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Review

Long-term pain, neuroinflammation and glial activation

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ABSTRACT

Nociceptive and neuropathic pain signals are known to result from noxious stimuli, which are converted into electrical impulses within tissue nociceptors. There is a complex equilibrium of pain-signalling and pain-relieving pathways connecting PNS and CNS. Drugs against long-term pain are today directed against increased neuronal excitability, mostly with less success.

An injury often starts with acute physiological pain, which becomes inflammatory, nociceptive, or neuropathic, and may be transferred into long-term pain. Recently a low-grade inflammation was identified in the spinal cord and along the pain pathways to thalamus and the parietal cortex. This neuroinflammation is due to activation of glial cells, especially microglia, with production of cytokines and other inflammatory mediators within the CNS. Additionally, substances released to the blood from the injured region influence the blood–brain barrier, and give rise to an increased permeability of the tight junctions of the capillary endothelial cells, leading to passage of blood cells into the CNS. These cells are transformed into reactive microglia. If the inflammation turns into a pathological state the astrocytes will be activated. They are coupled into networks and respond to substances released by the capillary endothelial cells, to cytokines released from microglia, and to neurotransmitters and peptides released from neurons. As the astrocytes occupy a strategic position between the vasculature and synapses, they monitor the neuronal activity and transmitter release. Increased release of glutamate and ATP leads to disturbances in Ca^{2+} signalling, increased production of cytokines and free radicals, attenuation of the astrocyte glutamate transport capacity, and conformational changes in the astrocytic cytoskeleton, the actin filaments, which can lead to formation and rebuilding of new synapses. New neuronal contacts are established for maintaining and spreading pain sensation with the astrocytic networks as bridges. Thereby the glial cells can maintain the pain sensation even after the original injury has healed, and convert the pain into long-term by altering neuronal excitability. It can even be experienced from other parts of the body. As astrocytes are intimate co-players with neurons in the CNS, more knowledge on astrocyte responses to inflammatory activators may give new insight in our understanding of mechanisms of low-grade inflammation underlying long-term pain states and pain spreading. Novel treatment strategies would be to restore glial cell function and thereby attenuate the neuroinflammation.

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Contents

1. Introduction

In recent years, research has focused on the roles of glial cells in the generation and maintenance of long-term pain, both nociceptive and neuropathic. Clarifying the mechanisms behind these phenomena may help us to identify new methods of treating long-term pain [1]. Nociceptive and neuropathic pain signals are known to result from noxious stimuli, which are converted into electrical impulses within tissue nociceptors [2]. There is a complex equilibrium of pain-signalling and pain-relieving pathways connecting the peripheral and central nervous systems (PNS, CNS) [3]. Drugs against long-term pain are today directed against increased neuronal excitability; unfortunately, in many cases with little success.

Recently, a low-grade inflammation was identified in the dorsal horns of spinal cord and along the pain pathways to the thalamus and further to the parietal cortex induced by peripheral nerve injuries [4]. This neuroinflammation is due to the activation of glial cells, especially microglia, and with the production of cytokines and other inflammatory mediators within the CNS [2,5,6]. At the injured region substances are released to the blood which influence the blood–brain barrier (BBB) and give rise to an increased permeability of the tight junctions of the capillary endothelial cells, leading to passage of blood cells into the CNS [7,8]. These cells are transformed into reactive microglia. If the inflammation becomes pathological, the astrocytes will be activated. As these cells are coupled into networks and respond both to substances released by the capillary endothelial cells and to cytokines and adenosine trisphosphate (ATP) their Ca^{2+} signalling system will be influenced [9].

Because the astrocytes occupy a strategic position between the vasculature and the synapses, they monitor neuronal activity and transmitter release. Increased releases of glutamate and ATP lead to disturbances in Ca^{2+} signalling, attenuation of the astrocyte glutamate transport capacity, and increased production of cytokines and free radicals, which can lead to the formation and rebuilding of new synapses [10]. New neuronal contacts are established for maintaining and spreading neurosignals, with the pain sensation-mediating astrocytic networks acting as bridges [11]. This may help to explain both how pain sensations can be long-term and how they can be experienced in other parts of the body.

2. Neuroinflammation

Activation of sensory fibres by “pain” activators cause large amounts of substance P, calcitonin gene-related peptide (CGRP), glutamate, serotonin, and ATP to be released from neurons in the spinal cord; the postsynaptic neurons are depolarized by the resulting influx of Ca^{2+} and the activation of the nitric oxide (NO) system. The postsynaptic neurons may be over-activated, and thus cause resting microglia to react by releasing cytokines, leading to cascading reactions in the astrocytic networks [3,12]. Substances released to the blood from the injured region influence the BBB [9]. The immune competent cells from the blood pass the partially opened BBB and are converted into microglia, which produce even more cytokines [13]. Inflammation induces changes in BBB permeability and the capillary endothelial cells release substances which influence the astrocytic endfeet, which are a part of the BBB [14]. The astrocytes, which form the astrocytic networks, are influenced by both cytokines and other inflammatory substances within the CNS and by factors released from activated capillary endothelial cells (see Fig. 1).

3. Blood–brain barrier alterations

Peripheral stimuli, such as inflammatory processes associated with pain, alter BBB tight-junction protein expression and BBB permeability [7]. To maintain the homeostasis of the brain’s microenvironment, the microvessels and astrocytes, the gliovascular unit, together with neurons, the neurovascular unit, acts as an integrated unit. Brain capillary endothelial cells are not fenestrated and are interconnected by tight junctions (Fig. 2). Endothelial cells produce vasodilators such as NO, chemotactic factors, and other signalling substances for the induction and maintenance of the gliovascular unit [14]. The astrocytic endfeet processes almost completely surround microvessels. They are enriched in ion channels, receptors, and the water-channel protein aquaporin-4. The gap junction protein connexin 43 (Cx43) is expressed extensively and astrocytic Ca^{2+} signalling may play a role in astrocytic functions related to the BBB. Cerebral bloodflow is modified during inflammation or other disturbances, which leads to opening of the BBB, changes in the tight-junction proteins occludin and claudin, and increases in glucose transporter expression. An increased concentration of intracellular Ca^{2+} [15], which is propagated from cell to cell in the form of oscillations or waves through the networks [16], results in release of glial products [17] and may be a link in astrocytic functions related to the BBB. Neuronal processes that originate from local interneurons or from intrinsic neurons contain many neurotransmitters and are closely associated with microvessels. In response to inflammation or pathological pain, pain transmitting neurons become sensitized and over-respond in releasing neurotransmitters [3].

Today there is considerable evidence that astrocyte interaction with cerebral endothelial cells helps determine the BBB function, in both morphology and protein expression [18]. Few studies have addressed glial–endothelial interactions or possible glial regulation of the BBB during inflammatory processes with pain states,

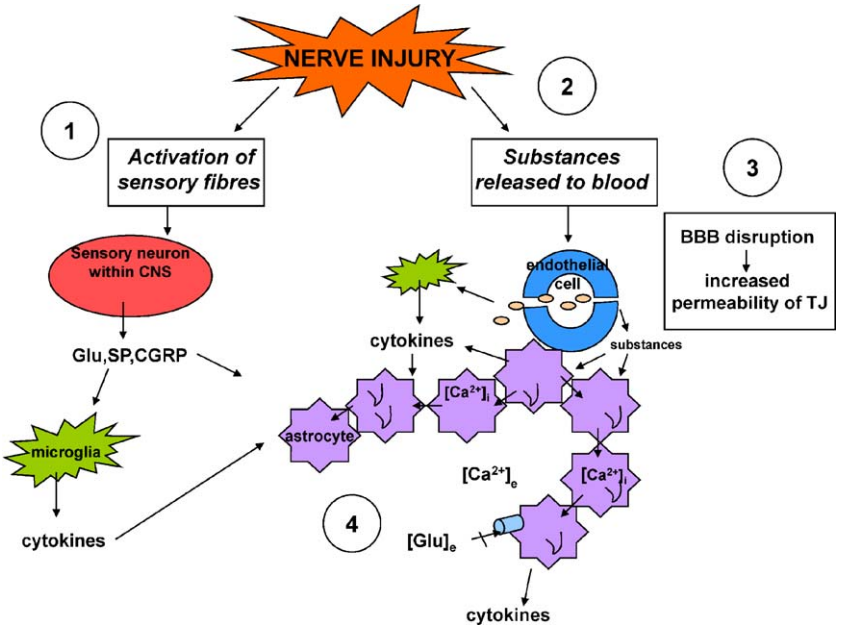

Fig. 1. Physiological parameters of importance in inflammation; after a nerve injury a low-grade neuroinflammation may originate in the spinal cord and could travel all the way along the activated sensory pathways within the CNS. Glutamate (Glu) substance P (SP), and calcitonin gene-related peptide (CGRP) are released from sensory neurons in the spinal cord and have an influence on the microglia to release cytokines. Both neurotransmitters and cytokines affect the astrocytes (1). Transmitters and peptides are also released in the periphery, transported in the blood and have an influence on the blood–brain barrier (BBB), giving rise to an increased permeability of tight junctions (TJ) of the endothelial cells of this barrier. This leads to an opening of the BBB, with passage of blood cells into the CNS. These cells are converted into activated microglia, which can release cytokines affecting the astrocyte networks (2). Due to the BBB disruption, substances from the capillary endothelial cells are also released and affect the astrocyte networks (3). The astrocyte networks are thereby affected by neurotransmitters released from neurons, cytokines released from reactive microglia within the nervous system, and microglia, which have been converted from blood cells; they are also affected by substances released from endothelial cells. This cascade of reactions influences the astrocyte networks in forms of changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) as well as in extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$), which can lead to Ca^{2+}-oscillations/-waves within the networks. The extracellular glutamate concentration ($[Glu]_e$) will also be affected (4).

as discussed in *in vivo* animal experiments [19]. Recent findings have, however, discussed the involvement of neuronal input from pain activity that leads to alterations in BBB permeability, where nociceptive signalling is in part responsible [20].

4. Astrocytic networks

Various soluble factors, some of them released upon neuronal activity, induce Ca^{2+}-mediated signalling in astrocytes and propagate Ca^{2+} fluctuations between astrocytes via gap junctions, in which Cx43 and ATP and its purinergic receptors are basic elements [16,21]. Via intercellular communication, astrocytes act as a syncytium for modulating neuronal and vascular function. The degree of coupling between astrocytes may influence neuronal activity; increased neuronal activity can upregulate gap junction-mediated communication and Cx43 expression and increase the propagation of Ca^{2+} waves in astrocytes [22]. Raised Cx43 levels have been observed in reactive astrocytes [23]. The Ca^{2+} signalling system can allow bloodflow to vary in a manner that is coupled to the intensity of neuronal activity [17].

Regulation of Ca^{2+} dynamics by transmitters and other soluble factors is thus a possible mechanism by which the astrocyte network detects changes in the CNS microenvironment and regulates brain activities, such as inflammatory processes, of importance for long-term pain sensation, regeneration, and memory formation under various physiological and pathophysiological conditions (Table 1).

5. Cytoskeleton

An intact actin cytoskeleton is required for the propagation of astrocytic Ca^{2+} waves ([21]; Lundborg C, Westerlund A, Björklund U, Biber B, Hansson E. Naloxone and IL-1ra restore GDNF-evoked Ca^{2+} transients in an *in vitro* model for inflammatory reactive astrocytes (unpublished)). Disruption of the cytoskeleton abolishes Ca^{2+} oscillations by changing the balance between the Ca^{2+}-regulating processes [24]. An intact cytoskeleton is dominated by F-actin filaments organised in stress fibres, which are rearranged with increased ring formations and more dispersed F-actin filaments when astrocytes are influenced by inflammatory stimuli. This leads to disturbances in intracellular Ca^{2+} signalling in the astrocytic networks ([13]; Lundborg et al.). A good relationship between astrocytic gap junctions and an intact cytoskeleton is required for the propagation of Ca^{2+} signalling in the network.

6. Glutamate release

Neuronal excitability releases glutamate into the neural synapse and metabotropic glutamate receptors on astrocytes, mGluR5, react with intracellular Ca^{2+} elevations, which result in the release of glutamate [25]. Astrocytes can modulate the synaptic transmission [17], and they are the predominant players in clearing the extracellular space. Astrocyte uptake of excessive extracellular glu-

Table 1
Elements influencing astrocytes during inflammation.

At inflammation, the astrocytic networks are affected by:
1. Microglia within the CNS
2. Substances released to the blood that
   - Have an influence on the BBB
   - Give rise to an increased permeability of tight junctions
   - Lead to passage of blood cells into the CNS, and these cells are then converted to microglia
3. Transmitters and peptides released from presynaptic neurons
4. Endothelial cells in the microvasculature that release substances, which influence the astrocytic processes covering the microvessels

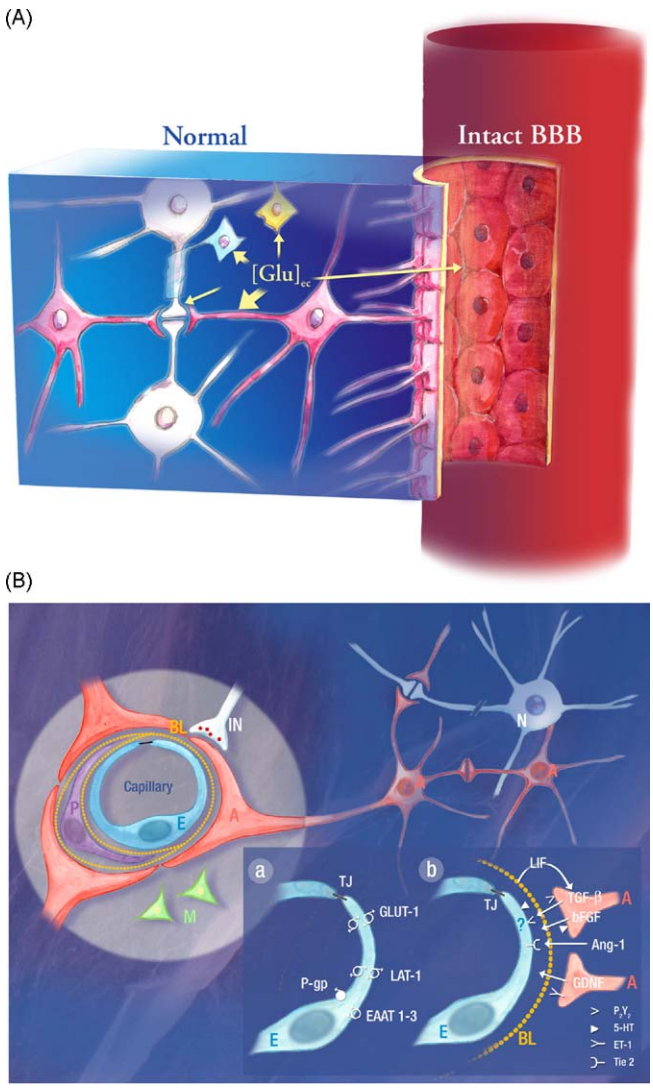

Fig. 2. Cellular constituents of the blood–brain barrier (BBB). (A) A brain capillary showing the main cell types with the potential to signal to each other. The astrocytic endfeet are apposed to the basal lamina. In the brain side are found neurons, astrocytes and microglia. Agents such as glutamate (Glu) can influence the cellular function. (B) The capillary is surrounded by an endothelial cell (E), surrounded by basal lamina (BL), a pericyte (P) and astrocytic endfeet (A). Astrocytes provide the cellular link to the neurons (N). The figure also shows microglial cells (M) and interneurons (IN). (a) A brain endothelial cell, linked with a tight junction (TJ), expressing a number of transporters: GLUT1, glucose transporter; LAT-1, L-system for large neutral amino acids; EAAT1-3, excitatory amino acid transporters; P-gp, P-gycoprotein. (b) An endothelial cell and astrocytic endfeet to demonstrate the bidirectional induction to establish and maintain the BBB. Some receptors and peptides of importance are shown. 5-HT, 5-hydroxytryptamine; P_2Y_2, purinergic receptor; TIE2, endothelium-specific receptor tyrosine kinase 2; ET1, endothelin 1; LIF, leukaemia inhibitory factor; TGFβ, transforming growth factor-β; bFGF, basic fibroblast growth factor; Ang-1, angiopoetin 1; GDNF, glial cell line-derived neurotrophic factor.

tamate by membrane-bound glutamate transporters, GLAST and GLT-1, plays a critical role in preventing glutamate excitotoxicity [26] (Fig. 1). Ionotropic glutamate receptors are also excited, while the NMDA receptor subunit NR2B is expressed in the astrocytes in states of increased neuronal excitability [27], and in inflammatory reactive astrocytes (Lundborg et al.). IL-1β is increased at neuroinflammation and induces phosphorylation of the NMDA receptor [23]. The astrocytes can, at least to some part, control the release of gliotransmitters and Ca^{2+} signalling, thereby also influencing to some degree the microvasculature.

7. Inflammatory receptors

Novel treatment strategies for long-term pain would be to restore glial cell dysfunction and thereby attenuate the neuroinflammation. From recent results, we have seen that Ca^{2+} signalling in astrocytes is changed when astrocytes are treated with inflammatory stimuli and they have become reactive. mRNAs for several TLRs are expressed in both microglia and astrocytes [28], and they have a role in CNS inflammation [29]. LPS induces stronger expression of TLR4 ([28]; Lundborg et al.), and LPS stimulated astrocytes lead to upregulation in the production of pro-inflammatory cytokines [30]. The balance between anti- and pro-inflammatory cytokines will be changed [31].

Signals that carry peripheral injury or inflammation signals can trigger glial activation in the CNS. Neurotransmitters or modulators as glutamate, brain-derived neurotrophic factor (BDNF), substance P, ATP, opioids, chemokines, and different peptides are released from neurons and reach receptors on microglia and astrocytes to produce glial activation [32]. Glutamate triggers stimulation of mGluR5 on the astrocytic plasma membrane leading to increased intracellular Ca^{2+} concentration and intracellular Ca^{2+} oscillations. The NMDA receptor subtype 2B (NR2B) is expressed on astrocytes after ischemia and anoxia [27] and on astrocytes co-cultured with endothelial cells (Lundborg et al.). Blocking receptors as TLR4, IL-1R, NR2B, which will be activated during inflammatory processes, would restore Ca^{2+} signalling in astrocytes and may lead to an increase or balance of endogenous substances, which are of importance in normal cell physiology.

8. Glial cell line-derived neurotrophic factor (GDNF) and cytokines

Glial cell line-derived neurotrophic factor (GDNF) has neuroprotective functions that imply that it modulates NMDA receptor activity. Pre-treatment with GDNF reduces NMDA-induced Ca^{2+} influx in cortical neurons [33]. Ifenprodil, an antagonist of the NMDA receptor's NR2B subunit, also restores GDNF-evoked Ca^{2+} transients, attenuating brain oedema and BBB breakdown [34]. Inflammatory reactive cultured astrocytes, which have been in contact with substances released from microvessel endothelial cells, show over-activated NMDA receptors and impaired Ca^{2+} signalling from GDNF-stimulated astrocytes.

Another mechanism to control inflammatory glial activation is through cytokine signalling. Astrocytes respond to ongoing synaptic activity by mobilizing intracellular Ca^{2+}, leading to the release of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6. Activated microglia are also a source of these pro-inflammatory substances. The inflammatory reactive glial cells can be neuroprotective by releasing anti-inflammatory cytokines, such as IL-10 and IL-4 [2]. Achieving a balance between overproduction of pro-inflammatory cytokines and decreased production of anti-inflammatory cytokines may be one way to regulate an inflammatory response [35].

9. Inflammatory experimental models

Inflammatory models of neuropathic pain have been developed in animals [36], and some exists for cellular systems. Experimental neuroinflammation is commonly induced with exposure to lipopolysaccharide (LPS), an endotoxin and very potent inflammatory activator [37,38], which stimulates the Toll-like receptor 4 (TLR4) of astrocytes and microglia [29] and is involved in the initiation of neuropathic pain [36].

We have developed an *in vitro* model consisting of astrocytes co-cultured with brain endothelial cells, which is pro-inflammatory

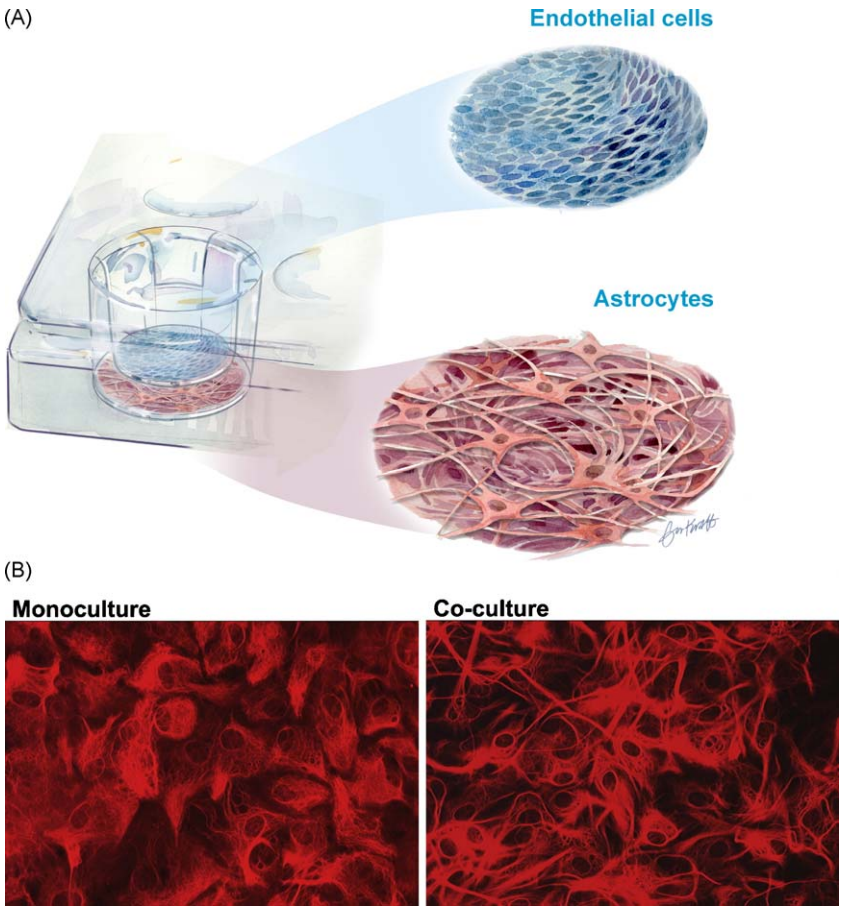

Fig. 3. An *in vitro* model of astrocytes co-cultured with adult rat brain microvascular primary cultures. (A) Astroglial cultures at 6 days *in vitro* were co-cultured with newly prepared microvascular cultures. The endothelial cells were grown in inserts above the astrocytic cultures. The cells from the two different cultures were never in contact. The cells were grown together for 9–11 days. (B) Morphological changes of the co-cultured astrocytes compared with astrocytes in monoculture. In the co-cultured astrocytes long, slender processes are visible. The astrocytes are stained for glial fibrillary acidic protein (GFAP) [12].

compared with astrocytes in monoculture [12,13]. These astrocytes exhibited a morphologically differentiated appearance with long processes (Fig. 3). The biological rationale for co-culturing astrocytes with endothelial cells is to study astrocytes that are influenced by substances released from the capillary endothelial cells of the BBB. It has been demonstrated that co-culturing of astrocytes and endothelial cells induces the modulation and differentiation of astrocytes [39]. Receptor agonists for amino acids, monoamines, purines, peptides, opioids, and nicotine induced higher Ca^{2+} amplitudes and/or more Ca^{2+} transients compared with astrocytes in monoculture [12,13]. This could imply that endothelial factors influence both astrocytes and the exchange of substances in the gliovascular system. To achieve an inflammatory reactive model of astrocytes, the co-cultured astrocytes are incubated with LPS and also often with IL-1β. Astrocytes produce high levels of IL-1β [23] under conditions of damage, stress and disease [40]. Activation with LPS is accompanied by increased levels of IL-1β [13]. LPS and IL-1β trigger signalling pathways and inflammatory mediators through TLR4 and/or IL-1 receptor (IL-1R1) activation in astrocytes. Activation of TLR4 has been demonstrated to be essential for the development of neuropathic pain states [41] and antagonism of TLR4 by naloxone can attenuate the pain sensation [42].

Research may approach the BBB as a dynamic system, where the periphery and the CNS are integrated and the transport of substances between blood and brain is important [9].

10. Pain memory and pain spreading

Because astrocytes occupy a strategic position between the vasculature and the synapses, they partly monitor neuronal activity and transmitter release. Increased release of glutamate and ATP leads to disturbances in Ca^{2+} signalling, attenuation of the astrocyte glutamate transport capacity, and increased production of cytokines and free radicals. Astrocyte activation may act as a bridge on the one hand between blood and the brain, and on the other between neuronal tracts, allowing the formation and establishment of new neuronal contacts. Regulation of Ca^{2+} waves in astrocytes may be important for the formation of synapses [10]. Thus, we could have an explanation at the cellular level of how pain sensation can be long-term and also of how pain may be experienced in other uninjured parts of the body [11,43].

11. Importance

Long-term pain is common in the population. The costs are considerable, whether measured in terms of long-lasting illness and the subsequent fall in economic production or measured in terms of human suffering. Unfortunately, current pharmacological treatment, directed toward neuronal over-excitability, is less than successful in dealing with long-term pain. Recent research has shown that neuronal over-excitability may be driven by low-grade neuroinflammation. The BBB seems to be important in two ways:

it is influenced by substances released to the blood, which affect the capillary endothelial cells and the astrocytic endfeet; and blood cells that are transported through the BBB are then converted to microglia. Both astrocytes and microglia contribute to neuroinflammation and are generators of neuronal activation. Interestingly, these new insights into the probable origin of long-term pain, focusing on microglial activation, may also explain the phenomenon of pain spreading over astroglial bridges both within the ipsilateral side and to the contralateral side of the body. A novel pharmacological treatment strategy would thus be directed towards the activated astrocytes and microglial cells as the source of the neuroinflammation.

Conflicts of interest

The author has no conflicts of interest to report.

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