

REVIEW

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Molecules in pain and sex: a developing story

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Abstract

Microglia are dynamic immune cells with diverse roles in maintaining homeostasis of the central nervous system. Dysregulation of microglia has been critically implicated in the genesis of neuropathic pain. Peripheral nerve injury, a common cause of neuropathic pain, engages microglia-neuronal signalling which causes disinhibition and facilitated excitation of spinal nociceptive pathways. However, recent literature indicates that the role of microglia in neuropathic pain is sexually dimorphic, and that female pain processing appears to be independent of microglia, depending rather on T cells. Despite this sex difference, pain signalling in the spinal cord converges downstream of microglia, as NMDAR-mediated facilitated excitation in pain transmitting neurons is consistent between males and females. Determining whether pain signalling is sexually dimorphic in humans and, further, addressing the sex bias in pain research will increase the translational relevance of preclinical findings and advance our understanding of chronic pain in women.

Keywords: Sex differences, Microglia, Neuropathic Pain, T cells, Spinal Cord

Introduction

Chronic pain is a highly prevalent and economically costly health condition [1, 2]. In the United States, at least 116 million adults have chronic pain with an estimated annual cost of \$560-\$635 billion [1]. Neuropathic pain, a common form of chronic pain, is caused by a lesion to or disease in the peripheral or central somatosensory nervous system [3]. Neuropathic pain is characterized by spontaneous pain, hyperalgesia and allodynia (pain hypersensitivity) [4]. Spontaneous pain is persistent or paroxysmal pain that is not elicited by a stimulus. Hyperalgesia is increased pain response to a noxious stimulus while allodynia (pain hypersensitivity) is pain in response to a normally innocuous stimulus.

Preclinical studies using rodent models of neuropathic pain have implicated spinal microglia as key mediators of peripheral nerve injury (PNI)-induced pain hypersensitivity [5–7]. However, evidence suggests that the role of microglia in neuropathic pain is sex-dependent [8]. This sexual dimorphism was only recently discovered as preclinical pain

research has generally excluded female subjects. The consensus was that oestrus caused increased variability, now shown not to be the case [9, 10]. Consequently, the overwhelming majority of preclinical pain research is conducted using only male rodents [11], a sex bias reflective of the biomedical field as a whole [12]. This sex bias poses serious issues for the translational relevance of preclinical pain research given that women represent the majority of patients with chronic pain, including neuropathic pain [2, 13, 14]. Additionally, women appear to be more sensitive to experimentally induced pain [15, 16]. Sex differences in response to pain treatment in humans have also been reported [16], such as increased morphine analgesia in females [17].

Microglia ontogeny and function

Microglia constitute an estimated 10% of centrally located cells and are the principal immune cell of the central nervous system (CNS) [18, 19]. As such, microglia maintain physiological homeostasis by responding directly to insults to CNS integrity such as traumatic brain injury, toxins, pathogens or other physiological stressors. Threats to the CNS produce characteristic microglial responses including proliferation, changes in morphology, antigen presentation and release of pro-inflammatory signalling molecules such as cytokines [19, 20]. Microglia share functional similarities

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to macrophages, which are innate immune cells involved in peripheral inflammation, but are distinct in cellular origin [21]. Microglia derive from primitive myeloid progenitors which originate in the yolk sac prior to embryonic day 8 and populate the developing CNS [22, 23]. Microglia cell populations are maintained and proliferate via self-renewal, without recruitment of peripheral precursors from the blood [24, 25].

Microglia were considered quiescent in the absence of CNS damage, reflected in classification of cells with long, ramified processes and small cell bodies as 'resting' microglia. In fact, microglia are highly dynamic with motile processes which extend and retract rapidly over a period of seconds to minutes within non-overlapping microdomains [7, 26, 27], providing constant environmental surveillance in addition to injury response. Consequently, CNS insult induces an immediate tropic response towards the injury site mediated by extracellular ATP signaling via P2Y₁₂ [26, 28]. The physiological functions of microglia have now been shown to be far more diverse, with fundamental roles in ensuring healthy CNS functioning through phagocytic clearing of cellular debris, responding to and modulating neuronal activity, influencing synaptic pruning and maturation as well as modulating synaptic plasticity [29–34]. Misregulation of this normal dynamic functioning of microglia can contribute to pathology associated with disease or injury, and a prime example of this is the role of microglia in nerve injury-induced chronic pain.

Spinal microglial reactivity after injury to peripheral nerves

PNI produces a stereotypical response in spinal microglia characterized by proliferation around central terminals and cell bodies of the peripherally damaged sensory and motor nerves respectively. The proliferative response is dependent on resident spinal microglia, as there is little, if any, infiltration of bone marrow-derived cells [35] or monocytes [36] after peripheral nerve injury. Although a striking and consistent response after PNI, it is unknown whether microglial proliferation in the dorsal horn is necessary for PNI-pain hypersensitivity. But, it is clear that the proliferation of microglia per se is not sufficient for that development of PNI-induced pain hypersensitivity [36–39]. Thus, a proliferative microglial response should not be considered as a proxy measure of pain [40]. Proliferation is accompanied by retraction of ramified processes and adoption of an amoeboid morphology and changes in expression of cell-surface proteins [41]. PNI activates *de novo* colony-stimulating factor 1 (CSF1) production in injured sensory neurons, which is transported to the spinal cord and binds to CSF1 receptors on microglia [25]. CSF1 activity simultaneously engages a membrane adaptor protein DAP12-independent pathway responsible for microglial

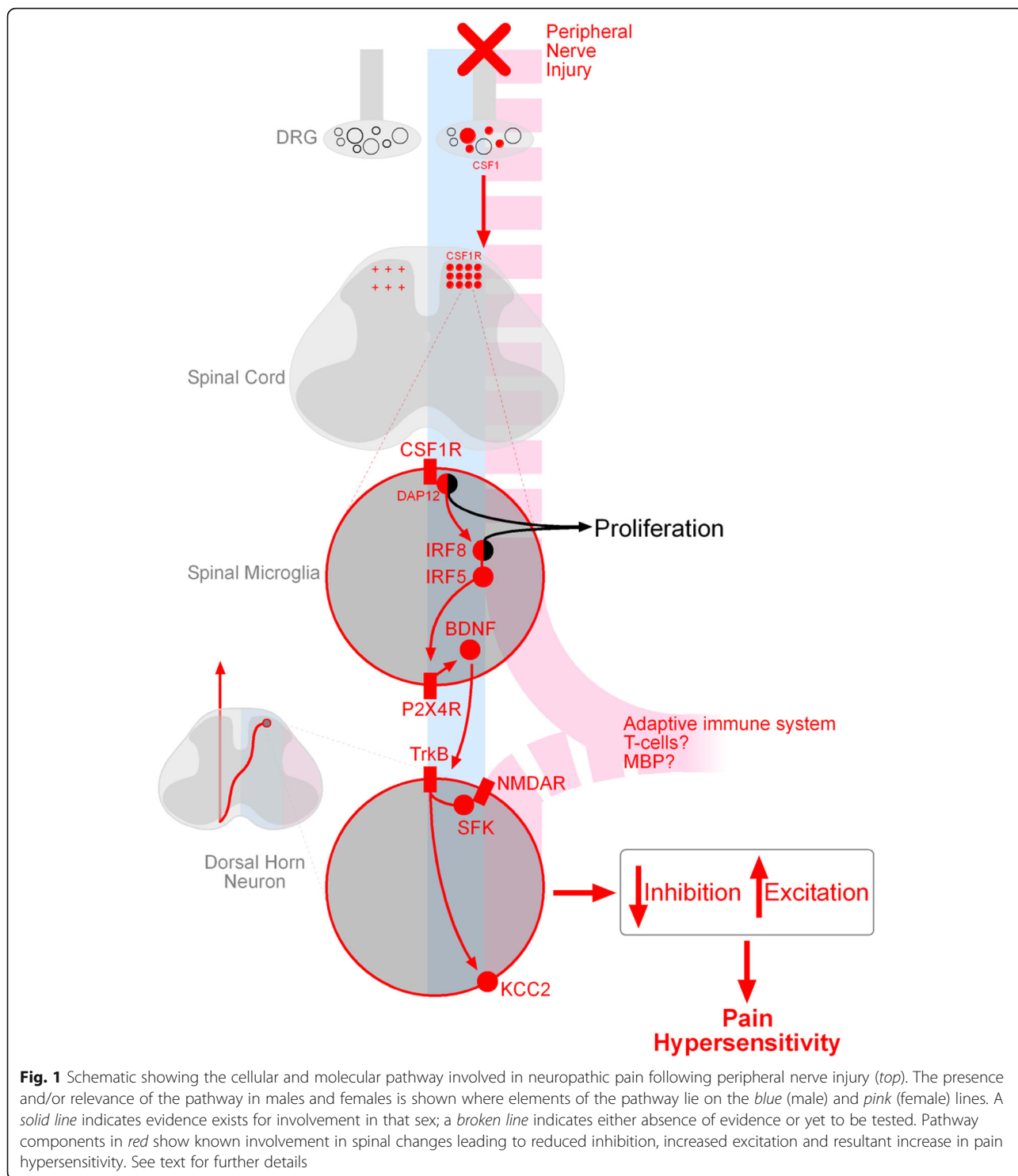
proliferation and a DAP12-dependent pathway mediating upregulation of microglial genes associated with pain hypersensitivity, including *Irf8* and *Irf5*. [25, 37, 42]. Increases in IRF8 expression after PNI activates IRF5 which binds specifically to the promoter of *P2rx4*, leading to an upregulation of P2X₄ receptor (P2X₄R) expression on microglia [37, 42] (see Fig. 1).

Similar to signals in ascending pathways such as CSF1, descending serotonergic signalling has also been implicated in inducing spinal microglia reactivity [43] and facilitation of pain hypersensitivity [43, 44]. Alleviation of pain hypersensitivity occurs after inhibition of spinal 5-HT₃ receptors in nerve-injured animals [43]. Furthermore, intrathecal application of a 5-HT₃ agonist results in glial reactivity and development of hypersensitivity in rats [43]. Additionally, spinal microgliosis due to peripheral inflammation can be attenuated by depletion of the 5-HT system [43].

Microglia signalling in the spinal cord

Upregulation and activation of microglial P2X₄R expression in the spinal dorsal horn has been found to be necessary for development of PNI-induced pain hypersensitivity [5, 7]: pharmacological suppression of spinal P2X₄R attenuates PNI-induced pain hypersensitivity [5], and P2X₄-deficient mice (*P2X₄^{-/-}*) show a lack of hypersensitivity after PNI [38]. In addition, adoptive transfer of ATP-stimulated microglia into naïve rats produces hypersensitivity [5] and sensitization of lamina I dorsal horn neurons [45] similar to that seen in nerve injured rodents. Activation of microglial P2X₄R, by ATP released from dorsal horn neurons [46], evokes an influx of extracellular calcium, phosphorylating p38 MAP kinase and results in release of brain derived neurotrophic factor (BDNF) [6, 47]. Microglial BDNF secretion is impaired in P2X₄-deficient mice [38]. Furthermore, deletion of microglial BDNF (*CX₃CR1^{CreER} x loxP-Bdnf*) both prevents and reverses PNI-induced hypersensitivity in mice [8].

Secreted BDNF stimulates neuronal TrkB receptors in the dorsal horn initiating downregulation of potassium chloride co-transporter KCC2 and a consequent shift in the transmembrane anion gradient [6, 48]. KCC2 maintains neuronal chloride extrusion, ensuring low intracellular chloride concentrations [49]. γ -aminobutyric acid type A (GABA_A) receptor functioning depends on the chloride gradient; thus, increased intracellular chloride due to KCC2 downregulation impairs GABA-mediated inhibitory synaptic transmission [50]. Pharmacological blockade or antisense knockdown of KCC2 produces hypersensitivity in naïve rodents [48]. Pharmacological activation of KCC2 reduces intracellular chloride concentration and alleviates hypersensitivity in nerve-injury models [51]. Reduced chloride extrusion capacity also promotes an efflux of HCO₃⁻ anions through GABA_A



channels which further enhances GABA-mediated disinhibition [52]. Carbonic anhydrase (CA) is a family of enzymes which catalyzes the synthesis of HCO_3^- [53]. Inhibition of CA alleviates nerve-injury induced hypersensitivity, likely by decreasing the depolarizing efflux of HCO_3^- [52]. The consequence of disinhibition is

therefore a net increase in excitability of lamina 1 neurons, which transforms spinal output and produces the hallmark symptoms of neuropathic pain in rodents [45, 48].

In addition to disinhibition, facilitated excitation produced by Src kinase-mediated enhancement of NMDAR

currents [54] contributes to hyperexcitability of lamina 1 projection neurons and associated pain hypersensitivity [55]. Src is a non-receptor protein tyrosine kinase with diverse physiological functions [56]. Src binding to the NMDAR complex via NADH dehydrogenase subunit 2 (ND2) increases NMDAR activity [57]. Enhancement of NMDAR function amplifies glutamatergic synaptic transmission, increasing the output of lamina 1 neurons. Uncoupling of Src kinase from the NMDAR complex blocks Src-mediated NMDAR activity enhancement [57]. Furthermore, intrathecal application of a peptide that disrupts Src binding alleviates nerve-injury induced pain hypersensitivity [55]. The factors mediating Src-ND2 binding after PNI are not fully understood. Microglial BDNF may be fundamental to this process as intrathecal BDNF in naïve rodents potentiates NMDARs in a Src family kinase-dependent manner [58] and potentiation of NMDAR currents due to nerve injury is dependent on BDNF signalling [59]. Cytokines can also enhance NMDAR function through Src activation [60]. Furthermore, chloride-mediated disinhibition is required for potentiation of NMDAR activity after nerve injury [59]. Thus, multiple signalling molecules may contribute to NMDAR-enhancement after PNI.

Sexually dimorphic role of microglia in pain

The microglia-neuronal signalling pathway was established through experiments using almost exclusively male rodents; thus, its role in pain processing was not established in females. Recent experiments using mice of both sexes have shown that microglia are not involved in mediating pain hypersensitivity in females with PNI, suggesting the existence of sexually dimorphic pain processing [8].

The first indication of sexually dimorphic pain processing was evidence showing that spinal TLR4s, which are expressed specifically on microglia in the CNS, contribute to PNI-induced pain hypersensitivity in male but not female mice [61]. TLR4 is a member of the Toll-like receptor family involved in response to pathogens such as bacterial lipopolysaccharide and consequent activation of the innate immune system [62]. The sexually dimorphic role of TLR4 prompted broader investigation into the relevance of microglia to neuropathic pain in female mice. Damage to a peripheral nerve produces spinal microglial reactivity in female mice comparable to that of males [8, 63]. However, application of intrathecal minocycline, propentofylline or fluorocitrate, which may inhibit processes in glial cells, or specific microglial lesioning (via intrathecal injection of saporin toxin conjugated to the MAC-1 receptor) in mice alleviates nerve injury-induced pain hypersensitivity in males only and are entirely ineffective in females, suggesting that microglia are not necessary for pain hypersensitivity in female mice [8]. Inhibition of spinal P2X4Rs attenuates pain hypersensitivity in male but not female

mice, confirming that microglia neuronal signalling does not contribute to pain processing in female mice [8]. p38 MAP kinase inhibition in the spinal cord is also ineffective in attenuating hypersensitivity in female mice [8]. While male microglial BDNF knockout mice ($CX_3CR1^{CreER} \times loxP-Bdnf$) display significant impairments in development and maintenance of PNI-induced hypersensitivity, pain processing is unaffected in female knockout mice [8]. Furthermore, pharmacological inhibition of spinal BDNF reverses hypersensitivity in males only, ruling out the possibility that non-microglia derived BDNF contribute to female pain processing [8]. Assessment of genes relevant to the microglia neuronal signalling pathway demonstrated that upregulation of *P2rx4* is exclusive to male mice, indicating that the P2X4R-dependent signalling pathway is not being engaged in female mice, which may be the key to the sex dependency of microglia in pain [8]. IRF8-IRF5 signalling lies upstream of P2X4R transcriptional upregulation [37, 42]; therefore, a sex difference in *P2rx4* levels after nerve injury may result from differential IRF8-IRF5 expression. However, PNI upregulates *Irf8* and *Irf5* expression equally in both sexes, which could account for the microglial proliferation observed in females [8]. Thus, the inference is that IRF5 mediated transcription of *P2rx4* is likely not occurring in females, which may be the key to the sex-dependency of microglia in pain.

Sexual dimorphism of the microglia-neuron signalling pathway has been confirmed elsewhere [64]. Inhibition of spinal p38 MAP kinase alleviates nerve injury-induced pain hypersensitivity in male but not female mice as well as rats, consistent with the more substantial p38 phosphorylation levels after injury in males [64]. Furthermore, spontaneous excitatory postsynaptic currents (EPSCs) are depressed only in male lamina IIo neurons during p38 MAP kinase blockade [64]. This sex difference appears to be spinally restricted as p38 MAP kinase inhibition through intraperitoneal and perineural application routes produces robust reversal in either sex [64]. The specificity of this sex difference is consistent with the sexually dimorphic role of spinal but not peripheral TLR4 in pain [61]. However, recent evidence has shown that spinal microglia are involved in mediating bone cancer pain in female rats [65], which suggests that sex differences in pain processing may not be consistent across injury models. The contribution of descending serotonergic circuitry to neuropathic pain in females has yet to be investigated, as previous work in investigating 5-HT₃ was conducted using male rodents only [43, 44].

Despite the absence of a role for microglia in mediating neuropathic pain in females, there seems to be mechanistic convergence at the neuronal level as antagonising NMDAR activity alleviates pain hypersensitivity in both sexes [8]. This suggests that despite a sex difference in upstream signalling, similar neuronal changes occur after

nerve injury, i.e. potentiation of synaptic NMDAR activity. Targeting convergent mechanisms between females and males is an alternative strategy to development of sex-specific therapies. It is still unknown whether the role for NMDARs in female pain processing is Src kinase dependent. Given the congruency in NMDAR involvement between females and males, it is possible that disinhibition due to KCC2 downregulation also contributes to pain behavior in both sexes. Investigation of whether impaired chloride extrusion mediates pain in females is critical given the interest in targeting KCC2 to treat neuropathic pain [51]. Orally administered drugs which rescue plasma membrane expression of KCC2 produce strong analgesia in rodent models of PNI without loss of motor function [51]. Such chloride extrusion enhancers remain to be tested in females. If KCC2-dependent disinhibition is consistent between the sexes, this would indicate that the sex difference in neuropathic pain processing is restricted to immune system functioning.

The underlying cause of the sexual dimorphism in pain processing remains unidentified; however, sex hormones represent a strong candidate [66]. Engagement of the microglial-dependent pathway appears to be contingent on the presence of high testosterone levels, regardless of sex [8]. Sex steroids have profound influences on immune functioning and may be responsible for many sex differences in pain, see Rosen et al., 2017 for a in-depth review of this topic [66]. Hormones also have been shown to regulate gene transcription [67, 68]. Thus, future investigation is required to determine whether sex steroids underlie the differential upregulation of *P2rx4* between males in females after nerve injury.

Critical role for adaptive immune cells in females

That pain hypersensitivity in female mice is independent of microglia, which are innate immune cells, led to considering the possibility that cells of the adaptive immune system may be necessary in females [8]. It has been previously reported that male mice lacking adaptive immune cells develop less hypersensitivity after nerve injury [69, 70]. In contrast, it was found that nerve-injured adaptive immune cell knockout mice (B6.129S7-*Rag1*^{tm1-Mom}/J and NU/J) display equivalent hypersensitivity to their wildtype counterparts, regardless of sex. However, interrogation of pain signalling mechanisms in these mice revealed that females lacking adaptive immune cells employ a microglia-dependent pathway in the mediation of pain hypersensitivity. Immune system reconstitution of female *Rag1* knockouts with spleenocytes causes a 'switch' to a microglia-independent pathway. Therefore, it is hypothesized that the presence of adaptive immune cells, likely T cells, is necessary to drive the non-microglia pathway (Fig. 1). T cells migrate into the

spinal cord after nerve injury and have been implicated as key regulators of hypersensitivity [69–71].

T cells of mouse and human lineage display a testosterone-dependent sex difference in expression of peroxisome proliferator activated receptors (PPARs), transcription factors integral in cytokine regulation [72]. Expression of PPAR α is augmented by testosterone, which boosts secretion of interleukin-17A [72]. Conversely, testosterone decreases expression of PPAR γ , which suppresses production of interferon- γ [72]. Infiltrating cells into the spinal cord after nerve injury appear to be primarily T helper type 1 (Th1) lymphocytes [73]. As interferon- γ is the prototypical Th1 cytokine, infiltrated T cells in nerve-injured male mice may secrete fewer pro-inflammatory mediators relative to females. Consistent with sexually dimorphic PPAR expression, intrathecal application of the PPAR α agonist, fenofibrate, attenuates nerve injury-induced hypersensitivity in male mice only, a drug effect abolished by castration [8]. Furthermore, intrathecal administration of the PPAR γ agonist, pioglitazone, reverses hypersensitivity after nerve injury in females, but not males [8]. Pioglitazone-mediated analgesia in females is attenuated by treatment with testosterone propionate [8]. In addition to sex differences in T cell phenotype, female mice also have higher peripheral and central T cell counts than male mice [8]. Combined, differential T cell numbers and cytokine secretion could lead to sexually dimorphic involvement of T cells in pain processing. A T cell driven mechanism could also directly suppress the microglia-dependent pathway through inhibition of *P2rx4* transcription.

Hypersensitivity resulting from a sciatic nerve injection of myelin basic protein (MBP) in female rats is also mediated by T cells [74]. MBP has also been implicated in the development of PNI-induced hypersensitivity in females [75]. Thus, MBP-mediated activation of T cells may represent a component of the microglia-independent pathway. T cells could release pro-inflammatory cytokines to produce neuronal changes, such as enhancement in NMDAR functioning, which result in hyperexcitability of spinal nociceptive circuitry. B cells may also represent a critical mediator of the microglial-independent pathway. However, nerve injury does not induce spinal infiltration of B cells in male mice [70] and little evidence has implicated B cells in pain processing. The exact role for adaptive immune cells and MBP in mediating pain in females requires further investigation.

Relevance to clinical pain in humans

The role of microglia in chronic pain in humans of either sex remains unclear. Significant differences exist in immune system functioning between rodents and humans, which poses issues for translation of preclinical findings to humans [76, 77]. Post-mortem analysis of spinal cord tissue has shown significant microglial and astrocytic

activation in a female patient with longstanding complex regional pain syndrome [78]. In post-mortem tissue of male patients with HIV-associated neuropathic pain, astrocytic but not microglial markers are upregulated in the spinal dorsal horn [79]. In-vivo imaging of patients of both sexes with chronic low back pain shows evidence of glial activation in the brain [80]. Female and male patients with peripheral nerve injury display activation of glial cells in the thalamus [81]. Thus, there is evidence that glial cells, including microglia, are activated in certain chronic pain conditions. This glial reactivity is observed in patients of both sexes [78–81], which is consistent with the pre-clinical data [8]. Given the dissociation between microglial proliferation and pain hypersensitivity, it cannot be concluded that microglia in humans of either sex are involved in chronic pain per se [40].

The preclinical literature on microglia and pain has sparked significant interest in targeting microglia in order to treat chronic pain in humans. However, a clinical trial of propentofylline, a glial modulating drug, failed to show efficacy in treating pain in patients with post-herpetic neuralgia [82]. It is possible that the dosing schedule of propentofylline may not have been sufficient to achieve adequate spinal glial inhibition. Additionally, post-herpetic neuralgia is not strictly a nerve lesion, which has been the primary preclinical model used to establish microglial involvement in pain processing. Alternatively, the clinical trial may indicate a lack of microglial involvement in neuropathic pain in humans. Whether the efficacy of propentofylline in treating neuropathic pain differs between women and men was not reported. Thus, a possible sexually dimorphic role of microglia in pain in humans cannot be ruled out.

Conclusions

Microglia have become a focus in the preclinical pain research field. There is clear evidence in rodent models of neuropathic pain that microglia are critical in mediating pain behavior associated with nerve injury. However, the evidence implicating microglia in pain comes almost exclusively from experiments using male rodents. Recent research has put into question our understanding of the role of microglia in pain processing. While microglia adopt a reactive phenotype following nerve injury in mice of both sexes, these cells are not involved in mediating neuropathic pain behavior in females. The cross-species generalizability of this sexual dimorphism remains to be fully explored, but preliminary evidence supports the existence of sex differences in pain signalling in rats with nerve injury. Sexually dimorphic pain processing denotes the importance of including animals of both sexes in preclinical research. The translational potential of preclinical pain research may be greatly improved with equal representation of male and female subjects and may lead to development of precision medicine for chronic pain patients.

Abbreviations

BDNF: Brain derived neurotrophic factor; CA: Carbonic anhydrase; CNS: Central nervous system; CSF1: Colony-stimulating factor 1; GABA_A: γ -aminobutyric acid type A; KCC2: Potassium chloride cotransporter 2; ND2: NADH dehydrogenase subunit 2; MBP: Myelin basic protein; P2X4R: P2X4 receptor; PNI: Peripheral nerve injury; PPAR: Peroxisome proliferator activated receptor

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References

- Pizzo P, Clark N. Relieving pain in America: a blueprint for transforming prevention, care, education, and research (Institute of medicine). Washington, DC: The National Academies Press; 2011.
- Bouhassira D, Lantéri-Minet M, Attal N, Laurent B, Touboul C. Prevalence of chronic pain with neuropathic characteristics in the general population. *Pain*. 2008;136(3):380–7.
- Merskey H, Bogduk N. Classification of chronic pain. 2nd ed. Seattle: IASP Press; 1994.
- Woolf CJ, Mannion RJ. Neuropathic pain: aetiology, symptoms, mechanisms, and management. *Lancet* (London, England). 1999;353(9168):1959–64.
- Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, Inoue K. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature*. 2003;424(6950):778–83.
- Coull JA, Beggs S, Boudreau D, Boivin D, Tsuda M, Inoue K, Gravel C, Salter MW, De Koninck Y. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature*. 2005;438(7070):1017–21.
- Beggs S, Trang T, Salter MW. P2X4R+ microglia drive neuropathic pain. *Nat Neurosci*. 2012;15(8):1068–73.
- Sorge RE, Mapplebeck JCS, Rosen S, Beggs S, Taves S, Alexander JK, Martin LJ, Austin J-S, Sotocinal SG, Chen D, et al. Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nat Neurosci*. 2015;18:1081–3.
- Klein SL, Schiebinger L, Stefanick ML, Cahill L, Danska J, de Vries GJ, Kibbe MR, McCarthy MM, Mogil JS, Woodruff TK, et al. Opinion: sex inclusion in basic research drives discovery. *Proc Natl Acad Sci U S A*. 2015;112(17):5257–8.

10. Prendergast BJ, Onishi KG, Zucker I. Female mice liberated for inclusion in neuroscience and biomedical research. *Neurosci Biobehav Rev*. 2014; 40:1–5.
11. Mogil JS, Chanda ML. The case for the inclusion of female subjects in basic science studies of pain. *Pain*. 2005;117(1–2):1–5.
12. Beery AK, Zucker I. Sex bias in neuroscience and biomedical research. *Neurosci Biobehav Rev*. 2011;35(3):565–72.
13. Fillingim RBK, Ribeiro-Dasilva MC, Rahim-Williams B, Riley 3rd JL. Sex, gender, and pain: a review of recent clinical and experimental findings. *J Pain*. 2009; 10(5):447–85.
14. Torrance NSB, Bennett MI, Lee AJ. The epidemiology of chronic pain of predominantly neuropathic origin. Results from a general population survey. *J Pain*. 2006;7(4):281–9.
15. Mogil JS. Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. *Nat Rev Neurosci*. 2012;13(12):859–66.
16. Bartley EJ, Fillingim RB. Sex differences in pain: a brief review of clinical and experimental findings. *BJA*. 2013;111(1):5–8.
17. Niesters M, Dahan A, Kest B, Zacny J, Stijnen T, Aarts L, Sarton E. Do sex differences exist in opioid analgesia? A systematic review and meta-analysis of human experimental and clinical studies. *Pain*. 2010;151(1):61–8.
18. Aguzzi A, Barres BA, Bennett ML. Microglia: scapegoat, saboteur, or something else? *Science (New York, NY)*. 2013;339(6116):156–61.
19. Streit WJ, Mrak RE, Griffin WS. Microglia and neuroinflammation: a pathological perspective. *J Neuroinflammation*. 2004;1(1):14.
20. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci*. 1996;19(8):312–8.
21. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958–69.
22. Ginhoux FGM, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM, Merad M. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science (New York, NY)*. 2010;330(6005):841–5.
23. Fogg DK, Sibon C, Miled C, Jung S, Aucouturier P, Littman DR, Cumano A, Geissmann F. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science (New York, NY)*. 2006;311(5757):83–7.
24. Ajami B, Bennett JL, Kriegler C, Tetzlaff W, Rossi FMV. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci*. 2007;10(12):1538–43.
25. Guan Z, Kuhn JA, Wang X, Colquitt B, Solorzano C, Vaman S, Guan AK, Evans-Reinsch Z, Braz J, Devor M, et al. Injured sensory neuron-derived CSF1 induces microglial proliferation and DAP12-dependent pain. *Nat Neurosci*. 2016;19:94–101.
26. Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB. ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci*. 2005;8(6):752–8.
27. Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science (New York, NY)*. 2005;308(5726):1314–8.
28. Haynes SE, Hloppeter G, Yang G, Kurpius D, Dailey ME, Gan WB, Julius D. The P2Y12 receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci*. 2006;9(12):1512–9.
29. Li Y, Du XF, Liu CS, Wen ZL, Du JL. Reciprocal regulation between resting microglial dynamics and neuronal activity in vivo. *Dev Cell*. 2012;23(6):1189–202.
30. Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, Giustetto M, Ferreira TA, Guiducci E, Dumas L, et al. Synaptic pruning by microglia is necessary for normal brain development. *Science (New York, NY)*. 2011; 333(6048):1456–8.
31. Roumier A, Bechede C, Poncer JC, Smalla KH, Tomasello E, Vivier E, Gundelfinger ED, Triller A, Bessis A. Impaired synaptic function in the microglial KARAP/DAP12-deficient mouse. *J Neurosci*. 2004;24(50):11421–8.
32. Parkhurst CN, Yang G, Nanan I, Savas JN, Yates 3rd JR, Lafaille JJ, Hempstead BL, Littman DR, Gan WB. Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. *Cell*. 2013;155(7):1596–609.
33. Neumann H, Kotter MR, Franklin RJ. Debris clearance by microglia: an essential link between degeneration and regeneration. *Brain*. 2009;132(Pt 2):288–95.
34. Salter M, Beggs S. Sublime microglia: expanding roles for the guardians of the CNS. *Cell*. 2014;158(1):15–24.
35. Tashima R, Mikuriya S, Tomiyama D, Shiratori-Hayashi M, Yamashita T, Kohro Y, Tozaki-Saitoh H, Inoue K, Tsuda M. Bone marrow-derived cells in the population of spinal microglia after peripheral nerve injury. *Sci Rep*. 2016;6:23701.
36. Gu N, Peng J, Murugan M, Wang X, Eyo UB, Sun D, Ren Y, DiCicco-Bloom E, Young W, Dong H, et al. Spinal microgliosis Due to resident microglial proliferation is required for pain hypersensitivity after peripheral nerve injury. *Cell Rep*. 2016;16(3):605–14.
37. Masuda T, Iwamoto S, Yoshinaga R, Tozaki-Saitoh H, Nishiyama A, Mak TW, Tamura T, Tsuda M, Inoue K. Transcription factor IRF5 drives P2X4R + -reactive microglia gating neuropathic pain. *Nat Commun*. 2014;5:3771.
38. Ulmann L, Hatcher JP, Hughes JP, Chaumont S, Green PJ, Conquet F, Buell GN, Reeve AJ, Chessell IP, Rassendren F. Up-regulation of P2X4 receptors in spinal microglia after peripheral nerve injury mediates BDNF release and neuropathic pain. *J Neurosci*. 2008;28(44):11263–8.
39. Tozaki-Saitoh H, Tsuda M, Miyata H, Ueda K, Kohsaka S, Inoue K. P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. *J Neurosci*. 2008;28(19):4949–56.
40. Mapplebeck JCS, Beggs S, Salter MW. Sex Differences in Pain: A Tale of Two Immune Cells. *Pain*. 2015; Publish Ahead of Print.
41. Beggs S, Salter MW. The known knowns of microglia-neuronal signalling in neuropathic pain. *Neurosci Lett*. 2013;557 Pt A:37–42.
42. Masuda T, Tsuda M, Yoshinaga R, Tozaki-Saitoh H, Ozato K, Tamura T, Inoue K. IRF8 is a critical transcription factor for transforming microglia into a reactive phenotype. *Cell Rep*. 2012;1(4):334–40.
43. Guo W, Miyoshi K, Dubner R, Gu M, Li M, Liu J, Yang J, Zou S, Ren K, Noguchi K, et al. Spinal 5-HT3 receptors mediate descending facilitation and contribute to behavioral hypersensitivity via a reciprocal neuron-glial signaling cascade. *Mol Pain*. 2014;10(1):35.
44. Wei F, Dubner R, Zou S, Ren K, Bai G, Wei D, Guo W. Molecular depletion of descending serotonin unmasks its novel facilitatory role in the development of persistent pain. *J Neurosci*. 2010;30:8624–36.
45. Keller AF, Beggs S, Salter MW, De Koninck Y. Transformation of the output of spinal lamina I neurons after nerve injury and microglia stimulation underlying neuropathic pain. *Mol Pain*. 2007;3:27.
46. Masuda T, Ozono Y, Mikuriya S, Kohro Y, Tozaki-Saitoh H, Iwatsuki K, Uneyama H, Ichikawa R, Salter MW, Tsuda M, et al. Dorsal horn neurons release extracellular ATP in a VNUT-dependent manner that underlies neuropathic pain. *Nat Commun*. 2016;7:12529.
47. Trang T, Beggs S, Wan X, Salter MW. P2X4-receptor-mediated synthesis and release of brain-derived neurotrophic factor in microglia is dependent on calcium and p38-mitogen-activated protein kinase activation. *J Neurosci*. 2009;29(11):3518–28.
48. Coull JA, Boudreau D, Bachand K, Prescott SA, Nault F, Sik A, De Koninck P, De Koninck Y. Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature*. 2003;424(6951):938–42.
49. Payne JA. Functional characterization of the neuronal-specific K-Cl cotransporter: implications for [K⁺]_o regulation. *Am J Physiol Cell Physiol*. 1997;273(5):C1516–25.
50. Hübner CA, Stein V, Hermans-Borgmeyer I, Meyer T, Ballanyi K, Jentsch TJ. Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. *Neuron*. 2001;30(2):515–24.
51. Gagnon M, Bergeron MJ, Lavertu G, Castonguay A, Tripathy S, Bonin RP, Perez-Sanchez J, Boudreau D, Wang B, Dumas L, et al. Chloride extrusion enhancers as novel therapeutics for neurological diseases. *Nat Med*. 2013; 19(11):1524–8.
52. Asiedu M, Ossipov MH, Kaila K, Price TJ. Acetazolamide and midazolam act synergistically to inhibit neuropathic pain. *Pain*. 2010;148(2):302–8.
53. Maren TH. Carbonic anhydrase: chemistry, physiology, and inhibition. *Physiol Rev*. 1967;47(4):595–781.
54. Salter MW, Kalia LV. Src kinases: a hub for NMDA receptor regulation. *Nat Rev Neurosci*. 2004;5(4):317–28.
55. Liu XJ, Gingrich JR, Vargas-Caballero M, Dong YN, Sengar A, Beggs S, Wang SH, Ding HK, Frankland PW, Salter MW. Treatment of inflammatory and neuropathic pain by uncoupling Src from the NMDA receptor complex. *Nat Med*. 2008;14(12):1325–32.
56. Parsons SJ, Parsons JT. Src family kinases, key regulators of signal transduction. *Oncogene*. 2004;23(48):7906–9.
57. Gingrich JR, Pelkey KA, Fam SR, Huang Y, Petralia RS, Wenthold RJ, Salter MW. Unique domain anchoring of Src to synaptic NMDA receptors via the mitochondrial protein NADH dehydrogenase subunit 2. *Proc Natl Acad Sci U S A*. 2004;101(16):6237–42.
58. Chen W, Walwyn W, Ennes HS, Kim H, McRoberts JA, Marvizon JC. BDNF released during neuropathic pain potentiates NMDA receptors in primary afferent terminals. *Eur J Neurosci*. 2014;39(9):1439–54.

59. Hildebrand ME, Xu J, Dedek A, Li Y, Sengar AS, Beggs S, Lombroso PJ, Salter MW. Potentiation of synaptic GluN2B NMDAR currents by Fyn kinase is gated through BDNF-mediated disinhibition in spinal pain processing. *Cell Rep*. 2016;17(10):2753–65.
60. Viviani B, Bartesaghi S, Gardoni F, Vezzani A, Behrens MM, Bartfai T, Binaglia M, Corsini E, Di Luca M, Galli CL, et al. Interleukin-1beta enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. *J Neurosci*. 2003;23(25):8692–700.
61. Sorge RE, LaCroix-Fralish ML, Tuttle AH, Sotocinal SG, Austin JS, Ritchie J, Chanda ML, Graham AC, Topham L, Beggs S, et al. Spinal cord toll-like receptor 4 mediates inflammatory and neuropathic hypersensitivity in male but not female mice. *J Neurosci*. 2011;31(43):15450–4.
62. Miller SI, Ernst RK, Bader MW. LPS, TLR4 and infectious disease diversity. *Nat Rev Microbiol*. 2005;3(1):36–46.
63. Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain*. 2000;87(2):149–58.
64. Taves S, Berta T, Liu DL, Gan S, Chen G, Kim YH, Van de Ven T, Laufer S, Ji RR. Spinal inhibition of p38 MAP kinase reduces inflammatory and neuropathic pain in male but not female mice: Sex-dependent microglial signaling in the spinal cord. *Brain Behav Immun*. 2015;55:70–81.
65. Yang Y, Li H, Li T-T, Luo H, Gu X-Y, Lü N, Ji R-R, Zhang Y-Q. Delayed activation of spinal microglia contributes to the maintenance of bone cancer pain in female wistar rats via P2X7 receptor and IL-18. *J Neurosci*. 2015;35(20):7950–63.
66. Rosen S, Ham B, Mogil JS. Sex differences in neuroimmunity and pain. *J Neurosci Res*. 2017;95(1–2):500–8.
67. Krum SA. Direct transcriptional targets of sex steroid hormones in bone. *J Cell Biochem*. 2011;112(2):401–8.
68. Farza H, Salmon AM, Hadchouel M, Moreau JL, Babinet C, Tiollais P, Pourcel C. Hepatitis B surface antigen gene expression is regulated by sex steroids and glucocorticoids in transgenic mice. *Proc Natl Acad Sci U S A*. 1987;84(5):1187–91.
69. Costigan M, Moss A, Latremoliere A, Johnston C, Verma-Gandhu M, Herbert T, Barrett L, Brenner G, Vardeh D, Woolf C, et al. T-cell infiltration and signaling in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity. *J Neurosci*. 2009;29(46):14415–22.
70. Cao L, DeLeo J. CNS-infiltrating CD4+ T lymphocytes contribute to murine spinal nerve transection-induced neuropathic pain. *Eur J Immunol*. 2008;38(2):448–58.
71. Sweitzer SM, Hickey WF, Rutkowski MD, Pahl JL, DeLeo JA. Focal peripheral nerve injury induces leukocyte trafficking into the central nervous system: potential relationship to neuropathic pain. *Pain*. 2002;100(1–2):163–70.
72. Zhang MA, Rego D, Moshkova M, Kebir H, Chruscinski A, Nguyen H, Akkermann R, Stanczyk FZ, Prat A, Steinman L, et al. Peroxisome proliferator-activated receptor (PPAR)alpha and -gamma regulate IFNgamma and IL-17A production by human T cells in a sex-specific way. *Proc Natl Acad Sci U S A*. 2012;109(24):9505–10.
73. Draleau K, Maddula S, Slaiby A, Nutile-McMenemy N, De Leo J, Cao L. Phenotypic identification of spinal cord-infiltrating CD4(+) T lymphocytes in a Murine model of neuropathic pain. *J Pain Relief*. 2014;Suppl 3:003.
74. Ko JS, Eddinger KA, Angert M, Chernov AV, Dolkas J, Strongin AY, Yaksh TL, Shubayev VI. Spinal activity of interleukin 6 mediates myelin basic protein-induced allodynia. *Brain Behav Immun*. 2016;56:378–89.
75. Liu H, Shiryayev SA, Chernov AV, Kim Y, Shubayev I, Remacle AG, Baranovskaya S, Golubkov VS, Strongin AY, Shubayev VI. Immunodominant fragments of myelin basic protein initiate T cell-dependent pain. *J Neuroinflammation*. 2012;9:119.
76. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, Richards DR, McDonald-Smith GP, Gao H, Hennessy L, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A*. 2013;110(9):3507–12.
77. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol (Baltimore, Md : 1950)*. 2004;172(5):2731–8.
78. Del Valle L, Schwartzman RJ, Alexander G. Spinal cord histopathological alterations in a patient with longstanding complex regional pain syndrome. *Brain Behav Immun*. 2009;23(1):85–91.
79. Shi Y, Gelman BB, Lisinicchia JG, Tang SJ. Chronic-pain-associated astrocytic reaction in the spinal cord dorsal horn of human immunodeficiency virus-infected patients. *J Neurosci*. 2012;32(32):10833–40.
80. Loggia ML, Chonde DB, Akeju O, Arabasz G, Catana C, Edwards RR, Hill E, Hsu S, Izquierdo-Garcia D, Ji RR, et al. Evidence for brain glial activation in chronic pain patients. *Brain*. 2015;138(Pt 3):604–15.
81. Banati RB, Cagnin A, Brooks DJ, Gunn RN, Myers R, Jones T, Birch R, Anand P. Long-term trans-synaptic glial responses in the human thalamus after peripheral nerve injury. *Neuroreport*. 2001;12(16):3439–42.
82. Landry RP, Jacobs VL, Romero-Sandoval EA, DeLeo JA. Propentofylline, a CNS glial modulator does not decrease pain in post-herpetic neuralgia patients: in vitro evidence for differential responses in human and rodent microglia and macrophages. *Exp Neurol*. 2012;234(2):340–50.

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