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# Different immune cells mediate mechanical pain hypersensitivity in male and female mice

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**A large and rapidly increasing body of evidence indicates that microglia-to-neuron signaling is essential for chronic pain hypersensitivity. Using multiple approaches, we found that microglia are not required for mechanical pain hypersensitivity in female mice; female mice achieved similar levels of pain hypersensitivity using adaptive immune cells, likely T lymphocytes. This sexual dimorphism suggests that male mice cannot be used as proxies for females in pain research.**





It is now well appreciated that immunocompetent cells are important in pain pathophysiology[1]. Spinal glial cells are reactive to peripheral inflammation or nerve damage and can produce symptoms that include mechanical hypersensitivity (allodynia). Intrathecal injection of drugs disrupting glial functioning can prevent and/or reverse pain behavior in rodents[2], supporting this hypothesis.

These findings, as is standard in the pain field[3], represent the results of experiments that were overwhelmingly conducted on male rodents. We previously reported that the involvement of spinal toll-like receptor 4 (TLR4) in the production of mechanical allodynia was male specific[4]. Here we investigated the possibility that the underlying reason for this sex difference was that microglia, on which TLR4s are located, may not be required for pain processing in female mice.

We induced mechanical allodynia in mice of both sexes using spared nerve injury (SNI), a procedure producing persistent neuropathic pain. We injected mice intrathecally with the glial inhibitors minocycline, fluorocitrate or propentofylline 7 d after the nerve injury, and retested mechanical thresholds over the next 120 min. All three inhibitors produced robust, dose-dependent reversal of allodynia in male mice; no reversal of allodynia was observed in female mice at any dose (**Fig. 1a** and **Supplementary Fig. 1**). Similar results were observed for persistent inflammatory pain (**Supplementary Fig. 2**). Repeated systemic injections of minocycline also reversed mechanical allodynia in male, but not female, mice (**Supplementary Fig. 3**). Moreover, in mice tested 28 d post-SNI, minocycline reversed mechanical allodynia in males, but not females (sex × repeated measures: $F_{5,26} = 2.8$, $P = 0.04$; **Fig. 1a**). As previously demonstrated for TLR4 involvement in chronic pain[4], the sex difference appeared to be dependent on testosterone (**Supplementary Fig. 4**). Reactive microgliosis after SNI was broadly similar between sexes (**Supplementary Fig. 5**).

Although a common action of minocycline, fluorocitrate and propentofylline is to inhibit glial function, these compounds have many different unrelated actions. To determine whether spinal cord microglia themselves are necessary for allodynia in male, but not female, mice, we injected saporin toxin conjugated to macrophage antigen complex-1 (Mac-1, integrin CD11b/CD18 receptor) intrathecally to transiently deplete microglia. At 4 h post-injection, similar microglial depletion in both sexes (**Supplementary Fig. 6**) resulted in significant reversal of allodynia in male mice, but had no effect in females (sex × drug × repeated measures: $F_{4,80} = 4.7$, $P = 0.002$; **Fig. 1b**).

A microglial receptor essential for SNI-induced allodynia is the $P2X_4$ receptor ($P2X_4R$), as previously demonstrated in male rats via the reversal of allodynia by intrathecal TNP-ATP[5]. We replicated the effect in male mice, but found no effect of TNP-ATP in female mice ($t_{14} = 4.1$, $P = 0.001$; **Fig. 1c**). Allodynia was also reversed in male, but not female, mice by inhibitors of key signaling molecules in the spinal cord microglia-to-neuron pain pathway[6]: p38 MAPK[7] (SB203580; $t_{10} = 2.5$, $P = 0.03$) and BDNF[8] (Y1036 or TrkB-Fc; $t_{10} = 5.5$, $P < 0.001$ and $t_{14} = 3.4$, $P = 0.004$, respectively) (**Fig. 1c**). However, SNI-induced allodynia was equally reversed ($t_{10} = 0.6$, $P = 0.56$) in male and female mice by intrathecal NMDA receptor blockade using AP5 (**Fig. 1c**).

The behavioral sex difference was accompanied by an analogous sex difference in dorsal horn gene expression, whereby SNI upregulated *P2rx4* gene expression in male, but not female, mice (**Supplementary Fig. 7**). In contrast, SNI increased expression in dorsal horn of other genes associated with microglial reactivity (and upregulation of *P2rx4*)—*Itgam*, *Emr1*, *Irf5* and *Irf8*—equally in both sexes, consistent with the lack of a sex difference in SNI-induced microgliosis (**Supplementary Fig. 5**). Given that IRF5 and IRF8 are transcription factors upstream of *P2rx4*, the point of divergence between males and females is the injury-induced upregulation of $P2X_4R$ expression.

To further investigate the requirement for microglial BDNF in mice of both sexes, we created transgenic mice in which BDNF was deleted, in a tamoxifen-dependent manner, in CX3CR1-positive cells[9]. We took advantage of the difference in rates of cell turnover between central and peripheral CX3CR1-expressing populations, allowing the periphery to repopulate before testing. Thus, the animals that we tested lacked only the BDNF derived from microglia. These mice

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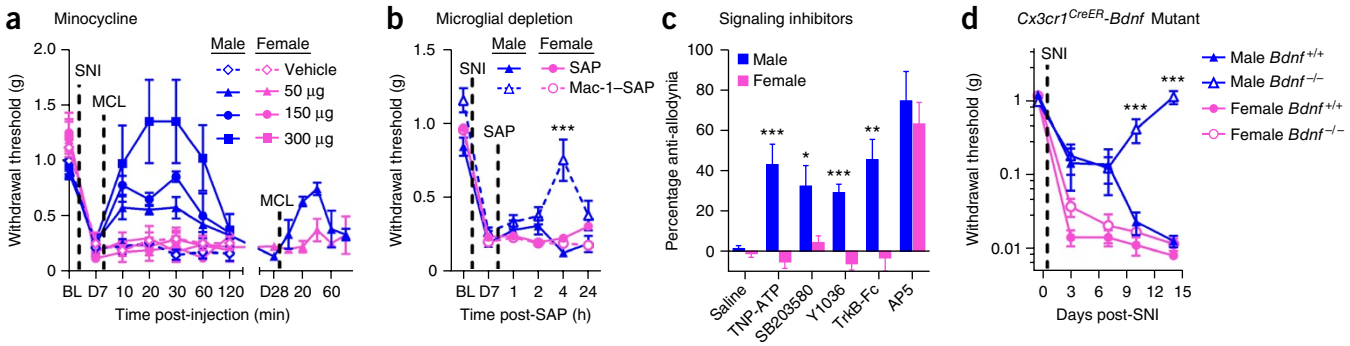


**Figure 1** Mechanical allodynia after nerve injury is reversed by microglial inhibition in male but not female mice. (**a**) Reversal of established SNI-induced mechanical allodynia by intrathecal minocycline (MCL) in male, but not female, mice (see also **Supplementary Fig. 1a**). Data are presented as mean ± s.e.m. Shown is 50% withdrawal threshold from von Frey filaments before surgery (BL), 7 d after surgery (pre-injection; D7) and 10–120 min post-injection of minocycline ($n$ = 4–5 mice per sex per dose). A different set of mice ($n$ = 4 mice per sex) were tested similarly 28 d post-SNI (D28, right). (**b**) Male, but not female, mice treated with Mac-1–saporin (SAP) displayed significantly reduced SNI allodynia at 4 h post-treatment. Data are presented as mean ± s.e.m. Shown is 50% withdrawal threshold from von Frey filaments before surgery (BL), 7 d after surgery, pre-injection (D7), and 1, 2, 4 and 24 h post-SAP ($n$ = 11 mice per sex per condition). (**c**) Intrathecal administration of the P2X inhibitor TNP-ATP, the p38 MAPK inhibitor SB203580, the NGF/BDNF inhibitor Y1036 or the BDNF-sequestering fusion protein TrkB-Fc all blocked SNI-induced allodynia in male, but not female, mice. The NMDA receptor antagonist AP5 blocked allodynia equally in both sexes. Data are presented as mean ± s.e.m. percentage of maximal anti-allodynia (Online Methods; $n$ = 8 mice per sex per drug). (**d**) Development of SNI-induced mechanical allodynia in male and female mice lacking CNS microglial BDNF (that is, with tamoxifen-induced Cre-*loxP*–mediated deletion of the *Bdnf* gene in CX3CR1-positive cells). Mutant ($Bdnf^{-/-}$) male mice failed to develop full allodynia displayed by female $Bdnf^{-/-}$ and wild-type ($Bdnf^{+/+}$) mice. Data are presented as mean ± s.e.m. absolute withdrawal threshold from von Frey filaments before and 3, 7, 10 and 14 d after surgery ($n$ = 4–8 mice per sex per genotype). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with corresponding female mice by $t$ test.

displayed normal reactive microgliosis in the dorsal horn in both sexes (**Supplementary Fig. 8**). Mutant male mice developed modest mechanical allodynia early after SNI surgery, but allodynia was absent by 14 d post-surgery. In contrast, female mutant mice displayed equivalent mechanical allodynia to wild-type mice (genotype × sex × repeated measures: $F_{4,92} = 11.9$, $P < 0.001$; **Fig. 1d**). To determine whether pre-existing SNI-induced mechanical allodynia may be reversed by depleting microglial BDNF in a sex-specific manner, we treated mutant mice with tamoxifen 1 week after SNI. We found that allodynia was reversed in male mutant mice, but not in female mutants or in wild-type mice of either sex (**Supplementary Fig. 9**).

We observed sex differences in baseline mechanical sensitivity and allodynia in some cases (**Supplementary Fig. 10**), but these differences did not affect the interpretation of the qualitative (all or none) sex differences in the microglial-dependence of allodynia. The fact that all of the interventions suppressing microglia signaling reversed allodynia in male, but not female, mice, and that female mice displayed equivalent levels of allodynia as males, demands the existence of an alternate pathway with an analogous role in female mice.

The demonstrated involvement of infiltrating T cells in mechanical allodynia in mice[10–12] suggests that female mice might preferentially use adaptive immune cells instead of microglia to produce allodynia after injury, which we tested using T cell–deficient (and B cell deficient) nude ($Foxn1^{nu}$) and $Rag1^{-/-}$ mice. In contrast with previous observations[10,11], we observed no consistent reduction of mechanical allodynia in nude or $Rag1^{-/-}$ mice (**Supplementary Fig. 10**). Wild-type mice (CD-1 and C57BL/6J strains, respectively) displayed the sex difference in glial inhibitor sensitivity described above. In contrast, both male and female nude and $Rag1^{-/-}$ mice were sensitive to glial inhibitor reversal of SNI or of complete Freund's adjuvant (CFA)-induced allodynia (all genotype × sex interactions $P < 0.05$; **Fig. 2a** and **Supplementary Figs. 11** and **12**). These data indicate that, in the absence of adaptive immune cells, female mice use the male, glial-dependent pathway, as was the case for testosterone-treated females. We previously observed this type of 'switching' from female to male systems in studies of opioid analgesia and hyperalgesia[13–15].

If the absence of adaptive immune cells causes female mice to use a glial-dependent pathway, then restoring these cells may enable the mice

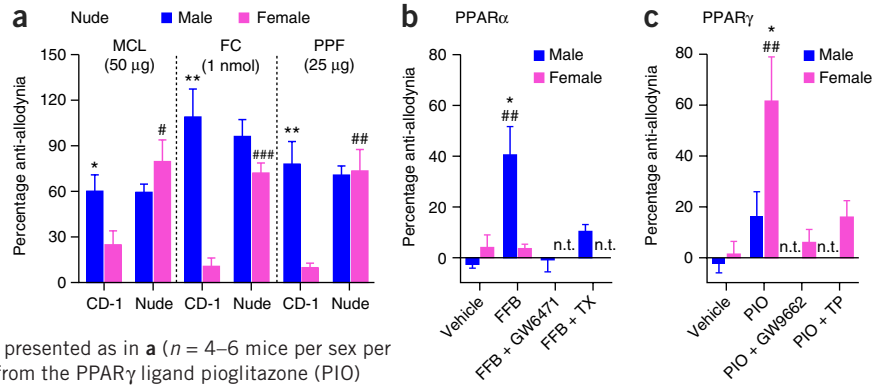


**Figure 2** Mechanical allodynia after nerve injury is mediated by adaptive immune cells in female mice. (**a**) Reversal of mechanical allodynia after SNI by intrathecally administered glial inhibitors minocycline (MCL), fluorocitrate (FC) and propentofylline (PPF) in male CD-1 mice and in immunocompromised nude mice of both sexes, but not in female CD-1 mice. Data are presented as mean ± s.e.m. percentage of maximal anti-allodynia ($n$ = 4–7 mice per sex per drug per genotype). (**b**) Male-specific reversal of allodynia from the PPARα ligand, fenofibrate (FFB) was blocked by the PPARα antagonist GW6471 and by castration (TX). Data are presented as in **a** ($n$ = 4–6 mice per sex per condition). (**c**) Female-specific reversal of allodynia from the PPARγ ligand pioglitazone (PIO) was blocked by the PPARγ antagonist GW9662 and by testosterone proprionate (TP). Data are presented as in **a** ($n$ = 7–10 mice per sex per condition). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with corresponding female mice by $t$ test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with same-sex wild-type (CD-1) or vehicle group by $t$ test. n.t. = not tested.

to switch to the glial-independent pathway. We performed adoptive splenocyte transfer in female *Rag1*$^{-/-}$ mice (**Supplementary Fig. 13a**), followed by evaluation of the effects of minocycline (50 μg) on CFA-induced allodynia. The adoptive splenocyte transfer had no effect on allodynia levels (**Supplementary Fig. 10**). However, female *Rag1*$^{-/-}$mice receiving the adoptive transfer were rendered insensitive to minocycline, in contrast with vehicle-treated *Rag1*$^{-/-}$ mice, which were sensitive to minocycline (**Supplementary Fig. 13b**).

We considered the possibility that the observed sex difference may derive from the almost twofold higher resident T-lymphocyte population in the periphery of female mice compared with males[16]. We confirmed that female CD-1 mice indeed had higher levels of lymphocytes and CD4$^+$ and CD8$^+$ cells in the blood than males (**Supplementary Fig. 14**). Furthermore, we found that, in the lumbar spinal cord 7 d after SNI, mRNA levels of the T-cell markers CD3e, CD4 and CD8a were higher in female CD-1 mice than in males (**Supplementary Fig. 15**).

A known immune system–related sex difference that may explain our observations is the reported sexually dimorphic expression of peroxisome proliferator activated receptors (PPARs) α and γ in mouse and human T cells[17]. Testosterone increases PPARα and decreases PPARγ expression in T cells, leading to decreased production of interferon-γ and higher production of interleukin-17A[17]. Both cytokines have been previously associated with pain[10,18], but a recent study observed that, after nerve injury, T cells infiltrating the spinal cord are predominantly Th1 (that is, interferon-γ releasing) cells[19]. Perhaps infiltrating T cells in male mice are less able to mediate hypersensitivity, resulting in the adoption of an alternative mechanism (that is, microglia). We observed that the PPARα agonist fenofibrate reversed SNI-induced allodynia in male, but not in female, mice (sex × drug: $F_{1,16} = 12.6$, $P = 0.003$), an effect blocked by the PPARα antagonist GW6471 (**Fig. 2b**). Conversely, the PPARγ agonist pioglitazone reversed SNI-induced allodynia in female, but not male, mice (sex × drug: $F_{1,35} = 3.9$, $P = 0.05$), an effect that was blocked by the PPARγ antagonist GW9662 (**Fig. 2c**). Notably, castration of male mice also prevented the anti-allodynic effect of fenofibrate (male only groups: $F_{3,15} = 10.8$, $P < 0.001$; **Fig. 2b**), and administrating testosterone propionate to female mice prevented the anti-allodynic effect of pioglitazone (female only groups: $F_{3,33} = 6.7$, $P = 0.001$; **Fig. 2c**).

These observations, made independently by multiple researchers in three different laboratories, suggest that there are two separable mechanisms of immune-neuronal interactions in the spinal cord of CD-1 and C57BL/6 mice. One important component of microglial pain processing is P2X$_4$R-induced release of BDNF by microglia[8], which we found to be essential only in male mice. We note that T cells can also release a host of other proalgesic mediators that might similarly enhance neuronal activity[1]. The topic of immune system involvement in chronic pain pathophysiology is one of the most active in the pain field; that this sex difference has not been observed until now is very surprising indeed. An important implication of our findings is that distinct strategies targeting neuroimmune signaling might be required for the treatment of chronic pain in men versus women.

## METHODS

Methods and any associated references are available in the online version of the paper.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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### AUTHOR CONTRIBUTIONS

R.E.S., S.B., R.-R.J., M.W.S. and J.S.M. conceived the study. R.E.S. designed most of the experiments, and J.C.S.M. and J.Z. designed certain experiments. R.E.S., J.C.S.M., S.R., S.T., S.B., J.K.A., L.J.M., J.-S.A., S.G.S., D.C., M.Y., X.Q.S., H.H., N.J.P., P.J.B., Y.T. and A.K. collected and analyzed data. R.E.S., J.C.S.M., S.B., M.W.S. and J.S.M. wrote the paper.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.



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## ONLINE METHODS

**Mice.** Subjects were naive, young adult (7–12 weeks old) mice of both sexes, except for one experiment using 4-week-old mice of both sexes. CD-1 (Crl:ICR) and nude CD-1 (Crl:CD1-*Foxn1*$^{nu}$) mice were purchased from Charles River Laboratories, C57BL/6J were purchased from The Jackson Laboratory, and *Rag1*$^{-/-}$ mice (*Rag1*$^{tm1Mom}$) were obtained from M. Saleh (McGill University). Castrated and ovariectomized CD-1 mice were purchased (surgeries performed by supplier) from Charles River Laboratories; at least 2 weeks elapsed from surgery to the start of testing. All mice were housed in standard polycarbonate cages in groups of 2–5 same-sex littermates in a temperature-controlled (20 ± 1 °C) environment (14:10-h light/dark cycle; lights on at 07:00 h); tap water and food (Harlan Teklad 8604) were available *ad libitum*. All procedures were approved by Downtown Animal Care Committee at McGill University, the SickKids Animal Care Committee at University of Toronto, and the Institutional Animal Care and Use Committee at Duke University and were consistent with national guidelines. In pharmacological studies, mice were assigned to experimental groups using within-cage randomization. Experimenters were blinded to drug and dose, and sex where possible; blinding to sex was not possible in behavioral experiments.

**von Frey testing.** Behavioral testing was performed by experimenters of both genders[20]; no differences were noted. The up-down method of Dixon[21] was used to estimate 50% withdrawal thresholds using nylon monofilaments (Stoelting Touch Test), calibrated monthly. All experiments took place during the light cycle, no earlier than 09:00 h and no later than 16:00 h. Mice were placed in custom-made Plexiglas cubicles (5 × 8.5 × 6 cm) on a perforated metal floor, and were permitted to habituate for at least 1 h before testing. Filaments were applied to the plantar surface of the hind paw for 1 s and responses were recorded. Two consecutive measures were taken on each hind paw at each time point, and averaged. Although data were collected on both hind paws, only data from the hind paw ipsilateral to the injury (neuropathic or inflammatory; see below) are presented, as no significant effects of sex, drug or genotype on the contralateral paw were observed in any experiment.

In one study, von Frey filaments were used to estimate absolute withdrawal thresholds. Mice were tested using an ascending series, starting with the lowest von Frey filament (0.008 g) until threshold was reached, determined as reflex withdrawal occurring twice in ten stimulations[22].

**Neuropathic surgeries.** Some mice received unilateral SNI[23] on the left side, 1 d after baseline von Frey testing. Surgery was performed under isoflurane/oxygen anesthesia. We spared the sural nerve, and thus von Frey testing before and after SNI occurred on the lateral aspect of the hind paw. In one experiment conducted in a different laboratory (SB203580), mice received unilateral chronic constriction injury[24] (CCI) on the left side instead, and were tested with von Frey filaments aimed at the mid-plantar hind paw. In most experiments mice were retested for mechanical allodynia on day 7 post-surgery; in one experiment (**Fig. 1a**), mice were retested on day 28 post-surgery. Microglial-specific *Bdnf* mutants (see below) were retested 3, 7, 10 and 14 d post-surgery in the prevention experiment, and 1, 4, 5, 6, 7 and 8 weeks post-surgery in the reversal experiment.

**CFA.** Some mice received unilateral injections of CFA (50% in 20 µl) into the plantar surface of the left hind paw. von Frey fibers before and after CFA were aimed at the mid-plantar hind paw. In all CFA experiments mice were retested for mechanical allodynia on day 3 post-injection.

**Intrathecal injections.** Immediately following post-SNI or post-CFA testing on day 7 or day 3, respectively, mice were removed from their cubicles, lightly anesthetized using isoflurane/oxygen and given intrathecal (i.t.) injections of drugs[25], in a volume of 5 or 10 µl over 30 s, using a 30-gauge needle.

**Drugs.** Minocycline (50–300 µg, i.t.), fluorocitrate (0.5–1.5 nmol, i.t.), propentofylline (25–75 µg, i.t.), TNP-ATP sodium salt (5.0 µg, i.t.), (2*R*)-amino-5-phosphonovaleric acid; (2*R*)-amino-5-phosphonopentanoate (AP5; 0.5 µg, i.t.) and fenofibrate (200 µg, i.t.) were purchased from Sigma. The p38 inhibitor, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580; 30 µg, i.t.) and Y1036 (5 µg, i.t.) were obtained from Calbiochem. Recombinant human TrkB-Fc (three i.t. injections of 0.5 µg, 24 h apart) was purchased from R&D Systems. Pioglitazone potassium salt (300 µg, i.t.) was purchased from Cedarlane. GW 9662 (2 mg per kg, intraperitoneal (i.p.)) was obtained from Tocris, and administered 90 min before pioglitazone. GW 6471 (10 mg per kg, i.p.) was obtained from Sigma, and administered immediately before fenofibrate. All drugs were dissolved in physiological saline or 10–20% dimethyl sulfoxide (vol/vol). Doses were determined in pilot experiments. In one experiment, minocycline was injected systemically at a dose of 25 mg per kg, i.p.

**Allodynia and anti-allodynia data transformation.** SNI- (day 7 post-surgery) and CFA- (day 3 post-injection) induced allodynia (**Supplementary Fig. 10**) was quantified as percentage of maximum possible allodynia using the formula: percentage allodynia = [(baseline threshold − post-injury threshold)/baseline threshold] × 100. Reversal of allodynia by drugs (that is, anti-allodynia) was quantified with respect to the area under the threshold-time curve (using the trapezoidal method) over the post-injection testing period (120 min in all cases except the SB203580 experiment, which was 6 h). Data are reported as percentage of the maximum possible anti-allodynia, calculated for each mouse as a ratio of its actual anti-allodynia compared to a hypothetical situation in which the drug brought withdrawal thresholds to their original baseline at all post-injection time points.

**Testosterone treatment.** Testosterone proprionate was purchased from Sigma and dissolved in polyethylene glycol. This solution was placed into an Alzet osmotic minipump (Model 2002, 0.5 µl h$^{-1}$ for 14 d) and implanted subcutaneously between the scapulae under isoflurane/oxygen anesthesia. The pump delivered an effective dose of 250 µg d$^{-1}$, and testing began after the full 14 d of treatment. In another experiment, testosterone (2 mg per kg, subcutaneous) was injected 48 h before pioglitazone.

**Iba1 immunohistochemistry.** Immediately following behavioral testing, mice were perfused transcardially with 4% paraformaldehyde (vol/vol) for subsequent immunohistochemical processing. Lumbar spinal cord was cryosectioned and stained with anti-ionized calcium-binding adaptor molecule 1 (Iba1) polyclonal antibody (1:1,000, Wako Chemicals, Cat. #019-19741), anti-NeuN (1:2,000, Millipore, Cat. #MAB377) and/or anti-GFAP (1:2,000, Sigma, Cat. #G3893). Spleen tissue was stained with Iba1 and anti-CD3 (1:500, Serotec, Cat. #MCA1477). YFP expression in *Cx3cr1*-Cre mice was revealed with anti-GFP (1:500 Santa Cruz, Cat. #sc390394). All tissue sections were subsequently incubated with appropriate fluorescently labeled secondary antibodies (1:1,000, Jackson; Cy2-conjugated donkey anti-rabbit #715-175-150, Cy3-conjugated donkey anti-rabbit #711-165-152, Cy5-conjugated donkey anti-rabbit #711-175-152, Cy3-conjugated donkey anti-mouse #715-165-150, Cy5-conjugated donkey anti-mouse #715-175-150), and confocal images were acquired using a Zeiss Axiovert microscope and PerkinElmer Volocity software. The number of Iba-1–positive microglial cells was quantified with Image Pro Plus software in a defined area of interest on the spinal cord dorsal horn (lamina I–III). The scorer was blinded to drug treatment.

**Mac-1–saporin lesioning.** Mac-1-saporin mouse/human toxin (15 µg in 8.8 µl) and saporin control (8.8 µg in 8.8 µl) were purchased in solution from Advanced Targeting Systems. Doses were determined from pilot experiments. Drugs were administered via i.t. injection as described above.

**Construction of microglial-specific Bdnf mutants (*Cx3cr1*$^{CreER}$ × *loxP-Bdnf*).** Mice expressing tamoxifen-inducible Cre recombinase (CreER) under the endogenous *Cx3cr1* promoter (a generous gift from W. Gan, New York University School of Medicine) were crossed with mice possessing *loxP* sites flanking the *Bdnf* coding region (*Bdnf*$^{tm3Jae/J}$; The Jackson Laboratory, Cat. #00439). Progeny heterozygous for CreER expression were crossed with *loxP-Bdnf* mice such that all experimental mice were homozygous for *loxP*-flanked *Bdnf*.

Cre expression was induced by oral administration of tamoxifen (two doses of 10 mg of tamoxifen in 0.5 ml corn oil 48 h apart, and 5% glucose (wt/vol) daily subcutaneously[9]). Non-tamoxifen–treated animals received corn oil as a vehicle control. Specificity was established using qPCR and FACS. To allow for repopulation of peripheral CX3CR1-expressing populations, tamoxifen was administered 1 month before SNI in the prevention experiment shown in **Figure 1d** and 1 week post-SNI, at the peak of behavioral allodynia, in the reversal experiment shown in **Supplementary Figure 9**.




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**Adoptive splenocyte transfer.** Spleens harvested from female wild-type (C57BL/6) mice using aseptic technique were placed in sterile, ice-cold RPMI-1640 medium (Sigma) and transferred to a cell culture hood for sterile disruption into a single-cell suspension. After centrifugation (800 *g* for 5 min at 4 °C), red blood cells were lysed by re-suspending cells in RBC lysis buffer (Sigma) and incubating on ice for 5 min. Buffer was diluted with cold RPMI and cells were centrifuged as above and resuspended in ice-cold RPMI, then counted on a hemocytometer using trypan blue (Sigma) exclusion as a measure of viability. Cells were centrifuged as above and resuspended in sterile 0.9% saline (vol/vol). Unfractionated splenocytes ($10^7$ cells in 0.15 ml) were injected into the tail vein of awake, loosely restrained recipient female *Rag1*$^{-/-}$ mice. Control female *Rag1*$^{-/-}$ mice received vehicle injection into the tail vein. CFA was injected 5 days later to both groups, and to wildtype mice of both sexes.

To confirm reconstitution of *Rag1*$^{-/-}$ mice with wildtype splenocytes, 12 d post-adoptive transfer, spleen tissue was immunolabeled with antibodies against Iba1 and CD3 (see above).

**FACS.** Blood samples were collected from naïve male and female CD-1 mice. Erythrocytes were removed from whole blood with ACK lysing buffer (Gibco). Leukocyte suspensions and blocking were prepared as described previously[26]. Samples were then stained with mouse CD4 (1:50, BD Pharmingen, Cat. #553048) and CD8 (1:50, eBioscience, Cat. #46-0081-82) antibodies conjugated by FITC and PE, respectively. Cellular events were acquired using a LSR Fortessa flow cytometer (BD). The number of CD4$^+$ or CD8$^+$ T cells was counted among $10^4$ events isolated from each blood sample. Data were analyzed using Flow Jo software.

**qPCR (microglial markers).** Male and female CD-1 mice were killed 7 d after SNI or sham surgery ($n$ = 8 mice/sex/surgical condition) and transcardially perfused with 10 ml RNAlater solution (Life Technologies). The ipsilateral dorsal horn from the sciatic territory of the lumbar spinal cord were dissected out and collected in RNAlater solution. RNA was isolated by digesting tissues in TRIZOL (Life Technologies) and cDNA synthesized using the SuperScript VILO cDNA kit (Life Technologies). 10 ng per reaction were used for RT-qPCR using pre-designed Taqman probes for *P2rx4* (#Mm00501787), *Emr1* (#Mm00802529), *Irf5* (#Mm00496477), *Irf8* (#Mm00492567) and *Itgam* (#Mm00434455). qPCR were performed for 40 cycles (95 °C for 1 s, 60 °C for 20 s). Levels of the target genes were normalized against the average of four housekeeping genes (*Abt1*, *Eef2*, *Gapdh* and *Hprt1*), and interpreted using the comparative ΔΔCt method.

**qPCR (T cell markers).** Lumbar spinal cords were harvested from male and female CD-1 mice ($n$ = 5–6 mice/sex), 7 d post-SNI surgery. Tissues were snap frozen in dry ice. Total RNA was extracted with the phenol-chloroform method. Reverse transcription was performed using with SuperScript III Reverse Transcriptase (Invitrogen) with 500 ng of total RNA. Real-time quantitative PCR reactions (in triplicate) were processed with a Rotor-Gene Q real-time PCR cycler (Qiagen) using SYBR Green mix from Qiagen (RT$^2$ SYBR Green FAST Mastermix). The levels of *Cd3*, *Cd4*, and *Cd8* were normalized against the housekeeping gene, *Gapdh*, and interpreted using the comparative Ct method. qPCR primers were designed based on gene sequences from GeneBank database on NCBI (*Cd3*: NM_007648.4; *Cd4*: NM_013488.2; *Cd8*: NM_001081110.2), and synthesized by IDT (Integrated DNA Technologies).

**Statistics.** A criterion α level of 0.05 was adopted in all experiments. Data were analyzed by *t* test (two-sided in all cases) or repeated-measures ANOVA, as appropriate, after determining the normality (Shapiro-Wilk test) and homoscedasticity (Levene's test) of the experimental data. *Post hoc* testing was performed using Tukey's test. In five cases, data points were excluded as statistical outliers (standardized residuals > 3); in no case does their inclusion alter experiment conclusions. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications in the field[27].

A **Supplementary Methods Checklist** is available.


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