Role of Endocannabinoids in Neuron-Glial Crosstalk

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Abstract: Evidence shows bidirectional crosstalk between neurons and glia, suggesting that glia play an active role in synaptic plasticity leading to chronic pain. Importantly, gliosis has been implicated in the development and maintenance of hyperalgesia or allodynia following chronic inflammation or nerve injury. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG), or the lipoamino acid *N*-arachydonoyldopamine (NADA), are fatty acid derivative neurotransmitters, named endocannabinoids (eCBs). These perform several biological actions, via the activation of cannabinoid type 1 and 2 (CB1/CB2) receptors belonging to the G-protein-coupled receptor family. The eCBs are produced *on demand* by neurons or glial cells and it has been suggested that they might be involved in the crosstalk between astrocytes, microglia, oligodendrocytes and neurons. In chronic pain, the modified glial or neural activity also seems to be associated with changes in eCB levels in pain processing areas either in the spinal cord or the brain. The activation of the eCB system in microglia or astrocytes could be crucial in modulating axonal growth and synaptogenesis at the base of neural phenotypic changes. Furthermore, changes in eCBs levels have been suggested to affect the destiny of cells: death or survival may depend on a specific pain condition. Thus, although eCBs are emerging as neurotransmitters responsible for the regulation of glia-neuron crosstalk in chronic pain, the precise mechanisms leading to eCB production, the origin and the time-course of eCB release, the eCB release switch from one cell type to the other and their movement or catabolism across the glial or neural cell membrane nevertheless still remain unknown. These issues together with alternative eCB targets will be addressed in the current review.

Key words: Glia-neuron crosstalk, endocannabinoids, anandamide, 2-arachidonoylglycerol, chronic pain.

INTRODUCTION

Neuropathic pain is a debilitating condition which has a serious impact on the quality of life. It is a devastating and difficult-to-manage consequence of injury to the peripheral or central nervous systems (PNS or CNS) that **results in** the enhanced transmission of pain messages [1, 2]. Consequently, noxious stimuli are perceived as more painful (hyperalgesia), whereas normal, harmless stimuli elicit pain (allodynia). Therefore, neuropathic pain constitutes a real dysfunction of the nervous system that is characterized by as yet poorly-defined neurophysiological changes. Very few pharmacological strategies exist to treat neuropathic pain, which is very often refractory even to morphine and its derivatives, possibly because it is associated with plastic rearrangements of nociceptive pathways at both spinal and supraspinal level.

Among the pharmacological strategies that have been suggested for neuropathic pain management, the activation of cannabinoid receptors, either directly by natural or synthetic agonists, or indirectly by selective inhibitors of the inactivation of endogenous cannabinoids *receptor ligands* (endocannabinoids), is widely supported by recent preclinical studies in animal models [3-5]. Evidence shows that cannabinoid receptor agonists can be effective in several animal models of neuropathic pain [3, 6-10]. However, in spite of evidence that neuropathic pain leads to increased endocannabinoid levels at spinal and supraspinal sites [11], the role of endocannabinoids within the brain "pain matrix" need to be further clarified.

Until recently, pain had been thought to arise primarily from the dysfunction of neurons. Recent evidence, however, suggests that neuroimmune changes might contribute to pain following injury to the nervous system as well. Glial cells involved in mediating inflammatory processes are resident within the spinal cord and include both astroglia and microglia**,** the latter of which has been directly implicated in the initiation of peripheral injury-induced pain [12]. Moreover, microglia have been shown to express cannabinoid receptors [13-16], and to produce and inactivate endocannabinoids [15, 17, 18].

THE ENDOCANNABINOID SYSTEM AND PATHOLOGICAL PAIN

Among the several neurotransmitters that have been suggested to be involved in neuropathic pain, endocannabinoids have been strongly highlighted and heavily investigated over the last decade. The endocannabinoid system consists of the G-protein coupled cannabinoid (CB) receptors, CB1 and CB2, the endogenous ligand anandamide (arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG), and their synthetic and metabolic machinery [19]. The CB1 receptor is localized preferentially in several brain areas such as periaqueductal grey (PAG), cerebellum, hippocampus, cortex). Despite the general opinion which had up until re-

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cently believed CB2 receptors to be exclusively expressed in peripheral tissues and inflammatory cells [20-23], there is now convincing evidence to suggest that they are also expressed in the CNS [24]. Indeed, an increased expression of CB2 receptors has been shown in microglial cells, as well as in astrocytes, in neuropathic pain conditions [25, 26]. It has been underlined that CB2 receptors play a crucial role in the regulation of central immune responses during neuropathic pain [27, 28]. Besides the well characterized CB1 and CB2 receptors, several reports have shown the existence of other receptors that represent potential targets to be included in the endocannabinoid system (eCBSS). Thus, the orphan G protein-coupled receptor 55 (also called GPR55) could represent a third CB receptor [29-32]. However, studies performed so far remain inconsistent and further efforts are required to link GPR55 to the eCBSS. Another receptor identified that could belong to the eCBSS is the abnormal-cannabidiol (abn-CBD) receptor (also known as anandamide receptor) [33]. This receptor, also found in microglial cells, is activated by abn-CBD while cannabidiol (CBD) antagonizes it [33-35, 15]. The endocannabinoid system includes lipid transmitters serving as endogenous ligands for the cannabinoid receptors, and the enzymes for their biosynthesis and inactivation. The first endocannabinoid to be discovered was the *N*-arachidonoyl-ethanolamine (AEA), also called anandamide from the Sanskrit "internal bliss" [36]. This finding was followed by the observation that an already known endogenous metabolite, 2-arachidonoyl-glycerol (2-AG), also exhibits high affinity with CB1 and CB2 receptors [36, 37]. Anandamide, which is synthesized by a phospholipase D (NAPE-PLD) specific for N-acylphosphatidylethanolamine binds both CB1 and CB2 receptors and behaves mostly as a partial agonist [38-40]. Biological inactivation of anandamide occurs through a rapid uptake followed by intracellular hydrolysis mediated by the enzyme fatty acid amide hydrolase (FAAH) [41]. 2-AG, which is more abundant than anandamide in the brain [42], binds to CB1 and CB2 with a lower affinity than anandamide but behaves like a full agonist since it shows higher intrinsic activity [37, 43, 42, 15]. 2-AG is synthesized by the enzyme diacylglycerol lipase (DGL) in a Ca^{2+}-dependent pathway [44, 45]. Other alternative mechanisms of 2-AG synthesis have also been proposed [46]. Biological inactivation occurs through uptake followed by hydrolysis mediated by the enzyme monoacylglycerol lipase (MGL). Other enzymes have been indicated for 2-AG metabolism, including cyclooxygenases (COXs), lipooxygenases (LOXs) and FAAH [47, 48].

The first evidence of the analgesic properties of cannabis was observed in 1899 by Ernest Dixon [49]. In the last few decades, scientists have focused their attention on the endocannabinoid system in the treatment of chronic and neuropathic pain. Indeed an increasing amount of evidence shows that the cannabinoid receptor system is involved in the pathogenesis of various pain states. CB1 receptor mediated analgesia is associated with adverse psychoactive effects such as sedation, dependence, cognitive impairment and psychotic-like behaviour [50, 51], due to the overall activation of this ubiquitous receptor. However, CB2 receptor stimulation is also effective in alleviating inflammatory [52-54, 10] and neuropathic pain [25, 55-59]. Intriguingly, the CB2-mediated antinociceptive effects seem devoid of any central action (which are CB1 receptor-mediated), and are likely mediated by several mechanisms, and peripheral sites of action of CB2 agonists in both inflammatory and neuropathic pain models have been recognized [52, 53, 55, 56]. Among these, a peripheral release of endogenous opioids from keratinocytes has also been shown [55, 56]. Conversely, other evidence shows that the release of endogenous opioids is not involved in CB2 agonist-mediated analgesia in a model of neuropathic pain, [57]. On the other hand, more recent studies have shown the involvement of CB2 receptors within the central nervous system in the analgesic effect of CB2 agonists in neuropathic pain models [60, 61]. These data are supported by reports demonstrating an up-regulation of CB2 receptor mRNA and/or protein in the spinal cord [25, 61, 28] in neuropathic pain conditions. In these studies, an up-regulation of CB2 receptors has been found on the activated microglia in the ipsilateral dorsal horn of spinal cord. Another strategy for obtaining analgesia while avoiding the central psychotic effect of cannabinoids is to target the EC turnover such as that of AEA [36], 2-AG [37, 42] and NADA [62, 63], whose increase inhibits nociception by acting on CB1 and CB2 receptors [4]. This approach would have the benefit of cannabinoid receptor activation at sites of high EC turnover without interfering with all CB1 receptors which can cause side effects. Indeed, endocannabinoids are synthesized on demand [64] in certain patho-physiological conditions including inflammatory and neuropathic pain [5, 65]. In neuropathic pain, changes in the levels of ECs and related compounds have been reported in several regions of ascending and descending pain pathways. Jhaveri and coworkers (2007) have shown that the levels of AEA and another endovanilloid compound *N*-oleoylethanolamine (OEA), but not of 2-AG, were higher in the ipsilateral hindpaw of neuropathic rats, compared to the ipsilateral hindpaw of sham rats. Similarly, levels of AEA and 2AG, but not palmitoyltanolamide PEA, were increased in the spinal cord, PAG and rostral ventromedial medulla (RVM) in neuropathic rats [11]. In the dorsal raphe nucleus, which has reciprocal projections to the PAG, levels of AEA, but not 2-AG, increased in the chronic constriction injury model of neuropathic pain [8]. Collectively, these studies provided evidence that the endocannabinoid system may be a suitable target for neuropathic pain treatment.

NEURON-MICROGLIA AND PATHOLOGICAL PAIN: A PUZZLING NETWORK

Spinal cord microglial cells are the earlier-activated cell type in response to peripheral inflammation or nerve injury. In physiological conditions, these cells show a "resting" phenotype which is believed to be responsible for the continuous immune surveillance of their milieu [66, 67]. After peripheral nerve injury "resting" microglia quickly change their phenotype and function, a process identified as "microglial activation" (Fig. **1**). This activation process consists of distinct cellular functions aimed at repairing damaged neural cells and eliminating debris from the damaged area [68]. Damaged cells release chemo-attractant molecules that both increase the motility (i.e. chemokinesis) and stimulate the migration (i.e. chemotaxis) of microglia, the combination of which recruits the microglia much closer to the damaged cells [69]. Microglial activation in the spinal cord can be promoted by sciatic nerve ligation [70], spinal nerve ligation [71], sciatic nerve inflammation [72], traumatic nerve tran-

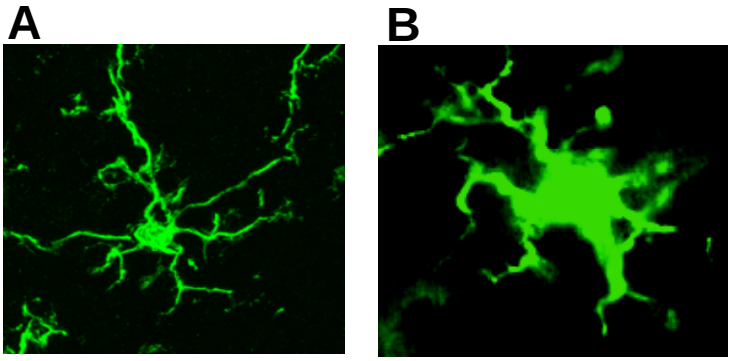

Fig. (1). A confocal image of resting microglia labeled with Iba-1 (**A**). Single activated microglial cell modified from Luongo et al., 2009 (**B**).

section [28] and autoimmune diseases such as autoimmune encephalomyelitis and neuritis **(EAE, EAN)** [73, 74]. Once microglia become activated, they can exert both pro-inflammatory or anti-inflammatory, neuroprotective functions depending on the combination of the stimulation of several receptors and the expression of specific genes [68, 18]. Thus, the activation of microglia following a peripheral injury can be considered as an adaptation to tissue stress and malfunction [75] that contributes to the development and subsequent maintenance of chronic pain [61, 76]. Spinal microglia respond quickly to injury, up-regulating cell surface proteins and increasing synthesis and the release of inflammatory mediators, including cytokines and proteases that can sensitize neurons, thereby establishing positive feedback which helps to facilitate nociceptive signalling [77]. Accordingly, the inhibition of microglial targets can reduce hypersensitivity in neuropathic pain states.

The signals responsible for neuronal-microglial and/or astrocytic communication are being extensively investigated as they may represent new targets for chronic pain management. The first candidates are substances released by activated nociceptive primary afferent fibers, such as glutamate and substance P (SP), which are able to activate microglia [78, 79]. Glutamate activates microglia by stimulating NMDA channels [79], although other mechanisms involving metabotropic glutamate receptors (mGluRs) cannot be ruled out since they appear expressed on microglial cells [80-82]. SP acts mostly by activating microglial neurokinin-1 (NK1) receptors. Many mechanisms have been proposed for neuron-microglia crosstalk. Among these, the fractalkine (FKN, CX3CL1), a member of CX3C class of chemokines and its receptor CX3CR1 have been extensively investigated [83]. FKN is constitutively expressed by spinal cord and sensory neurons in the dorsal root ganglia (DRGs) [84-86], while CX3CR1 is exclusively expressed by microglial cells [85] and, after peripheral nerve injury it is widely up-regulated in microglia [85]. FKN produces nociceptive behaviour by activating CX3CR1 on microglia and p38 mitogen-activated protein kinase (MAPK)-mediated pathways [86, 87]. A mechanism for a cleavage of neuronal membrane-bound FKN has been elegantly demonstrated [86]. Briefly, neuronal FKN is cleaved by cathepsin S (CatS), a proteolitic enzyme, which is expressed and released by activated microglia [86]. Same authors have demonstrated that the liberation of fractalkines in the dorsal horn requires CatS to be released from microglia [88]. However, the CX3CL1/CX3CR1 pathway, which represents a pro-nociceptive non adaptive process seems to perform a neuroprotective role in neurodegenerative diseases [89]. Another candidate for neuronal-microglial crosstalk is ATP, which is produced by neurons as well as by glial cells. ATP exerts its effect by activating the purinergic ionotropic P2X4 and P2X7, as well as the metabotropic P2Y6 and P2Y12 receptors on microglia [90]. P2X4 activation seems to be involved in the development of neuropathic pain by inducing the release of brain derived neurotrophic factor (BDNF) [91, 92]. P2X4 receptor activation occurs earlier than that of P2X7 channel due to the greater affinity of ATP to bind to P2X4 receptor. Indeed, P2X7 is involved in the maintenance of microglial activation. The P2X7 receptor appears to be a functionally unique ionotropic receptor among the P2X receptor family since its activation is able to stimulate the release of the pro-inflammatory cytokine interleukin-1β (IL-1β), as well as a variety of other proinflammatory cytokines. Recent studies have revealed that P2Y12R is also crucial in neuropathic pain induction and maintenance. It has been found that the expression of P2Y12R mRNA and protein are markedly enhanced in the spinal cord ipsilaterally to spinal nerve injury [93] or to sciatic nerve partial ligation [94]. The cellular location of this receptor in the spinal cord was heavily restricted to microglia and recently has been purported to participate in the motility of microglial cell bodies and processes [95]. It is therefore possible that P2Y12R activity in microglia affects their ability to extend the branched processes toward neighboring neurons of the pain matrix, which, in turn, may interfere with microglia-neuron communications. A metabotropic adenosine receptor A_{2A} has also been shown to be involved in the microglial process retraction occurring during microglial activation [96]. The up-regulation of Gs protein-coupled adenosine A_{2A} receptor on activated microglia seems to occur concomitantly to down regulation of Gi-protein coupled P2Y12 receptor [96]. Furthermore, the adenosine A1 and A3 receptors have been found to be expressed in microglial cells [97, 98]. Another chemokine implicated in neuron-glia communication is the chemokine (C-C motif) ligand 2 (CCL2, MCP-1), which is *de novo* expressed by sensory neurons as early as the day after peripheral injury [99]. Once released, CCL2 activates microglia via interaction with CCR2 receptors, and, accordingly, mice lacking CCR2 receptors display a reduction in nerve injury-induced tactile allodynia [100]. The action of the monocyte chemoattractant protein 1 (MCP-1) at the spinal level has also been demonstrated by the intrathecal

administration of an MCP-1 neutralizing antibody, which proved able to inhibit neuropathic pain symptoms [99].

ROLE OF ENDOCANNABINOIDS IN NEURON-GLIA INTERACTIONS

In the complex *scenario* of neuropathic pain, which involves microglial cell-induced synaptic plasticity, the endocannabinoid system may represent an interesting target for modulating microglia-neuron communication. Indeed, endocannabinoids could be released on demand by neurons as well as by astrocytes and microglial cells [101, 15]. It should be emphasized that microglial cells produce 20-fold higher amounts of endocannabinoids (expressed in picomoles per nanogram of protein) compared to neurons and astrocytes [15]. In particular, the role of 2-AG in microglial modulation has been investigated [15, 17, 28]. The increased level of 2-AG after noxious stimulation has been studied at spinal and supraspinal level in different models of chronic pain [11, 65]. Moreover, analgesic and neuroprotective effects of 2-AG have been reported in several models of brain injury [102, 5]. A recent study has highlighted the neuroprotective effect of 2-AG on the excitotoxic lesion on dentate gyrus granule cells via abnormal-cannabidiol-sensitive receptors on microglial cells [103]. It has been demonstrated that the production of 2-AG in microglial cells is a Ca^{2+}-dependent phenomenon that involves P2X7 receptor activation [104]. Importantly, the EAE model of multiple sclerosis does not lead to an increase in 2-AG [105], although the microglial CB2 receptors are functionally active [106]. This lack of 2-AG increase in the EAE has been explained by the abnormal release of interferon-gamma (IFNγ) due to a T cell "invasion" of the CNS which, in turn, impairs the functionality of P2X7 [105]. Intriguingly, 2-AG seems to play a role in the regulation of microglia proliferation and migration. Indeed, it has been shown that microglial cells are able to synthesize this endocannabinoid which increases their proliferation through the activation of CB2 receptors *in vitro* [17]. Moreover, 2-AG also stimulates the migration of microglial cells towards dying cells. Once again the CB2 receptors, as well as the abn-CBD receptors on microglia seem to be involved in these mechanisms [15]. Furthermore the role of the endocannabinoids on microglia has been investigated. Navarrete and coworkers have recently shown that NADA is a potent inhibitor of prostaglandin E2 (PGE2) synthesis and of free radical formation in primary lipopolysaccharide (LPS) stimulated microglial cells [107]. Consistently, anandamide is capable of enhancing the anti-inflammatory cytokine interleukin 10 (IL-10) and regulating other cytokine production such as IL-12 and IL-23 in activated microglia by targeting CB(2) receptors [108, 109].

PATHOLOGICAL OR PROTECTIVE: A HAMLETICAL QUESTION!

The pathological and protective roles of glia have recently been reviewed by Milligan and Watkins [76]. A recent study also defined the markers of two distinct phenotypes of microglia, pro-inflammatory (M1) and anti-inflammatory (M2) [110]. This evidence provides new tools for investigating the contribution of the immune response in neuropathic pain.

Recent reports, focused on understanding the mechanisms involved in neurodegenerative diseases, have suggested that microglia and astrocytes can also be neuroprotective by releasing several factors that have been demonstrated to have sensitizing actions in neuropathic pain conditions [111]. On this subject, we have already mentioned that the same pronociceptive pathway CX3CL1/CX3CR1 is neuroprotective in neurodegenerative diseases [89]. Both microglia and astrocytes can recognize 'danger signals' and can remove the pathogen or cellular debris through phagocytosis, which also represents the ongoing activity of these cells in healthy conditions. Indeed, astrocytes and microglia express pattern-recognition receptors that recognize surface proteins, thus priming the phagocytosis of 'altered' cells. This process seems to be associated with a down-regulation of pro-inflammatory cytokines to reduce damage to neighboring healthy tissue [112]. On this subject, a study with transgenic TNFα-knockout mice demonstrated that microglial TNFα, a known pro-inflammatory cytokine, was critical in the resolution of an inflammatory response and excitotoxic cell death. In addition, while the lack of TNFα reduced microglial activation within 6 hours, an exaggerated microglial activation was measured 4 days later [113]. These data have highlighted not only that a "dark side" of the glia exists, but that in some cases, preventing glial activation in the CNS is undesirable as it could amplify or create pathological pain. Thus, stimulating the anti-inflammatory features of glial activation represents a more powerful approach to controlling pain signaling than exclusively preventing glial activation. With this purpose cannabinoids are being investigated as therapeutic targets for inflammatory neurodegenerative diseases and neuropathic pain for their immunomodulatory properties [27, 28]. , Discrepancies among different studies have however highlighted that further efforts are necessary to be able to include the cannabinoid system in the neuron-glia crosstalk during the neuropathic pain establishment. Indeed, evidence that endocannabinoids stimulate the proliferation and migration of microglia may appear in contrast with their anti-inflammatory properties. Indeed, it is worth noting that another anti-inflammatory endogenous lipid mediator, palmitoylethanolamide (PEA), which exerts its action mostly by activating the peroxisome proliferator-activated receptor alpha (PPARα) [52], is also able to potentiate microglial cell motility [114]. Several studies in recent years have focused on CB2 receptor activation in different neuropathic pain models [27, 28, 115, 19]. Such studies have highlighted the role of CB2 receptor in the modulation of immune response involved in the development of neuropathic pain. CB2 receptor activation exerts antiallodynic and antihyperalgesic effects by modulating microglial responses. In one of our recent studies [28], we demonstrated that CB2 receptor stimulation induced an analgesic effect associated with a reduction in the pro-inflammatory (IFN-γ and IL-1β) and an enhancement of anti-inflammatory (IL-10) mediators within the spinal cord. In the study, we also demonstrated that the number of microglial profiles was not reduced by a CB2 receptor agonist. Moreover, the two 2-AG biosynthetic enzymes, DAGLα and MGL, were enhanced by the CB2 agonist treatment assuming that an enhanced turnover of 2-AG occurs. One can thus speculate that a critical role of endocannabinoids (i.e. 2-AG) on microglia is to shift their phenotype from pro- to anti-inflammatory, and to recruit more anti-inflammatory microglia to the site of injury (Fig. **2**) [18].

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