Symposium/Mini-Symposium

The Neuroimmunology of Chronic Pain: From Rodents to Humans

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Chronic pain, encompassing conditions, such as low back pain, arthritis, persistent post-surgical pain, fibromyalgia, and neuropathic pain disorders, is highly prevalent but remains poorly treated. The vast majority of therapeutics are directed solely at neurons, despite the fact that signaling between immune cells, glia, and neurons is now recognized as indispensable for the initiation and maintenance of chronic pain. This review highlights recent advances in understanding fundamental neuroimmune signaling mechanisms and novel therapeutic targets in rodent models of chronic pain. We further discuss new technological developments to study, diagnose, and quantify neuroimmune contributions to chronic pain in patient populations.

Key words: sex differences; TSPO; Nrf2; biomarkers; Fc gamma receptors; cannabinoids

Introduction

Acute pain serves an adaptive purpose to warn the organism of actual or impending tissue injury. Noxious mechanical, thermal, and chemical stimuli activate primary sensory neurons that transmit nociceptive information to the spinal and medullary dorsal horns. Here, peripheral sensory input is integrated in a complex network of secondary nociceptive neurons that project to supraspinal sites, interneurons, and inhibitory descending projections from brainstem sites (Peirs and Seal, 2016). Secondary projection neurons synapse with tertiary neurons in thalamic and parabrachial nuclei that project to cortical and subcortical regions that encode and perceive pain (Peirs and Seal, 2016). Persistent activation or malfunction of this nociceptive system gives rise to chronic pain. While chronic pain is generally considered to be maladaptive, this concept has recently been challenged (Crook et al., 2014; Lister et al., 2020).

Chronic pain is a pervasive problem affecting ∼20% of adults in developed nations (Breivik et al., 2013; Fayaz et al., 2016; Dahlhamer, 2018; Australian Institute of Health and Welfare, 2020). While not fatal, such disorders remain poorly treated and account for the greatest societal burden of disability and disease (Institute for Health Metrics and Evaluation, 2017; James et al., 2018). Some chronic pain conditions, such as arthritis, are characterized by ongoing peripheral nociceptive input related to peripheral inflammation, whereas others, such as neuropathic pain, are a consequence of abnormal functioning of the nervous system because of injury or disease. Yet other chronic pain conditions, such as persistent postsurgical pain, fibromyalgia, and low back pain, may result from a combination of processes. All forms of chronic pain are believed to be maintained, to varying extents, by peripheral sensitization (increased responsiveness and reduced spiking threshold of peripheral nociceptive neurons to stimulation of their receptive fields) (International Association for the Study of Pain, 2017) and central sensitization (increased responsiveness of nociceptive neurons in the CNS to their normal or subthreshold afferent input) (International Association for the Study of Pain, 2017). Research over the past three decades has revealed that such sensitization is not solely the result of direct neuronal communication, but requires cross-talk between neurons, glia, and immune cells (for comprehensive review, see Beggs et al., 2012; Grace et al., 2014, 2016; McMahon et al., 2015; Ji et al., 2016; Kato et al., 2016; Inoue and Tsuda, 2018; Haight et al., 2019; Malcangio, 2019). The field of pain neuroimmunology has nearly tripled its share of all pain research over the past 20 years (Fig. 1). This growth has been led in part by work published in the *Journal of Neuroscience* (Table
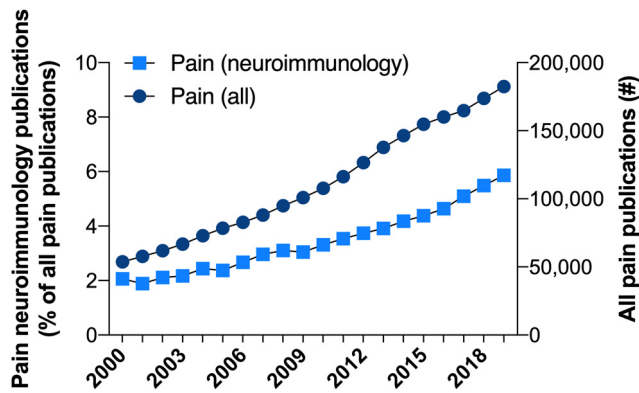
Figure 1. Annual publications on pain and pain neuroimmunology (2000-2019). Total numbers of publications related to pain (pain OR hyperalgesia OR allodynic OR allodynia OR hypernociception OR hypernociceptive OR nociception OR neuropathic) and pain neuroimmunology ((pain OR hyperalgesia OR allodynic OR allodynia OR hypernociception OR hypernociceptive OR nociception OR neuropathic) AND (astrocyte OR astrocytic OR astroglia OR microglia OR oligodendrocyte OR neuroimmune OR neuroimmunology OR neuroinflammation)), between 2000 and 2019, were tabulated from Dimensions using a PubMed limited filter. The pain neuroimmunology publications are expressed as a percentage of the total number of publications related to pain.

Table 1. Top 25 research journals publishing articles related to pain neuroimmunology (2000–2019)[a]

Name	Publications	Average citation rate
Journal of Neuroscience	952	81.05
Scientific Reports	884	13.19
Pain	723	52.71
Experimental Neurology	704	39.29
Brain, Behavior, and Immunity	659	42.19
Journal of Neuroinflammation	651	30.37
British Journal of Pharmacology	488	65.59
Molecular Neurobiology	463	24.00
Neurology	409	52.59
Glia	404	51.09
Frontiers in Pharmacology	394	17.13
Journal of Neuroscience Research	383	29.34
Neurobiology of Disease	320	44.33
Anesthesiology	304	39.79
Annals of Neurology	294	78.81
Proceedings of the National Academy of Sciences of the United States of America	286	81.93
Progress in Neurobiology	269	124.14
Journal of Pain	252	39.74
Frontiers in Immunology	249	26.68
Anesthesia and Analgesia	248	24.37
Pharmacological Research	219	32.11
Journal of Physiology	210	48.62
Neuron	203	142.13
FASEB Journal	201	62.19
Brain	195	95.65

[a]Research journals ranked in the top quartile(s) of their categories are included (web of science). Search terms are provided in Figure 1.

1). In this review, we summarize recent and select advances that will be covered in a mini-symposium on the neuroimmunology of chronic pain.

Immune phenotyping after injury: a focus on sex differences

Orthopedic injury or surgery presents a unique challenge because the initiating event may result in polytrauma to muscle, bone, and nerves (Beswick et al., 2012; Mehta et al., 2015).

Moreover, it is estimated that each year in the United States 100,000 bone fractures heal poorly. Accordingly, persistent limb pain is common after such injuries. Because high levels of acute pain increase the risk of developing chronic pain (Hah et al., 2019), likely because of phenomena, such as hyperalgesic priming of primary afferent neurons (Parada et al., 2003; Reichling and Levine, 2009), effective treatment to improve recovery from injury is imperative. Unfortunately, the key components of the multicellular response to injury and how these components can be manipulated to improve outcomes remain unclear (Kehlet and Dahl, 2003).

One important contributor to the development of chronic pain after injury is activation of the immune system. Peripheral injury mobilizes both innate and adaptive branches of the immune system to resolve tissue damage, but persistent immune activation can be detrimental and contribute to delayed healing (Grace et al., 2014; Loi et al., 2016). Notably, immune-system contributions to chronic pain may differ in males and females. Indeed, males are more susceptible to infection from diverse pathogens, reflecting in part a hypoactivation of the innate immune system in males (vom Steeg and Klein, 2016). In contrast, women have a higher prevalence of autoimmune diseases resulting from inappropriate activation of the adaptive immune system (Jacobson et al., 1997). It is therefore crucial to understand sex differences in postinjury immune responses that may result in increased vulnerability to chronic pain, and may carry important treatment implications.

There are some indications from the literature that there are sex-specific immune mechanisms contributing to chronic pain. For example, several studies have highlighted that microglia, the innate immune cells of the CNS, may only maintain pain in males (Sorge et al., 2011, 2015; Agalave et al., 2020). However, not all groups have observed this sexual dimorphism (Peng et al., 2016). Moreover, peripheral macrophage inhibition seems to specifically reverse pain behaviors in male, but not female, mice (Rudjito et al., 2020), suggesting that the immune contribution to pain is likely cell- and location-specific (Lopes et al., 2017).

To evaluate sex differences more comprehensively in the whole-system immune response to injury and its relation to pain progression, Tawfik et al. (2020b) recently characterized a mouse model of orthopedic trauma. The model consists of unilateral tibial fracture with internal fixation and associated injury to the tibialis anterior muscle. These mice exhibit mechanical hypersensitivity in the hindpaw that lasts for >5 weeks after injury, making it an appropriate model for studying pain mechanisms. High-dimensional immune profiling was performed at various time points using cytometry by time-of-flight mass spectrometry (Tawfik et al., 2020a). Intracellular signaling pathways in 21 immune cells spanning all major innate and adaptive cell types, as well as individual cell type frequency were assessed, for a total identification of 273 unique immune features. In order to probe this complex, high-parameter dataset, we performed multivariate modeling of the innate and adaptive immune cell responses using a regression method that minimized false positives (Tawfik et al., 2020a). Results suggested that males and females exhibit unique immune profiles: females had a greater neutrophil and dampened T regulatory cell response in the acute postinjury period, as well as heightened CD4 T memory cell mitogen-activated protein kinase responses in the subacute postinjury period (Tawfik et al., 2020a). T regulatory cells function as a brake on the immune system, specifically limiting autoimmune reactions (Sharma and Rudra, 2018), whereas CD4 T memory cells become activated after antigen presentation and ensure a more
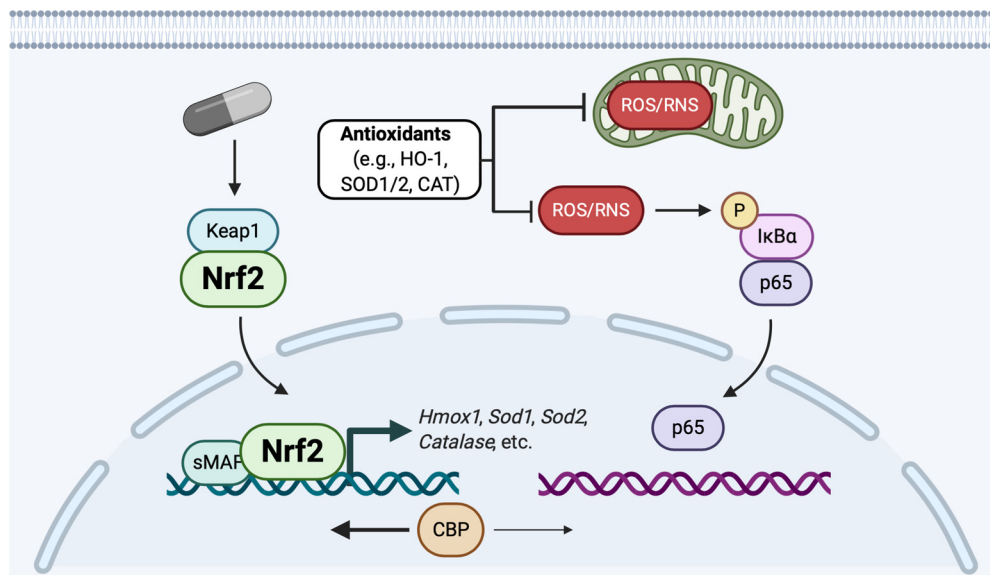
Figure 2. Nrf2 activation alleviates nitro-oxidative stress and neuroinflammation. Pharmacological agents, such as dimethyl fumarate and sulforaphane, can induce nuclear translocation of Nrf2 by disrupting its cytosolic complex with Kelch-like ECH-associated protein 1 (Keap1). Nrf2 binds to DNA, aided by small musculoaponeurotic fibrosarcoma (sMAF) proteins, increasing expression of a suite of antioxidant genes, for example, those encoding heme oxygenase-1 (HO-1), superoxide dismutase (SOD) 1 and 2, catalase (CAT), and others. Antioxidants scavenge ROS/RNS that otherwise facilitate IκBα phosphorylation, inducing NFκB-dependent proinflammatory gene expression. NFκB p65 subunit-DNA binding is further prevented through competition with Nrf2 for CREB-binding protein (CBP). Antioxidants also scavenge ROS/RNS generated by dysfunctional mitochondria. Although not depicted, antioxidants further reduce neuroinflammation by scavenging ROS/RNS that otherwise activate mitogen activated protein kinases.

efficient secondary immune response (Gasper et al., 2014). In combination, these findings indicate an enhanced adaptive immune response to injury in females, with a specific role for T-cell subsets.

A recent comprehensive review highlights several studies in both mice and humans that support a contribution of T cells to pain (Laumet et al., 2019). The causative role of T-cell subsets in sex-specific responses to injury, and ultimately vulnerability to chronic pain, has been investigated in one prior preclinical study (Sorge et al., 2015). In this study, the PPARγ agonist pioglitazone reversed nerve injury-induced pain behaviors only in females, presumably through suppression of interferon-γ, a cytokine produced in greater amounts by female T cells (Zhang et al., 2012). In contrast, other groups have found that pioglitazone attenuates neuropathic pain behaviors in males (Griggs et al., 2015; Lyons et al., 2017; Khasabova et al., 2019). Therefore, this remains an area of active research.

The details of the immune response to injury and how it contributes to the transition from acute to chronic pain are a complex and exciting area for future inquiry. As such research moves from preclinical to clinical studies, future results will open new avenues for the exploration of sex-specific treatment paradigms for patients with chronic pain.

Regulatory role of nuclear factor erythroid 2-related factor 2 (Nrf2) in neuroimmune–nitro-oxidative stress interactions
Neuroinflammatory signaling is intertwined with other pathologic processes underlying neuropathic pain, including overproduction of reactive oxygen and nitrogen species (ROS/RNS) (nitro-oxidative stress) and mitochondrial dysfunction (Salvemini et al., 2011; Janes et al., 2012; Little et al., 2012; Bennett et al., 2014; Symons-Liguori et al., 2016). For example, danger-associated molecular patterns can activate pattern recognition receptors, such as Toll-like receptors, to drive transcription of inducible nitric oxide synthase and activation of NAPDH oxidases that produce nitric oxide and ROS (Grace et al., 2016; Kato et al., 2016; Lacagnina et al.,

2018). Reciprocally, ROS/RNS induce expression of proinflammatory mediators by activating mitogen-activated protein kinases and nuclear factor κB (NFκB), both directly and via receptors like TRPM2 expressed by glia and leukocytes (Grace et al., 2016). Injury-induced ROS/RNS can damage mitochondria in nociceptive pathways, causing the organelles to leak ROS and danger-associated molecular patterns that activate inflammasomes and Toll-like receptors (Grace et al., 2016, 2018; Kato et al., 2016; Próchnicki et al., 2016; Lacagnina et al., 2018). Collectively, inflammatory mediators and ROS/RNS promote sensitization through direct activation of neuronal ion channels, as well as neuromodulation and dysfunctional synaptic plasticity via well-characterized mechanisms, described previously in detail (Salvemini et al., 2011; Beggs et al., 2012; Grace et al., 2014, 2016; McMahon et al., 2015; Ji et al., 2016; Inoue and Tsuda, 2018; Haight et al., 2019; Malcangio, 2019). Simultaneously resolving neuroinflammation and nitro-oxidative stress could be an improved strategy for relief of neuropathic pain.

Nrf2, also known as NFE2L2, is a potential therapeutic target to alleviate neuroinflammation and nitro-oxidative stress (Fig. 2). The transcription factor increases expression of a suite of antioxidant and cyto-protective genes in response to oxidants (Dodson et al., 2018; Cuadrado et al., 2019). Subsequent detoxification of ROS/RNS reduces downstream inflammatory signaling. In addition, Nrf2 exerts direct anti-inflammatory actions by attenuating NFκB activity (Wardyn et al., 2015). While this endogenous regulator normally buffers nitro-oxidative stress, for unknown reasons, the Nrf2 pathway fails to adequately detoxify pathologic levels of ROS/RNS after injury.

Notably, there is evidence that pharmacological activation of Nrf2 can alleviate neuropathic pain in preclinical models. For example, using a model of peripheral nerve injury, Grace and colleagues recently showed that dimethyl fumarate reverses allodynia and hyperalgesia in reflex and operant assays (Li et al., 2020). At the same time, dimethyl fumarate induced nuclear translocation (activation) of Nrf2 in the DRGs, which contains

the cell bodies of sensory neurons, and increased expression of antioxidant target genes and enzyme activity (superoxide dismutase, glutathione) (Li et al., 2020). Confirming Nrf2 as a major therapeutic target in both sexes, the antinociceptive effects of dimethyl fumarate were lost in male and female $Nrf2^{-/-}$ mice or when dimethyl fumarate was coadministered with the Nrf2 inhibitor trigonelline (Li et al., 2020). These results complement previous work showing another well-known Nrf2 activator, sulforaphane, both prevented and dose-dependently reversed mechanical allodynia and thermal hyperalgesia induced by peripheral nerve injury or the chemotherapy, oxaliplatin (Kim et al., 2010; C. Wang and Wang, 2017; Ferreira-Chamorro et al., 2018; Yang et al., 2018), and that the antinociceptive efficacy of sulforaphane was lost in $Nrf2^{-/-}$ mice (Yang et al., 2018).

Nrf2 activators may alleviate neuropathic pain by normalizing mitochondrial and microglial function. Dimethyl fumarate and sulforaphane reversed mitochondrial dysfunction in sensory neurons caused by peripheral nerve injury and by the chemotherapeutic agent, oxaliplatin, respectively (Yang et al., 2018; Li et al., 2020). While the mechanisms linking mitochondrial damage to increased neuronal excitability or spontaneous activity are not yet fully understood, normalizing mitochondrial function attenuates evoked and ongoing pain (Bennett et al., 2014). Dimethyl fumarate and sulforaphane also attenuated injury-induced microglial activation and increases in proinflammatory cytokines and ROS in the pain neuraxis (Kim et al., 2010; C. Wang and Wang, 2017; Ferreira-Chamorro et al., 2018; Li et al., 2020). These pharmacological data from several groups indicate that Nrf2 activation alleviates neuropathic pain and underlying mechanisms in preclinical models.

The protective role of Nrf2 in neuropathic pain is an emerging area of investigation, with several questions to be addressed in future studies. Evidence is mixed regarding whether Nrf2 is appreciably activated after peripheral nerve injury (Yang et al., 2018; Li et al., 2020). However, Nrf2 still serves a protective role, as mechanical allodynia induced by oxaliplatin treatment was exacerbated in $Nrf2^{-/-}$ mice compared with WT mice (Yang et al., 2018). Spatiotemporal analysis of the endogenous role of Nrf2 after injury is still required. The cells for whom Nrf2 activation is protective, and the neuroanatomical locations in which they reside, are still to be identified. Such investigation could support Nrf2 as a therapeutic target, as it sits at the nexus of several major mechanisms that underlie neuropathic pain.

Contributions of autoantibodies and neuronal Fcγ receptors to joint pain in arthritis

Pain is one of the most problematic symptoms for patients with rheumatoid arthritis (RA). Pain in RA has traditionally been attributed to the inflammatory process in the joint, but it is becoming increasingly clear that other mechanisms are also at play. During the period immediately before diagnosis, individuals frequently suffer from joint pain, often without signs of joint inflammation (de Hair et al., 2014). Furthermore, pain still persists in a sizable proportion of RA patients for whom other RA symptoms, including joint inflammation, are medically controlled (Taylor et al., 2010). Thus, joint pain uncoupled from apparent disease activity is a pervasive problem and represents a fundamental gap in our mechanistic understanding of pain in autoimmune disorders.

A joint pathology similar to human RA can be induced in rodents by immunizing animals with collagen Type II, a structural protein mainly found in articular cartilage, or by transferring monoclonal anti-collagen II antibodies (Holmdahl et al., 1986; Terato et al., 1992; Lindh et al., 2014). Using the collagen-antibody-induced arthritis model (Nandakumar et al., 2003), Svensson and colleagues observed that pain-related behaviors develop before any signs of joint inflammation and remains for weeks after the inflammation has subsided (Bas et al., 2012; Agalave et al., 2014; Su et al., 2015). Other antibodies binding to cartilage, such as cartilage oligomeric matrix protein, were also found to elicit mechanical hypersensitivity uncoupled from visual, histologic, and molecular indications of inflammation in mice (Bersellini Farinotti et al., 2019). Because cartilage is not innervated, the anti-cartilage antibodies must act on other targets to mediate pronociceptive effects in the pre-inflammatory stage. Mice lacking functional complement 5 or treated with a complement 5 receptor antagonist still developed pain behaviors induced by anti-collagen II antibody. This suggests that cartilage-antibody-induced pain-related behaviors do not depend on joint inflammation or complement 5 (and thereby terminal/lytic complement), but instead on tissue antigen recognition, local immune-complex formation, and activation of neuronally expressed Fcγ receptors.

Fcγ receptors are bound by immunoglobulin G (IgG) antibody and may activate (e.g., Fcγ receptors I, III, and IV) or inhibit (e.g., Fcγ receptor IIb) cells based on the cytoplasmic tyrosine-based motif associated with the receptor. Recent work from several groups has demonstrated that nociceptors express Fcγ receptor I that are activated by IgG after they have bound antigen and formed an antibody-antigen complex (immune complex) (Qu et al., 2011, 2012; L. Wang et al., 2019). Svensson and colleagues discovered that not only the activating Fcγ receptor I, but also the inhibitory Fcγ receptor IIb, are present in the peripheral terminals of primary afferents in uninjured mice. Focusing on the phase before inflammation, preformed collagen II antibody-antigen immune complexes directly activated cultured DRG WT neurons, but not neurons lacking activating FcγRs. In line with this observation, anti-collagen II antibodies and collagen II immune complexes did not induce mechanical hypersensitivity in Fc receptor γ-chain$^{-/-}$ mice (which lack cell surface expression and signaling of all activating Fcγ receptors), or mice lacking activating Fcγ receptors in neurons. Furthermore, anti-collagen II antibodies that retain their ability to bind collagen II but either lack the Fc region or have a reduced affinity for Fcγ receptors were not pronociceptive, indicating that the Fc-Fcγ receptor interaction is critical for development of collagen II antibody-induced pain-related behaviors. Fcγ receptor III and IV deficiency did not prevent collagen II antibody responses, which supports an important role of Fcγ receptor I in the direct action of immune complexes on nociceptors. It is unlikely that Fcγ receptor IIb is coupled to enhancement of neuronal excitability because Fc receptor γ-chain deficient mice were protected against the pronociceptive actions of cartilage-associated antibodies despite expression of Fcγ receptor IIb (Bersellini Farinotti et al., 2019). Nevertheless, the presence of Fcγ receptor IIb in sensory neurons is interesting and warrants further investigation, as the receptor could be linked to inhibitory mechanisms in neurons during established disease.

In summary, studies from Svensson and colleagues show that cartilage antibody immune complexes, which are highly correlated with early RA and joint pathology, serve as key triggers for pain behavior in the early phase of the disease via activation Fcγ receptor I on nociceptors, without generating histologic or biochemical signs of inflammation. These studies point to a functional coupling between autoantibodies and pain transmission, which may be at play before and subsequent to flares of active disease. Together, the identification of novel contributions of

autoantibodies to persistent pain may aid in the development of new treatment strategies, not only for pain in RA, but also for pain in other diseases associated with autoantibody production, such as Sjögren's syndrome, systemic lupus erythematosus, and Guillain–Barré syndrome.

Cannabinoid receptor Type 1 (CB1R) as a neuroimmune therapeutic target for chronic pain

Preclinical studies in the neuroimmunology of chronic pain have revealed numerous potential therapeutic targets. Among these, CB1R has been identified as a viable candidate in controlling pain and inflammation (Kunos et al., 2009; Milligan et al., 2020). Global CB1R KO studies have demonstrated the necessity of CB1R action for both endogenous and therapeutically induced pain inhibition (Sideris et al., 2016; Bajic et al., 2018). Although these studies have established the analgesic function of CB1R, they have failed to distinguish the most critical loci for analgesic action. CB1R is one of the most abundantly expressed GPCRs in the entire nervous system, and studies are mixed regarding the loci of CB1R analgesic action (Martin et al., 1995; Fox et al., 2001; Meng and Johansen, 2004; Agarwal et al., 2007; Pernía-Andrade et al., 2009; Skaper and Di Marzo, 2012; Lu and Mackie, 2016). Indeed, the function of cannabinoids is governed by their site of action, and overlapping expression patterns in the nervous system make it difficult to tease out the analgesic, metabolic, and psychoactive sites of actions of cannabinoids.

How CB1R specifically modulates pain processing is an ongoing area of study and there is intense debate regarding whether peripheral or CNS activation of CB1R is more critical to produce analgesic effects (Milligan et al., 2020). In the periphery, CB1R is expressed on DRG neurons and various other cell types (Mackie, 2005; Ständer et al., 2005), while in the CNS, GABAergic interneurons in the dorsal horn of the spinal cord, brainstem, and amygdala are putative sites of CB1R action (Navarrete et al., 2020). While sensory neurons have the capacity to powerfully regulate immune responses (Pinho-Ribeiro et al., 2017), it is unclear whether CB1R expression by sensory neurons or macrophages regulates inflammation after injury (Amaya et al., 2006). This is an important distinction, as the anti-inflammatory effects of CB1R through expression on immune cells is a recent finding (Cinar et al., 2017; Jourdan et al., 2017; Joffre et al., 2020).

The identity of the peripheral cell that is responsible for immune modulation is a major source of contention in translational studies (Kunos et al., 2009; Pacher and Kunos, 2013). Despite the fact that cannabinoids have a long history in medicinal use, major drawbacks, including psychotropic effects, have led to extended lines of enquiry aimed at parsing central effects from peripheral analgesic actions (Pacher et al., 2006). Interestingly, the debate has led to the development of peripherally restricted cannabinoids, designed to circumvent various unwanted central effects (Seltzman et al., 2016). However, peripherally restricted synthetic CB1R agonists have not produced analgesia in clinical trials (Kalliomäki et al., 2013a,b). To date, the outcome of clinical and preclinical studies is mixed with an overwhelming majority of clinical trials of cannabinoid agonists failing because of analgesic efficacy, kidney damage, and absence of longitudinal data (Kunos et al., 2009; Kunos and Tam, 2011; Pacher and Kunos, 2013; Finnerup et al., 2015). Identifying the CB1R cell types that mediate the analgesic actions of cannabinoids is imperative to realizing the anti-inflammatory and analgesic potential of CB1R agonists (DeMarco and Nunamaker, 2019).

Neuroinflammation in human chronic pain states

Despite a large preclinical literature demonstrating a key role of neuroinflammation in the CNS in animal pain models (glial activation and attendant production of proinflammatory mediators) (Beggs et al., 2012; Grace et al., 2014; McMahon et al., 2015; Ji et al., 2016; Inoue and Tsuda, 2018; Malcangio, 2019), the role of neuroinflammation in human pain is still unknown. Sampling of CSF and analysis of postmortem spinal cord samples suggest that glial activation may occur in patients with various types of chronic pain (Brisby et al., 1999; Del Valle et al., 2009; Kadetoff et al., 2012; Shi et al., 2012; Kosek et al., 2015; Bäckryd et al., 2017), but the ability to "visualize" neuroinflammation in living patients long remained elusive. In the last few years, the use of PET with radioligands targeting the 18 kDa translocator protein (TSPO) has begun to fill this gap. TSPO is a five-transmembrane domain protein expressed on mitochondria (Papadopoulos et al., 2006) and thus is found in cells in addition to glia (Batarseh and Papadopoulos, 2010; Wei et al., 2013). Nevertheless, TSPO can serve as a marker of neuroinflammation because this protein, for reasons that are not fully understood, is dramatically upregulated in activated microglia and astrocytes. Indeed, a strong colocalization between TSPO upregulation and activated glial cells has been found across multiple preclinical and human studies of various disorders, including neurodegeneration. Hence, TSPO is intensively used to image neuroinflammation (Banati et al., 2000; Ji et al., 2008; Cosenza-Nashat et al., 2009; Alshikho et al., 2018; Lois et al., 2018; Barletta et al., 2020).

In preclinical studies of arthritis, complex regional pain syndrome, and lumbar radiculopathy, TSPO was upregulated concomitantly with glial activation (Hernstadt et al., 2009; Wei et al., 2013; Cropper et al., 2019; Guilarte, 2019), supporting the use of TSPO as a marker of glial activation. Using a second-generation TSPO radioligand ([^{11}C]PBR28), Loggia et al. have demonstrated increased TSPO signal in the brains of patients with chronic low back pain, fibromyalgia, migraine and veterans suffering from Gulf War Illness, as well as in the spinal cord of patients with lumbar radiculopathy (Fig. 3) (Loggia et al., 2015; Albrecht et al., 2018, 2019a,c; Alshelh et al., 2020). The signal appears to exhibit specific spatial distribution across disorders. For instance, thalamic signal elevation is the most consistent finding for chronic low back pain (an observation that was recently replicated in an independent cohort) (Torrado-Carvajal et al., 2020), whereas cortical regions are mainly involved in other conditions, such as fibromyalgia (Loggia et al., 2015; Albrecht et al., 2019a). Furthermore, along with the primary somatosensory cortex (S1), the TSPO signal was elevated in the lumbar spine cortical representation in chronic low back pain, in a ventrolateral aspect of S1 compatible with the face area in migraine, and in a large portion of the sensorimotor strip in patients suffering from widespread body pain (fibromyalgia) (Loggia et al., 2015; Albrecht et al., 2019a,c). Together, these observations suggest that neuroinflammation, as assessed by TSPO signal elevation (1) might be a pervasive phenomenon observed across multiple, etiologically heterogeneous human pain disorders and (2) might present itself in disorder-specific spatial distributions, paralleling the specific body distribution of the pain experienced by each patient group.

Certainly, the biological and clinical significance of the observed TSPO signal elevations in chronic pain disorders remains to be elucidated. First, while this signal might correlate "spatially" with the body distribution of pain disorders, to date, the relationship between TSPO signal elevations and disorder severity has been inconsistent. For instance, brain TSPO signal was found to be positively correlated with frequency of migraine attacks in migraineurs, with fatigue severity in patients with
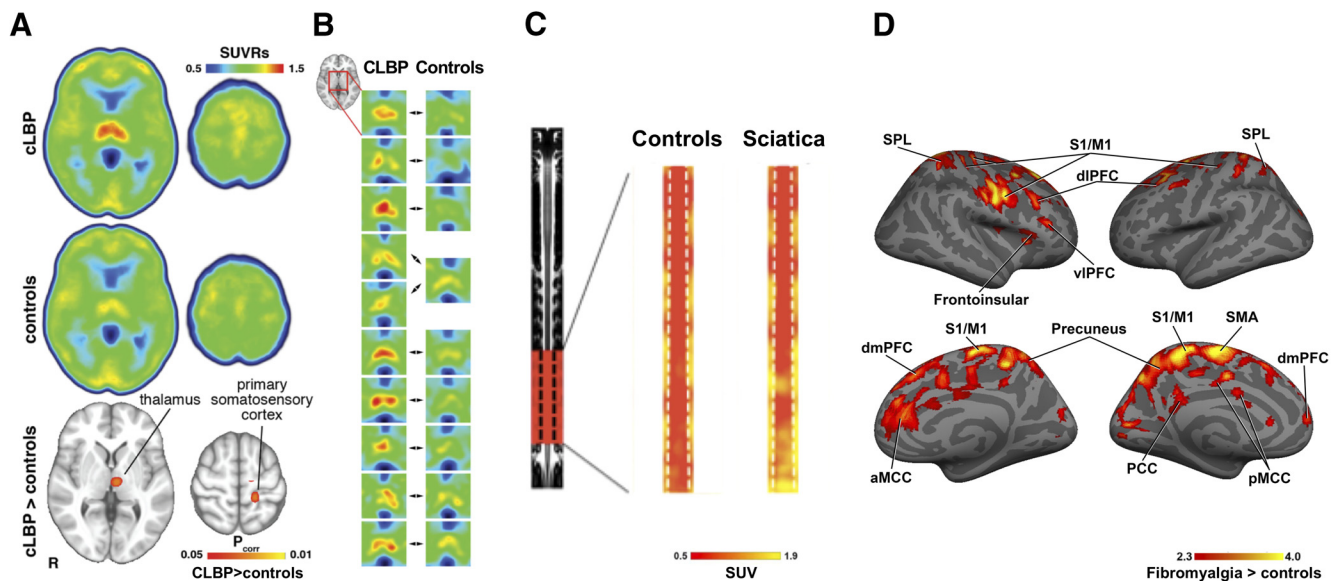
Figure 3. TSPO ([^{11}C]PBR28) signal increases in chronic pain patients. **A**, Brain TSPO signal elevation in chronic low back pain (cLBP) patients (median images and group comparison). **B**, Individual data showing consistently higher thalamic [^{11}C]PBR28 signal in patients, compared with sex-, age-, and binding affinity-matched controls (Loggia et al., 2015). **C**, [^{11}C]PBR28 signal elevations in the lower spinal cord segments in patients with radicular LBP ("Pain target") compared with reference region ("Pain reference") and healthy controls ("Control") (Albrecht et al., 2018). **D**, [^{11}C]PBR28 signal elevation in patients with fibromyalgia (Albrecht et al., 2019a).

fibromyalgia, and with depressive scores in chronic low back pain patients with comorbid negative affect (Albrecht et al., 2019a,b,c). At the same time, this signal was reported to be either not associated with, or even inversely related to, pain severity in chronic low back pain and arthritis (Loggia et al., 2015; Forsberg et al., 2019). Moreover, while TSPO upregulation in neuroinflammatory responses consistently colocalizes with microglia, an accompanying astrocytic component has been observed in some cases (Rupprecht et al., 2010; Wei et al., 2013; Liu et al., 2016). Furthermore, TSPO does not appear to differentiate immune phenotypes (i.e., proinflammatory vs anti-inflammatory), although some evidence suggests that TSPO upregulation may favor the resolution of neuroinflammation, possibly through the stimulation of steroidogenesis (Batarseh and Papadopoulos, 2010; Wei et al., 2013; Bae et al., 2014; M. Wang et al., 2014).

While many questions remain to be answered, a growing literature nonetheless suggests that neuroinflammation occurs in clinical chronic pain states, adding further weight to the preclinical support of glial modulation as a therapeutic strategy. Importantly, because pain-related TSPO upregulation has been described in both human and preclinical pain studies, the study of this protein potentially offers major reverse-translational opportunities, whereby human imaging results can inform mechanistic evaluations of the role of TSPO in animals. These approaches, together with the development of novel radioligands targeting more specific immune cell subtypes and phenotypes (Narayanaswami et al., 2018), are likely to lead to significant advances in our understanding of the role of neuroinflammation in human chronic pain.

Development of neuroimmune biomarkers of pain
Chronic pain has a complex, multisystem etiology, involving interactions between genes and environment. The advent of precision medicine that allows personalization of treatments in fields, such as cancer based on mechanistic biomarkers of complex phenotypes, have not yet been applied to the treatment of

Table 2. Ratio of publications to patents and clinical trials for pain neuroimmunology and pain (2000–2019)[a]

	Pain (neuroimmunology)		Pain (all)	
	Total numbers	Ratio of publications to X	Total numbers	Ratio of publications to X
Publications	201,683	1.00	4,422,911	1.00
Patents	43,198	4.66	435,852	10.15
Clinical trials	111	1816.96	12,284	360.05

[a]Search terms are provided in Figure 1. Data were tabulated from dimensions using a PubMed limited filter.

chronic pain. Major technological advances have already occurred to aid in the management of other disorders of the nervous system. For example, imaging platforms, such as fMRI, PET, and measurements from EEG, have changed the way diseases, such as epilepsy, are diagnosed and treated (Patel et al., 2019). However, these advances are somewhat isolated, and the identification and quantification of pain are still reliant on subjective diagnosis and empirical treatment selection. This clinical predicament creates a significant burden on the individual and profound health economic waste, with patients waiting up to 1 year after experiencing symptoms before presenting to a physician, and then taking >2 years and presenting to up to four different physicians before receiving a diagnosis for some complex chronic pain conditions (Choy et al., 2010).

The research activities of the Australian Research Council Centre of Excellence for Nanoscale BioPhotonics have sought to identify novel biomarkers of pain and to create measurement technologies that will allow pain diagnosis and direct precision medicine treatment of chronic pain. The criteria for such technologies are sensitivity and precision but must also deliver actionable information within a clinically meaningful timeframe and cost-effective strategy. Such measurements of chronic pain need to account for the sensory and emotional dimensions of chronic pain in addition to the aforementioned gene-environment-multisystem biology etiology of diseases of the CNS. The team rationalized that the pain state of an individual could be

quantified from peripheral blood because *a priori*, peripheral blood represents the accumulated environmental and genetic state of the individual. These collective changes in cellular state and proteome content of the blood are impacted by the emotional and physiological state of the individual causally linked to the sensory and emotional dimensions of chronic pain, given that peripheral immune cells support pain mechanisms in the CNS (Grace et al., 2011a,b; Wohleb et al., 2013; Sawicki et al., 2018). The hypothesis is supported by work showing that the *ex vivo* activity of peripheral immune cells can be used to stratify patients into chronic pain and healthy populations (Kwok et al., 2012; Evans et al., 2020).

Hyperspectral imaging of biology is emerging as an analytical tool that can be harnessed to improve research efficiency, lead to novel discoveries, and guide point-of-care decisions. Simultaneous integration of the emission spectra from multiple excitation wavelengths can provide substantial quantitative information about native fluorophores in individual cells in a mixed cell population. This analytical approach can then be used to rapidly phenotype entire cell populations in a label-free approach. For example, markers of mitochondrial oxidative stress/mitochondrial function/dysfunction (free NADH, bound NADH, flavoproteins, including flavin adenine dinucleotide-containing flavoproteins pyruvate dehydrogensase, ketoglutumate dehydrogenase, and electron transfer flavoproteins, retinoids, e.g., A2E, lipofuscin, and cytochrome *c*) can be quantified using hyperspectral analysis and linked to pain behaviors (Staikopoulos et al., 2016; Mahbub et al., 2019; Habibalahi et al., 2020) Alternatively, unsupervised analysis can be used to explore the *n*-dimensional data frames for spectral features that are predictive of pain states with nonlinear machine learning used to extract the clinically relevant signal. Preclinical and clinical trials are identifying hyperspectral signatures from a simple blood sample or a complex spinal cord tissue that can delineate healthy patients from those with chronic pain. Importantly, the technology and consumables can be produced at a very cost-effective unit per measurement; and owing to the ability to use microfluidics within sample processing, the test results can be available within minutes. Excitingly, this approach is also allowing the identification of the discrete subpopulations of cells, both centrally and peripherally, that are driving the spectral diagnosis and could be associatively and/or mechanistically linked to the exaggerated pain state.

In conclusion, since the first publications linking neuroinflammation to chronic pain more than two decades ago (Garrison et al., 1991; Svensson et al., 1993; Meller et al., 1994; Watkins et al., 1997; Colburn et al., 1999), the dividing line between the nervous and immune systems has become increasingly blurred. Sensory neurons express classical immune receptors, such as Fcγ receptors, that enable them to directly transduce signals from immune cells. Secreted immune mediators, such as ROS/RNS, directly activate ion channels that are classically expressed by sensory neurons. Sensory neurons can reciprocally regulate immune cell activity following stimulation of a variety of receptors, including CB1Rs. Connecting all of these discoveries is an increasing appreciation for how the immune response to injury is differentially regulated between the sexes.

Despite these continuing advances, the field of pain neuroimmunology is not yet translating basic science to the clinic at the same rate as the pain field in general; there is a fivefold difference in the ratio of publications to clinical trials (Table 2). This could

be explained by the relative immaturity of the subfield. Excitingly, however, patents related to pain neuroimmunology have been filed at more than double that of the broader pain field over the past 20 years (Table 2). Facilitated by the recent insights afforded by PET imaging into how central neuroinflammation is manifested in patients with chronic pain, together with the promise of peripherally accessible neuroimmune biomarkers of pain, we may be on the cusp of exciting discoveries. This decade may see an explosion in translational activity through active recruitment of clinicians and technologists into the field of chronic pain neuroimmunology.

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