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Experimental autoimmune prostatitis induces microglial activation in the spinal cord

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Abstract

Background—The pathogenesis of chronic prostatitis/chronic pelvic pain syndrome is unknown and factors including the host's immune response and the nervous system have been attributed to the development of CP/CPPS. We previously demonstrated that mast cells and chemokines such as CCL2 and CCL3 play an important role in mediating prostatitis. Here, we examined the role of neuroinflammation and microglia in the CNS in the development of chronic pelvic pain.

Methods—Experimental autoimmune prostatitis (EAP) was induced using a subcutaneous injection of rat prostate antigen. Sacral spinal cord tissue (segments S4–S5) was isolated and utilized for immunofluorescence or QRT-PCR analysis. Tactile allodynia was measured at baseline and at various points during EAP using Von Frey fibers as a function for pelvic pain. EAP mice were treated with minocycline after 30 days of prostatitis to test the efficacy of microglial inhibition on pelvic pain.

Results—Prostatitis induced the expansion and activation of microglia and the development of inflammation in the spinal cord as determined by increased expression levels of CCL3, IL-1 β , Iba1, and ERK1/2 phosphorylation. Microglial activation in mice with prostatitis resulted in increased expression of P2X4R and elevated levels of BDNF, two molecular markers associated with chronic pain. Pharmacological inhibition of microglia alleviated pain in mice with prostatitis and resulted in decreased expression of IL-1 β , P2X4R, and BDNF.

Conclusion—Our data shows that prostatitis leads to inflammation in the spinal cord and the activation and expansion of microglia, mechanisms that may contribute to the development and maintenance of chronic pelvic pain.

Keywords

prostatitis; pelvic pain; CPPS; neuroinflammation; microglia

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Introduction

Chronic Prostatitis/Chronic Pelvic Pain Syndrome (CP/CPPS) is a chronic pain syndrome with unknown etiology affecting millions of men each year. Clinical studies suggest that multiple factors play a role in the development of pain in prostatitis, including psychological factors, the nervous system, and the immune response [1]. However, a dearth of basic science studies in CP/CPPS has made it difficult to determine the underlying cause of pain in patients with prostatitis. Our lab recently developed two animal models of prostatitis that develop pelvic pain symptoms similar to patients with prostatitis [2, 3]. Using these animal models, we were able to show that pelvic pain in prostatitis required the presence of mast cells and involved the expression of the chemokines CCL2 and CCL3 [4, 5]. Pain was abrogated in CCL2^{-/-} and CCL3^{-/-} mice induced with EAP and, despite their well-established role in immune cell recruitment, CCL2^{-/-} and CCL3^{-/-} mice displayed no significant defects in immune cell recruitment to the prostate [5]. These results suggested that their effects on pelvic pain were through actions at extraprostatic locations. In this context, CCL2 and CCL3 expression in the nervous system has been shown to play a critical role in the development of neuropathic pain in models of peripheral nerve injury (PNI) [6].

In the CNS, activated glial cells can express CCL2 and CCL3 to promote neuropathic pain development [7–9]. Among the glial cells, microglia has been shown to be an important mediator in the development of chronic pain through their expression of the purinergic receptor P2X4R [10]. Expression of P2X4R and the concomitant release of brain-derived neurotrophic factor (BDNF) by activated microglia are required for the transition from acute to chronic pain [11]. In microglia, CCL2 has been shown to be involved in the trafficking of the P2X4R to the cell surface [12] and CCL3 can induce the expression of proinflammatory cytokine IL-1 β [13].

In this study, we hypothesized that prostatitis in EAP would lead to changes in the CNS at the level of the spinal cord. We sought to identify a role for CCL3 in the CNS, similar to that described for CCL2, and to identify cellular mediators of chronic pelvic pain. We demonstrate that EAP is associated with increased expression of CCL3 and its cognate receptors, CCR1 and CCR5, in the sacral spinal cord, induces markers of neuroinflammation such as IL-1 β , and is associated with increased microglial activation and expression of neuromodulators such as BDNF. Pharmacological inhibition of microglial activation was shown to attenuate the maintenance of chronic pelvic pain. These results show that neuroinflammation at the CNS and activation of microglia play an important role in the maintenance of chronic pelvic pain in EAP and potentially in CP/CPPS

Materials and Methods

Animals and Induction of EAP

Mice were housed in pathogen-free conditions in the animal care facilities at Northwestern University and all procedures conducted were approved by Northwestern University's Animal Care and Use Committee. EAP in C57BL/6 and NOD ShiLtJ mice was induced by subcutaneous injection of rat prostate antigen (rat prostate lysates) with adjuvant behind the

shoulder. EAP and chronic pain was allowed to establish for 30 days and verified by testing for tactile allodynia using von Frey filaments as described previously.

Isolation of mRNA and cDNA preparation

Sacral spinal cords were isolated from mice and made into single cell suspensions by digesting in papain and dispase for 10 minutes each. Cells were lysed and mRNA was isolated using the SV Total RNA Isolation System (Promega) per the manufacturer's protocol. mRNA was reverse transcribed into cDNA for real time PCR analysis using the Super Script III Reverse Transcriptase system (Invitrogen). The primers used were as follows: CCL3: 5' – actgctgctgcttctctaca – 3', 5' – atgacactggctgggagcaaa – 3'; CCR1: 5' – ttgcttccatgcctgcctata – 3', 5' – tccactgcttcaggctcttg – 3'; CCR5: 5' – ggatttcaagggtcagttc – 3', 5' – aaccttcttctgagatctgg – 3'; Iba1: 5' – gtcctgaagcgaatgctgg – 3', 5' – catttcaagatggcagatc – 3'; IL-1 β : 5' – gtgtgtgacgttccattaga – 3', 5' – ttagaaacagtccagccatac – 3'; 5' – gacgagaggttcagtgtatgtag – 3'; BDNF: 5' – tggctgacacttttgaccac – 3', 5' – caaaggcacttgactgctga – 3'.

Flow cytometry

Sacral spinal cords were isolated from naïve and EAP mice and digested with papain and dispase to generate single cell suspensions. Cells were washed twice with FACS buffer (2% FBS in PBS) and stained for F4/80 (clone BM8, ebioscience) and CD11b (clone M1/70, ebioscience) at 1:100 dilution each for 30 minutes in the dark. Cells were washed twice in FACS buffer and analyzed using the Accuri C6 cytometer and CFlow software.

Immunofluorescence

Naïve or EAP mice were anesthetized and a transcardial perfusion was performed with 4% paraformaldehyde to fix tissue. Spinal cords were isolated and freshly frozen for immunohistochemical analysis. For immunofluorescence, sections were stained for CCL3, CD11b (both Abcam, Cambridge MA), and Iba1 (Santa Cruz Biotechnology) overnight at 4°C in the dark at 1:50 dilutions followed by Texax Red- or FITC-conjugated secondaries (Santa Cruz Biotechnology, Dallas, TX) diluted 1:200. Slides were mounted in mounting media containing DAPI (Vectorshield, Vector Laboratories, Burlingame CA). Images were taken with a Leica DMI 6000B inverted microscope and deconvolved with AutoQuant Deconvolution algorithms by Media Cybernetics.

Treatment of EAP mice

NOD mice were induced with EAP as previously described. After 30 days, mice were treated with minocycline (40mg/kg by intraperitoneal injections (i.p.) twice on the first day and then once daily for a week. Tactile allodynia testing was measured every other day until the end of the experiment. Upon termination of the experiment, sacral spinal cords were isolated for real time RT-PCR and ELISAs. ELISA for IL-1 β was performed according to the manufacturer's protocol with 100 μ g of protein per sample being loaded per well and samples were run in triplicates.

Results

Mice with EAP have increased activated microglia

Astrocytes and microglia are involved in the generation of neuropathic pain and microglia have been implicated as key players in the generation of chronic pain [14, 15]. We therefore examined the spinal cord of EAP-induced mice to identify changes in numbers and activation status of microglia. Segments of the sacral spinal cord (S1–S4) in EAP-induced mice that correspond to afferent innervation of the prostate gland were dissociated and relative changes in microglial numbers were identified using flow cytometry for CD11b, a surface marker for microglia. As shown in Figure 1A, EAP mice had almost twice the number of CD11b+ cells than naïve mice (right panel). To determine if microglia in EAP-induced mice were activated compared to naïve mice we determined the transcript levels of Iba1, a marker for activated microglia. We found that mice with EAP had 2–3 fold increase in Iba1 transcripts compared to naïve mice (Figure 1B). To confirm these results, we performed double immunofluorescence for CD11b and Iba1 on sacral spinal cord sections from naïve and EAP mice. CD11b+Iba1+ cells were present in greater numbers in the dorsal horn region of the sacral spinal cord in EAP mice compared to naïve mice (Figure 1C). These results indicate the increased abundance and activation of microglia in the spinal cord of mice with EAP.

Microglia have been shown to mediate the transition from acute to chronic pain through their expression of the purinergic ATP receptor P2X4R and the subsequent release of BDNF by P2X4R+ microglia [17]. We therefore examined the expression levels of P2X4R in the spinal cord in EAP mice compared to naïve mice using immunoblots. EAP mice had an approximately three-fold increase in P2X4R expression compared to naïve mice (Figure 1D, 1E). These results show that the increased numbers of microglia present in EAP mice are in a heightened state of activation with implications for the development of chronic pelvic pain in EAP.

EAP induced CCL3, CCR1 and CCR5 expression in microglia

Microglia have been shown to express CCL3 upon activation and CCL3 expression in the spinal cord has been correlated with the development of pain [8, 9, 18]. Additionally, increases in Iba1+ microglia at the site of injury and microglia-specific expression of CCL3 is linked to the development of persistent inflammation [19]. Therefore, we wanted to determine if EAP induced prolonged expression of CCL3 or its receptors. Sacral spinal cord sections from EAP mice were stained using double immunofluorescence for CD11b and CCL3 to determine if microglia express CCL3. CCL3 staining was observed with CD11b+ cells and Iba1+ cells, indicating that microglia in EAP-induced mice were capable of expressing CCL3 (Figure 2A, 2B). Next, we determined if the expression of CCL3 and its receptors, CCR1 and CCR5, were increased at the mRNA transcript level in the spinal cord of EAP mice compared to naïve mice. CCL3 was increased by 6-fold while CCR1 and CCR5 expression was 10- and 5-fold higher, respectively, in mice with EAP (Figure 2C). While these differences were observed in the sacral spinal cord of EAP mice, no difference was seen when comparing spinal cord isolated from the thoracic region (Figure 2C, bottom), showing that EAP-induced changes were localized to the sacral region of the spinal cord.

Our data suggest that microglia are a source of CCL3 in the sacral spinal cord and may contribute to the development of pelvic pain in with EAP.

EAP induces spinal cord inflammation

Microglial activation in the central nervous system contributes to the inflammatory environment found in a number of diseases [20–22] and we determined if prostatitis also resulted in an inflammatory environment in the spinal cords of mice with EAP. We examined the sacral segments of the spinal cord that correspond to afferent innervation of the prostate gland for markers of neuroinflammation. Activation and phosphorylation of extracellular signal-regulated kinase (ERKs) is associated with spinal inflammation and the development of pain [23, 24]. Mice with EAP had increased levels of ERK1/2 phosphorylation in the sacral spinal cord at 30 days compared to naïve mice (Fig. 3A) suggesting that EAP is associated with increased activation and signaling of the different cell types at the spinal cord. We next examined mRNA expression of a number of proinflammatory mediators associated with neuroinflammation, including the cytokines IL-1 β and TNF α , and the growth factor BDNF (brain-derived neurotrophic factor). Sacral spinal cords from naïve and EAP mice were isolated at day 30 and the levels of IL-1 β , TNF α , and BDNF transcripts were determined by real time RT-PCR. IL-1 β was approximately 12 fold higher in EAP mice than their naïve counterparts while BDNF was approximately 22 fold higher in EAP mice (Figure 3B). Conversely, TNF α was not detected in the spinal cords of either naïve or EAP mice. These results demonstrate that EAP induces a proinflammatory environment in the spinal cord that is characterized by the presence of cytokines and neurotrophic factors that are known to play a role in the development and maintenance of chronic pain.

Therapeutic inhibition of microglial activation attenuates pelvic pain in EAP

To establish a direct role for microglia in the development and/or maintenance of chronic pain in mice with prostatitis, we inhibited microglia in mice with EAP by treating them with minocycline, a CNS-permeable tetracycline antibiotic commonly used to inhibit microglial activation [26]. Mice were induced with EAP resulting in significantly higher response frequency to von Frey fiber stimulation at day 30 (Figure 4A). At this time mice were treated with minocycline or PBS for seven days and tactile allodynia was measured every other day. While pelvic pain response frequency remained high and unchanged in EAP mice treated with PBS, EAP mice treated with minocycline had a significant reduction in pelvic pain following treatment (Figure 4B).

We next confirmed the inhibition of microglial activation upon minocycline treatment by examining the mRNA expression levels of P2X4R. Minocycline-treated mice had reduced levels of P2X4R expression indicating a suppression of microglial activation (Figure 5A). We next examined the effect of microglial inhibition on expression of the proinflammatory cytokine IL-1 β . IL-1 β levels were found to be significantly lower in EAP mice treated with minocycline compared to PBS treated controls (Fig. 5B). Similarly, mRNA transcript level of IL-1 β , and BDNF were reduced upon inhibition of microglial activation (Fig. 5C). Conversely, only a minimal difference was observed in CCL3 and CCR1 expression while no difference was observed in the levels of CCR5 between PBS- or minocycline-treated

mice (Figure 5C). Taken together, our data demonstrate that microglia may play an important role in the development and/or maintenance of chronic pain in mice with prostatitis by regulating the expression of mediators that promote the development of chronic pain.

Discussion

The etiology of prostatitis remains elusive. As such, the underlying cause of pain in patients with CP/CPPS is undefined but is believed to include components of the immune and nervous systems [1]. Our lab previously demonstrated that the host's immune system is critical for the development of pain in mice with prostatitis. Mast cells are necessary for the development of pain in mice with prostatitis and T cells, especially the Th1 and Th17 subset of T cells, can induce pain in naïve mice [4, 27]. Having demonstrated that pain from prostatitis is at least in part attributed to the actions of the immune system in the periphery, we turned our attention to the effects of prostatitis on the nervous system in light of our previous work demonstrating that CCL2 and CCL3, two chemokines traditionally involved in recruiting leukocytes, are increased in mice with prostatitis [5]. Coincidentally, CCL2 and CCL3 are also involved in neuropathic pain development in mice with peripheral nerve injury [6]. Increased expression of CCL2 and CCL3 are observed in PNI models of pain in mice and we posited that prostatitis is reminiscent of these peripheral pain models and share similar mechanisms of pain induction. To that end, we show in this paper that microglia are centrally important for the development of chronic pain in mice with prostatitis. Activated microglia express P2X4R and release BDNF and these two factors have recently been shown to regulate the transition from acute to chronic pain [17]. Microglia activation can persist for long periods [28], suggesting that enhanced susceptibility to chronic pain from prostatitis may result from an inability to return microglia to their homeostatic state. This is evident in our EAP mice where inhibition of microglia with minocycline reduced pain in mice with prostatitis by approximately 20%. Markers of microglial activation, P2X4R and BDNF were reduced while minimal differences were observed in CCL3, CCR1, and CCR5. Astrocytes and infiltrating lymphocytes are also able to express CCL3, CCR1, and CCR5 [29–31] and may account for the lack of change in their expression. We postulate that chronic pain as a result of prostatitis is dependent on events that occur in the visceral organs, peripheral nervous system, and central nervous system (Figure 6). Our previous data showing that Th1 and Th17 cells induce pain in naïve mice [27] suggest that inflammation in the prostate contributes to the generation of pain in mice with prostatitis. We hypothesize that inflammation in the prostate leads to the expression of many cytokines and chemokines, including CCL2 and CCL3 [5] and both of these chemokines are known to lower the threshold of activation for neurons in the dorsal root ganglia (DRG) [32, 33], a sensory tissue in the peripheral nervous system, making the host more susceptible to neuropathic pain. Our present data suggests that microglial activation is one component of a whole series of events that contributes to the development and maintenance of chronic pelvic pain in prostatitis (Figure 6).

Activation of neurons in the DRG can lead to the release of neurotransmitters and result in the activation of glial cells in the spinal cord, including microglia [34]. We determined that spinal cords from mice with EAP were inflamed based on their CCL3, IL-1 β , and phospho-

ERK1/2 expression levels, a phenotype that favors the development of neuropathic pain [35]. Interestingly, TNF α was not detected in either naïve or EAP mice after 30 days. In chronic constriction injury (CCI) models, TNF α expression was dramatically increased and peaked in the first seven days after induced-injury to induce expression of P2X4R but its effects are decreased after the acute phase of pain is over [36, 37]. In an experimental autoimmune encephalitis (EAE) model, a mouse model of multiple sclerosis that also results in inflammation in the CNS, TNF α appears to be required early in the induction of EAE and to recruit neutrophils [38]. Thus, in our model of prostatitis that focuses on the chronic stage of prostatitis, it may be that by 30 days, *de novo* expression of TNF α has ceased and the chronic pain experienced by mice with EAP may be due to the activities related to P2X4R and the release of BDNF.

We present herein that pain due prostatitis is similar to how pain is generated upon peripheral nerve injury. Nerve growth factor (NGF) is increased in mice with prostatitis [4] and may be indicative of ongoing neuronal repair due to neuronal damage that may have risen due to prostatitis, presumably as a result of inflammation. Our hypothesis that prostatitis results in conditions similar to PNI pain models that favor the development of chronic pain is independent of neuronal damage so long as the expressed factors are present to initiate the cascade of events leading to chronic pain. In the case of prostatitis, we believe that the high levels of CCL2 and CCL3 is part of the signaling cascade that ultimately lead to activation of microglia and inflammation in spinal cord. Peripheral nerve injury has been shown to promote the deterioration of the blood-spinal cord barrier [39, 40], allowing for the influx of immune cells as part of the proinflammatory environment that promotes neuropathic pain development [41]. It will be useful to determine if such is the case in mice with prostatitis. In particular, the type of macrophage/microglia recruited can help resolve or exacerbate neuronal damage in the spinal cord [42]. Together, our data present a novel pathway that leads to the development of chronic pain in mice prostatitis and may serve as a therapeutic target to help alleviate pain in those patients affected by prostatitis.

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Abbreviations

CP/CPPS	Chronic prostatitis/chronic pelvic pain syndrome
EAP	experimental autoimmune prostatitis
DRG	dorsal root ganglion

REFERENCES

1. Pontari MA, Ruggieri MR. Mechanisms in prostatitis/chronic pelvic pain syndrome. *J Urol.* 2008; 179(5 Suppl):S61–S67. [PubMed: 18405756]

2. Rudick CN, et al. Uropathogenic *Escherichia coli* induces chronic pelvic pain. *Infect Immun*. 2011; 79(2):628–635. [PubMed: 21078846]
3. Rudick CN, Schaeffer AJ, Thumbikat P. Experimental autoimmune prostatitis induces chronic pelvic pain. *Am J Physiol Regul Integr Comp Physiol*. 2008; 294(4):R1268–R1275. [PubMed: 18287220]
4. Done JD, et al. Role of mast cells in male chronic pelvic pain. *J Urol*. 2012; 187(4):1473–1482. [PubMed: 22341813]
5. Quick ML, et al. CCL2 and CCL3 are essential mediators of pelvic pain in experimental autoimmune prostatitis. *Am J Physiol Regul Integr Comp Physiol*. 2012; 303(6):R580–R589. [PubMed: 22814670]
6. Kiguchi N, Kobayashi Y, Kishioka S. Chemokines and cytokines in neuroinflammation leading to neuropathic pain. *Curr Opin Pharmacol*. 2012; 12(1):55–61. [PubMed: 22019566]
7. Menetski J, et al. Mice overexpressing chemokine ligand 2 (CCL2) in astrocytes display enhanced nociceptive responses. *Neuroscience*. 2007; 149(3):706–714. [PubMed: 17870246]
8. Kiguchi N, et al. CC-chemokine MIP-1 α in the spinal cord contributes to nerve injury-induced neuropathic pain. *Neurosci Lett*. 2010; 484(1):17–21. [PubMed: 20692319]
9. Okamura T, et al. Neuronal injury induces microglial production of macrophage inflammatory protein-1 α in rat corticostriatal slice cultures. *J Neurosci Res*. 2012; 90(11):2127–2133. [PubMed: 22791363]
10. Tsuda M, et al. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature*. 2003; 424(6950):778–783. [PubMed: 12917686]
11. Trang T, et al. 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):3518–3528. [PubMed: 19295157]
12. Toyomitsu E, et al. CCL2 promotes P2X4 receptor trafficking to the cell surface of microglia. *Purinergic Signalling*. 2012; 8(2):301–310. [PubMed: 22222817]
13. Kiguchi N, et al. Macrophage inflammatory protein-1 α mediates the development of neuropathic pain following peripheral nerve injury through interleukin-1 β up-regulation. *Pain*. 2010; 149(2):305–315. [PubMed: 20223588]
14. Hains BC, Waxman SG. Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. *J Neurosci*. 2006; 26(16):4308–4317. [PubMed: 16624951]
15. Shi Y, et al. Chronic-pain-associated astrocytic reaction in the spinal cord dorsal horn of human immunodeficiency virus-infected patients. *J Neurosci*. 2012; 32(32):10833–10840. [PubMed: 22875918]
16. Ayoub AE, Salm AK. Increased morphological diversity of microglia in the activated hypothalamic supraoptic nucleus. *J Neurosci*. 2003; 23(21):7759–7766. [PubMed: 12944504]
17. Beggs S, Trang T, Salter MW. P2X4R+ microglia drive neuropathic pain. *Nat Neurosci*. 2012; 15(8):1068–1073. [PubMed: 22837036]
18. Knerlich-Lukoschus F, et al. Spatiotemporal CCR1, CCL3(MIP-1 α), CXCR4, CXCL12(SDF-1 α) expression patterns in a rat spinal cord injury model of posttraumatic neuropathic pain. *Journal of Neurosurgery: Spine*. 2011; 14(5):583–597. [PubMed: 21332278]
19. Kohno H, et al. CCL3 Production by Microglial Cells Modulates Disease Severity in Murine Models of Retinal Degeneration. *The Journal of Immunology*. 2014
20. Getts DR, et al. Ly6c+ "inflammatory monocytes" are microglial precursors recruited in a pathogenic manner in West Nile virus encephalitis. *J Exp Med*. 2008; 205(10):2319–2337. [PubMed: 18779347]
21. Sun YE, et al. Intrathecal injection of spironolactone attenuates radicular pain by inhibition of spinal microglia activation in a rat model. *PLoS One*. 2012; 7(6):e39897. [PubMed: 22768159]
22. Murphy AC, et al. Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis. *Brain, Behavior, and Immunity*. 2010; 24(4):641–651.
23. Zhang X, et al. ERK MAP kinase activation in spinal cord regulates phosphorylation of Cdk5 at serine 159 and contributes to peripheral inflammation induced pain/hypersensitivity. *PLoS One*. 2014; 9(1):e87788. [PubMed: 24498195]

24. Zhuang ZY, et al. ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain*. 2005; 114(1–2):149–159. [PubMed: 15733640]
25. Piao ZG, et al. Activation of glia and microglial p38 MAPK in medullary dorsal horn contributes to tactile hypersensitivity following trigeminal sensory nerve injury. *Pain*. 2006; 121(3):219–231. [PubMed: 16495005]
26. Yoon SY, Patel D, Dougherty PM. Minocycline blocks lipopolysaccharide induced hyperalgesia by suppression of microglia but not astrocytes. *Neuroscience*. 2012; 221:214–224. [PubMed: 22742905]
27. Quick ML, et al. Th1-Th17 cells contribute to the development of uropathogenic *Escherichia coli*-induced chronic pelvic pain. *PLoS One*. 2013; 8(4):e60987. [PubMed: 23577183]
28. Leinders M, et al. Up-regulation of spinal microglial Iba-1 expression persists after resolution of neuropathic pain hypersensitivity. *Neurosci Lett*. 2013; 554:146–150. [PubMed: 24021808]
29. Tanabe S, et al. Murine astrocytes express a functional chemokine receptor. *J Neurosci*. 1997; 17(17):6522–6528. [PubMed: 9254664]
30. Carpentier PA, et al. Differential activation of astrocytes by innate and adaptive immune stimuli. *Glia*. 2005; 49(3):360–374. [PubMed: 15538753]
31. Simpson J, et al. Expression of the β -chemokine receptors CCR2, CCR3 and CCR5 in multiple sclerosis central nervous system tissue. *Journal of Neuroimmunology*. 2000; 108(1–2):192–200. [PubMed: 10900353]
32. Zhang N, et al. A proinflammatory chemokine, CCL3, sensitizes the heat- and capsaicin-gated ion channel TRPV1. *Proc Natl Acad Sci U S A*. 2005; 102(12):4536–4541. [PubMed: 15764707]
33. Jung H, et al. Monocyte chemoattractant protein-1 functions as a neuromodulator in dorsal root ganglia neurons. *Journal of Neurochemistry*. 2008; 104(1):254–263. [PubMed: 17944871]
34. Thacker MA, et al. CCL2 is a key mediator of microglia activation in neuropathic pain states. *European Journal of Pain*. 2009; 13(3):263–272. [PubMed: 18554968]
35. Xanthos DN, Sandkuhler J. Neurogenic neuroinflammation: inflammatory CNS reactions in response to neuronal activity. *Nat Rev Neurosci*. 2014; 15(1):43–53. [PubMed: 24281245]
36. Zhang H, Zhang H, Dougherty PM. Dynamic effects of TNF- α on synaptic transmission in mice over time following sciatic nerve chronic constriction injury. *Journal of Neurophysiology*. 2013; 110(7):1663–1671. [PubMed: 23864372]
37. Zhang Z-J, et al. Chemokine contribution to neuropathic pain: Respective induction of CXCL1 and CXCR2 in spinal cord astrocytes and neurons. *PAIN*. 2013; 154(10):2185–2197. [PubMed: 23831863]
38. Christy AL, et al. Mast cell activation and neutrophil recruitment promotes early and robust inflammation in the meninges in EAE. *Journal of Autoimmunity*. 2013; 42(0):50–61. [PubMed: 23267561]
39. Denieffe S, et al. Classical activation of microglia in CD200-deficient mice is a consequence of blood brain barrier permeability and infiltration of peripheral cells. *Brain, Behavior, and Immunity*. 2013; 34(0):86–97.
40. Echeverry S, et al. Peripheral Nerve Injury Alters Blood–Spinal Cord Barrier Functional and Molecular Integrity through a Selective Inflammatory Pathway. *The Journal of Neuroscience*. 2011; 31(30):10819–10828. [PubMed: 21795534]
41. Z. Adzemovic M, et al. Imatinib Ameliorates Neuroinflammation in a Rat Model of Multiple Sclerosis by Enhancing Blood-Brain Barrier Integrity and by Modulating the Peripheral Immune Response. *PLoS ONE*. 2013; 8(2):e56586. [PubMed: 23437178]
42. Kigerl KA, et al. Identification of Two Distinct Macrophage Subsets with Divergent Effects Causing either Neurotoxicity or Regeneration in the Injured Mouse Spinal Cord. *The Journal of Neuroscience*. 2009; 29(43):13435–13444. [PubMed: 19864556]

Figure 1. Activated microglial cells are increased in mice with prostatitis

Single cell suspension of sacral spinal cords from naive and EAP mice were stained for microglial marker CD11b. Data is representative of 5 independent experiments (A). Sacral spinal cords were isolated from naive and EAP mice and analyzed for microglial activation marker (Iba1) and expressed as fold change over naive and is a combination of three independent experiments (B). Sections from sacral spinal cords from EAP mice were stained for Iba1 (green) and CD11b (red). Inset depicts region of spinal cord shown in Figure (C). Sacral spinal cords were isolated and the expression of P2X4R was determined by

immunoblotting and band densities were measured by densitometry. $p=0.0045$ by the Student's t test (D, E).

Figure 2. Microglia express CCL3 in mice with prostatitis

Sacral spinal cord sections were stained for DAPI (blue), CD11b (green), and CCL3 (red) (A). Sacral spinal cord was stained for Iba1 (green) and CCL3 (red) in EAP mice. Bar represents 10 μ m (B). RNA was isolated from sacral (C, top) or thoracic (C, bottom) spinal cords of naïve and EAP mice and real time RT-PCR was performed to determine expression levels of CCL3, CCR1 and CCR5. Data is expressed as fold change over naïve and is a combination of three separate experiments.

Figure 3. Spinal cords of EAP mice are more activated and inflamed than naive mice
Cell lysates from sacral spinal cords were analyzed by Western blotting for phospho- and total ERK1/2 (A, top). Quantification was performed by densitometry and $p=0.0018$ using the Student's t test (A, bottom). Data is representative of two independent experiments with at least 4 mice per group. Sacral spinal cords were isolated from naive and EAP mice and mRNA were isolated for semiquantitative real time RT-PCR analysis of IL-1 β , and BDNF expression. Data is a combination of three independent experiments and expressed as fold change over Naive (B).

Figure 4. Inhibition of microglia reduces pain in mice with prostatitis

Mice were induced with EAP for 30 days. After 30 days, mice were treated with minocycline and pain was measured using von Frey filaments as described in Materials and Methods. After 30 days, mice with prostatitis had increased pain as determined by tactile allodynia when compared to naïve mice (A). Treatment with minocycline reduces the total number of responses. * $p=0.0278$ using Student's t test (B).

Figure 5. Microglial inhibition decreases expression levels of pain-associated mediators mRNA were isolated from sacral spinal cord and analyzed for P2X4R expression by real time RT-PCR. Data is representative of two independent experiments with 5 mice per group (A). Sacral spinal cords from PBS- or minocycline- treated mice were isolated and an ELISA was performed for IL-1 β . Data is a combination of two experiments with 5 mice per group per experiment. $p=0.0260$ using the Mann-Whitney test (B). mRNA was isolated from sacral spinal cords of PBS- or minocycline- treated mice for real time qRT-PCR analysis. Data is representative of two independent experiments with 5 mice per group (C).

Figure 6. Multiple factors lead to the development of pain in prostatitis

Our proposed model of neuropathic pain development in mice with prostatitis. Prostatitis induces expression of many proinflammatory cytokines, leading to increased sensitivity and activation of neurons in the DRG. Activated neurons in the DRG release neurotransmitters that activate microglia and induces inflammation in the spinal cord. Activated microglia send pain signals to the brain via BDNF and may contribute to the disruption in the blood-spinal cord barrier, allowing for an influx of leukocytes that may further contribute to inflammation and pain development in the spinal cord.