive phenotype, also referred to as reactive astrocytosis, in response to peripheral injury. This process is often referred to as astroglial activation. By immunostaining against glial fibrillary acidic proteins, an intermediate cytoskeleton filament protein selectively localized to matured astrocytes, hypertrophy of astrocytes are clearly visible in the spinal cord dorsal horn and spinal trigeminal nucleus following a variety of injuries. Injury-related astroglial activation correlates with behavioral hyperalgesia and conversely, astroglial inhibition attenuates pain hypersensitivity. Astrocytes have a close anatomical relationship with neurons. Interactions between astrocytes and neurons contribute to the mechanisms of chronic pain. Astroglial activation is accompanied by initiation of cellular signal transduction pathways that lead to transcriptional gene regulation and release of a variety of chemical mediators or gliotransmitters, down-regulation of glutamate transporter activity that directly affects synaptic transmission, changes in gap junction proteins by which calcium waves spread, and alterations of the blood brain barrier. These coordinated changes in astroglial functions in turn modulate neuronal activity and facilitate pain transmission.

Astroglia is a major non-neuronal cell type in the central nervous system (CNS). Morphologically, astroglia are stellate-shaped (star) and can be classified into protoplasmic and fibrous astrocytes. Protoplasmic astrocytes are found in grey matter and have many branching processes whose small terminals (end-feet) enwrap neuronal somata, dendrites and synapses. Fibrous astrocytes are localized in white matter and have long unbranched processes, the tip of which may form pedicles (vascular feet) that come into contact with blood vessels. Astrocytes interconnect by connexin 43 (Cx43), an astrocytic gap junction protein, and form a functional syncytium, a network of coupled astrocytes. It is believed that waves of calcium ions can spread between astrocytes through the astrocytic syncytium. Similar to other glial cell subtypes, microglia and oligodendrocytes, astroglia have long been thought to mainly have supportive roles in CNS neuronal activity. Astroglia help to maintain the CNS's extracellular environment by scavenging ions such as potassium and neurotransmitters such as glutamate. Astrocytes also store glycogen for energy metabolism, form cellular scar tissue for repairing neural tissue damage and facilitate CNS development. Beyond these supporting roles, however, recent studies have shed new

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light on our understanding of astroglia biology. Ample evidence suggests that astroglia actively participate in synaptic activity [1] and potentially contribute to neurological disorders including chronic pain [2,3].

1. Injury, astroglial activation and pain hypersensitivity

Similar to microglia, astroglia exhibit a hyperactive phenotype, also referred to as reactive astrocytosis, in response to peripheral injury. This process is often referred to as astroglial activation. By immunostaining against glial fibrillary acidic proteins (GFAP), an intermediate cytoskeleton filament protein selectively localized to matured astrocytes, hypertrophy of astrocytes are clearly visible in the spinal cord dorsal horn following a variety of injuries including chronic constriction injury of the sciatic nerve [4], spinal nerve ligation [5], nerve root injury models of lumbar radiculopathy [6], experimental bone cancer [7,8], spinal cord injury [9], complete Freund's adjuvant (CFA)-induced inflammation [10], formalin-induced nociception [11], acute cardiac injury induced by intracardial injection of formalin [12], and snake venom-induced pain [13]. Orofacial inflammation induces astroglial activation in the regions of the spinal trigeminal complex with a time course correlating with hyperalgesia [14]. Astroglial activation after peripheral injury also occurs at multiple supraspinal levels related to pain transmission and modulation [10,15–18].

Injury-related astroglial activation correlates with behavioral hyperalgesia. Colburn et al. [19] compared the effect of seven different nerve injuries at the L5 spinal level: spinal nerve cryoneurolysis, spinal nerve tight ligation, dorsal root cryoneurolysis, dorsal root tight ligation, dorsal root transection, ventral root tight ligation, and sham laminectomy/dural incision. It was found that spinal nerve or dorsal root injury always led to mechanical allodynia and glial activation. In contrast, ventral root lesions did not induce hyperalgesia and produced only sporadic glial responses. Interestingly, spinal glial responses to dorsal root lesions were almost exclusively astrocytic. Colburn et al. [19] concluded that astrocytic activation was always observed following spinal nerve or dorsal root axonal injury and reliably coexisted with the behavioral response.

Targeted expression of chemical mediators in astrocytes through genetic manipulation further supports their role in pain and hyperalgesia. In mice in which expression of murine tumor necrosis factor (TNF), a proinflammatory cytokine, was targeted to astrocytes using a glial GFAP-TNF fusion gene, mechanical allodynia was significantly enhanced after L5 spinal nerve transection [20]. In mice overexpressing CC chemokine ligand 2 (CCL2 or monocyte chemoattractant protein-1, MCP-1) in astrocytes and Schwann cells under control of GFAP promoter, CFA induced greater edema and enhanced and prolonged hyperalgesia when compared to control mice [21].

Astroglial inhibition attenuates pain hypersensitivity. Fluorocitrate interrupts citric acid cycle by its inhibition of aconitase. Although not selective against aconitase of different cellular origin *per se*, fluorocitrate selectively inhibits the citric acid cycle of astrocytes due to a more avid uptake by astrocytes. The subsequent energy failure results in selective disruption of the function of astrocytes [22]. Fluorocitrate has been used to assess the role of astrocytes in pain. Intrathecal administration of fluorocitrate (1 nmol) results in a significant attenuation of the pain hypersensitivity produced by intraplantar zymosan [23] and blocks formalin-induced nocifensive behavior [24]. Microinjection of fluorocitrate into the spinal trigeminal complex (0.1–1 nmol) attenuates masseter inflammatory hyperalgesia [14]. Injection of fluorocitrate (1–100 fmol) into the rostral ventromedial medulla (RVM) reverses descending facilitation of orofacial hyperalgesia induced by constriction injury of the infraorbital nerve [17]. It should be noted that fluorocitrate might also produce neuronal damage. Intrastriatal injection of 1 nmol fluorocitrate produced ultrastructural alterations of astrocytes without affecting neurons while a larger dose at 2 nmol also affected neuronal structures and metabolism [25]. In the RVM, we have found that a dose $\leq$0.1 pmol is necessary to avoid an apparent effect on neurons [17].

2. Cellular mechanisms underlying the involvement of astroglia in pain hypersensitivity

Astrocytes have a close anatomical relationship with neurons. A single cortical astrocyte enwraps on an average of four neuronal somata and contacts 300–600 neuronal dendrites [26]. The concept of "gliapse" at which neuron–glial and glia–neuronal interactions would occur was proposed in the early 1960s to include glial cells (astrocytes) in the neuronal network to form a fundamental functional unit of the brain [27]. Studies have shown that synaptic strength may be modulated by gliotransmission through a "tripartite" synapse that includes pre- and post-synaptic membranes and extrasynaptic astrocytic contacts [1,28]. Most recent studies favor the view that interactions between astrocytes and neurons contribute to the mechanisms of synaptic plasticity and CNS disorders.

The cellular mechanisms of astrocyte–neuronal interactions and contribution of astrocytes to pain hypersensitivity is beginning to be understood. Although GFAP has been used extensively as a marker of astroglial activation, little is known about the functional significance of its increased expression in astrocytes following peripheral tissue or nerve injury. Additional markers and cellular events, however, have provided some insight. Astroglial activation is accompanied by initiation of cellular signal transduction pathways that lead to transcriptional gene regulation and release of a variety of chemical mediators or gliotransmitters, down-regulation of glutamate transporter activity that directly affects synaptic transmission, changes in their gap junction proteins by which calcium waves spread, and alterations of the blood brain barrier. These coordinated changes in astroglial functions in turn modulate neuronal activity and facilitate pain transmission. Some recent evidence on cellular mechanisms of a role of astroglia in pain hypersensitivity is briefly discussed below (see Refs. [2,3,29] for further information).

2.1. Astrocytes as a potential source of inflammatory cytokines: role of IL-1β

The inflammatory cytokines are among a group of chemical mediators that can be released by activated glia and have been implicated in persistent pain states [30]. Interleukin (IL)-1β, a

prototype proinflammatory cytokine, is involved in a variety of diseases with pain conditions such as rheumatoid arthritis, inflammatory bowel disease, osteoarthritis, multiple sclerosis and neuropathy [31]. Interestingly, recent studies show that IL-1β is selectively induced in astrocytes in animal models of bone cancer pain [8], masseter inflammation [14], infraorbital nerve injury [17] and intracerebral hemorrhage [32]. These results suggest that astrocytes are a source of secreted IL-1β after injury. Following L5 spinal nerve injury, matrix metalloproteinase (MMP)-2 in astrocytes cleaves pro-IL-1β into bioactive IL-1β and this event correlates with the maintenance of neuropathic pain behavior in rats [33].

IL-1β may be a messenger of astroglia to neurons through signal coupling with N-methyl-D-aspartate (NMDA) receptors. IL-1 receptors colocalize with the NR1 subunit of the NMDA receptor [14]. IL-1β enhances NMDA receptor-mediated intracellular calcium release [34]; and facilitates inward currents and inhibits outward currents induced by NMDA in hippocampal neurons [35]. Direct application of IL-1β enhances NMDA receptor phosphorylation similar to that following tissue inflammation [14]. In primary mouse neuronal cultures, IL-1β activates Src kinase that further triggers the phosphorylation of the NMDAR NR2B subunit [36]. The IL-1 receptor antagonist attenuates pain hypersensitivity through preventing NMDA receptor phosphorylation in rats [37]. Thus, astroglial activation and associated IL-1β release play a critical role in the mechanisms of hyperalgesia. The cellular events that lead to IL-1β release after astroglial activation is less clear. The potential role of the inflammasome, a multiprotein complex that mediates the activation of caspase-1 that facilitates secretion of proinflammatory cytokines IL-1β and IL-18, should be investigated [38].

2.2. Astrocytic MCP-1 in neuropathic pain

Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a chemokine that is expressed in primary sensory neurons and involved in persistent pain [39]. MCP-1 may also be present in astrocytes [40]. Recent studies show that spinal nerve ligation induced MCP-1 upregulation in the spinal cord, primarily in spinal cord astrocytes [41]. Spinal administration of MCP-1 neutralizing antibody attenuated neuropathic pain and application of MCP-1 induced hyperalgesia [41]. Activation of MCP-1 is apparently down stream to c-jun-N-terminal kinase (JNK) in spinal astrocytes [5]. Both nerve injury-induced MCP-1 upregulation and pain were suppressed by a JNK inhibitor, D-JNKI-1 [41]. In cultured astrocytes, TNFα transiently activated JNK via TNF receptor-1 and induced MCP-1 [41], suggesting an astrocytic signaling pathway involving TNF receptor-JNK-MCP-1. Interestingly, TNFα has been shown in microglia and macrophages but is absent in astrocytes; and the MCP-1 receptor CCR2 is localized in microglia/neuron, but not in astrocytes [41,42]. MCP-1 potentiates NMDA- and AMPA-induced currents in spinal dorsal horn neurons [41]. These findings suggest interactions between microglia and astroglia and neuron and glia.

2.3. NFκB in astrocytes

Nuclear factor kappa-B (NFκB) is a transcription factor that regulates transcription of a variety of genes including inflammatory cytokines and chemokines in glia. A common downstream event of IL-1R/toll-like receptor (TLR) signaling is phosphorylation of the NFκB subunit followed by its translocation to the nucleus. The activation of NFκB is preceded by phosphorylation of inhibitory kappa-B (IκB) induced by cytokines. As a mechanism of feedback control of NFκB activity, IκB is also upregulated upon NFκB activation.

IκBα mRNA expression was markedly induced in the spinal cord, mainly in astrocytes and endothelial cells, following intrathecal administration of the human immunodeficiency virus-1 envelope glycoprotein, gp120 [43]. Intrathecal administration of NFκB inhibitors partially attenuated gp120-induced allodynia and reversed pain hypersensitivity induced by sciatic nerve inflammation [43]. IL-18R is upregulated in spinal astrocytes after nerve injury and the functional inhibition of IL-18 signaling pathways suppressed nerve injury-induced allodynia and decreased the phosphorylation of NFκB in astrocytes and the induction of astroglial markers [44]. Recent studies have shown that low levels of activity of catechol-O-methyltransferase (COMT), an enzyme involved in catecholamines metabolism is related to pain hypersensitivity [45]. This altered pain may be related to NFκB-mediated inhibition of COMT expression in astrocytes [46]. These findings suggest that the increased NFκB transcriptional activity in astrocytes may underlie molecular mechanisms of pain hypersensitivity.

2.4. Astrocytic gap junction proteins

Communication between astrocytes can be mediated by specific gap junction proteins, which spread calcium waves between astrocytes after stimulation and may also convey astrocyte-to-neuron signaling [47]. The gap junction may play a role in spinal nociceptive processing [48]. Spataro et al. [49] showed that intrathecal administration of the gap junction decoupler carbenoxolone reversed mirror image mechanical allodynia after nerve injury, while leaving ipsilateral mechanical allodynia unaffected, suggesting that spinal gap junctions are potentially involved in spread of pain facilitation. Following masseter muscle inflammation, astrocytic gap junction protein connexin 43 (Cx43) was upregulated in astrocytes in the subnuclei interpolaris/caudalis (Vi/Vc) trigeminal transition zone, a subregion of the spinal trigeminal complex involved in trigeminal pain processing [14]. Cx43 also increased in the trigeminal ganglion satellite glial cells in rats with chronic constriction injury of the infraorbital nerve [50]. Injection of gap junction decouplers into the Vi/Vc site attenuated masseter hyperalgesia/allodynia after inflammation (Wang et al., unpublished observation). Sciatic nerve stimulation led to an increase in Cx43 dephosphorylation in the spinal cord dorsal horn [51], suggesting that neural input can regulate gap junctional coupling between astrocytes. Cx43 dephosphorylation may counter phosphorylation-related inhibition of gap junction channel communication [52].

The involvement of astrocytic gap junction proteins in pain facilitation may be understood by their role in inter-astrocytic cellular communication, which is accomplished by direct exchange of small molecules. A particular relevant phenomenon is that the calcium surge in astrocytes can propagate through gap junctions [47]. This apparent calcium signaling between astrocytes, however, is mainly observed in

cultured cells and whether it is applicable to *in vivo* conditions is unclear. Recent two-photon microscopy studies suggest that the spread of calcium waves between astrocytes is limited, most likely only involving immediate neighboring astrocytes [53]. Other signaling molecules such as IP_3 may also diffuse through astrocytic gap junctions to spread excitation [1]. Nevertheless, there is a positive correlation between coordination of calcium activity in neighboring astrocytes and neuronal discharge [53], supporting a role of astrocytic gap junction proteins in neuronal hyperexcitability and enhanced astrocytic activity after injury.

2.5. Excitatory and inhibitory amino acids transporters

Astrocytic glutamate transporters (mainly GLT-1) uptake glutamate into astrocytes to help to maintain an appropriate level of extracellular glutamate concentration. Models of neuropathic pain have been associated with a decrease in GLT-1 expression ([3], Review). Presumably, a reduction in GLT-1 activity may lead to a build up of glutamate concentration in the synaptic cleft, leading to neuronal hyperexcitability and hyperalgesia. Interestingly, the often-used glial modulator/inhibitor propentofylline produces multiple effects on astrocytes. In primary astrocytic cultures that exhibit an activated phenotype, propentofylline suppresses lipopolysaccharide (LPS)-induced chemokine release but induces GLT-1 expression and glutamate uptake [54]. Propentofylline may also be able to restore glutamate transporter activity *in vivo*. In transgenic mice with double transgenic GLT-1-enhanced green fluorescent protein/GLAST-Discosoma Red promoter, propentofylline reversed the reduced promoter activation in the spinal dorsal horn after spinal nerve injury [55]. These cellular actions of propentofylline are consistent with its anti-allodynic effect [56].

Expression of astrocytic GLT-1 is regulated by neuronal activity. Transcriptional activation of GLT-1 depends on neuron-stimulated kappa B-motif binding phosphoprotein (KBBP) that binds the GLT-1 promoter [57]. Nervous tissue injury reduced astroglial KBBP expression and led to transcriptional dysfunction of astroglial transporter expression [57].

Astrocytes are also responsible for removing gamma-aminobutyric acid (GABA), an inhibitory amino acid transmitter, after its release from GABAergic presynaptic terminals. High affinity GABA transporters are localized in astrocytes. After facial inflammation, the GABA transporter GAT immunoreactivity increased in glial cells in the spinal trigeminal nucleus on the side of injury [58]. Thus, the GABA transporter changes in the opposite direction after injury compared to glutamate transporters. The increased expression of GAT may facilitate removal of GABA and lead to a reduced GABAergic inhibition. This effect is consistent with central hyperexcitability and hyperalgesia after tissue injury.

2.6. Astroglial glutamate—glutamine shuttle

After being taken up by astrocytes, glutamate is converted to glutamine. Glutamine is then transported back to presynaptic terminals where it is converted to glutamate to replenish the transmitter pool. While this astroglial glutamate—glutamine shuttle is critical in maintaining normal synaptic excitability, hyperactivity of the shuttle may contribute to increased pain. Inhibition of the astrocytic glutamine shuttle by application of methionine sulfoximine, an inhibitor of astroglial glutamine synthetase that catalyzes conversion of glutamate to glutamine, hyperexcitability of trigeminal nociceptive neurons is attenuated [59]. Consistently, inhibition of presynaptic uptake of glutamine by the neuronal system A transporter suppressed mustard-oil-induced sensitization of medullary dorsal horn nociceptive neurons [60]. These effects are likely a result of reduced supply of glutamate neurotransmitters due to inhibition of the astroglial glutamate—glutamine shuttle.

2.7. D-Serine

Astrocytes may release so called gliotransmitters to affect neuronal activity and it has now been recognized that neurotransmission is not the only form of signal transmission in the brain. Glutamate may be released from astrocytes through exocytosis, although the specific mechanisms are still unclear [1]. Glial-derived glutamate may have an impact on neuronal hyperexcitability through an effect on extrasynaptic NMDA receptors [28]. However, recent work suggests that calcium-dependent glutamate release from astrocytes may not directly affect neuronal activity *in situ* [61]. D-Serine, a co-agonist of the NMDA receptor, may also be released from astrocytes [62]. Intrathecal administration of a D-serine inhibitor, D-amino acid oxygenase, attenuated mechanical hypersensitivity induced by titanic sciatic stimulation [63]. The role of astroglia-derived excitatory amino acids to central sensitization and hyperalgesia should be further studied.

2.8. Calcium-binding proteins in astrocytes

S100β is a calcium-binding peptide produced mainly by astrocytes. Astrocytic localization of S100β is limited to the soma, which is distinctly different from widespread distribution of GFAP in branches and cell body of astrocytes. About 30—40% of the S100β immunoreactivity overlaps with GFAP [15,64] and can be used as an alternative functional marker of astroglial activity. S100β mRNA and protein levels are upregulated in the spinal cord after hind paw inflammation, L5 spinal nerve ligation and spinal cord injury [10,64,65]. The increased GFAP immunoreactivity in rostral ventromedial medulla after injury of the infraorbital nerve is accompanied by an increase in S100β immunoreactivity [17]. The pain hypersensitivity induced by nerve injury is attenuated in S100β knockout mice and enhanced in S100β expressing animals [64]. These findings support a role of enhanced astroglial activity in the development of persistent pain.

2.9. Endothelin B receptors

Astrocytes express endothelin B (ET_B) receptors [66,67], a subtype of G protein-linked receptors mediating effects of endothelins. After compression injury of the spinal cord, the ET_B receptor immunoreactivity is increased in both protoplasmic and fibrous astrocytes, which coincides with upregulation of GFAP, or astroglial activation [66]. Interestingly, the upregulation of ET_B receptors appears selective in

astrocytes since the ET_B receptor immunoreactivity is reduced in ependymal cells and does not change in vascular endothelial cells. The increased ET_B receptor function after injury is consistent with a role of endothelins in astroglial responses to injury. Application of endothelin-1 induces hypertrophy of astrocytes in the normal optic nerve and the ET receptor antagonist blocks hypertrophy of astrocytes in the crushed optic nerve [67]. Endothelin-1 also affects S100β expression in astrocytes [68]. Since endothelins can be secreted from vascular endothelial cells, they are likely hematogenous messengers that bind to ET_B receptors on astrocytes and contribute to astroglial activation after injury.

2.10. Blood brain barrier

Astrocytes have contact with brain blood vessels via their end-feet and processes and play an important role in maintaining normal function of the blood brain barrier (BBB), although in most vertebrates, the principal anatomic site of the BBB is located within the endothelium ([69], review). The BBB permeability is apparently increased after nerve injury and peripheral inflammation, which is correlated with astroglial activation and pain hypersensitivity [70,71]. The water channel protein aquaporin-4 is distributed in the end-foot membrane of astrocytes to mediate water movements between brain cellular compartments including interstitial and vascular spaces and contributes to the maintenance of BBB properties. The aquaporin-4 mRNA is upregulated in the spinal cord after spinal cord injury and central neuropathic pain [65]. It is likely that astroglial activity plays a role in perturbed BBB function that may precipitate brain responses to injury and pain hypersensitivity.

Recognition of the contribution of astroglia and their interactions with neurons to pain hypersensitivity prompts new treatment for chronic pain conditions. In addition to direct inhibition of neuronal activity, a variety of non-neuronal components of the brain may now also be targeted for pain relief. It is hoped that more selective agents that block astroglial activation after injury will soon be developed and proven effective in treating chronic pain.

Conflict of interest statement

The author declares no conflict of interest.

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