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## Pathological pain and the neuroimmune interface

Peter M. Grace<sup>1,2</sup>, Mark R. Hutchinson<sup>1,2</sup>, Steven F. Maier<sup>1</sup>, and Linda R. Watkins<sup>1</sup>

<sup>1</sup>Department of Psychology and Neuroscience, and The Center for Neuroscience, University of Colorado Boulder, Boulder 80309–0345, USA

<sup>2</sup>School of Medical Sciences, University of Adelaide, Adelaide 5005, Australia

### Abstract

Reciprocal signalling between immunocompetent cells in the central nervous system (CNS) has emerged as a key phenomenon underpinning pathological and chronic pain mechanisms. Neuronal excitability can be powerfully enhanced both by classical neurotransmitters derived from neurons, and by immune mediators released from CNS-resident microglia and astrocytes, and from infiltrating cells such as T cells. In this Review, we discuss the current understanding of the contribution of central immune mechanisms to pathological pain, and how the heterogeneous immune functions of different cells in the CNS could be harnessed to develop new therapeutics for pain control. Given the prevalence of chronic pain and the incomplete efficacy of current drugs — which focus on suppressing aberrant neuronal activity — new strategies to manipulate neuroimmune pain transmission hold considerable promise.

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Pain has been associated with the immune system since the time of Celsus, who identified pain (*dolor*) as a cardinal sign of acute inflammation. Though acute pain may occur in relative isolation from broader syndromes, it was identified in the mid-twentieth century as one of a constellation of adaptive behaviours, collectively termed the sickness response.

The idea that pain and immunity might be associated beyond an acute response first arose from clinical observations in the 1970s that patients with chronic pain exhibited other symptoms, in addition to hyperalgesia, that parallel the classical systemic sickness response — including lethargy, depression and anxiety. The concomitance of sickness behaviours with chronic pain is therefore suggestive of underlying immune activity. Efforts to identify the origin and nature of the immune mediators involved soon followed, leading to the discovery that elevated peripheral levels of interleukin-1 $\beta$  (IL-1 $\beta$ ) both induced hyperalgesia per se and mediated sickness-induced hyperalgesia<sup>1,2</sup>. Although peripheral sensitization of pain fibres at local tissue sites of inflammation has a key role in heightening pain from those regions, these peripheral observations were soon extended with the discovery of a central nervous system (CNS) mechanism of action for IL-1 $\beta$  and other cytokines<sup>3,4</sup>. This research

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Correspondence to P.M.G. peter.grace@colorado.edu.

#### Competing interests statement

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was accompanied by a growing appreciation that the release of cytokines in the CNS, and the behavioural effects that occur subsequent to this, might not be solely dependent on peripheral signalling (for example, sensory vagal nerve stimulation and/or cytokine release from peripheral immune cells), but could also be due to the local release of cytokines by glia residing within the CNS<sup>5</sup>. Glia were first implicated when increased expression of astrocyte- and microglia-associated activation markers was observed in the lumbar spinal cords of rats with peripheral nerve injury<sup>6,7</sup>. The contribution of spinal glia and their pro-inflammatory products to allodynia and hyperalgesia was then demonstrated when spinal intrathecal injection of inhibitors of gliosis or IL-1 receptor antagonist (IL-1Ra) was found to attenuate the pain behaviours that are associated with diverse pain models<sup>8–11</sup>.

Since these seminal observations, there have been major advances in understanding how glia and immune cells in the CNS respond to pain stimuli and contribute to pathological pain. In this Review, we detail these advances in the context of the neuroimmune interface, which is integral to an appreciation of central immune signalling. It should be noted that neuroimmune interactions are crucial to inflammation-induced peripheral sensitization and pathophysiological changes at the site of peripheral nerve injury. However, this Review is restricted to the composition of the neuroimmune interface in the CNS, and how its constituent cells become reactive and contribute to chronic pain. The protective role of immune signalling in neuropathic pain, together with methods to pharmacologically target the neuroimmune interface for superior pain control are also discussed.

## Physiological pain processing

Pain (either nociceptive pain or inflammatory pain) is protective and adaptive, warning the individual to escape the pain-inducing stimulus and to protect the injured tissue site during healing. The basic scientific understanding of sensory processing and modulation has been dramatically improved by the development of pain assays that recreate some elements of clinical pain syndromes (BOX 1).

### Box 1

#### Preclinical pain assays and measures

The development of preclinical pain assays — the experimental procedures by which pain is induced in the subject — has typically occurred over several distinct phases<sup>154</sup>. Initial studies of pain used acute assays, involving the application of a noxious stimulus (which may be thermal, mechanical, electrical or chemical) to an accessible body part (usually the hindpaws, tail or abdomen); or inflammatory assays that directly activate nociceptors (for example, treatment with formalin or capsaicin) or the immune system (for example, treatment with complete Freund's adjuvant or carrageenan). In order to study the unique pathophysiological mechanisms of neuropathic pain that arise owing to differing adaptations resulting from injury to nervous — compared with other somatic — tissues, as well as increased pain duration, three main assays were developed: first, chronic constriction injury, in which loose ligatures are tied around the sciatic nerve to produce damage to some of the axons by inducing swelling and then strangulation<sup>155</sup>; second, partial sciatic nerve ligation, in which a tight ligature is applied through

approximately one-half of the proximal sciatic nerve<sup>156</sup>; and finally, spinal nerve ligation, involving tight ligation of the lumbar spinal nerves (L4 and L5), close to the dorsal root ganglion<sup>157</sup>. These assays have undergone continual development, and have been adapted to study orofacial pain and heterogeneous pain thresholds<sup>154,158</sup>. The recognition that existing assays can model extremely rare pain syndromes, but lack face validity for the more common pain syndromes, has led to more direct attempts to mimic the pain associated with specific disease states, often by attempting to induce the disease, injury or the physiological state itself (for example, chemotherapy-induced neuropathic pain or multiple sclerosis<sup>159,160</sup>)<sup>154</sup>.

Furthermore, modern studies of chronic pain use acute assays to quantify hypersensitivity, which is the most common preclinical end point. The pain measures typically used are evoked spinal reflexes (for example, the von Frey test for mechanical allodynia and the Hargreaves test for thermal hyperalgesia), spino-bulbospinal reflexes (for example, jumping or abdominal stretching), or simple innate behaviours (for example, licking, guarding or vocalization). As part of the general critique of pain models, the validity of current pain measures is presently being re-examined, and alternative measures are being developed to reflect supraspinal pain processing, such as operant measures (for example, conditioned place aversion<sup>161</sup>), spontaneously-emitted behaviours (for example, facial grimacing or suppressed voluntary wheel running<sup>162,163</sup>), as well as complex states affected by chronic pain (for example, altered social interactions or sleep disruptions<sup>164</sup>)<sup>154,165</sup>.

Painful stimuli (for example, mechanical, thermal and chemical) are initially transduced into neuronal electrical activity and conducted from the peripheral stimulus site to the CNS along a series of well-characterized peripheral nociceptive sensory neurons (first-order primary afferent neurons). The nociceptive signal is then transmitted at central synapses through the release of a variety of neurotransmitters that have the potential to excite second-order nociceptive projection neurons in the spinal dorsal horn or hindbrain (FIG. 1). This process of nociception can occur through several mechanisms involving glutamate and neuropeptides (for example, substance P or calcitonin gene-related peptide (CGRP)). Glutamate activates postsynaptic glutamate AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and kainate receptors on second-order nociceptive projection neurons. Interestingly, these receptor systems are not all engaged equally in response to different types of pain. Modification of the nociceptive signal can occur at the level of the spinal cord through activation of local GABAergic (that produce  $\gamma$ -aminobutyric acid) and glycinergic inhibitory interneurons.

Second-order nociceptive projection neurons project to supra-spinal sites, which further project to cortical and subcortical regions via third-order neurons, enabling the encoding and perception of the multidimensional pain experience. Second-order nociceptive projection neurons can be further modulated through the activation of descending serotonergic and noradrenergic projections to the spinal cord, which can influence the response to and perception of pain (FIG. 1). For recent reviews that describe these processes in detail, see REFS 12,13.

## Pathological pain processing

Pain can extend beyond its protective usefulness, lasting for a period of weeks to years — well beyond the resolution of the initial injury. In this case, pain is maladaptive and is believed to result from abnormal functioning of the nervous system. In the United States alone, such persistent pain is estimated to affect ~37% of the population; representing an economic burden of up to US\$635 billion per year<sup>14</sup>. Perhaps the most well-studied example is neuropathic pain, which is relatively common and arises as a direct consequence of a lesion or disease affecting the somatosensory system.

Intense, repeated and sustained activity of first-order neurons elicits well-characterized changes in neuronal and biochemical processing at central synapses and descending projections, transitioning these sites into a pain-facilitatory state<sup>12,13</sup>. In the spinal dorsal horn, these changes are collectively known as central sensitization and windup. These processes involve the phosphorylation of a range of receptors, including NMDA (*N*-methyl-D-aspartate), AMPA and/or kainate receptors, which increases synaptic efficacy by altering channel opening time, increasing burst firing, removing the Mg<sup>2+</sup>-mediated channel blockade at the NMDA receptor, and promoting trafficking of receptors to the synaptic membrane<sup>15</sup>. Under such circumstances, low-threshold sensory (A $\beta$ ) fibres that are activated by innocuous stimuli are able to activate high-threshold nociceptive neurons, owing to either a strengthened excitatory input or the lowered excitation threshold of nociceptive projection neurons<sup>15</sup>. Central sensitization is maintained by ongoing stimuli, such as spontaneous activity arising from sensory fibres or locally released immune mediators (see below), which are responsible for the persistence and spread of neuropathic pain beyond the initial injury site.

Although the importance of these neuronal pain facilitation mechanisms is undisputed, not all symptoms and mechanisms can be explained solely by such neuronal mechanisms. These neuronal pathophysiological mechanisms are being supplemented by an appreciation for the role of central immune signalling, such that neuropathic pain is now considered as a neuroimmune disorder<sup>16</sup>.

## The neuroimmune interface

Seminal studies towards the end of the last century have led to an appreciation of the bidirectional signalling that occurs between neurons and a host of immunocompetent cells present in the CNS, including glia (microglia, astrocytes and oligodendrocytes), endothelial cells, perivascular macrophages and infiltrating T cells. These cells are now known to be far more than passive bystanders or components of the extracellular matrix, and have been shown to modulate neurotransmission within the CNS. Such a relationship may be best described as the neuroimmune interface.

The majority of research has focused on the role of astrocytes and microglia, and to a lesser extent on that of T cells and endothelial cells. Astrocytes are the most abundant cell type in the CNS. In addition to providing structural support, promoting formation of the blood–CNS barrier and regulating cerebral blood flow, astrocytes contribute to synaptic transmission,

provide trophic support and promote repair of neuronal systems. They also maintain homeostasis in the extracellular environment by regulating the concentration of neurotransmitters and ions in the synaptic cleft<sup>17</sup>. Microglia are the tissue-specific phagocytes of the CNS. They exhibit constitutive and regional heterogeneity throughout the parenchyma, presumably to coordinate diverse responses to insult<sup>18</sup>. Under a basal surveillance state, the cytoarchitecture of microglia allows them to continuously sample the extracellular space for perturbations<sup>19</sup>. Transition to a state of reactive gliosis involves changes in cell number, morphology, phenotype and motility, the expression of membrane-bound and intracellular signalling proteins (for example, mitogen-activated protein kinases (MAPKs)), and the release of immunoregulatory products, such as cytokines and chemokines. A common marker of astrogliosis is increased expression of glial fibrillary acidic protein (GFAP), which is indicative of altered morphology. Microgliosis is often correlated with increased expression of CD11b and allograft inflammatory factor 1 (AIF1; also known as IBA1)<sup>20,21</sup>. It should be noted that the functional relationship between the expression of such markers and nociceptive hypersensitivity is currently unclear, and in certain circumstances there seems to be no connection between the two<sup>22</sup>.

Endothelial cells respond to chemokines such as CC-chemokine ligand 2 (CCL2; also known as MCP1) and CX<sub>3</sub>C-chemokine ligand 1 (CX<sub>3</sub>CL1; also known as fractalkine) by increasing expression of receptors and tethering proteins that facilitate transendothelial migration of monocytes and T cells across the blood–CNS barrier. CCL2 and CX<sub>3</sub>CL1 also attract monocytes and T cells to the CNS, where they interact with local immunocompetent cells to facilitate the propagation and differentiation of the immune response, which eventually leads to modification of neuronal activity<sup>23,24</sup>. The presence of specific T cell subpopulations at the neuroimmune interface is not well established and has primarily been defined on the basis of cytokine expression. For example, the respective T helper 1 (T<sub>H</sub>1), T<sub>H</sub>2 and T<sub>H</sub>17-associated cytokines interferon- $\gamma$  (IFN $\gamma$ ), IL-4 and IL-17 have been detected in rodent lumbar spinal cords after peripheral nerve injury or inflammation<sup>25–28</sup>. A specific role for regulatory T (T<sub>Reg</sub>) cells at the neuroimmune interface has not yet been established, but systemic expansion of T<sub>Reg</sub> cells was found to attenuate nerve injury-induced nociceptive hypersensitivity<sup>29</sup>.

Although not systematically characterized, the nature of the precipitating injury or insult is likely to influence the cell populations that are recruited to the spinal dorsal horn. For example, pain is associated with both spinal cord injury and peripheral nerve injury. However, infiltration of neutrophils into the spinal cord is only associated with spinal cord injury, which could be due to differential expression of cell adhesion molecules<sup>30,31</sup>. Interestingly, nociceptive thresholds have been successfully predicted on the basis of peripheral immune cell activity in preclinical studies, and in both healthy volunteers and patients with chronic pain<sup>32–35</sup>. Finally, neurons not only respond to neurotransmitters derived from other neurons, but also express a wide range of constitutive and inducible classical immune signalling receptors and ligands, and are capable of modulating local immune activity via both contact-dependent and contact-independent mechanisms<sup>36,37</sup>.

Transition of immunocompetent cells in the spinal dorsal horn to a reactive phenotype has been implicated in neuropathic pain<sup>8–11,23,38–42</sup>. Considerable investigation has addressed

key questions of how such central immune signalling is initiated after a peripheral nerve injury, and the mechanisms by which it contributes to central sensitization.

## The neuroimmune interface in pathological pain

Following peripheral nerve injury, high-threshold activity or damage of first-order neurons initiates central immune signalling at the level of innervation<sup>43–45</sup>. Although astrocytes and T cells may respond to neuronal signals, microglia have been identified as the first responders to a host of neuronally-derived mediators, including matrix metalloproteinase 9 (MMP9), neuregulin 1, chemokines, ATP and endogenous danger signals (damage-associated molecular patterns (DAMPs; also known as alarmins))<sup>46,47</sup>. Upon detection of such signals, microglia transition into a state of reactive gliosis. Peripheral immune cell infiltration and astrogliosis soon follow, owing to the release of cytokines, chemokines, DAMPs and inflammatory mediators from reactive microglia<sup>23,42</sup> (FIG. 2).

### Chemokines, ATP and initiation of central immune signalling

Following the transduction of noxious stimuli, ATP and chemokines — such as CCL2, CX<sub>3</sub>CL1 and CCL21 (also known as SLC and 6CKine) — are released from central terminals in the spinal dorsal horn, and induce signalling via G protein-coupled receptors (GPCRs) and ligand-gated ion channels. The expression of CCL2 and CX<sub>3</sub>CL1 is not only inducible, but these chemokines can also be constitutively expressed by first-order neurons<sup>48–52</sup>. Genetic or pharmacological manipulation of the receptors for CCL2 and CX<sub>3</sub>CL1 attenuates microgliosis and the ensuing development of nociceptive hypersensitivity after peripheral nerve injury<sup>24,48–53</sup>. CX<sub>3</sub>CL1 may be a secondary signal to microgliosis, as cleavage of this chemokine from neuronal membranes typically requires the release of cathepsin S (a lysosomal protease), which is produced by P2X<sub>7</sub> receptor (P2X<sub>7</sub>R)-stimulated microglia<sup>51,54</sup>. MMP9 and MMP2 produced by neurons and astrocytes may also promote CX<sub>3</sub>CL1 liberation<sup>55</sup>. Interestingly, the pro-inflammatory role of CX<sub>3</sub>CL1 has largely been defined within the pain field, as this neuronally tethered chemokine is commonly viewed as a homeostatic regulator that maintains a ‘surveillance’ phenotype in microglial cells. However, it has been posited that CX<sub>3</sub>CL1 can have different functions in its tethered and cleaved states<sup>56</sup>. CCL21 has also been implicated in nociceptive hypersensitivity. It is released from the central terminals of injured neurons, and stimulates and attracts local microglia and infiltrating T cells<sup>57,58</sup>.

Resident microglia also express a range of ionotropic and metabotropic purinergic receptors — P2X and P2Y purinoceptors, respectively — that shape microgliosis<sup>59</sup>. Hence, neuronal release of ATP, either as a neurotransmitter or as a consequence of cell damage, is a crucial molecular substrate for early microgliosis<sup>60,61</sup>. P2X<sub>4</sub>R and P2X<sub>7</sub>R have been implicated in neuropathic pain by experiments that blocked receptor function pharmacologically and by genetic ablation<sup>22,40,62–64</sup>. However, certain developmental factors should be considered when interpreting knockout data and hypo-functional variants. For example, nitric oxide production by endothelial cells is impaired in P2X<sub>4</sub>R-deficient mice<sup>65</sup>. This makes it difficult to interpret the direct effects of P2X<sub>4</sub>R activation on microglia using these animals, as disruption of endothelial cell function will perturb the cellular milieu in the CNS after

peripheral nerve injury. Furthermore, some P2X<sub>7</sub>R-deficient mouse strains express a splice variant, which allows them to retain P2X<sub>7</sub>R function<sup>66</sup>. Intrathecal adoptive transfer of ATP-stimulated microglia into naive rats, which induced robust allodynia, showed that these effects are localized to microglia<sup>39,40</sup>. Furthermore, activation of P2X<sub>7</sub>R, expressed by microglia, induced the release of IL-1 $\beta$  and cathepsin S, which are key contributors to nociceptive hypersensitivity<sup>54,67</sup>. The development of allodynia also correlates temporally with an increase in spinal microglial cell expression of P2X<sub>4</sub>R<sup>22,40</sup>. P2Y<sub>12</sub> receptor (P2Y<sub>12</sub>R) and P2Y<sub>13</sub>R expression by microglia rapidly increases after peripheral nerve injury, and pharmacological or genetic disruption of signalling through these receptors prevents the development of allodynia<sup>68,69</sup>. These data provide compelling evidence that P2 receptor activation is crucial to microgliosis and the aetiology of neuropathic pain.

### Toll-like receptors and the initiation of central immune signalling

Damaged first-order sensory neurons may also release extracellular matrix molecules and DAMPs, which are detected by Toll-like receptors (TLRs) expressed by immunocompetent cells within the CNS<sup>70,71</sup>. TLRs respond to diverse invading pathogens and DAMPs, and rely on receptor dimerization, such as that of the co-receptor CD14 with myeloid differentiation protein 2 (MD2; also known as LY96), to achieve specificity in agonist recognition<sup>71</sup>. Spinal DAMPs implicated in rodent pain models include high mobility group box 1 (HMGB1), fibronectin and the heat shock proteins HSP60 and HSP90. Neuronal HMGB1 expression is increased in the spinal cord after peripheral nerve injury, and temporally correlates with the development of allodynia in bone cancer and diabetic neuropathy assays<sup>72-74</sup>. Such pain is attenuated by intrathecal administration of an HMGB1-specific neutralizing antibody<sup>73,74</sup>. Fibronectin has been shown to upregulate spinal microglial cell expression of P2X<sub>4</sub>R<sup>59</sup>. After peripheral nerve injury, HSP60 is upregulated in the spinal cord and intrathecal HSP90 inhibition reverses allodynia<sup>75,76</sup>.

To date, only TLR2 and TLR4 have been implicated in gliosis after peripheral nerve injury. Increased TLR4 expression correlates temporally with the development of nociceptive hypersensitivity after peripheral nerve injury<sup>77</sup>. In addition, spinal expression of CD11b and GFAP is decreased in nerve-injured mice with genetic disruption of *Tlr4* or *Cd14*, which is indicative of attenuated gliosis in these animals<sup>72,77,78</sup>. This is not simply a consequence of disrupted TLR4 signalling at the site of nerve injury, as nerve injury-induced allodynia can be prevented or reversed by intrathecal administration of TLR4 antagonists<sup>79,80</sup>. Furthermore, gliosis and nociceptive hypersensitivity is attenuated in nerve-injured TLR2-deficient mice, and pro-inflammatory cytokine expression is abolished in TLR2-deficient microglia treated with conditioned media from damaged sensory neurons<sup>43</sup>. Activation of alternative immune signalling pathways in the absence of TLRs should also be considered. For example, compared with wild-type controls, TLR4-deficient mice have elevated levels of T<sub>H</sub>1- and T<sub>H</sub>2-type cytokines during infection<sup>81</sup>. Furthermore, TLR4-deficient mice still develop allodynia, although unlike in wild-type mice it can no longer be inhibited by IL-1Ra<sup>82</sup>. These reports indicate that caution must be exercised when interpreting the influence of TLRs on nociception when the conclusions are solely drawn from knockout data.

Provocative data also suggest that neurons may be capable of directly sensing DAMPs, owing to the expression of a wide range of TLRs and TLR adaptor proteins by primary sensory afferents, such as trigeminal sensory neurons and dorsal root ganglia (DRG) neurons in both humans and rodents (for a review, see REF. 71). At present, expression of TLRs in spinal dorsal horn second-order projection neurons remains to be shown. Neuronal TLR expression has not only been demonstrated at the mRNA level and through the use of antibodies (which currently lack suitable specificity, making antibody-based detection suspect), but also by binding assays and measures of function, such as patch clamp electrophysiology<sup>71,83,84</sup>. In DRG neurons, TLR3 expression increased after injury, and activation of TLR4, TLR7 and TLR9 in DRG neurons elicited intracellular calcium accumulation, inward currents, sensitization of transient receptor potential cation channel subfamily V member 1 (TRPV1), and production of IL-1 $\beta$ , CCL5, CXCL10 and PGE2 (REFS 71,83,84). Although neuronal TLR7 has been shown to signal through MYD88, the intracellular signalling events that are downstream of other neuronal TLRs, as well as their consequences for action potential generation, have not yet been fully characterized<sup>85</sup>. However, it is clear that the TLR signalling pathways required by innate immune cells are not necessarily identical to those of neuronal TLRs. For example, in mice, DRG neurons require myeloid differentiation factor 1 (MD1; also known as LY86) and CD14 for functional TLR4 signalling, whereas TLR4 signalling by innate immune cells requires MD2 and CD14 (REF. 86).

### Intracellular signalling pathways underlying gliosis

Given the diverse array of neuronal and immune cell-derived signals described above, it is no surprise that a wide range of intracellular signalling pathways can be activated, leading to gliosis and the production of immune mediators<sup>41</sup>. MAPK signalling pathways (such as those involving extracellular signal-regulated kinase (ERK), p38 and JUN N-terminal kinases (JNK)) in glia have an important role in intracellular signalling, leading to the activation of transcription factors, such as nuclear factor- $\kappa$ B (NF- $\kappa$ B), that promote the expression of a wide array of genes encoding pro-inflammatory products (such as cytokines and brain-derived neurotrophic factor (BDNF)). In various models of peripheral pain, spinal microglia show increased phosphorylation of SRC family kinases (namely, SRC, LYN and FYN), which are upstream of ERK<sup>28,87,88</sup>. Furthermore, specific inhibition of SRC family kinases in microglia attenuates nerve injury-induced allodynia by preventing long-term potentiation in spinal dorsal C-fibres<sup>88,89</sup>. Adaptations in intracellular signalling may also contribute to the transition from acute to chronic pain (BOX 2).

#### Box 2

##### Acute to chronic pain transition and the neuroimmune interface

The neurobiological mechanisms underlying the transition from adaptive acute pain to maladaptive chronic pain are not yet well understood, as most people recover from a lesion or disease affecting the somatosensory system without going on to develop chronic neuropathic pain. For example, only ~9% of patients (uncorrected for age) with acute herpes zoster virus infection continue to experience pain 1 year after the initial



infection<sup>166</sup>. This raises an intriguing question: what makes these patient subsets different?

One hypothesis is that unregulated gliosis may sustain central sensitization. For instance, G protein-coupled receptor kinase 2 (GRK2; also known as  $\beta$ -adrenergic receptor kinase 1) is an enzymatic regulator of the homologous desensitization of many G protein-coupled receptors (GPCRs) that are involved in pain signalling (for example, receptors for chemokines and prostaglandins and some glutamate receptors). Such desensitization of GPCRs by GRK2 protects cells against overstimulation. Peripheral inflammation or nerve injury induces a 35–40% reduction in GRK2 expression in lumbar dorsal horn microglia, as a secondary adaptation of interleukin-1 receptor 1 (IL-1R1)-mediated signalling in these cells<sup>167–169</sup>. This decrease in GRK2 expression prolongs nociceptive signalling, as shown by several studies in which peripherally induced hyperalgesia was prolonged in mice with a specific deletion of GRK2 in microglia, macrophages and granulocytes<sup>169</sup>. Downregulation of GRK2 may lead to uncontrolled stimulation of microglia and, therefore, to the exaggerated and prolonged production of pro-inflammatory mediators.

Another answer may lie in the immunological history of patients with chronic neuropathic pain. There is emerging evidence that after a primary immune challenge, microglia may retain enhanced transcriptional activity or epigenetic modifications that confer a heightened response to subsequent immune challenges<sup>20</sup>. Such immune priming (also called immune training or postactivation) by stress, ageing, illness or injury followed by a somatosensory lesion or disease (or vice versa) may lead to chronic pain in clinical populations, and has been modelled in several preclinical studies. Prior treatment with corticosterone potentiated lipopolysaccharide (LPS)-induced allodynia and increased spinal cord levels of interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-6 (*REFS* 170,171). Such potentiated allodynia was inhibited by co-administration of the IL-1 receptor antagonist protein (IL-1Ra)<sup>170</sup>. In a similar manner, prior exploratory abdominal surgery (laparotomy) also potentiated LPS-induced allodynia, which was attenuated by minocycline (a broad-spectrum tetracycline antibiotic and anti-inflammatory agent) administered either at the time of surgery or LPS administration<sup>172</sup>. Prior treatment with morphine (which induces microgliosis; *BOX 3*) has also been shown to potentiate allodynia induced by hindpaw or *masseter* inflammation, as well as peripheral nerve injury<sup>173</sup>. Although it is likely that such primed microglia retain enhanced transcriptional activity, these changes may only be subtle and the intracellular mechanisms involved are not well understood.

### Box 3

#### Opioids and central immune signalling

Opioids remain the gold standard therapy for acute and chronic pain. However, their clinical use is limited by issues with tolerance, paradoxical hyperalgesia and their abuse potential. Although these adverse effects can be partly explained by neuronal mechanisms, the emerging role of central immune signalling is revolutionizing opioid

pharmacology<sup>136,174</sup>. In addition to stereoselectively signalling at classical opioid receptors, morphine is now known to non-stereoselectively signal at Toll-like receptor 4 (TLR4) by binding to myeloid differentiation factor 2 (MD2)<sup>136,174,17</sup>. Hence, in addition to being activated by opioid receptor active (–)-isomers, TLR4 is also non-stereoselectively activated by opioid receptor inactive isomers, such as (+)-morphine and the metabolite morphine-3-glucuronide, and may be selectively antagonized by opioid receptor inactive (+)-naloxone and (+)-naltrexone<sup>80,136,174</sup>. The initiation of central immune signalling by TLR4 activation has marked consequences for opioid pharmacodynamics, including opposition of analgesia, probably by induction of central sensitization, and also opioid reward, craving, reinstatement and withdrawal<sup>174,176</sup>. A recent study has also shown that  $\mu$ -opioid receptor signalling on microglia induced brain-derived neurotrophic factor (BDNF) release, downregulating the potassium-chloride cotransporter KCC2 and disrupting the anion reversal potential<sup>177</sup>. Such alterations were found to contribute to hyperalgesia, but not tolerance.

## Pain enhancement at the neuroimmune interface

Glia and immune cells exert their influence on neural pain processing circuitry via soluble mediators that are released for months after injury as a consequence of gliosis<sup>90</sup>. A recent study has also raised the intriguing possibility that neurons may be autonomous ‘immune sensors’, acting independently of glial and immune cell influences. Pain induced by *Staphylococcus aureus* was shown not to be initiated by an immune cell intermediary, but rather by direct activation of first-order nociceptive neurons by bacterially derived *N*-formylated peptides signalling at formyl peptide receptors, as well as by  $\alpha$ -haemolysin<sup>91</sup>. Nonetheless, mediators derived from glia and immune cells diffuse and bind to receptors on presynaptic and postsynaptic terminals in the spinal dorsal horn to modulate excitatory and inhibitory synaptic transmission. This results in nociceptive hypersensitivity that is characteristic of central sensitization (FIG. 3). Notably, compared with classical neurotransmitters, immune mediators can modulate spinal cord synaptic transmission at substantially lower concentrations<sup>41</sup>.

## Enhanced excitatory synaptic transmission

Immune mediators, such as tumour necrosis factor (TNF), IL-1 $\beta$ , IFN $\gamma$ , CCL2 and reactive oxygen species (ROS) can directly modulate excitatory synaptic transmission at central terminals, principally by enhancing glutamate release<sup>92–96</sup>. Such effects are mediated by a functional coupling between IL-1 receptors and presynaptic NMDA receptors, and presynaptic TRPV1 and transient receptor potential cation channel subfamily A member 1 (TRPA1) activation that leads to Ca<sup>2+</sup>-dependent glutamate release<sup>95–98</sup>. Immune mediators such as TNF, IL-1 $\beta$ , IL-17, prostaglandin E2 (PGE2), CCL2 and CXCL1 also directly sensitize postsynaptic terminals of central synapses by several key mechanisms. These include the increased trafficking and surface expression of AMPA receptors (including those permeable to Ca<sup>2+</sup>), which renders neurons vulnerable to excitotoxicity, as well as the phosphorylation of the NR1 and NR2A or NR2B subunits of the NMDA receptor<sup>27,99–105</sup>. Excitatory synaptic transmission is also indirectly enhanced following astroglial gliosis, as the

spinal astrocyte glutamate transporters excitatory amino acid transporter 1 (EAAT1) and EAAT2 are persistently downregulated after peripheral nerve injury, leading to excitotoxicity and nociceptive hypersensitivity<sup>106,107</sup>.

### **Diminished inhibitory synaptic transmission**

The nociceptive pathway is regulated at multiple levels through numerous inhibitory systems. Interestingly, a reduction or loss of inhibitory synaptic transmission (known as disinhibition) in the spinal cord pain circuit has also been implicated in the genesis of central sensitization and chronic pain<sup>15</sup>. TNF, IL-1 $\beta$ , IL-6, CCL2, IFN $\gamma$  and ROS decrease inhibitory signalling in the spinal dorsal horn by reducing the release of GABA and glycine from interneurons and inhibitory descending projections<sup>92,108–110</sup>. Such immune mediators also contribute to disinhibition at the postsynaptic membrane of central synapses. BDNF is released as a consequence of microgliosis and, on activation of the neuronal cognate receptor TRKB (also known as NTRK2), downregulates the principal neuronal potassium-chloride cotransporter, KCC2, thereby increasing the intracellular Cl<sup>-</sup> concentration in lamina I neurons<sup>38,39</sup>. This positive shift of the anion reversal potential weakens GABA<sub>A</sub> and glycine channel hyperpolarization, and even causes GABA-evoked depolarization in a minority of neurons<sup>38,39</sup>. The ultimate consequence of such disinhibition is an increase in nociceptive hypersensitivity, and an unmasking of responses to innocuous peripheral inputs<sup>38,39,111</sup>.

### **Protective role of central immune signalling**

A complex network of regulatory mechanisms actively controls central immune signalling after neuronal insult. These mechanisms include the production of anti-inflammatory mediators and the polarization of specialized cells with an anti-inflammatory phenotype to prevent uncontrolled inflammation, as well as coordination of temporal and diverse classic pro-inflammatory mechanisms. An imbalance in such regulatory mechanisms might contribute to the development of chronic pain, presenting therapeutic opportunities to enhance neuroprotection and neuroregeneration while suppressing destructive inflammation.

### **Protective anti-inflammatory mechanisms and phenotypes**

A reactive phenotype is not synonymous with a pro-inflammatory and pro-nociceptive phenotype. That is, depending on the stimulating signal, there are subpopulations of reactive immune cells with an anti-inflammatory phenotype. Although the existence of a parallel anti-inflammatory microglial cell phenotype remains controversial, these anti-inflammatory populations include alternatively activated macrophages (also known as M2 macrophages), and T<sub>H</sub>2 and T<sub>Reg</sub> cells that contribute to the resolution of nociceptive hypersensitivity after nerve injury<sup>18,23,26,29,112,113</sup>. A hallmark of such anti-inflammatory cell types is their production of anti-inflammatory mediators, such as IL-1Ra, IL-4 and IL-10 in the spinal dorsal horn. The factors driving the recruitment and differentiation of such anti-inflammatory phenotypes are not well understood. Nociceptive hypersensitivity associated with spinal gliosis is attenuated by intrathecal administration of IL-1Ra, elevation of spinal IL-10 levels, and by systemic administration of glatiramer acetate, which increases expression of IL-4<sup>+</sup> and IL-10<sup>+</sup> T cells in the spinal dorsal horn<sup>8,26,114,115</sup>. Aside from the

effects of IL-1Ra on excitatory postsynaptic current (EPSC) frequency and NR1 subunit phosphorylation, it remains to be shown whether other anti-inflammatory cytokines directly regulate central sensitization<sup>96,101</sup>. However, the anti-nociceptive effects may be indirect, as IL-4 and IL-10 inhibit the synthesis of pro-inflammatory cytokines and chemotactic factors by microglia and T cells, and regulate cell phenotype and the expression of MHC class II and co-stimulatory molecules<sup>116,117</sup>. In addition to these mediators, cells with an anti-inflammatory phenotype express enzymes that promote extracellular matrix repair and MAPK phosphatases, as well as receptors that suppress pro-inflammatory signalling (for example, IL-1R2 and IL-10R). Microglia also express scavenger receptors and co-stimulatory molecules (such as CD86) that can promote the development of regulatory adaptive immune responses<sup>18,23,116,118,119</sup>.

### Protection mediated by pro-inflammatory mechanisms

The display of pro-inflammatory phenotypes by reactive innate and adaptive immune cells is not necessarily synonymous with nociceptive hypersensitivity and tissue destruction; a rapid, well-regulated immune response to neuronal insult is important for the regulation of gliosis and clearance of tissue debris to promote neuroregeneration. TNF seems to have a more prominent role than IL-1 $\beta$  in promoting neuronal regeneration in the CNS, whereas both cytokines have crucial roles in peripheral neuronal regeneration<sup>120,121</sup>. In a model of nitric oxide-induced neurotoxicity, neuronal cell death and demyelination were increased in TNF-deficient mice relative to wild-type controls<sup>120</sup>. Furthermore, although microgliosis was attenuated within 6 hours in these mice, it was exacerbated 4 days later. Hence, TNF per se may be required for the control of secondary damage, or the sequence of the early reactive cascade in general may be crucial for such control. In addition to temporal action, several other factors may influence neuroprotection in general, including the receptor subtype involved (for example, TNFR1 or TNFR2, IL-1R1 or IL-1R2) and other stimulating signals that accompany insult detection<sup>63,64,67,122</sup>.

Finally, after neuronal insult, reactive microglia phagocytose apoptotic cells and debris, and eliminate the source of DAMPs, leaving healthy tissue intact. Such a nuanced and regulated reactive response may be coordinated through microglial cell recognition of particular inhibitory molecules that are expressed by healthy cells within the CNS. The molecules involved in inhibiting innate immune cells may be humoral (for example, semaphorin) or membrane-bound (for example, CD22, CD47, CD200 and CD200R)<sup>36,37</sup>. Cells that have undergone apoptosis lose expression of these inhibitory molecules and increase their expression of 'altered-self molecular patterns' (for example, nucleic acids, sugars, oxidized low-density lipoproteins and altered membrane electrical charge), allowing reactive microglia to selectively phagocytose such cells<sup>37</sup>. These mechanisms have not been directly implicated in nerve injury-induced central immune signalling, but may be dysregulated in persistent pain.

### The neuroimmune interface and pain control

The fundamental role of neurons in neuropathic pain is undisputed. However, the strongest pain management strategies currently in use focus on suppressing aberrant neuronal activity

and lack suitable efficacy<sup>123</sup>. Given the key role of the neuroimmune interface in persistent pain, there is interest in targeting these mechanisms for pain management. There are several strategies that could be used to target the neuroimmune interface: first, direct inhibition of local pro-inflammatory signalling; second, stimulation of local protective anti-inflammatory mechanisms; third, inhibition of specific immune mediators; and finally, antagonism of specific cytokine or chemokine receptors.

Cytokines and chemokines in the CNS have been targeted preclinically using the TNF inhibitor etanercept and IL-1Ra to successfully attenuate nociceptive hypersensitivity<sup>8,124</sup>. However, clinical translation of such therapies may be limited by potential disruption of the beneficial neuroprotective and neuroregenerative effects of TNF and IL-1, and by the inability of such molecules to penetrate the blood–CNS barrier. Although the beneficial effects of pro-inflammatory mediators could be left intact by selectively targeting receptor subtypes that mediate damaging pro-inflammatory processes (for example, TNFR1 or IL-1R1), such approaches have not yet been demonstrated for pain, but are likely to be too specific to address the widespread adaptations and possible redundancy of cytokines and pain processing pathways<sup>33</sup>. Hence, the first two cellular approaches present the greatest potential for clinical translation and are focused on below (summarized in TABLE 1).

### **Inhibition of pro-inflammatory signalling at the neuroimmune interface**

Extensive preclinical studies of the anti-inflammatory drugs minocycline, propentofylline, ibudilast (MN-166; MediciNova) and methotrexate (which inhibit gliosis and T cell activation through various mechanisms; TABLE 1), have shown anti-allodynic and anti-hyperalgesic efficacy in neuropathic pain models, however, some of these drugs have had mixed clinical success<sup>23,42,125–129</sup>. Ibudilast has shown signs of efficacy in neuropathic pain and multiple sclerosis<sup>126</sup>. Ibudilast is in Phase II of clinical development for medication overuse headache (ClinicalTrials.gov identifier: NCT01317992) and progressive multiple sclerosis with neuropathic pain as a secondary end point (NCT01982942).

There are several possible reasons for these mixed results, such as the inherent design flaws that have been highlighted for trials involving propentofylline and minocycline<sup>130,131</sup>. It is therefore important that prior failures are taken into consideration and that a consensus is reached on clinical trial design for drugs targeting the neuroimmune interface. It is also possible that current neuroimmune modulators retain confounding off-target effects that limit efficacy. For example, minocycline has been shown to increase the expression and phosphorylation of Ca<sup>2+</sup>-permeable AMPA receptors in mice, which will oppose anti-allodynic and anti-hyperalgesic efficacy<sup>132</sup>. Furthermore, neuroimmune modulators such as minocycline, propentofylline and ibudilast may lack selectivity for specific glial and immune cell phenotypes, leading to suppression of neuroprotective and neuroregenerative effects. The results of an early study suggested that minocycline had non-selective actions on microglial cell subpopulations as, despite attenuating allodynia induced by intrathecal administration of HIV-1 gp120 envelope protein, minocycline also suppressed the expression of *IL1RN* (which encodes IL-1Ra) and *IL10* mRNA<sup>10</sup>. However, only a single study to date has directly characterized the effect of neuroimmune modulators on the phenotype of microglia, in which minocycline selectively inhibited expression of M1, but not M2,

microglia markers in the mutant superoxide dismutase 1 (SOD1<sup>G93A</sup>) mouse model of amyotrophic lateral sclerosis (ALS)<sup>133</sup>. Hence, it is necessary that further studies are undertaken to define the selectivity of phenotype-specific effects of neuroimmune modulators, as broad-spectrum inhibition may be detrimental to recovery.

The ambitious but attractive, approach is to antagonize specific stimulatory signals that drive destructive and pro-nociceptive processes, while simultaneously allowing for the coordination of a normal immune response to insult via anti-inflammatory and regulated pro-inflammatory phenotypes. Preclinical data suggest that the TLR4 and purinergic receptor signalling cascades in microglia may fulfil such criteria<sup>59,71</sup>. Several novel TLR4 antagonists have shown efficacy in preclinical neuropathic pain models, including rifampin, FP-1 and the opioid receptor-inactive isomers (+)-naloxone and (+)-naltrexone<sup>79,80,134–136</sup>. In addition, TLR4 antagonism may be exploited to improve the efficacy and adverse effect profile of opioids (BOX 3). Given the observed preclinical efficacy, the purinergic receptors P2X<sub>4</sub>R or P2X<sub>7</sub>R are also potential therapeutic targets for neuropathic pain<sup>22,40,63,64</sup>. Interestingly, some antidepressants currently indicated for neuropathic pain, such as amitriptyline and paroxetine, possess significant TLR4 and P2X<sub>4</sub>R inhibitory activity, respectively<sup>137,138</sup>. An alternative direct approach may be to target downstream signalling pathways, such as p38 MAPK, which drives microglia towards a reactive state. A recent trial demonstrated oral analgesic efficacy for a selective p38 inhibitor, dilmapiomod (SB681323; GlaxoSmithKline), in patients with nerve trauma, radiculopathy or carpal tunnel syndrome<sup>139</sup>.

Pro-resolution lipid mediators, such as the resolvins, protectins and lipoxins, are emerging as potential therapies that have direct effects on pathological neuroimmune transmission. Biosynthesized during the resolution of acute inflammation, such mediators were originally isolated from exudates in both rodents and humans<sup>140</sup>. Pro-resolution mediators signal at their cognate GPCRs, such as CHEMR23 (also known as CMKLR1; which is activated by resolvin E1) and *N*-formyl peptide receptor 2 (FPR2; also known as ALX) which is activated by resolvin D1 and lipoxin A4), and are widely expressed by neurons, glia and peripheral immune cells<sup>140</sup>. These mediators have marked effects on nociceptive hypersensitivity in diverse pain models, not by modifying basal pain thresholds like analgesics, but by restoring normal sensitivity through modification of aberrant neuroimmune transmission<sup>140,141</sup>.

### **Stimulation of anti-inflammatory signalling at the neuroimmune interface**

There are several potential therapeutics that may attenuate pathological neuroimmune signalling by stimulating anti-inflammatory mechanisms to restore homeostasis in the spinal dorsal horn microenvironment. The adenosine GPCRs A<sub>2A</sub> and A<sub>2B</sub> are expressed by peripheral immune cells and glia, and pharmacologically targeting these receptors with a single intrathecal injection of specific agonists reverses established peripheral nerve injury-nociceptive hypersensitivity for more than 4 weeks<sup>114,115</sup>. This therapeutic effect is dependent on the activation of protein kinase A (PKA) and protein kinase C (PKC), leading to elevated IL-10 and decreased TNF levels. Without excluding a contribution from recruited or other resident cells, these pathways were specifically identified in resident microglia and astrocytes. In addition, ceftriaxone and glatiramer acetate may restore homeostasis either by

normalizing astrocyte expression of EAAT2 or by increasing the recruitment of IL-4-producing and IL-10-producing T cells to the spinal dorsal horn, respectively<sup>26,107</sup>.

Anti-inflammatory cytokine gene therapy with either naked DNA, or a range of vectors, is yet another approach<sup>142</sup>. IL-4 and IL-10 gene therapies have proven effective in models of peripheral inflammation and nerve injury, spinal cord injury, paclitaxel neuropathy and experimental encephalomyelitis<sup>42,142</sup>. Such efficacy is correlated with reduced levels of phosphorylated p38, TNF, IL-1 $\beta$  and PGE2 in spinal microglia, as well as decreased GFAP expression by astrocytes. Avoiding some of the safety issues associated with viral vectors, long-term reversal of peripheral nerve injury-induced nociceptive hypersensitivity has been achieved using synthetic polymer vectors to encapsulate and release the IL-10 transgene upon intrathecal injection<sup>142,143</sup>.

## Clinical evidence for the neuroimmune interface

Several lines of correlative and indirect evidence exist in support of glial cell involvement in clinical chronic pain conditions. In a post-mortem study, astrocyte but not microglial cell activation markers were upregulated in the dorsal spinal cords of patients with HIV who had been experiencing chronic pain, and astrocytes from these individuals also showed an increased density and hypertrophic morphology<sup>144</sup>. The patients also displayed increased phosphorylation of ERK, p38 and JNK, as well as elevated levels of TNF and IL-1 $\beta$ , in the dorsal spinal cord<sup>144</sup>. Another post-mortem study reported increased microgliosis and astrogliosis in the spinal cord grey matter of a patient with longstanding complex regional pain syndrome, although this study may have been confounded by the effects of prolonged opioid use by the patient<sup>145</sup> (BOX 3). However, these findings are supported by observations that the levels of IL-1 $\beta$  and IL-6 are increased in the cerebrospinal fluid of some patients with complex regional pain syndrome<sup>146</sup>. Clearly, there is a paucity of human data on pathological and pharmacological glial responses, as highlighted and discussed by several groups<sup>41,129,130</sup>. Endeavours to characterize the functional differences between rodent and human glia are of paramount importance to inform translational pharmacology.

P2X7R is expressed by all myeloid cells, including microglia, and single nucleotide polymorphisms in *P2RX7* that affect pore formation and channel function are associated with variability in chronic pain in two clinical populations<sup>62</sup>. Hyper- or hypo-functional variants were associated with increased or decreased pain ratings, respectively, in cohorts of patients with post-mastectomy and osteoarthritis pain compared to pain-free controls<sup>62</sup>. However, as impaired P2X7R function is also associated with reduced bone mineral content and density in mice, differences in pathology might also explain the correlation between osteoarthritis pain and *P2RX7* polymorphism<sup>147</sup>.

In a recent study in healthy volunteers, intravenous pretreatment with a low dose of endotoxin was shown to enhance flare, hyperalgesia and allodynia induced by intradermal capsaicin, and correlate with increased plasma levels of IL-6 (REF. 148). The observed large effect size and the fact that allodynia and hyperalgesia are centrally mediated, suggests the induction of central sensitization, probably due to an indirect effect of endotoxin mediated through a peripheral cytokine cascade.

As described above, microglia have been pharmacologically targeted in clinical chronic pain populations. A recent study showed that minocycline attenuated hyperalgesia in a small cohort of patients with sciatica<sup>128</sup>. Ibudilast also showed signs of efficacy in relieving pain associated with painful diabetic neuropathy and complex regional pain syndrome<sup>126</sup>. In contrast to these promising studies, propentofylline failed to improve self-reported pain scores, compared with placebo, in patients with postherpetic neuralgia<sup>129</sup>. Trials such as these have been undertaken on the assumption that possible functional differences between human and rodent glia would not limit the efficacy of drugs such as propentofylline in attenuating gliosis in humans. In addition to this assumption, several design flaws were inherent in the propentofylline trial, including selection of a pain syndrome for which no data exist to support glial cell involvement, and a lack of confirmation of whether the drug reached therapeutic concentrations at the target site. Furthermore, as preclinical studies indicate that it is easier to prevent than reverse neuropathic pain, propentofylline dosing may have been prematurely concluded before reversal of long-standing pain could have been achieved<sup>130</sup>. The CC-chemokine receptor 2 (CCR2) antagonist AZD2423 (AstraZeneca) showed a trend towards reduction in paroxysmal pain and paresthesia and dysesthesia, suggesting efficacy for some sensory components of pain<sup>149</sup>. As with many clinical trials for pain, intra- and inter-individual variability was high, which may be overcome by enriching for a specific subset of patients. Furthermore, target site efficacy was not evaluated. These issues further highlight the need to develop methods to enable detection of glial responses in humans.

Imaging may be one approach to detect real-time gliosis in patients with chronic pain. Although not completely specific for these cells, the peripheral benzodiazepine binding site is expressed by reactive microglia and allows them to be imaged in magnetic resonance imaging scans using the selective radioligand [<sup>11</sup>C](R)-PK11195. Binding of [<sup>11</sup>C](R)-PK11195 to microglia in the human thalamus is increased for many years after peripheral nerve injury<sup>150</sup>, however, further investigation is required to validate these findings.

Finally, it is worth noting that neuropathic pain is often comorbid with diseases in which dysregulation of central immune signalling is better established, such as multiple sclerosis and ALS<sup>151,152</sup>. In addition, depression, anxiety and drug abuse — common comorbidities of chronic pain — have a central immune basis<sup>153</sup>. These converging lines of evidence provide further indirect support for dysfunctional central immune signalling as a major contributor to chronic pain in clinical populations.

## Conclusions and future directions

More than two decades of research has seen the accumulation of a wealth of data supporting a major role for central immune signalling in preclinical models of pain, with microglia and astrocytes being among the best characterized members of the neuroimmune interface. However, future studies are likely to uncover prominent roles for other resident and infiltrating immunocompetent cells in the spinal dorsal horn. Although the early study of central immune signalling focused on the pro-nociceptive effects of reactive phenotypes and immune mediators, the field is beginning to appreciate pleiotropic roles for such ‘pro-inflammatory’ phenotypes, in addition to those of specialized anti-inflammatory reactive



phenotypes. Mirroring the general trend within preclinical pain research, most investigations have exclusively focused on the contribution of central immune signalling to the sensory component of pain. Given that patients with chronic pain experience many other symptoms, including disability, anxiety, depression, cognitive dysfunction and sleep loss (some of which can be preclinically modelled), investigation of the respective neuroimmune correlates will yield great insight.

The existence and precise mechanisms of central immune signalling in humans have not yet been conclusively established. A considerable challenge for the future is to determine whether pathological neuroimmune signalling contributes to chronic pain in clinical populations. However, clinical trials of therapeutics that target the neuroimmune interface are a possible approach. A further challenge for drug development is to integrate the basic scientific data on nuanced central immune signalling in order to develop agents that normalize pathological neuroimmune transmission, without altering neuroprotective and neuroregenerative pathways.

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## Glossary

### **Celsus**

Aulus Cornelius Celsus was a first century Roman encyclopaedist who gathered extensive writings from the Greek empire and translated them into Latin. In his great work *De Medicina*, he characterized the four cardinal signs of inflammation: heat, pain, swelling and redness.

### **Sickness response**

A defence mechanism triggered by the recognition of anything foreign to the host. An organized constellation of responses initiated by the immune system but co-ordinated and partially created by the brain, including physiological responses (for example, fever, increased sleep, hyperalgesia and allodynia), behavioural responses (for example, decreased social interaction, sexual activity, and food and water intake), and hormonal responses (increased release of classic hypothalamo–pituitary–adrenal and sympathetic hormones).

### **Hyperalgesia**

Increased pain from a stimulus that normally provokes pain. A form of nociceptive hypersensitivity.

### **Peripheral sensitization**

Increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields, such as that developing upon inflammation.

### **Glia**

Resident non-neuronal cells in the nervous system first described by Rudolf Virchow in 1856, that maintain the structural integrity of the nervous system, provide trophic support to neurons, insulate one neuron from another, and destroy pathogens and clear debris. Astrocytes and microglia are among the most thoroughly characterized glial cells and are immunocompetent.

**Allodynia**

Pain in response to a stimulus that does not normally provoke pain. A form of nociceptive hypersensitivity.

**Gliosis**

The transition from a role in physiological maintenance or surveillance to a reactive phenotype that is characterized by changes in cell number, morphology, phenotype, motility, protein expression and by the release of immunoregulatory products.

**Neuroimmune interface**

Proposed to describe the bidirectional, modulatory signalling between immune cells and neurons. We argue that such interfaces are formed in the central nervous system and can explain the sensory adaptations underlying pathological pain.

**Central immune signalling**

The process and consequences of immune mediator release by reactive immunocompetent cells in the central nervous system.

**Nociceptive pain**

Physiological pain produced by intense noxious stimuli that activate high-threshold nociceptor neurons.

**Inflammatory pain**

Occurs in response to tissue injury and the subsequent inflammatory response.

**First-order primary afferent neurons**

Sensory neurons the cell bodies of which lie in the trigeminal or spinal dorsal root ganglia, with projections that transmit nociceptive signals from the periphery (the peripheral terminal) to the spinal cord (the central terminal).

**Central synapses**

The synapses between first-order neurons, which form the presynaptic terminal, and nociceptive projection neurons, which form the postsynaptic terminal.

**Second-order nociceptive projection neurons**

Neurons projecting from the medullary dorsal horn or the superficial laminae of the spinal dorsal horn to the brainstem or thalamic nuclei.

**Spinal dorsal horn**

Two longitudinal subdivisions of grey matter in the posterior part of the spinal cord that receive terminals from afferent fibres originating from each side of the body that encode several types of sensory information, including nociception.

**Hindbrain**

Also known as the rhombencephalon. An area rostral to the spinal cord that gives rise to the cerebellum, pons and medulla.

**Nociception**

The neural process of encoding noxious mechanical, thermal and/or chemical stimuli that occurs in afferent fibres, which send signals to the central nervous system. Pain is expressed as the complex emotional and behavioural response to the central integration of nociceptive signals.

**Third-order neurons**

Sensory neuronal projections originating from thalamic nuclei.

**Central sensitization**

A period of facilitated transmission in nociceptive projection neurons that is characterized by a decreased activation threshold and an increased responsiveness to nociceptive stimuli.

**Windup**

Cumulative increases in membrane depolarization elicited by repeated C-fibre stimulation.

**Masseter**

A facial muscle that has a major role in chewing.

**Blood–CNS barrier**

A barrier formed by astrocyte end-feet and the tight junctions between endothelial cells lining blood vessels that excludes constituents of the systemic circulation from entry into the central nervous system (CNS). It forms an important boundary between the sensitive microenvironment of the CNS and the relatively volatile environment of the systemic circulation.

**Trigeminal sensory neurons**

Neurons found in the trigeminal nerve that mediate facial sensation and motor functions, including biting and chewing.

**Dorsal root ganglia (DRG)**

The cell bodies of sensory neurons are collected together in paired ganglia that lie alongside the spinal cord. Each cell body is encapsulated by satellite glia, with the entire ganglia being surrounded by a capsule of connective tissue and a perineurium.

**Brain-derived neurotrophic factor**

(BDNF). A neurotrophin expressed at high levels in the central nervous system that is vital for the growth and survival of neurons, and has been implicated in many forms of synaptic plasticity.

**C-fibres**

Small diameter, unmyelinated primary afferent sensory fibres, with small cell bodies in the dorsal root ganglion. Some C-fibres are mechanically insensitive, but most are polymodal, responding to noxious, thermal, mechanical and chemical stimuli.

**Lamina I neurons**

The most superficial aspect of the spinal dorsal horn from which second-order nociceptive projection neurons originate.

**Reversal potential**

The membrane potential at which chemical and electrical drive are equal and opposite, such that there is no net flow of ions across the membrane. The direction of flow reverses above and below this potential.

**Anti-allodynic and anti-hyperalgesic**

Compounds that oppose allodynia and hyperalgesia to restore basal sensory thresholds.

**Radiculopathy**

A condition arising from a compressed spinal nerve root that is associated with pain, numbness, tingling or weakness along the course of the nerve.

**Carpal tunnel syndrome**

A syndrome arising from compression of the median nerve — which runs from the forearm into the palm of the hand — that is associated with itching numbness, burning and/or tingling.

**Complex regional pain syndrome**

An idiopathic chronic pain condition that usually affects an arm or leg and typically develops after an injury, surgery, stroke or heart attack, but the pain is out of proportion to the severity of the initial injury, if any.

**Capsaicin**

The active component of chilli peppers. It selectively binds to transient receptor potential cation channel subfamily V member 1 (TRPV1) on nociceptive and heat-sensing neurons.

**Postherpetic neuralgia**

Neuropathic pain that occurs in some patients following infection with the varicella zoster virus, predominantly affecting the face.

**Peripheral benzodiazepine binding site**

GABA<sub>A</sub> receptors in the central nervous system (CNS) represent the primary site of action for benzodiazepines, such as diazepam. The peripheral benzodiazepine binding site was originally discovered in the periphery a secondary binding site, but has since been identified in the CNS.

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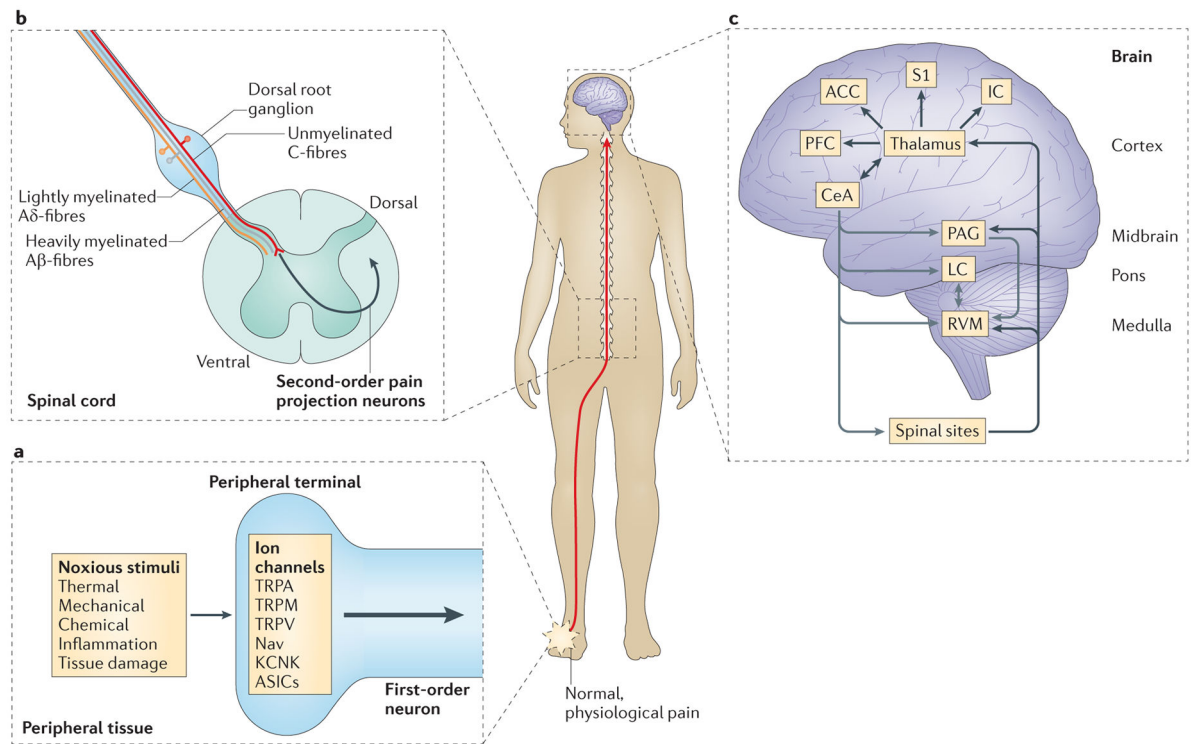
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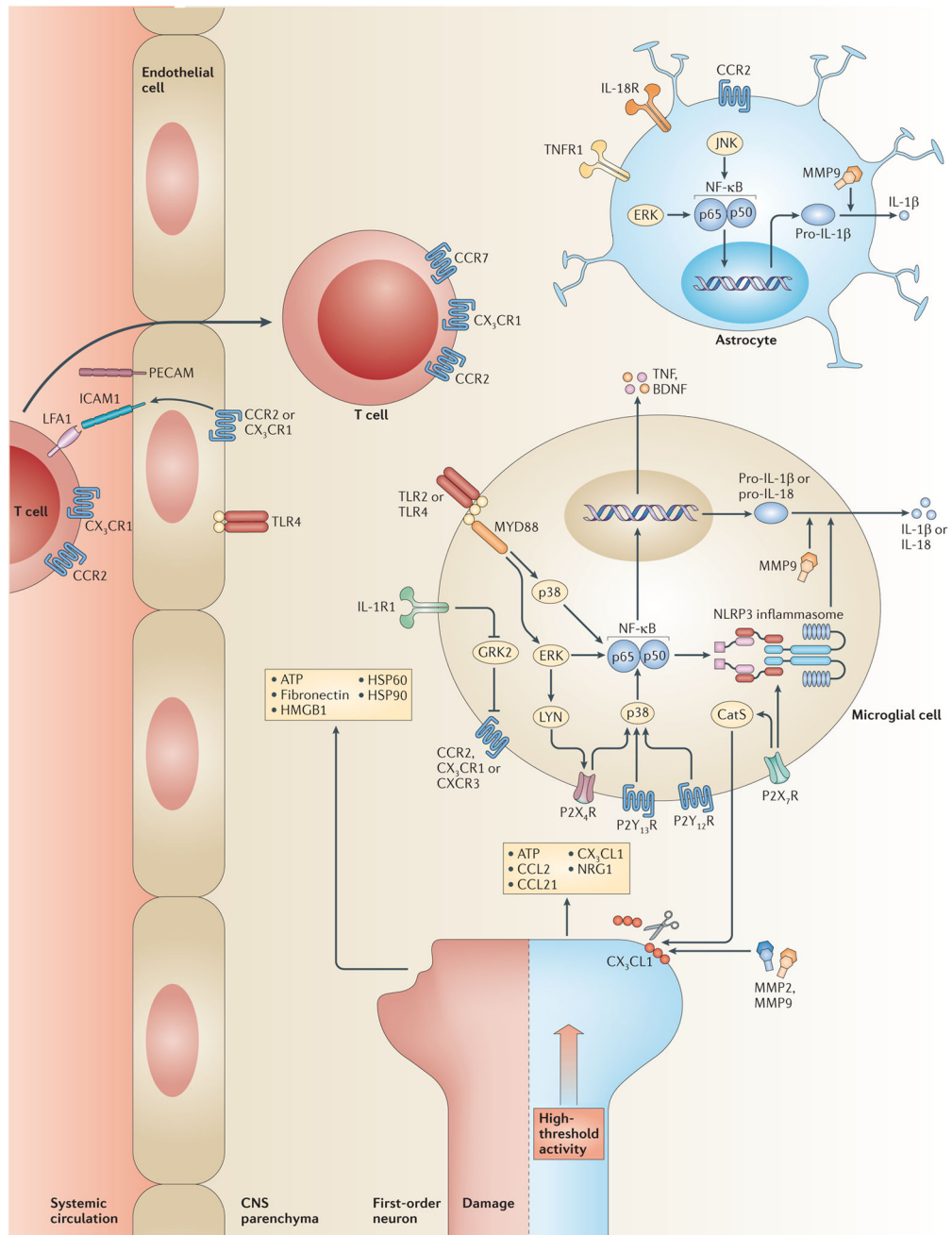
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### Figure 1. Physiological pain processing

**a** | Nociceptive signals are transmitted from the periphery by nociceptive sensory neurons (first-order primary afferent neurons) the peripheral terminals of which are clustered with ion channels, including transient receptor potential channel subtypes (TRPA, TRPM and TRPV), sodium channel isoforms (Nav), potassium channel subtypes (KCNK) and acid-sensing ion channels (ASICs). The transduction of external noxious stimuli is initiated by membrane depolarization due to the activation of these ion channels. **b** | Action potentials are conducted along the axons of nociceptive A $\beta$ - and C-fibres, through the cell body in the dorsal root ganglion to the axonal terminals, which form the presynaptic element of central synapses of the sensory pathway in the spinal dorsal horn or hindbrain. The central terminals of A $\beta$ - and C-fibres synapse with interneurons and second-order nociceptive projection neurons, primarily within the superficial laminae of the spinal dorsal horn. The axons of second-order nociceptive projection neurons decussate at the spinal cord level, joining the ascending fibres of the anterolateral system, and project to brainstem and thalamic nuclei, transferring information about the intensity and duration of peripheral noxious stimuli. **c** | No single brain region is essential for pain, but rather pain results from the activation of a distributed group of structures. Third-order neurons from the thalamus project to several cortical and subcortical regions (black arrows) that encode sensory-discriminative (for example, somatosensory cortex (S1)), emotional (for example, anterior cingulate cortex (ACC), amygdala (CeA) and insular cortex (IC)), and cognitive (for example, pre-frontal cortex (PFC)) aspects of pain. Several brainstem sites are also known to contribute to the descending modulation of pain (grey arrows) including the periaqueductal grey (PAG), locus coeruleus (LC) and rostral ventromedial medulla (RVM).

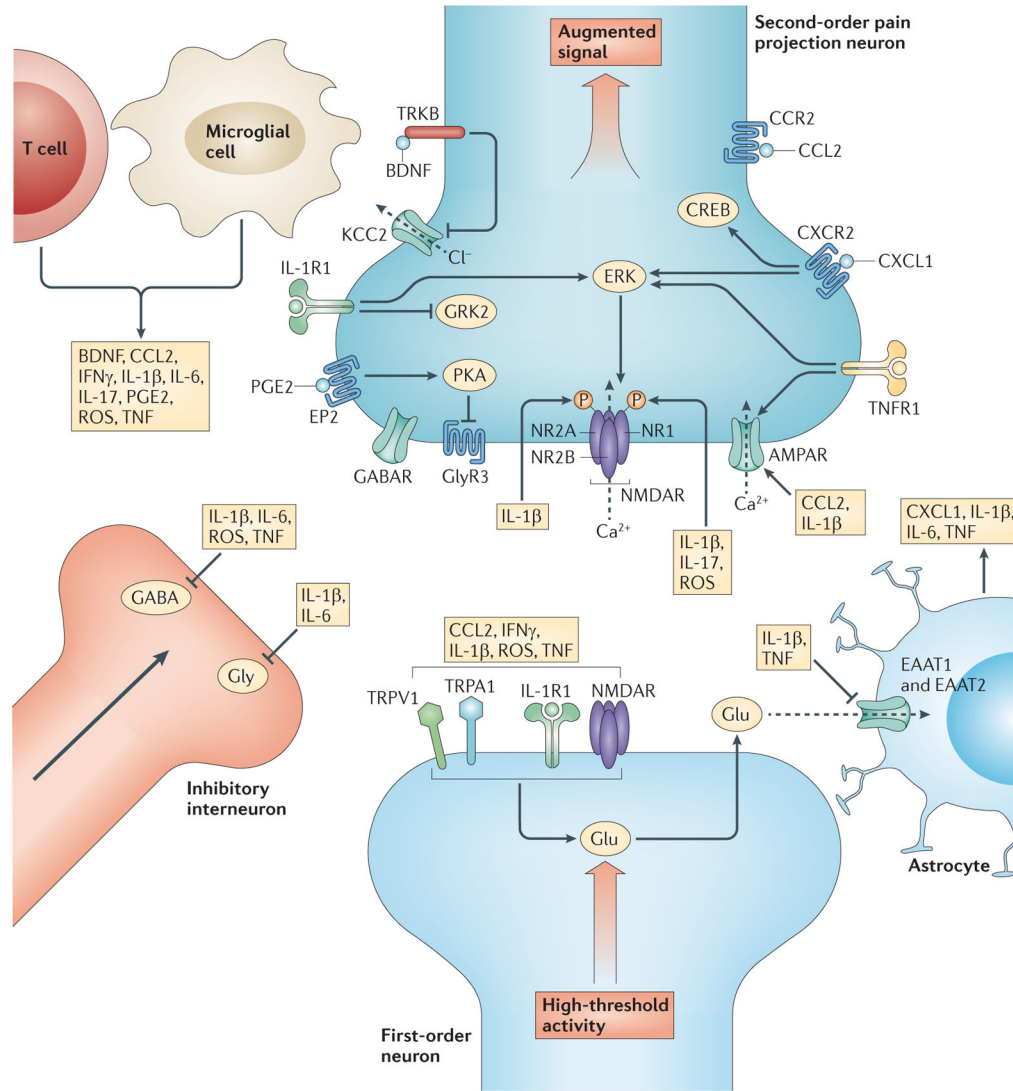


### Figure 2. Initiation of central immune signalling

Damage to or high-threshold activation of first-order neurons by noxious stimuli induces the release of ATP, CC-chemokine ligand 2 (CCL2), CCL21 and neuregulin 1 (NRG1), as well as endogenous danger signals to initiate central immune signalling in the dorsal horn of the spinal cord. CCL2 is released and rapidly upregulated by neurons following depolarization, and signals via its cognate receptor CC-chemokine receptor 2 (CCR2) on microglia. CX<sub>3</sub>C-chemokine ligand 1 (CX<sub>3</sub>CL1) is liberated from the neuronal membrane by matrix metalloproteinase 9 (MMP9) and MMP2 and by microglial cell-derived cathepsin S (CatS) and signals via CX<sub>3</sub>C-chemokine receptor 1 (CX<sub>3</sub>CR1), which is expressed by microglia.

CCR2 and CX<sub>3</sub>CR1 signalling also induces the expression of integrins (such as intercellular adhesion molecule 1 (ICAM1) and platelet endothelial cell adhesion molecule (PECAM1)) by endothelial cells, allowing the transendothelial migration of T cells. Neuronal cell release of CCL21 stimulates local microglia via CCR7, and infiltrating T cells are activated via CXCR3. ATP induces microgliosis via the purinergic receptors P2X<sub>3</sub>R, P2X<sub>7</sub>R, P2Y<sub>12</sub>R and P2Y<sub>13</sub>R. Microglia express Toll-like receptor 2 (TLR2) and TLR4 leading to activation of the MYD88 pathway after detection of endogenous danger signals, such as heat shock protein 60 (HSP60), HSP90, high mobility group box 1 (HMGB1) and fibronectin. Following the detection of such signals, many intracellular pathways are recruited including SRC family kinases (SRC, LYN and FYN), MAPKs (extracellular signal-regulated kinase (ERK), p38 and JUN N-terminal kinase (JNK)) and the inflammasome. This leads to phenotypic changes, increased cell motility and proliferation, altered receptor expression, the activation of transcription factors, such as nuclear factor- $\kappa$ B (NF- $\kappa$ B), and the production of inflammatory mediators (such as interleukin-1 $\beta$ , (IL-1 $\beta$ ), IL-18, tumour necrosis factor (TNF) and brain-derived neurotrophic factor (BDNF)). Interleukin-1 receptor (IL-1R1) signalling reduces microglial cell expression of G protein-coupled receptor kinase 2 (GRK2), which is a negative regulator of G protein-coupled receptors (GPCRs), including chemokine receptors. This leads to sustained GPCR signalling, and the exaggerated and prolonged production of pro-inflammatory mediators. The release of soluble mediators also provides a feedback mechanism by which further immune cells are activated.





**Figure 3. Neuroimmune enhancement of nociception**

Soluble mediators released by reactive immunocompetent cells in the central nervous system diffuse and bind to receptors on presynaptic and postsynaptic terminals in the spinal dorsal horn to modulate excitatory and inhibitory synaptic transmission, resulting in nociceptive hypersensitivity. Tumour necrosis factor (TNF), interleukin-1 $\beta$  (IL-1 $\beta$ ), CC-chemokine ligand 2 (CCL2), reactive oxygen species (ROS) and interferon- $\gamma$  (IFN $\gamma$ ) increase glutamate (Glu) release from central terminals, partly due to the activation of transient receptor potential channel subtypes (TRPV1 and TRPA1), and functional coupling between interleukin-1 receptor 1 (IL-1R1) and presynaptic ionotropic glutamate receptors (NMDAR). TNF, IL-1 $\beta$ , IL-6 and ROS decrease GABA ( $\gamma$ -aminobutyric acid) and glycine (Gly) release by inhibitory interneurons. IL-1 $\beta$  and CCL2 also increase AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid) signalling, and TNFR1 signalling increases the expression of Ca<sup>2+</sup>-permeable AMPA receptors (AMPARs). TNF increases NMDAR activity through the phosphorylation (P) of extracellular signal-regulated kinases (ERKs), while IL-17 and ROS induce phosphorylation of the NR1 receptor subunit in spinal cord

neurons. IL-1 $\beta$  increases the calcium permeability of NMDAR through activation of SRC family kinases and also via phosphorylation of the NR1, NR2A and NR2B subunits. CXCL1 signalling via CXCR2 induces rapid activation of neuronal ERK and CREB. Brain-derived neurotrophic factor (BDNF) signalling via TRKB downregulates the potassium-chloride cotransporter KCC2, increasing the intracellular Cl<sup>-</sup> concentration and weakening the inhibitory GABA<sub>A</sub> and glycine channel hyperpolarization of second-order nociceptive projection neurons. Prostaglandin E2 (PGE2) signalling at the EP2 receptor activates protein kinase A (PKA), thereby inhibiting glycinergic neurotransmission via GlyR3. IL-1R1 signalling reduces neuronal expression of G protein-coupled receptor kinase 2 (GRK2), a negative regulator of G protein-coupled receptors (GPCRs), including chemokine and prostaglandin receptors, leading to sustained neuronal GPCR signalling. TNF and IL-1 $\beta$  downregulate astrocyte expression of the Glu transporters excitatory amino acid transporter 1 (EAAT1) and EAAT2, leading to enhanced glutamatergic transmission. CCR2, CC-chemokine receptor 2; GABAR, GABA receptor.

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**Table 1**

## Modulators of the neuroimmune interface

Drug name	Cellular targets	Mechanism of action	Clinical use
Amitriptyline	Microglia	Disrupts TLR4 signalling by binding to MD2	Depression
ATL313 (Santen Pharmaceutical)	Microglia and astrocytes	<ul style="list-style-type: none"> <li>Adenosine A<sub>2A</sub> receptor agonist</li> <li>PKA and PKC activation</li> </ul>	NA
BAY 60–6583 (Bayer HealthCare)	Microglia and astrocytes	Adenosine A <sub>2B</sub> receptor agonist	NA
Ceftriaxone	Astrocytes	Increases EAAT2 expression by inhibiting NF- $\kappa$ B activity	<ul style="list-style-type: none"> <li>Bacterial meningitis</li> <li>Lyme disease</li> </ul>
Dilmapiomod	Microglia	Selective p38 MAPK inhibition	NA
FP-1	Microglia	TLR4 antagonist	NA
Glatiramer acetate	T cells	Promotes generation of anti-inflammatory T cell phenotypes	Multiple sclerosis
Ibudilast (MN-166; MediciNova)	Microglia, astrocytes and T cells	Non-selective phosphodiesterase inhibitor	<ul style="list-style-type: none"> <li>Asthma</li> <li>Post-stroke dizziness</li> </ul>
Methotrexate	T cells	Suppresses expression of cell adhesion molecules	<ul style="list-style-type: none"> <li>Breast cancer</li> <li>Rheumatoid arthritis</li> </ul>
Minocycline	Microglia, T cells and neurons	<ul style="list-style-type: none"> <li>Inhibits NF-<math>\kappa</math>B translocation to the nucleus</li> <li>Inhibits NFAT1</li> </ul>	Acne vulgaris
Paroxetine	Microglia	P2X <sub>4</sub> R antagonist	Depression
Propentofylline (Aventis Pharma)	Microglia, astrocytes and neurons	<ul style="list-style-type: none"> <li>Inhibits cAMP and cGMP phosphodiesterases</li> <li>Adenosine reuptake inhibitor</li> </ul>	Ischaemic stroke
Resolvin D1 (Resolvix Pharmaceuticals)	Microglia and neurons	<ul style="list-style-type: none"> <li>Attenuates pro-inflammatory cytokine release</li> <li>Inhibits TRPV1</li> <li>Reverses NMDAR subunit phosphorylation</li> </ul>	NA
Resolvin E1 (Resolvix Pharmaceuticals)	Microglia and neurons	<ul style="list-style-type: none"> <li>Attenuates pro-inflammatory cytokine release</li> <li>Inhibits TRPV1</li> <li>Attenuates glutamate release</li> <li>Reverses NMDAR subunit phosphorylation</li> </ul>	NA

Drug name	Cellular targets	Mechanism of action	Clinical use
Rifampin	Microglia	Disrupts TLR4 signalling by binding to MD2	Tuberculosis
(+)-naloxone	Microglia	Disrupts TLR4 signalling by binding to MD2	NA
(+)-naltrexone	Microglia	Disrupts TLR4 signalling by binding to MD2	NA

A<sub>2A</sub>, adenosine receptor 2A; A<sub>2B</sub>, adenosine receptor 2B; cAMP, cyclic AMP; cGMP, cyclic GMP; EAAT2, excitatory amino acid transporter 2; MAPK, mitogen-activated protein kinase; MD2, myeloid differentiation protein 2; NA, not applicable (no current clinical application); NFAT1, nuclear factor of activated T cells; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NMDAR, ionotropic glutamate receptor; P2X<sub>4</sub>R, P2X purinoceptor 4; PKA, protein kinase A; PKC, protein kinase C; TLR4, Toll-like receptor 4; TRPV1, transient receptor potential cation channel subfamily V member 1