Review

The mechanisms of microgliosis and pain following peripheral nerve injury

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A B S T R A C T

Microglia are the resident macrophages in the central nervous system (CNS). Any insult to the CNS homeostasis will induce a rapid change in microglia morphology, gene expression profile and functional behaviour. These responses of microglia have been collectively known as 'microgliosis'. Interestingly, damage to the nervous system outside the CNS, such as axotomy of a peripheral nerve, can lead to microgliosis in the spinal cord. There is a variation in the degree of microgliosis depending on the model of nerve injury employed for instance this response is more marked following traumatic nerve injury than in models of chemotherapy induced neuropathy. Following peripheral nerve injury nociceptive inputs from sensory neurons appear to be critical in triggering the development of spinal microgliosis. A number of signalling pathways including growth factors such as Neuregulin-1, matrix metalloproteases such as MMP-9 and multiple chemokines enable direct communication between injured primary afferents and microglia. In addition, we describe a group of mediators which although not demonstrably shown to be released from neurons are known to modulate microglial phenotype. There is a great functional diversity of the microglial response to peripheral nerve injury which includes: Cellular migration, proliferation, cytokine release, phagocytosis, antigen presentation and recruitment of T cells. It should also be noted that in certain contexts microglia may have a role in the resolution of neuro-inflammation. Although there is still no direct evidence demonstrating that spinal microglia have a role in neuropathic pain in humans, these patients present a pro-inflammatory cytokine profile and it is a reasonable hypothesis that these cells may contribute to this inflammatory response. Modulating microglial functions offers a novel therapeutic opportunity following nerve injury which ideally would involve reducing the pro-inflammatory nature of these cells whilst retaining their potential beneficial functions.

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Microglia are the resident macrophages and the only immune cells in the central nervous system (CNS). Unlike neurons and macroglia, microglia have a mesodermal origin as they are derived from myeloid precursor cells which enter the developing CNS during embryogenesis (Ginhoux et al., 2010; Ransohoff and Perry, 2009). Under the influence of the CNS microenvironment microglia develop fine and long processes and protrusions which are continually surveying their microenvironment (Nimmerjahn et al., 2005). Local cell division at a very low level maintains the number of resident microglia in the brain of rodents (Lawson et al., 1992). When the tightly regulated CNS homeostasis is disturbed by any kind of insult, microglia rapidly change their morphology, gene expression profile and functional behaviour. The local density of microglia increases at the site of injury through migration of these cells from other sites at the CNS and through local proliferation. These responses of microglia have been collectively known as ‘microgliosis’ (reviewed in Hanisch and Kettenmann, 2007). Interestingly, not only damage to the CNS parenchyma elicits this microglial response; also damage to the nervous system outside the CNS, such as axotomy of a peripheral nerve, can lead to microgliosis both within the dorsal horn of the spinal cord where the injured sensory afferents terminate and within the ventral horn around the cell bodies of injured motor neurons (Eriksson et al., 1993).

Microgliosis following peripheral nerve injury has been shown to contribute to the development of neuropathic pain. Blocking the microglial response with minocycline, a second-generation tetracycline can prevent nerve injury induced hypersensitivity in rats (Ledeboer et al., 2005; Lin et al., 2007; Raghavendra et al., 2003). Furthermore, it has been shown that microglia that have been activated in vitro using ATP can elicit pain related responses in naïve rats when they have been injected intrathecally (Coul et al., 2005; Tsuda et al., 2003).

In this review we will focus on the different aspects of the microglial response to peripheral nerve injury and how this may contribute to the development of neuropathic pain.

Microglial cells respond to neuronal damage

A number of different models of peripheral nerve injury have been developed in order to understand the pathogenesis of neuropathic pain. These involve the administration of a range of injurious stimuli including traumatic, metabolic, toxic and infectious. In all of these models pronounced abnormalities of sensory function are observed which mirror those observed in many neuropathic pain patients. Microgliosis is seen in virtually all models of traumatic nerve injury but may not be universal to all forms of nerve injury as it is less apparent in certain models of chemotherapy induced neuropathy.

Microgliosis in traumatic neuropathic pain models

The response of microglia to traumatic nerve injury has been widely studied in a number of different models. These involve axon transection usually to the sciatic nerve, one of its contributory spinal nerves or its distal branches (Fig. 1a). The activating transcription factor 3 (ATF3) is induced in virtually all DRG neurons and motoneurons that are axotomized and therefore it is regarded as a neuronal marker of nerve injury and is widely expressed in these models (Tsujino et al., 2000). In the spinal nerve ligation (SNL) model a tight ligature is applied to two spinal nerves, L5 and L6, close to the dorsal root ganglion. A modified version is now widely used in were only the L5 spinal nerve is ligated and cut (Kim and Chung, 1992). In this model microglia accumulate at the ipsilateral side to the injury in the L5 segment of the spinal cord in both the dorsal and the ventral horn (Fig. 1b, Calvo et al., 2010; Tsuda et al., 2003). Microglial morphology changes from a “resting state” in which the cell body is small and bear long and thin processes into a “effector state” in which the cells present an ameboid shape (Figs. 1c–d). This morphological change begins as early as 24 h following nerve injury, microgliosis is well established by 3 days post injury and has been observed to be maintained up to 84 days following spinal nerve ligation (Coyle, 1998). In the chronic constriction injury (CCI) model a catgut chromic suture is loosely ligated around the entire sciatic nerve producing damage to some of the sciatic axons by inducing swelling and stranulation (Bennett and Xie, 1988). The partial sciatic nerve injury (PNI) consists in placing a tight ligature through about half the proximal sciatic nerve, usually to the sciatic nerve, one of its contributory spinal nerves or it’s distal branches (Daulhac et al., 2006, 2011; Talbot et al., 2010; Tsuda et al., 2008; Wodarski et al., 2009). In this model of rapid onset diabetes, rats develop a painful neuropathy within 1–2 weeks of STZ administration (Courteix et al., 1993). It has been suggested that STZ can cause direct neuronal damage to DRG cells independently of the damage induced by hyperglycaemia (Pabbi et al., 2008). Moreover it has been shown that STZ causes selective injury to myelin and axons in the hippocampus in association with activation of microglia (Shoham et al., 2003). It is therefore difficult to determine to what extent microgliosis may be due to the neurotoxic effects of STZ per se and to what extent due to hyperglycaemia/insulin deficiency (Romanovsky et al., 2006). In contrast to the STZ model inbred rat strains which develop slower onset diabetes
typically display delayed manifestations of either painful and anaesthetic neuropathy, or anaesthetic neuropathy only (Gabra et al., 2005; Obrosova, 2009). Only one study has investigated the involvement of microglia in such models in mice (db/db mice) and they observed no microgliosis (Liao et al., 2011). It will be interesting to further investigate whether microgliosis occurs in such models in rats.

Bone cancer pain models

In bone cancer pain models not only there is bony destruction but also increased ATF3 expression in DRG cells (Dore-Savard et al., 2010; Ghilardi et al., 2005; Peters et al., 2005) which provides strong evidence for neuronal injury. The presence of microgliosis in models of bone cancer pain has been controversial as some groups have described it (Lan et al., 2010; Zhang et al., 2005) but others have not (Hald et al., 2009). This variation may well be due to differences in the exact model used and in particular in the degree of neuronal damage.

Models of HIV-induced painful neuropathy

HIV infection can cause a painful distal symmetrical polyneuropathy. The HIV coat protein, glycoprotein 120 (gp120) via binding to chemokine receptors in neurons can induce neuropathic pain like behaviours and has been shown to induce changes in the sensory neuron phenotype including expression of ATF3, caspase-3, nerve growth factor (NGF), and galanin (Wallace et al., 2007b). Gp120 administration has therefore been used as a model of HIV-induced painful neuropathy. This glycoprotein can cause microgliosis when administered intrathecally (Milligan et al., 2001) and perineurally (Herzberg and Sagen, 2001; Wallace et al., 2007a).

Chemotherapy induced painful peripheral neuropathy

The painful peripheral neuropathies produced by chemotherapeutic drugs are characterised by distal axonal injury, with partial degeneration of the intraepidermal nerve fibres (IENF) that form the terminal receptor arbours of the sensory axons that innervate the epidermis. Zheng and colleagues recently demonstrated that low doses of the cancer chemotherapeutic agents paclitaxel, oxaliplatin, and vincristine, and the anti-retroviral agent, 2′,3′-dideoxycytidine (ddC) caused IENF retraction, neuropathic pain like behaviours but did not caused microglial hypertrophy or microglial accumulation in the spinal cord (Zheng et al., 2011). Sweitzer et al. described a very mild microgliosis following vincristine treatment (Sweitzer et al., 2006). Similarly, Wallace and colleagues found no ATF-3 activation on DRG cells of ddC treated animals and only a very mild microglial response in the spinal cord (Wallace et al., 2007a). High doses of paclitaxel have been shown to induce microgliosis in the spinal cord (Peters et al., 2007) however in the study of Zheng and colleagues they used low doses of this chemotherapeutic which did not induce ATF-3 in DRG neurons (Flatters and Bennett, 2006) nor did it induce a microglial response. Grouped together these findings suggest that cytotoxic agents given at a dose which produces a ‘dying back’ neuropathy of sensory neurons but without a severe reaction at the level of the cell body do not produce a major microgliosis. There may, therefore be a threshold of stress or injury that a neuron needs to achieve before microglia can be recruited into a pro-inflammatory response.

Microgliosis following peripheral nerve injury

Microgliosis is dependent on primary afferent derived injury signals

Following peripheral nerve injury the accumulation of reactive microglia into the site of injury is orchestrated through different
signals which appear in the spinal cord micro-environment and which are released from neurons, astrocytes and other immune cells. Many heterogeneous signals appear to contribute to microgliosis however evidence suggests that these are initially triggered by the injury response of damaged primary afferents. Noxious inputs from injured or neighbouring uninjured sensory neurons projecting to the dorsal horn appear to be critical in the development of spinal microgliosis. Electrical activity in different classes of sensory neurons is important in transmitting this signal. For instance, stimulation of uninjured C-fibres is capable of inducing central sensitization and can elicit microgliosis in the areas where these fibres terminate. This C-fibre stimulation and consequent microgliosis are sufficient to evoke significant and long-lasting changes in mechanical sensitivity (Hathway et al., 2009). However, the development of microgliosis following nerve injury is not exclusively dependent on C-fibre activity. It has been shown that blockade of the peripheral input of TRPV1-positive fibres (Aδ and C-fibres) is not enough to prevent nerve injury-induced spinal microgliosis. However, bupivacaine which induces a complete sensory blockade resulted in a significant inhibition of the microglial response. Therefore, the peripheral input from large myelinated fibres also seems to have an important role in the development of microgliosis (Suter et al., 2009).

Using stereological techniques Beggs and Salter demonstrated that following peripheral nerve injury the accumulation of microglia mainly occurred at the central territory of peripherally axotomized afferents although it could also extend into areas of uninjured terminal afferents. However, this microgliosis was always restricted to the central terminals of the intact branch of the sciatic nerve. The central terminals of adjacent but independent nerves (such as the saphenous) remained unaffected (Beggs and Salter, 2007). An injury signal is therefore transmitted to the spinal cord by damaged primary afferents and neighbouring uninjured afferents which share the same nerve trunk. Moreover, it can be argued that microgliosis occurs as a consequence of the altered functional state of sensory neurons and not just as a result of degeneration of nerve terminals. This has been shown by comparing the effects of dorsal rhizotomy (i.e. transection of the dorsal root proximal to the DRG) to the effects of peripheral nerve axotomy. After dorsal rhizotomy microgliosis occurs but it is predominantly concentrated in the dorsal funiculus (and not the dorsal horn) at lumbar and thoracic levels, most likely as part of the immune response to Wallerian degeneration of the lesioned axons (Colburn et al., 1999). In contrast, after peripheral nerve axotomy accumulation of reactive microglia is seen at the areas where injured and neighbouring uninjured afferents fibres terminate (Beggs and Salter, 2007) within the dorsal horn. It is very possible that ongoing activity and subsequent neurotransmitter release from primary afferent terminals or transported factors from the cell body of these sensory neurons may be providing microglia with activation signals.

A number of signalling pathways which enable direct communication between injured primary afferents and microglia have been recently described. Some of these pathways involve complex two-way relationships between injured neurons and microglia and in many cases injury modulates the activity dependent release of secreted proteins or promotes the cleavage of a transmembrane precursor.

Neuregulin-1

Neuregulin-1 (NRG1) is one of a family of growth factors (NRG1-4) which has a key role in neural development (reviewed in Mei and Xiong, 2008). NRG1 binds to the tyrosine kinase receptors erbB3 and erbB4. These receptors subsequently heterodimerize with erbB2 which lacks a ligand-binding domain but is a key co-receptor in mediating signal transduction. Microglia, express the NRG1 receptors erbB2, 3, and 4. Primary afferents express a number of NRG1 isoforms and in culture systems NRG1 can be released in response to both neuronal activity and neurotrophin treatment. We have found that in vivo following spinal nerve ligation (SNL) the soluble form of NRG1 is released from primary afferents in the dorsal horn and activates the erbB2 receptors in microglia. Blockade of erbB2 or sequestration of endogenous NRG1 following SNL reduced the accumulation of microglia in the dorsal horn as well as mechanical pain-related hypersensitivity and cold allodynia (Calvo et al., 2010).

Metalloproteinase-9

Another neuronaly derived molecule involved in the development of microgliosis is the metalloproteinase-9 (MMP-9). After peripheral nerve injury, MMP-9 shows a rapid and transient upregulation in injured DRG primary sensory neurons. Spinal MMP-9 injection in naive animals resulted in increased expression of CD11b (or complement receptor 3a, which is expressed in microglia) and in p38MAPK phosphorylation in microglia together with increased mechanical hypersensitivity. On the other hand, MMP-9 inhibition or knock-down decreased nerve induced microgliosis and mechanical allodynia (Kawasaki et al., 2008a). It has been hypothesised that MMP-9 cleaves IL-1β which can then act in microglia. Indeed, spinal MMP-9 treatment in naive animals increases the cleaved forms of IL-1β in the DRG.

CCL2

The chemokine monocyte chemoattractant protein 1 (MCP-1, also known as CCL2) binds with high affinity to the chemokine receptor CCR2, a G-protein-coupled receptor, and stimulates monocyte migration (Bacon et al., 2002). CCL2 is constitutively expressed in small and medium diameter size neurons in DRG under naive conditions and following peripheral nerve injury it is up-regulated and transported to the central terminals of primary afferents in the dorsal horn. Microglial cells express CCR2, suggesting a potential communication pathway between injured primary afferents and dorsal horn microglia (Abbodie et al., 2003; Thacker et al., 2009; Zhang et al., 2007). Exogenous spinal administration of CCL2 induces spinal microgliosis and mechanical allodynia, both effects which are dependent on CCR2 expression. Similarly, following peripheral nerve injury, CCR2 knockout mice fail to develop tactile allodynia (Zhang et al., 2007). However, CCL2 also directly affects DRG neurons demonstrating that the role of this chemokine might be diverse and not only restricted to the activation of microglia (reviewed in White et al., 2007).

CCL-21

The chemokine CCL-21 has been implicated as a neuron-glial signalling molecule in CNS injury. CCL21 is exclusively expressed in damaged neurons (Biber et al., 2001; de Jong et al., 2005). A recent study revealed that this was also the case following peripheral nerve injury. Using immunochemistry the authors showed that CCL21 is rapidly expressed in injured unmyelinated sensory neurons and transported to their central terminals in the dorsal horn. They also found that this chemokine is crucial for the P2X4 up-regulation seen in microglia following peripheral axotomy. Mice deficient for CCL21 failed to up-regulate microglial P2X4 receptor expression and did not develop tactile allodynia (Biber et al., 2011). CCL21 can signal through two receptors: CCR7 and CXCR3 and microglia express both of them. However, neither receptor knock out lead to a reduction in P2X4 in microglia. Therefore, it is not yet clear if CCL-21 acts directly on microglia via another yet unidentified receptor or if it is an indirect effect mediated through other cell types. Either way, it seems that CCL-21 exerts an important role in the response of microglia to peripheral axotomy.

Fractalkine

The chemokine fractalkine is expressed on neurons in the dorsal horn of the spinal cord and in the DRG and the CX3CR1 receptor for fractalkine is predominantly expressed by microglia in the spinal cord (Verge et al., 2004). The chemokine domain of fractalkine can be shed from neurons via the protease cathepsin S which is secreted
by microglia in a P2X7 dependent way (Clark et al., 2010b). In neuropathic dorsal horn slices ex vivo, noxious electrical stimulation of dorsal roots evokes soluble fractalkine release and high levels of soluble fractalkine are detected in the CSF of neuropathic animals (Clark et al., 2009). Indeed, following SNL there is a reduction of the membrane-bound fractalkine in the DRG, suggesting a cleavage and release of this chemokine after axotomy (Zhuang et al., 2007). Intrathecal administration of fractalkine results in thermal and mechanical hypersensitivity that can be prevented by the absence of the CX3CR1 receptor or by pre-treatment with neutralising antibodies against CX3CR1 or fractalkine itself (Clark et al., 2007; Milligan et al., 2005a; Zhuang et al., 2007). Therefore, it is suggested that following peripheral nerve injury a positive loop is formed in which microglia release cathepsin S that cleaves neuronal fractalkine that further acts in microglial CX3CR1 receptor.

**Signals in spinal cord micro-environment regulating microglial responses**

There are a number of mediators that are known to modulate microglial phenotype after peripheral nerve injury, but that have not been demonstrably shown to be released from neurons. These molecules are present in the microenvironment surrounding microglia and many are known to be products of tissue injury including ATP, misfolded or aggregated proteins, nuclear factors not normally present in the extracellular matrix, reactive oxygen species, complement components and NO. Although their origins may not be entirely clear at the moment, these molecules have been well-characterised in their capacity to stimulate microglia.

**Activation through pattern of recognition receptors (PRRs)**

As part of their response to microorganisms microglia recognise some conserved structural motifs present in pathogens through PRRs. Similarly, PRRs can also recognise non-infectious endogenous factors that are normally sequestered intracellularly and therefore hidden from the immune system unless cellular injury occurs. It has been shown that following peripheral nerve injury recognition of these endogenous danger signals occurs through PRRs that include Toll-like receptors (TLRs) and Nod-like receptors (NLRs).

Microglia express the TLRs 1 to 9 (Jack et al., 2005; Olson and Miller, 2004). The TLRs 2, 3 and 4 have been implicated in turning microglial into a pro-inflammatory phenotype after peripheral nerve injury (Kim et al., 2007; Obata et al., 2008; Tanga et al., 2005). In TLR4-null mice and rats treated with anti-sense oligonucleotides to produce TLR4 knockdown, L5 nerve transection elicits an attenuated behavioural hypersensitivity and a decreased expression of spinal microglial markers and pro-inflammatory cytokines when compared with their respective genetically unaltered controls (Tanga et al., 2005). Antisense knockdown of TLR3 suppressed nerve injury-induced tactile allodynia, attenuated microgliosis, decreased p38MAPK phosphorylation in the spinal cord and decreased the release of IL-1β, IL-6 and TNFα (Obata et al., 2008). Kim et al. showed that TLR2 knockout mice demonstrate impaired induction of microgliosis and astrocytosis, decreased up-regulation of pro-inflammatory cytokines and reduced mechanical and thermal hypersensitivity after nerve injury (Kim et al., 2007).

Unlike membrane-bound TLRs, which sense danger signals on the cell surface or in endosomes, NLRs recognise stress-related molecules in the cytoplasm (Chakraborty et al., 2010). Activation of NLRs leads to the assembly of a complex of interacting proteins called the ‘inflammasome’ (Martinon et al., 2002). When the inflammasome is assembled it associates with the adaptor protein ASC (apoptosis-associated speck-like protein), which in turn recruits caspase-1 (Franchi et al., 2009). Caspase-1 cleaves pro-IL-1β and pro IL-18 for their functional activation into mature forms which can then be release from immune cells. Following peripheral nerve injury it has been observed that the inflammasome components are up-regulated in microglia (DLHB and Amanda Ellis unpublished observation).

**Nucleotides**

Accumulating evidence indicates that nucleotides play an important role in neuropathic pain through microglia P2 purinergic receptors. P2 purinoceptors are divided into two families, ionotropic receptors (P2X) and metabotropic receptors (P2Y). P2X receptors contain intrinsic pores that open through ATP binding. P2Y receptors are activated by nucleotides and couple to intracellular second-messenger systems through G-proteins. Nucleotides are thought to be released or leaked from non-excitable cells as well as neurons in pathological conditions and to stimulate different functions in microglia through purinergic receptors (Inoue, 2008).

ATP-mediated activation of ionotropic purinergic receptors has been shown to promote the release of sensitising agents such as TNFα, IL-1β and IL-6 from microglia (Farber and Kettenmann, 2006; Hide et al., 2000; Shigemoto-Mogami et al., 2001). Tsuda et al. showed that P2X4 receptors are up-regulated and specifically expressed by microglia in the spinal nerve transaction model (Tsuda et al., 2003). Activation of these receptors leads to release of BDNF, which causes a shift in neuronal excitability (Coull et al., 2005). Intrathecal P2X4 antisense treatment significantly reduced nerve injury-induced tactile allodynia (Tsuda et al., 2003). The P2X7 receptor has also been involved in stimulating the secretion of cytokines from microglial cells (Clark et al., 2010a). The metabotropic receptor P2Y12 have been shown to modulate microglial motility and neuropathic pain while P2Y6 has been involved in the regulation of phagocytosis in these cells (see more details in the next sections; Tsuda et al., 2010).

**Interferon γ**

IFN-γ is produced predominantly by natural killer cells as part of the innate immune response, and by effector T cells once antigen-specific immunity develops (Schoenborn and Wilson, 2007). IFN-γ efficiently up-regulates the MHC-II antigen presenting pathway and thus promotes CD4+ T cell recruitment (Gottfried-Blackmore et al., 2009). IFN-γ is significantly up-regulated after peripheral nerve injury and IFN-γR-null mutant mice exhibit significantly diminished mechanical allodynia following axotomy (Costigan et al., 2009). IFN-γ in the spinal cord is primarily expressed by T-cells (Schoenborn and Wilson, 2007; Schroder et al., 2004). Tsuda et al. demonstrated that IFN-γ stimulates microglia through the IFNRF and that interfering with this signalling inhibits neuropathic mechanical hypersensitivity (Tsuda et al., 2009).

**The functional diversity of the microglial response**

Increased knowledge of microglial function over the last decade has changed our view of these cells. We no longer describe them in the binary terms of being in either a ‘resting state’ in which they have low processes, small soma and express low levels of activation-associated molecules or an ‘activated state’ in which they increase in numbers, retract their processes and display an amoeboid appearance. It is now clear that ‘resting microglia’ are not really in a quiescent or dormant state but they are scanning and monitoring their microenvironment. Similarly, the ‘activated state’ is composed of many different functions that can be present simultaneously or in different combinations in a cell. Therefore, the response of microglia to nervous system damage is better represented as a transition from one state to another in which microglia change their functional repertoire rather an ‘all or nothing’ event.

Several different functions have been described in microglia (Fig. 2). In this review we will focus on functions that have been suggested to be present in the response to peripheral nerve injury.

**Migration**

When the CNS homeostasis is disturbed microglial cells accumulate at the site of injury through proliferation and migration. Microglial migration has been suggested to be a key step in microglialosis.
and consequent development of nerve induced hypersensitivity. For instance, it has been shown in vitro that one of the mechanisms of action of minocycline, an antibiotic which potently prevents microgliosis and injury induced hypersensitivity, may be through inhibition of migration (Nutile-McMenemy et al., 2007). Migration of these cells is thought to be orchestrated by various chemotactic agents that are released at the site of injury and through dilution in the neighbouring areas they form gradients that attract microglia. Some of the chemotactic molecules that contribute in microglial migration and accumulation at the site of injury following peripheral nerve injury have been described such as: purines, NRG1, complement components, and chemokines.

ATP and ADP are thought to leak from cells of the dorsal spinal cord following peripheral axotomy. These nucleotides can diffuse rapidly and it has been shown that they can induce membrane ruffling and chemotaxis of microglia. This effect was found to be dependent on the constitutively expressed G protein-coupled receptor P2Y12 (Honda et al., 2001). Using P2Y12 receptor knockout mice, Haynes et al. found that lack of P2Y12 receptors in vivo significantly delayed the ability of microglia to extend branches and migrate towards local tissue damage (Haynes et al., 2006). Following peripheral nerve injury the P2Y12 receptor is up-regulated in microglial cells and its pharmacological or genetic inhibition results in a decreased nerve injury-induced mechanical allodynia (Tozaki-Saitoh et al., 2008). Ohsawa et al. have demonstrated in vitro that P2X4 receptors also mediate chemotaxis as pharmacological antagonism or gene knockdown suppressed microglial chemotaxis through a mechanism independent of P2Y12 (Ohsawa et al., 2007).

Migrating microglia extend their processes towards the chemotactrant. However, it is well known that effector microglia retract their processes to convert into an amoeboid shape. Orr et al. have shown with in vitro studies that once microglia arrive to the site of injury they switch into an amoeboid morphology and the chemotactic response to ATP is reversed; purines no longer attract cells and can even repel them. Degradation of extracellular ATP by ectonucleotidases led to activation of the adenosine A2A receptor in microglia which was up-regulated at the same time that P2Y12 was down-regulated. Activation of adenosine A2A receptor led to process retraction and adoption of an amoeboid morphology by microglia (Orr et al., 2009). This probably allows microglia to stop migrating and thus stay at the level of the spinal cord where injured terminal afferents terminate. Indeed, Loram et al. showed that a single injection of an adenosine receptor 2A agonist two weeks after nerve injury (CCI) could reverse neuropathic pain like behaviour. This was accompanied by a reduction in microglia and astrocytes in the spinal cord (Loram et al., 2009). It will be interesting to investigate how this pathway is involved in microgliosis following peripheral nerve injury.

NRG1 has been recently implicated in microglial migration. Using a Boyden chamber and a ‘chequerboard analysis’ we have found that NRG1 could increase microglial chemotaxis in a dose dependant manner. This effect was dependent on signalling via the erbB2 receptor and the PI3K/Akt pathway (Fig. 3; Calvo et al., 2011). In vivo experiments have shown that peripheral nerve injury results in the release of NRG1 within the dorsal horn of the spinal cord and in activation of erbB receptors on microglia which leads to accumulation of these cells at the site of injury (Calvo et al., 2010). Furthermore, following intrathecal injection of NRG1 we observed an accumulation of microglia within the dorsal horn which was accompanied by mechanical and cold hypersensitivity (Calvo et al., 2010; Lacroix-Fralish et al., 2008).

Chemokines, like the name suggests, are chemotactic cytokines. As such, the chemokines monocyte chemoattractant protein 1 (MCP-1, also known as CCL2) and fractalkine have been implicated in microglial migration to the site of injury after peripheral nerve injury. CCL2 is known to be a potent monocyte chemoattractant (Cross and Woodroffe, 1999) and has been shown to be involved in monocyte recruitment in CNS diseases where the BBB is altered (Lu et al., 1998). Using irradiation and transplantation of GFP" bone marrow progenitor cells, Zhang et al. showed that after peripheral nerve injury, in addition to proliferation of resident microglia, bone marrow derived monocytes infiltrate the spinal cord, proliferate, and differentiate into microglia. This infiltration could be prevented by inhibiting CCL2 with a neutralising antibody. In addition, they utilised selectiveCCR2 knock-out in resident microglia or bone marrow derived monocytes and found that, although total CCR2 knock-out mice do not develop microgliosis or mechanical pain-related hypersensitivity, CCR2 expression in either resident microglia or peripheral monocytes is sufficient for the development of nerve injury-induced mechanical hypersensitivity (Zhang et al., 2007). However, it should be noted that experiments using irradiation and bone marrow transplantation are controversial as there are concerns about their results being confounded by the increase in BBB permeability induced by irradiation and by the artificial presence of bone marrow pluripotent cells that can cross the BBB in the circulation (Ajami et al., 2007; Mildner et al., 2007; Soulet and Rivest, 2008).

Fractalkine may also play a role in microglial migration following peripheral nerve injury. In vitro studies using a Boyden chamber have shown that fractalkine triggers the directed migration of microglia. Fractalkine mediates immediate increases in intracellular calcium mobilisation and induces actin rearrangement and shape change in microglia (Maciejewski-Lenoir et al., 1999). However, fractalkine as CCL2 is also chemoattractant to peripheral immune cells such as macrophages and leukocytes (Imai et al., 1997) and as such it may have a role in recruiting inflammatory cells in the periphery, as well as recruiting microglia in the CNS.

Finally, it is known that the complement component C5a and its receptor are up-regulated in spinal microglia following nerve injury (Griffin et al., 2007). C5a has been previously shown to be a potent...
chemoattractant for microglia (Yao et al., 1990). The acute application of C5a to primary culture microglia immediately induced intense ruffling of cell membranes, followed by lamellipodia extension within a few seconds (Nolte et al., 1996). Interestingly, it has been shown that the stimulatory effects of ATP and C5a were additive, suggesting that each chemoattractant stimulated migration through independent molecular mechanisms (Miller and Stella, 2009). Nevertheless, the exact role of the complement in microglial migration to the site of injury following peripheral nerve damage has not yet been investigated.

**Proliferation**

A key aspect of the microglial response to damage is an increase in the cell population at the site of injury that is achieved by proliferation and by migration of resident cells. It has been shown that following peripheral nerve injury microglia in the dorsal horn undergo extensive proliferation (Fig. 4. Echeverry et al., 2008; Liu et al., 2000). However, until recently relatively little was known about the mediators that induce this response.

NRG1 which is released in the spinal cord after nerve injury was found to significantly increase microglial proliferation in vitro. This effect could be prevented when inhibiting the erbB2 receptor and was dependent on the phosphorylation of ERK1/2 intracellular pathway (Calvo et al., 2011). Intrathecal injection of NRG1 produced an increase in microglial proliferation in the spinal cord which was accompanied by peripheral nerve injury ERK is activated acutely (from day 1 to 2) which are activated by upstream kinase MEK1/2. ERK1/2 activation on microglia is thought to be essential for the proliferative actions of a number of growth factors such as GM-CSF and corticotropin-releasing hormone (Suh et al., 2005; Wang et al., 2003). Following peripheral nerve injury ERK is activated acutely (first hours) in dorsal horn neurons, sub-acutely (from day 2 post-injury) in microglia and more chronically (from day 10 onwards) in astrocytes (Zhuang et al., 2005). It seems that this early ERK1/2 activation in microglia is playing an important role in the mitotic response of microglia to NRG1 as well as other proliferative factors. Moreover, as mentioned before, NRG1 also increases microglial migration in vitro. The accumulation of these cells within the dorsal horn following nerve injury is therefore likely to represent the stimulation of both proliferation and migration by NRG1.

Macrophage-colony stimulating factor (M-CSF) has been implicated in the microglial mitotic response following facial nerve axotomy. M-CSF is up-regulated following axotomy and this coincides with the time shift were microglia proliferation. In vitro experiments showed that M-CSF increased BrdU incorporation into the nucleus of primary microglia and this can be blocked by neutralising M-CSF (Yamamoto et al., 2010). Furthermore, animals in which there is a frameshift mutation in M-CSF have reduced numbers of brain microglia and demonstrate impaired microglial proliferation following facial nerve axotomy (Raivich et al., 1994). However, the role of M-CSF has not yet been explored in animal models of neuropathic pain.

Although IFN-γ has been implicated in several studies as a mitotic inhibitor in macrophages (Schroder et al., 2004), it has been reported that intrathecal IFN-γ administration also leads to an increase in microglial proliferation (Tsuda et al., 2009).
Cytokine release

Once stimulated through ‘danger’ stimuli microglia secrete cytokines, nitric oxide and BDNF, which directly affect synaptic transmission through enhancing dorsal horn neuron excitability (Coull et al., 2005; Kawasaki et al., 2008b). These cytokines released by microglia can induce central sensitization via distinct mechanisms (Ji and Suter, 2007). TNF-α enhances excitatory synaptic transmission by increasing the frequency of spontaneous excitatory postsynaptic currents and the amplitude of AMPA- or NMDA-induced currents. IL-6 inhibits spinal cord inhibition by reducing the frequency of spontaneous inhibitory postsynaptic currents and the amplitude of GABA and glycine induced currents. IL-1β can both enhance excitatory synaptic transmission and reduce inhibitory synaptic transmission (Kawasaki et al., 2008b). Microglia activated by ATP through the P2X4 receptor release BDNF (Trang et al., 2009). After axotomy release of BDNF from microglia results in a depolarizing shift in the anion reversal potential in spinal lamina I neurons, so that the polarity of currents activated by GABA is reversed (Coull et al., 2005). Together, these results suggest a role of molecules released by microglia in regulating synaptic plasticity and neuronal excitability (Zeilhofer, 2008).

Many different stimuli can lead to cytokine secretion by microglia. For instance, activation of TLR-3 and 4 leads to IL-1β, IL-6 and TNFα secretion (Obata et al., 2008; Tanga et al., 2005). ATP binding to different receptors in microglia stimulates cytokine release: P2X7 receptors activation leads to secretion of TNFα (Hide et al., 2000), CXCL2 (Shiratori et al., 2010), and IL-1β (Clark et al., 2010a); and an unidentified P2Y receptor stimulates IL-6 secretion (Shigemoto-Mogami et al., 2001). Two mitogen-activated kinases (MAPKs) have been implicated in promoting the microglial pro-inflammatory responses following axotomy. One is p38MAPK, which is regarded as a stress-induced kinase (Ji and Suter, 2007) and a number of cytokine receptors (TNFα receptor, CX3CL1) and purinoreceptors (P2X4) converge on its phosphorylation (Ji and Suter, 2007; Trang et al., 2009). This pathway is important for the synthesis of several pro-inflammatory mediators by microglia such as Cox-2 (Svensson et al., 2003), IL-1β (Clark et al., 2006; Ji and Suter, 2007), BDNF (Coull et al., 2005; Trang et al., 2009) and INOS (Sung et al., 2005). P38 MAPK is activated in microglia after SNL and contributes to neuropathic pain development (Jin et al., 2003; Tsuda et al., 2004).

Another MAPK, ERK1/2, has been implicated in microglial proliferation and the release of a number of pro-inflammatory mediators such as TNFα and NO (Bhat et al., 1998; Hide et al., 2000; Romero-Sandoval et al., 2009; Tichauer et al., 2007).

Phagocytosis

One of the key functions of microglia within the CNS is the clearance of neurotoxic molecules, cellular debris or microbes via phagocytosis to maintain neural networks (Napoli and Neumann, 2009). It is uncertain whether this microglial function plays a role in restoring transmission through clearance of degenerating presynaptic terminals following peripheral nerve injury. Synaptic stripping, a process in which microglia selectively remove synapses from injured neurons has been hypothesised to occur in motoneurons after facial and hypoglossal nerve axotomy and during inflammation in the cortex (Blinzing and Kreutzberg, 1968; Svensson and Aldskogius, 1993; Trapp et al., 2007). We know relatively little regarding the fate of synapses within the dorsal horn following nerve injury however there is evidence of degeneration of primary afferent terminals within the superficial laminae of the spinal cord (Knyihar-Csillik et al., 1987). Furthermore in the spinal cord, microglia engulfment of injured and uninjured myelinated axons via P2Y12 signalling has been described after peripheral axotomy (Maeda et al., 2010). Although we do not have direct evidence for the removal of synapses within the dorsal horn or for the clearance of cellular debris by microglia, it is reasonable to think that phagocytosis might play a role in restoring homeostasis after peripheral nerve injury.

Nucleotides leaking from necrotic cells seem to have the ability to stimulate phagocytosis. Indeed, it has been shown that UDP induces microglial phagocytosis in vitro through activation of P2Y6 receptor. Hippocampal neuronal damage in vivo induces an increase in extra-cellular UDP and an up-regulation of P2Y6 receptors in microglia which stimulates phagocytosis (Koizumi et al., 2007). This pathway however, has not yet been studied in the context of neuropathic pain development.

Another molecule putatively involved in microglial phagocytosis is the complement receptor 3 (CR3, all known as Mac-1), which is a heterodimeric receptor consisting of CD11b and non-covalently associated CD18. CR3 is widely expressed on both phagocytically active
cells and microglial cells in the CNS. It is involved in the complement-mediated phagocytosis of pathogens (Akiyama and McGeer, 1990; Le et al., 2002). Moreover, the classical complement cascade (specifically, the C1q component) has been involved in removal of neuronal debris in the developing brain and synapse elimination in the degenerating CNS (Stevens et al., 2007). These effects of the complement through CR3 activation in microglial cells have not been investigated in the context of neuropathic pain.

Antigen presentation and recruitment of T cells

Major histocompatibility complex class II (MHC II) molecules are a set of polymorphic genes responsible for presenting antigenic peptides to T lymphocytes which are found only on specialised cell types that are collectively known as antigen-presenting cells (APCs). APCs phagocytose extracellular antigens and present them in the lymph node through MHC II molecules to T-cells inducing their proliferation and production of cytokines. Although it is thought that microglia cannot exit the CNS after ingesting an antigen to enter the draining lymph nodes to stimulate naïve cells, they can express MHC II when they have been stimulated, as it occurs in many forms of CNS pathology (reviewed in Perry, 1998). Indeed, it has been shown that microglia express MHC II following peripheral nerve injury and that this contributes to injury-induced allodynia as MHC II knock-out mice exhibit attenuated allodynia following spinal nerve transection (Sweitzer et al., 2002). Another means of communication between microglia and CD4+ lymphocytes is via a CD40-CD154 interaction. After L5 spinal nerve ligation there is a significant increase of CD40+ microglia in the ipsilateral side of the lumbar spinal cord. CD40 knock-out mice exhibited significantly less mechanical hypersensitivity (Cao et al., 2009).

The antigen presenting feature of microglia seems to have a functional relevance as T cells have been observed to infiltrate the spinal cord following nerve injury (Sweitzer et al., 2002). Moreover, T cell infiltration has been shown to contribute to injury-induced pain behaviours: Mice that lack T cells or functional T cells display a significantly decreased mechanical hypersensitivity after axotomy, which can be reversed by transferring exogenous CD4+ lymphocytes (Cao and DeLeo, 2008; Costigan et al., 2009). Th1 lymphocytes (pro-inflammatory) secrete interferon γ (IFN-γ) which acts in microglia stimulating upregulation of P2X4 receptors. Mice that lack the IFN-γ receptor showed significant attenuation of nerve injury-evoked microglialosis and allodynia (Tsuda et al., 2009). Glatiramer acetate is an immunomodulatory drug that act partially through shifting the population of T cells from pro-inflammatory Th1 cells into regulatory Th2 cells. One study in which this drug was administered following nerve injury found that changing the balance between Th1 and Th2 cells resulted in a reduction in microgliosisis and allodynia (Leger et al., 2011). Taken together these studies suggest that presentation of antigens by microglia and the consequent T-cell recruitment contributes to the development and maintenance of axotomy-induced allodynia.

Resolution of inflammation

The immune response to nervous system damage is controlled by a highly complex and intricate network of regulatory mechanisms. Among these regulatory components are the anti-inflammatory cytokines and molecules (IL-4, IL-10, IL-13, TGFβ, resolvins, lipoxins, cannabinoids), specific cytokine inhibitors (IL-1 receptor antagonist), the activation of inhibitory signalling proteins such as inhibitor of NF-κB, complement inhibitors and MAPK phosphatases (Opal and DePalo, 2000; Romero-Sandoval et al., 2009; Soehnlein and Lindbom, 2010; Xu et al., 2010). Under physiologic conditions, these regulatory mechanisms limit the potentially damaging effects of persistent or excessive inflammatory reactions. Pathological conditions such as chronic pain may arise when these regulatory mechanisms are insufficient to control the proinflammatory response. Less is known about the role of these regulatory mechanisms in the context of neuropathic pain. Microglial cells can produce anti-inflammatory cytokines but their contribution in regulation of nerve-induced neuroinflammation in largely unknown. In one study IL-10 was administered intrathecal-ly which briefly reversed CCI-induced mechanical allodynia and thermal hyperalgesia (Milligan et al., 2005b). This same group found that IL-10 gene therapy attenuated paclitaxel-induced mechanical allodynia (Ledeboer et al., 2007). Interestingly, an adenosine 2A receptor agonist was shown to reduce microgliosis and nerve injury induced hyperalgesia after CCI. The authors found that the effect was through IL-10 as a neutralising antibody prevented the effect of the agonist on neuropathic pain (Loram et al., 2009). Therefore, the antiinflammatory effects of adenosine might not only be mediated by blocking microglial migration but also by increasing IL-10 release. Another regulatory mechanism is the endocannabinoid system. CB2 receptors are expressed in spinal microglia and perivascular cells and are up-regulated in neuropathic animals (Romero-Sandoval et al., 2008). Endocannabinoid levels are also increased after peripheral nerve injury (Guasti et al., 2009) and their action through CB2 receptors reduces hypersensitivity and microgliosis contributing to the local containment of neuropathic pain (Romero-Sandoval et al., 2008). Activation of the CB2 receptor in cultured primary microglia led to activation of the mitogen-activated protein kinase-phosphatase-3 (MKP-3) which inhibited the ERK pathway in these cells. ERK dephosphorylation led to a decrease in TNFα expression and a decrease migration towards ADP in microglia, therefore reducing the pro-inflammatory response in these cells (Romero-Sandoval et al., 2009).

Other mechanisms of regulation of inflammation following peripheral axotomy may be through apoptosis of excessively activated microglia. In a careful stereological analysis of the dorsal horn at 4 weeks following nerve injury, Polgar et al. found that TUNEL-positive cells (a marker of apoptosis) were present throughout the grey and white matter and virtually all were labelled with Iba1 (a marker of microglia) (Polgar et al., 2005). However, no further study have determine the fate of microglia following nerve injury-induce spinal inflammation.

Regulatory mechanisms offer a great opportunity for therapy as they can modulate the immune response without abolishing the neuroprotective effects that it might have. It is therefore of great interest to better understand how these mechanisms operate following peripheral nerve injury and how their manipulation can prevent or treat neuropathic pain.

Is microgliosis involved in human chronic pain?

Animal models of neuropathic pain have clearly demonstrated that microgliosis is involved in pain pathogenesis. However, we are still lacking direct evidence conclusively demonstrating that spinal microglia also has a role in the pathophysiology of neuropathic pain in humans. A PET scan study in humans using a radio-labelled ligand for the peripheral benzodiazepine receptor suggested the presence of microgliosis in the thalamus of amputees with longstanding phantom limb pain (Banati et al., 2001). Because the resolution of this technique is relatively poor to show small structures like the spinal cord, the classical complement cascade (specifically, the C1q component) has been involved in removal of neuronal debris in the developing brain and synapse elimination in the degenerating CNS (Stevens et al., 2007). These effects of the complement through CR3 activation in microglial cells have not been investigated in the context of neuropathic pain.
with complex regional pain syndrome have been shown to have elevated intrathecal levels of IL-6 and CCL2, and low levels of IL-4 and IL-10 (Alexander et al., 2007). In patients with non-inflammatory polyneuropathy, serum TNFα has been shown to be elevated in those with allodynia compared to those without (Ludwig et al., 2008). Hence, a pro-inflammatory cytokine profile seems to be associated with neuropathic pain in humans and it is a reasonable hypothesis (although not conclusively demonstrated) that glial cells may contribute to this inflammatory response.

Conclusions

There is now a significant body of evidence that microglial cells are active participants in the generation of neuropathic pain. There is a great functional diversity in the microglial response to nerve injury and we are now developing increasing knowledge as to the specific signalling pathways involved in regulating different aspects of microglial function. The detailed study of different models of neuropathic pain would suggest that the importance of these cells in pain generation may differ depending on the exact form of nerve injury used; they are likely to be ‘prime movers’ driving persistent pain following traumatic nerve injury but may have less of a role in toxic neuropathies induced by chemotherapeutic agents. This issue will need to be given serious consideration in terms of patient recruitment if clinical trials involving microglial modulation are to be contemplated. Therapies targeted at microglia are an exciting prospect and ideally would target the deleterious pro-inflammatory actions of these cells whilst maintaining beneficial functions.

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References


