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Title: Sex-dependent mechanisms of chronic pain: A focus on microglia and P2X4R

Authors and Affiliations:

Katherine Halievski^{1, 2}, Shahrzad Ghazisaedi^{1, 2, 3}, Michael W. Salter^{1, 2, 3}

1. Neurosciences and Mental Health Program, The Hospital for Sick Children, Toronto ON
2. The University of Toronto Centre for the Study of Pain, Toronto ON
3. The Department of Physiology, University of Toronto, Toronto ON

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Corresponding author:

Michael W. Salter
The Hospital for Sick Children
686 Bay St.
Toronto, ON
M5G 0A4 Canada
tel: (416) 813-6272
fax: (416) 813-7921
michael.salter@sickkids.ca

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brain-derived neurotrophic factor (BDNF)
chemokine (C-C motif) ligand (CCL)
colony-stimulating factor 1 (CSF-1)
interleukin-6 (IL-6)
interferon regulatory factor (IRF)
lipopolysaccharide (LPS)
N-methyl-D-aspartate receptor (NMDAR)
periaqueductal gray (PAG)
peripheral nerve injury (PNI)
purinergic receptor, ionotropic (P2XR)
prostaglandin E2 (PGE-2)
vesicular nucleotide transporter (VNUT)

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Abstract: For over two decades, purinergic signaling in microglia has persisted in the spotlight as a major pathomechanism of chronic pain. Of the many purinoreceptors, the P2X4R of the ionotropic family has a well-described causal role underlying chronic neuropathic pain. This review will first briefly examine microglial P2X4R signaling in the spinal cord as it relates to chronic pain through a historical lens, followed by a more in-depth examination of recent work, which has revealed major sex differences. We also discuss the generalizability of sex differences in microglial and P2X4R signaling in other pain conditions, as well as in non-spinal regions. Finally, we speculate on remaining gaps in the literature as well as what can be done to address them with the ultimate goal of using our collective knowledge to treat chronic pain effectively and in both sexes.

Significance Statement. Effective treatments are lacking for chronic pain sufferers, and this may be explained by the vast sex differences underlying chronic pain mechanisms. In this Minireview, we focus on the roles of microglia and P2X4R in chronic pain, with specific attention to the circumstances under which these pathomechanisms differ between males and females. By delineating the ways in which pain occurs differently between the sexes, we can start developing successful therapies for all.

Keywords: purinergic receptors, pain, sexual dimorphism, microglia

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1. A brief history of microglial P2X4R signaling in pain hypersensitivity

Chronic pain is a debilitating condition that affects up to 20% of the population and has an economic burden of \$43 billion dollars per year in Canada alone (Lynch, 2011; Schopflocher et al., 2011). Unfortunately, existing treatments for chronic pain are not wholly effective and have significant side effects (Fornasari, 2017). Chronic pain is not phenotypically or mechanistically homogenous and may arise from many causes. To determine the best ways in which to treat patients with chronic pain, we can start first by examining the recognized pathomechanisms of chronic pain. One well-defined pathomechanism is via purinergic signaling (Bernier et al., 2018). Purinergic receptors are activated by purine nucleosides or nucleotides, such as ATP, and include ionotropic P2X receptors, of which there are seven types and metabotropic P2Y receptors, of which there are eight (Burnstock, 2018). The P2X4 receptor (P2X4R) class of receptor was first linked to chronic pain signaling by Tsuda et al. (2003) and has been, to date, one of the best characterized signaling pathways for different types of chronic pain (Inoue, 2019).

The initial evidence of P2X4R underlying chronic pain was the demonstration of reversal of hypersensitivity caused by traumatic peripheral nerve injury (PNI) by inhibiting P2X4Rs, and increased P2X4R expression on microglia (Tsuda et al., 2003), a prominent cell type in the central nervous system. A functional consequence of increasing P2X4R expression and activation in microglia is the phosphorylation of p38-MAPK, which induces transcription, translation, and release of brain-derived neurotrophic factor (BDNF) from microglia (Trang et al., 2009). Then, microglial BDNF serves as the messenger molecule that drives disinhibition and hyperexcitation of dorsal horn neurons (Coull et al., 2005), ultimately resulting in pain hypersensitivity. That the requisite BDNF derives from microglia is shown by the lack of pain hypersensitivity in mice in which the *Bdnf* gene is disrupted selectively in microglia (Sorge et al., 2015). BDNF activation of the TrkB receptor in neurons leads to downregulation of the K⁺-Cl⁻ co-transporter KCC2 expression (Coull et al., 2003) and N-methyl-D-

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aspartate receptor (NMDAR) phosphorylation (Hildebrand et al., 2016). The spinal cord microglia P2X4R-BDNF to neuronal KCC2/NMDAR pathway is not only engaged following traumatic peripheral nerve injury but also mediates pain hypersensitivity caused by morphine (Ferrini et al., 2013) and in a model of herpetic pain (Matsumura et al., 2016). For a more in-depth overview of this signaling pathway of chronic pain, please see reviews on this topic (Salter and Beggs, 2014; Mapplebeck et al., 2017; Inoue, 2018).

In parallel to studies focusing on the P2X4R pathway within microglia as it relates to pain hypersensitivity caused by PNI, researchers linked aberrant microglial function more generally to pathological pain (Marchand et al., 2005). Since P2X4Rs are strongly expressed by microglia, the two fields could then inform each other about possible overlapping mechanisms. A critical discovery that prompted the two fields to merge was the finding of an apparent sex specificity in the use of microglia during chronic pain: in a report by Sorge and colleagues (2011), the TLR4 agonist, lipopolysaccharide (LPS), which is primarily expressed by microglia in the central nervous system, caused pain behaviours in mice. However, this only occurred in males. Female mice showed no response to intrathecal LPS administration. These findings led the authors to further characterize the sex-dependency of microglial P2X4R and BDNF pathway as it relates to chronic pain and to address the question of how *female* biology results in the same degree of pain hypersensitivity in the absence of a contributory role from microglia. Nevertheless, conditions under which microglia may mediate pain in females remain to be determined. In the following section, we describe what we have learned to date regarding specific nodes of the P2X4R sex-dependent pathway in chronic pain.

2. What do we know about sex differences in spinal microglia and P2X4R signaling?

Since the discovery that P2X4R signaling in microglia is not an underlying driver of chronic pain hypersensitivity in females, where the pathway diverges between sexes has been identified (Sorge

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et al., 2015; Mapplebeck et al., 2018). Namely, there is a male-specific upregulation of P2X4R after PNI, which induces pain hypersensitivity, and this phenomenon is consistent across species and pain models. Using quantitative reverse-transcriptase PCR of the injured side of the dorsal spinal cord, an analysis at the mRNA level revealed that *P2rx4* transcripts were upregulated in male, but not in female, mice after PNI (Sorge et al., 2015). In a separate study, microglial P2X4R protein levels were measured in rats after nerve injury: fluorescence-activated cell sorting-isolated CD11b+ cells from the spinal cord showed upregulation of P2X4R via Western blot, in males but not females (Mapplebeck et al., 2018). Furthermore, in primary cultures of rat microglia, ATP stimulation alone increased P2X4R levels, but again only in male-derived cells (Mapplebeck et al., 2018). Together, these data demonstrate that the P2X4R is upregulated in males but not in females after a chronic pain-inducing PNI.

The upregulation of the *P2rx4* message is controlled by the transcription factors interferon regulatory factor-5 (IRF5) and IRF8. IRF8 is an essential modulator of microglial reactivity and it directly regulates IRF5 expression (Masuda et al., 2012; Masuda et al., 2014). IRF5 then binds directly to the *P2rx4* promoter to drive gene expression (Masuda et al., 2014; Mapplebeck et al., 2018). When IRF5 is depleted in mouse spinal cord, *P2rx4* levels are not upregulated after PNI and, correspondingly, such mice have reduced pain (Masuda et al., 2014). To address whether there are any sex differences in the levels of *Irf8* and *Irf5* mRNA during chronic pain, the spinal cords of males and females were probed. Surprisingly, *Irf8* and *Irf5* transcripts were upregulated equally in both sexes (Sorge et al., 2015). This suggested that the divergence between sexes in *P2rx4* mRNA expression occurs only after *Irf8/5* regulation. Indeed, IRF5 binds to the *P2rx4* promoter region with a higher affinity in male- compared to female-derived primary microglia as well as in the whole spinal cord of male rats compared to females (Mapplebeck et al., 2018). Thus, differential binding of IRF5 at the *P2rx4* promoter region in males versus females appears to underlie the sexually dimorphic upregulation of

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microglial P2X4R after PNI. While the mechanisms regulating the differential binding of IRF5 to *P2rx4* remain unknown, one possibility may be due to epigenetic modifications of the *P2rx4* promoter.

It is possible that under certain circumstances the *P2rx4* promoter may become accessible even in females. For example, Matsumura et al. (2016) developed a female mouse model of postherpetic neuralgia, a painful condition associated with complications from shingles (Hadley et al., 2016). They showed that inhibiting P2X4R with NP-1815-PX blunted pain in females with herpetic pain hypersensitivity. In this model there was microgliosis in the dorsal horn and the BDNF sequestering agent, TrkB-Fc, also suppressed the pain hypersensitivity implying that the microglial P2X4R-BDNF pathway was activated. Whether there are other circumstances where P2X4R may be upregulated in microglia in females, for example opioid-induced hyperalgesia, remains to be determined.

The endogenous ligand for P2X4Rs expressed in spinal microglia is presumed to be ATP (Tsuda et al., 2005). The source of ATP has been determined to be neurons within the dorsal horn itself, rather than primary sensory neurons, microglia, or astrocytes (Masuda et al. 2016). Levels of ATP are heightened in the spinal cord after PNI due to an increase of vesicular nucleotide transporter (VNUT) in dorsal horn neurons, at least in males: mice lacking VNUT do not show PNI-induced increases in ATP and hypersensitivity is suppressed (Masuda et al., 2016). Conversely, viral-driven expression of VNUT in dorsal horn neurons in VNUT-deficient mice rescues the increase in ATP and the hypersensitivity induced by PNI. Currently, it is unknown whether the VNUT increase is sex-differentiated. Thus, it would be interesting to determine whether this is true in females and what biological consequences this might have.

Microglia from male, but not female, rats exhibit ATP-stimulated increases phosphorylation of p38-MAPK and an increase in the expression and release of BDNF (Mapplebeck et al., 2018), consistent with male-specific expression of P2X4Rs. In a separate study, a male-specific increase in phosphorylated p38-MAPK was also observed in microglia, and inhibition of p38-MAPK via

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intrathecal drug administration reduced PNI- and formalin-induced pain in male, but not female, rodents (Taves et al., 2016). It was later demonstrated that the key isoform driving pain in a sex-dependent manner is p38 α (Luo et al., 2018). Together, these data suggest that male microglia are uniquely prepared to mediate pain-inducing signaling. Indeed, male microglia that have been pre-exposed to ATP and then applied to naïve rat spinal cord *in vivo* induce a pain-like response, but female microglia that have been pre-treated with ATP do not have this effect (Mapplebeck et al., 2018). It is not clear whether and how female microglia respond to ATP stimulation at the level of molecular mediators. Nevertheless, female microglia may be involved in the maintenance of pain in females under certain conditions, as described below (Vacca et al., 2014; Yang et al., 2015; Peng et al., 2016).

Given the distinct microglial response to ATP in males and females, it was important to determine whether outcomes downstream of microglia signaling—i.e. the changes in neurons that underlie chronic pain—also occur in females, since the sexes exhibit indistinguishable behavioural sensitization after PNI. Earlier evidence implied that neurons underlie chronic pain in males and females, based on the finding that inhibiting NMDARs via intrathecal administration of AP5 suppressed pain similarly in females and males (Sorge et al., 2015). However, whether the loss of KCC2 function is also involved in females was not known until recently. Increased BDNF from microglia culminates in the reduction of KCC2 expression with a consequent loss of chloride gradient regulation of dorsal horn neurons in males (Coull et al., 2005). Recently, it was shown that KCC2 is downregulated in females as it is in males (Mapplebeck et al., 2019), despite the lack of congruency in the steps immediately preceding the male-typical mechanism of chronic pain, such as the requirement for microglial BDNF. Thus, we now know that after peripheral injury, microgliosis occurs in males and females and that there is an upregulation of transcription factors IRF8 and IRF5, but the critical point of divergence between the sexes is the binding of IRF5 to the *P2rx4* promoter. In the framework of this canonical microglia-P2X4R pathway, typically seen in males, and the microglia-P2X4R independent

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pathway, typically seen in females, converge at the level of KCC2 downregulation. It remains unclear how females achieve KCC2 downregulation that results in pain hypersensitivity, although the adaptive immune system is hypothesized to be involved (Sorge et al., 2015). For a pictorial representation of this pathway in males and females, see Mapplebeck et al. (2016) and (Mapplebeck et al., 2017).

Several questions remain about sex differences in the sequelae leading up to P2X4R expression and after BDNF binding to TrkB on neurons. For example, sensory neurons express colony-stimulating factor 1 (CSF-1) after nerve injury and this induces microglial activation and proliferation as well as mechanical hypersensitivity in males (Guan et al., 2016). In a recent report, Yu and colleagues (2020) found that CSF-1 from primary sensory neurons also induce proliferation of macrophages in the dorsal root ganglion after peripheral nerve injury in males, but not in females. To what extent microgliosis in the *female* spinal cord (Sorge et al., 2015; Mapplebeck et al., 2018) is mediated by CSF-1 after nerve injury, and what the functions of those microglia are, is still an open question.

The role of sex hormones is also important to address. While there is evidence that high circulating male sex hormones are not required to induce pain from male ATP-stimulated microglia (Mapplebeck et al., 2018), there is evidence that *in vivo* testosterone treatment to females does enable the P2X4R-mediated-microglia response to occur, at least in a chronic inflammatory model of pain (Sorge et al., 2015). Differences in pain models may explain why sex hormones may be critical for a microglia-dependent phenotype in one case but not another—more work certainly needs to be done to clarify these processes.

Finally, it continues to be a critical point of interest regarding whether P2X4R sex differences in chronic pain also occur in humans with pain and whether we can use this knowledge to develop treatments specific for each individual. It is possible that P2X4R signaling in the context of chronic pain is sexually dimorphic in humans, as surface P2X4R expression on blood eosinophils is higher in males compared to females during healthy conditions (Paalme et al., 2019). Moreover, *P2RX4* level is

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elevated in circulating leukocytes after moderate exercise in chronic fatigue syndrome patients with comorbid fibromyalgia syndrome (Light et al., 2009). Whether this finding is related to the pain symptoms per se remains to be determined. Given the lack of studies examining P2X4R in humans in the context of chronic pain, it is imperative for the field to fill this gap if we are to translate knowledge from rodent studies to the clinic. Particularly in the context of this review, it will be interesting to determine whether expression of P2X4R increases in a sexually dimorphic manner during chronic pain in humans as it does in rodent microglia (Mapplebeck et al., 2018).

3. Generalizability of spinal microglial- and/or P2X4R-dependency in different pain conditions

The evidence above is based on studies that detailed sex-specific behavioural sensitization following PNI: P2X4R-mediated pain mechanisms of pain hypersensitivity. In this section, we describe evidence as to whether sex differences in the P2X4R-mediated pathomechanism occur in pain states other than nerve-injury induced pain (also see Table 1), as well as more generally examine the evidence for microglia involvement in pain in females.

Hyperalgesic priming is a model of chronic pain in which an initial pain-inducing injury causes a heightened response to a subsequent injury, even after the first one has apparently healed. Specifically, once animals with the initial injury—the priming injury—no longer show a pain-like response, a second injury, which normally would not cause pain hypersensitivity, is applied. In “primed” animals, the normally sub-threshold second stimulus results in hypersensitivity, indicating that there has been a change in the nociceptive circuitry to make the animal now sensitive to a normally non-painful stimulus.

Using a hyperalgesic model with intraplantar soluble interleukin-6 (IL-6) receptor as the molecule to establish priming and prostaglandin E2 (PGE-2) to instigate it, Paige and colleagues (2018)

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examined sex differences in the role of P2X4R and p38-MAPK, the same signaling mediators that are sex-differentiated in chronic neuropathic and inflammatory pain (see Section 2). They found that when P2X4R or p38-MAPK signaling was inhibited at the time of the initial injury via intrathecal TNP-ATP or skenipnone, respectively, the subsequent injury no longer resulted in a hyperalgesic response, indicating that the changes to nociceptive circuitry due to the initial injury were prevented. Similar to the nerve injury pain models, this intervention at P2X4R and p38-MAPK signaling suppressed the hyperalgesic responses only in males. Notably, it was critical that the intervention be administered at the time of the initial injury, as later administration at the time of the second injury did not prevent the hyperalgesia. A further point regarding the mechanism leading to persistent pain in this model of hyperalgesic priming is that the P2XR blocker, TNP-ATP, *did* suppress the development of the initial injury response in females and males suggesting that P2X4R inhibition did have an effect. But, for females, this effect was only transient as they still remained “primed” for the second injury—i.e. TNP-ATP did not prevent the long-term consequences. As TNP-ATP is not totally specific to P2X4R (North and Jarvis, 2013), the prevention of the initial sensitization and/or of the priming may be mediated by other P2XRs. Thus, based on this work P2X4R and p38-MAPK signaling is important in the establishment of hyperalgesic priming, but only in males, whereas both of these signaling molecules are important for males and females for the expression of the initial injury.

To address the downstream step in the P2X4R-p38-MAPK pathway of pain within hyperalgesic priming, the same group of researchers asked whether BDNF signaling underlies the sex-dependent response in hyperalgesic priming (Moy et al., 2019). Again using IL-6 as the inducer of priming and PGE-2 to unmask the priming, they found that inhibiting BDNF signaling using intrathecal TrkB-Fc, which sequesters BDNF, prevented priming in male mice only. When this experiment was done in rats, the sex difference disappeared, and females were also rescued from hyperalgesic priming. The mechanisms underlying this form of hyperalgesic priming then must be different between species, at

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least for females. Given that microglial BDNF is critical for male-specific neuropathic pain (Sorge et al., 2015), the Moy et al. (2019) study asked whether microglial BDNF was also required for the acute and priming phases of IL-6 and PGE-2 induced pain, but found that removing BDNF from microglia had no observable effect on the pain phenotype. Together, these data further emphasize the need to study any purported pain mechanism across multiple pain conditions and species in order to determine its generalizability as well as unique response profile to different pathological states.

In a different pain priming paradigm, hindpaw incision during early life followed by a second incision in later life, animals show enhanced hyperalgesia and longer lasting pain (Walker et al., 2009). Moriarty et al. (2019) used this paradigm to study the sex-dependent involvement of microglia and p38-MAPK signaling in the establishment of incision-primed hyperalgesia in rats. They found that when microglia were inhibited at the time of neonatal incision injury via systemic minocycline treatment, or intrathecal p38-MAPK inhibition, using SB203580, that incision in later life no longer caused enhanced hyperalgesia, but only in males not in females. The authors further linked microglia to this sex-specific phenomenon by showing that after the second incision in adulthood, gene expression of microglial markers increased in females to a greater extent than in males, who were rescued by early life minocycline treatment. Together with the above examples, this suggests that even during early life, in male rodents use microglia in priming injury to produce persistent and latent pain states. It will be interesting to determine whether P2X4R signaling is also involved in neonatal incision pain priming, as it is involved in adult primed states (Paige et al., 2018) and in chronic pain conditions (Inoue, 2019).

P2X4Rs could be involved in pain hypersensitivity independent of microglia via expression and activation of these receptors in other types of cell (Ulmann et al., 2010). Under such alternative (non-microglia dependent) situations, P2X4R may then be involved in driving pain in females. For example, Lalisie and colleagues (2018) showed that a whole-body genetic knockout of P2X4R in a chronic inflammatory model, intraplantar complete Freund's adjuvant, prevented mechanical hypersensitivity

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in both males and females. The authors concluded that primary sensory neurons are the key source of P2X4R which induces hypersensitivity in this inflammatory model. P2X4Rs have also been found to mediate activity-induced muscle pain via muscle macrophages: blocking P2X4R signaling in the muscle, or specifically in muscle macrophages, reduced hyperalgesia in both male and female mice (Oliveira-Fusaro et al., 2020). Thus, depending on the particular set of conditions and initiating stimuli, females also show pain hypersensitivity mediated by P2X4Rs.

Microglia may also be involved in females to drive pain under certain conditions. One report using a chronic constriction injury model of pain showed that microglial activation is correlated to hypersensitivity, with female mice showing longer maintenance of activated microglia and pain compared to males (Vacca et al., 2014). Additionally, in a spinal transection model of neuropathic pain, females were protected from pain after ablation of CX3CR1-positive cells, which include microglia and myeloid cells (Peng et al., 2016). Interestingly, during early pregnancy, female mice appear to rely on microglia to drive inflammatory and neuropathic pain, as minocycline was capable of reversing pain during this window (Rosen et al., 2017). In a separate study, mice with nerve injury-induced pain did not exhibit sex differences in gene expression in purified sensory neurons nor in microglia, including for *P2rx4* transcripts (Lopes et al., 2017). Together, these data suggest that sex differences in the involvement of P2X4R signaling, and possibly microglia more broadly in pain, are dependent on the type of pain condition being examined and the state of the animal itself. Although microglia and P2X4R signaling show a sex-dependent involvement in many chronic pain conditions, it remains essential to elucidate the details under which conditions there are sex differences, and what underlies the ability of female microglia to contribute to pain in one circumstance but not another.

4. Sex differences in microglia and P2X4R signaling

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As described above, spinal microglia and P2X4R signaling underlie multiple pain states, and in many cases, are sex-differentiated. Other than the spinal cord, central nervous system regions involved in pain-signal transmission also exhibit basal sex differences in microglia, which may further underlie sex-specific responses after PNI.

Microglia have multiple functions in the healthy and diseased central nervous system (Kettenmann et al., 2011; Salter and Stevens, 2017). They can respond to the stimuli such as injury by producing pro-inflammatory cytokines and they also can produce anti-inflammatory cytokines and show neuroprotective effects (Lucin and Wyss-Coray, 2009). However, sex differences in microglia state during development or after an injury remain unclear. During early development in mice and rats, male and female brain microglia differ in number (Mouton et al., 2002; Schwarz et al., 2012; Lenz et al., 2013), and in expression of cytokines and chemokines (Schwarz et al., 2012). The sex differences in these early-life stages might be due to the neonatal testosterone surge since there are limited sex differences in microglial density and morphology *prior* to this developmental period (Schwarz et al., 2012). Neonatal androgens are converted to estradiol by the local aromatase enzyme in the brain and result in brain masculinization and defeminization (McCarthy et al., 2009). Just after birth, during the neonatal testosterone surge (Clarkson and Herbison, 2016), microglia in males have higher chemokine levels of chemokine (C-C motif) ligand 4 (CCL4) and CCL20 compared to females, which may drive an increase in microglial cell number in males at early ages (Schwarz et al., 2012). Indeed, four days after birth, neonatal male mice have more microglia in the CA1 and CA3 of the hippocampus, the dentate gyrus, the parietal cortex, and the amygdala (Schwarz et al., 2012). Thus, the basal microglial reprogramming window may overlap with the critical hormonal period, specifically with the androgen surge. In the preoptic area of the rat brain, a region responsible for adult male sexual behaviour, males exhibited increased secretion of a pro-inflammatory mediator (PGE-2) from microglia compared with females due to the higher level of estradiol in males during this critical period; this was necessary and

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sufficient for the masculinization of neuronal dendritic spine density and male copulatory behaviour (Amateau and McCarthy, 2004; Lenz et al., 2013; Rahimian et al., 2019). Therefore, microglia that express PGE-2 and its receptor might be essential for studying developmental sex differences in the nervous system. Studies indicate the sex difference in microglia number exists after puberty: in adolescence and adulthood, female mice have higher number of total (Mouton et al., 2002) and activated (Schwarz et al., 2012) microglia than males.

With recent advancements in sequencing technology, our understanding of sex differences in microglia has been transformed. Multiple studies on microglia transcriptome (Guneykaya et al., 2018; Villa et al., 2018), translome (Kang et al., 2018), and proteome (Guneykaya et al., 2018) showed that microglia in the healthy adult brain is sexually differentiated. Two transcriptomic studies in mice found that male-derived microglia are more pro-inflammatory (Guneykaya et al., 2018; Villa et al., 2018), and that female-derived microglia exhibit a neuroprotective-like transcriptome (Villa et al., 2018). On the other hand, a different study found that female microglia express more genes involved in the inflammatory response (Thion et al., 2018). Although all studies showed that microglia are sexually differentiated, whether males or females differ in proinflammatory transcriptome signature is debated. Such contrasting findings highlight the sensitivity of microglia transcriptome to the technical isolation, developmental stage, and the source of microglia. For reviews on this topic see (Hanamsagar and Bilbo, 2016; Kodama and Gan, 2019; Villa et al., 2019).

Interestingly, Villa and colleagues showed that female microglia maintain a sex-specific transcriptome when implanted into the male brain (Villa et al., 2018), indicating the programmed long-term gene regulation in these cells and irrespective of the circulating hormonal environment. Therefore, it is interesting to understand the mechanisms of gene regulation like epigenetic modulation in the context of organizational sex differences in microglia. Despite the lack of consistency in the

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directionality of the sex differences, all studies to date comparing male and female microglia have unequivocally found sex differences in the transcriptomic signature, and these depend on age.

Environmental stimuli like presence of LPS or antibiotic treatment further influence a sex-specific response in the microglial transcriptome. The study by Hanamsagar and colleagues (2017) showed that intraperitoneal injection of LPS to mice accelerates male microglial maturation. However, the same LPS challenge did not affect female-derived microglia (Hanamsagar et al., 2017). Other environmental stimuli, such as antibiotic treatment or the effect of the maternal microbiome on microglial maturation, have been studied. Thion et al. (2018) observed maternal microbiome depletion induced a sex-specific response. Male microglia were more vulnerable to the absence of a microbiome during early developmental stages, but female microglia were more sensitive in adulthood (Thion et al., 2018). Thus, environmental stimuli affect microglial function in a sex-specific way depending on the developmental stage.

The basal sex difference in purinergic receptor expression may determine the phenotypic state of microglia. Guneykaya et al. (2018) showed in the healthy mouse brain, translation of purinergic receptors P2X4R, P2X7R, and P2Y12R was significantly higher levels in microglia in males as compared with females. In a separate study, Crain and colleagues showed a sex difference in the expression of multiple purinergic receptors in freshly isolated microglia cells from mouse brain. Specifically, females express lower levels of *P2rx1*, *P2rx4*, *P2ry12* and *P2ry13* in adulthood and higher levels of *P2rx3* (adulthood) and *P2rx5* and *P2ry4* (only at early ages postnatal day 3) compared to males (Crain et al., 2009). Sex-dependent P2X4R expression might explain the sexually distinct pathways of P2X4R involvement in painful or disease conditions. For example, a study in ischemic stroke recovery showed that global knockout of P2X4R provided acute neuroprotection, i.e. reduced infarct volume, in both sexes compared to the wild type mice (Verma et al., 2017). However, when the P2X4R knock-out was specific to myeloid cells, only females showed acute neuroprotection after

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stroke. Observed sex differences were not related to the activational effects of estrogen, suggesting this sex difference is under the influence of the organizational effects of gonadal hormones or sex chromosomes (Verma et al., 2017).

Sex differences in brain microglia as they relate to pain modulation have been studied in the rat periaqueductal gray (PAG). Doyle et al. (2017) showed that naïve, female rat microglia have a more activated morphology compared to male, despite no sex difference in the number of microglia in PAG. They also examined how these basal sex differences remodel after activation of TLR4 which is primarily expressed in microglia. Interestingly, the sex difference in microglial activation at baseline potentiated after TLR4 activation was induced with LPS treatment. Doyle and colleagues also concluded that microglial TLR4 in the PAG contributes to the attenuated response to morphine observed in females, since blocking TLR4 in the PAG enhanced morphine efficacy in females but not in males (Doyle et al., 2017). This underlying sex-differentiated neural response may explain why morphine is less effective in females compared to males (Cepeda and Carr, 2003).

Collectively, multiple studies show sex differences in microglia during healthy states and across different brain regions. These sex differences are sensitive to age, developmental stage, and environment. In a disease situation sex differences in number, morphology, and gene expression of microglia remodels. In order to fully understand the role of microglia in disease conditions, such as chronic pain, it is crucial to know the basal state of the sex difference as well as the sex-specific responses to environmental challenges.

5. Summary

Microglia are critical for maintaining a healthy nervous system (Salter and Stevens, 2017), and when these cells malfunction due to disease or injury, they can cause pathological conditions such as chronic pain. As sex differences are normally present in microglia cell number, morphology, and gene

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expression, it stands to reason that sex differences are similarly present in microglia-dependent pathologies. Indeed, microglia are disease-mediating in models of chronic inflammatory and neuropathic pain, but in a sex-specific manner. As reviewed here, data from studies link microglial dysfunction to chronic pain in only male rodents. To date, there has been a focus on the purinergic receptor P2X4R as the male-biased microglial mediator of chronic pain. While there are a few examples of microglia possibly being involved in chronic pain in females, there is no report of microglial inhibition reducing pain in females *but not in males*. Thus, microglia-dependent chronic pain is male-biased and females likely use alternative pathways, potentially T lymphocytes—currently, these female-biased pathways are understudied, and there is a great need to understand them further.

Additional noteworthy gaps remain in the area of sex differences in microglia-P2X4R signaling of chronic pain. What role do sex hormones play? What causes IRF5 to differentially bind to the *P2rx4* promoter between sexes after nerve injury? What makes P2X4R/microglia involved in female pain under certain conditions but not others? Do microglia in the females dorsal spinal cord expand after nerve injury due to CSF-1, like in males? And what role do expanded microglia in females play, if not pain? It is also of great interest to the broader pain field how, where, and under which circumstances P2X4Rs and microglia underlie pathological pain in humans, and whether these contributions are sexually differentiated.

We hope that by continuing the discussion of sex-specific mechanisms of chronic pain, researchers will recognize that it is vital to study and report on both sexes in preclinical work so that ultimately clinical therapies for chronic pain are designed for efficacy in every person.

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Table 1. The role of P2X4R in various pain conditions.

| Pain Condition | Subjects studied | Key Finding | Reference |
|------------------------------------------------------------------------|-------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|
| Activity-induced muscle pain | Male and female C57BL/6 mice | Immunohistochemical staining shows P2X4R is upregulated in muscle macrophages, but the data were not split by sex. Intramuscular injection of the P2XR4 blocker 5-BDBD, or genetic knockdown of P2X4R with a macrophage-specific miRNA, prevented hyperalgesia in both sexes. | (Oliveira-Fusaro et al., 2020) |
| Peripheral diabetic neuropathy | Male Wistar rats | P2X4R protein is upregulated in the dorsal root ganglion, as detected by Western blot. Intrathecal or ganglionar injection of a P2X4R antagonist (PSB-15417), or of intrathecal P2X4R antisense oligodeoxynucleotide, reversed mechanical hypersensitivity. | (Teixeira et al., 2019) |
| Neuropathic pain (spared nerve Injury and chronic constriction injury) | Male and female Sprague-Dawley rats | Male rats, but not females, with spared nerve injury had increased P2X4R protein expression in microglia of the dorsal spinal cord. P2X4R antagonist (TNP-ATP) attenuated allodynia in rats with chronic constriction injury, but only in males and not females. | (Mapplebeck et al., 2018) |

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| Inflammatory pain (intraplantar injection of complete Freund's adjuvant) | Male and female C57BL/6 mice | P2X4R expression was increased in sensory neurons of the dorsal root ganglion in male mice; females were not tested in this experiment. Nevertheless, P2X4R-deficient female mice did not exhibit hypersensitivity to CFA, unlike their wild-type counterparts. | (Lalisse et al., 2018) |
| Hyperalgesic priming (intraplantar injection of soluble interleukin-6 receptor followed by prostaglandin E2) | Male and female Swiss Webster mice | Intrathecal injection of TNP-ATP (P2X4R inhibitor) suppressed the initial response to soluble interleukin-6 receptor (IL-6r) in both sexes. Intrathecal TNP-ATP blocked subsequent hyperalgesia to prostaglandin E2 only when given at the time of initial IL-6r administration, and only in males. TNP-ATP did not reverse established priming when given after prostaglandin E2 administration. | (Paige et al., 2018) |
| Partial sciatic nerve ligation | Male and female C57BL/6 mice | No sex difference was found in <i>P2rx4</i> transcript levels in purified peripheral afferent neurons or in isolated microglia after nerve injury. | (Lopes et al., 2017) |
| Herpetic pain | Female C57BL/6 mice | P2X4R mRNA level in the spinal dorsal horn and immunoreactivity in spinal microglia were increased. Selective P2X4R | (Matsumura et al., 2016) |

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| | | antagonist (NP-1815-PX) had an anti-allodynic effect when administered intrathecally. | |
| Spared nerve injury | Male and female CD1 mice | <i>P2rx4</i> mRNA level is upregulated in dorsal horn spinal cord of male mice, but not in females. | (Sorge et al., 2015) |
| Cancer-induced bone pain | Female Sprague-Dawley rats | P2X4R mRNA and protein was upregulated in spinal microglia. Silencing receptor activity with intrathecal P2X4R siRNA attenuated mechanical hypersensitivity. | (Jin et al., 2014) |
| Inflammatory pain (intraplantar injection of complete Freund's adjuvant, carageenan, or formalin) | Male C57BL/6 mice | P2X4R deficient mice show reduced hypersensitivity and impaired production of PGE2 after intraplantar complete Freund's adjuvant or carageenan. P2X4R knockout mice also exhibit modest reductions to acute pain formalin-induced pain, but hypersensitivity at baseline on the hotplate assay. | (Ulmann et al., 2010) |
| Chronic fatigue syndrome comorbid with fibromyalgia syndrome | Male and female human subjects | After moderate exercise, chronic fatigue syndrome (CFS) and CFS-fibromyalgia syndrome patients showed elevated level of P2RX4 in circulating leukocytes compared with control subjects. Baseline P2RX4 | (Light et al., 2009) |

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| | | expression did not differ between patients and controls. | |
| Inflammatory pain (intraplantar injection of complete Freund's adjuvant or formalin, or intraperitoneal injection of acetic acid) and neuropathic pain (L4 spinal nerve transection) | Male C57BL/6 mice | P2X4R knockout mice show reduced mechanical, but not cold, allodynia caused by nerve injury. P2X4R knockout also exhibit attenuated inflammatory pain. P2X4R knockout does not affect baseline mechanical or thermal measures, nor does it affect acute models of pain (visceral acetic acid or intraplantar formalin). | (Tsuda et al., 2009) |
| Inflammatory pain (formalin Injection to dorsal paw) | Male Lewis rats | P2X4R+ immunoreactivity was increased in the spinal cord dorsal horn 7 days after intraplantar formalin injection. | (Guo et al., 2005) |
| Spinal nerve injury | Male Wistar rats | P2X4R protein was selectively upregulated in spinal cord microglia. Knockdown of P2X4R with P2rx4 antisense oligodeoxynucleotide or P2X4R inhibition | (Tsuda et al., 2003) |

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| | | with TNP-ATP suppressed mechanical allodynia. Intraspinal injection to naïve rats of ATP-stimulated microglia produced allodynia. | |
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