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Injured sensory neuron–derived CSF1 induces microglial proliferation and DAP12-dependent pain

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Although microglia have been implicated in nerve injury–induced neuropathic pain, the manner by which injured sensory neurons engage microglia remains unclear. We found that peripheral nerve injury induced *de novo* expression of colony-stimulating factor 1 (CSF1) in injured sensory neurons. CSF1 was transported to the spinal cord, where it targeted the microglial CSF1 receptor (CSF1R). Cre-mediated sensory neuron deletion of *Csf1* completely prevented nerve injury–induced mechanical hypersensitivity and reduced microglial activation and proliferation. In contrast, intrathecal injection of CSF1 induced mechanical hypersensitivity and microglial proliferation. Nerve injury also upregulated CSF1 in motoneurons, where it was required for ventral horn microglial activation and proliferation. Downstream of CSF1R, we found that the microglial membrane adaptor protein DAP12 was required for both nerve injury– and intrathecal CSF1–induced upregulation of pain-related microglial genes and the ensuing pain, but not for microglial proliferation. Thus, both CSF1 and DAP12 are potential targets for the pharmacotherapy of neuropathic pain.

Neuropathic pain is a severe chronic pain condition that is characterized by ongoing mechanical hypersensitivity, where normally innocuous stimuli provoke intense pain[1,2]. Given that traditional pharmacotherapies are largely ineffective against neuropathic pain[3], the search continues for mechanism(s) by which nerve damage triggers pain. There is now considerable consensus that nerve damage alters pain transmission circuitry in the spinal cord dorsal horn[2] and that microglia, the tissue-resident macrophages in the CNS[4,5], are important contributors to this process[6–9]. What underlies the activation of microglia, however, is still unclear. Notably, although activation of microglia was readily demonstrated after damage to the peripheral branch of the primary sensory neuron, microglia appeared to be unresponsive to transection of the central branch, namely the dorsal root[10] (**Fig. 1a**). Thus, injured sensory neurons in dorsal root ganglia (DRG) must release a signal that communicates with and activates spinal cord microglia[1].

Although a host of studies have sought sensory neuron–derived factors, it is still unclear how injured neurons initiate microglia activation. For example, fractalkine (CX3CL1), a chemokine that is cleaved from the membrane of sensory neurons after peripheral nerve injury[11], requires cathepsin S (CatS), a protease released by already activated microglia[8]. Thus, fractalkine may contribute to the maintenance of, but cannot be the initiating signal for, microglia activation. Although the chemokines CCL2 and CCL21 have been reported to be induced in sensory neurons after nerve injury[12,13], CCR2, the receptor for CCL2, is not expressed in microglia[14], and deletion of CCL21 has no effect on nerve injury–induced microglia activation or proliferation[13]. Neuregulin-1 (NRG-1) has also been implicated, but NRG-1 is not induced in sensory neurons after nerve injury[15]. Another view holds that ATP released after nerve injury binds to the microglial P2X4 purinergic receptor to initiate microglia activation[6,16]. However, nerve injury–induced microglia activation is intact in P2X4 knockout mice[17], and the source of ATP after nerve injury that binds the receptor has never been unequivocally identified[6].

In addition to being activated, the microglia population expands after nerve injury[18]. Whether this expansion results from proliferation of local microglia or from infiltration of circulating monocytes is unclear. As both resident microglia and infiltrating monocytes express common markers, addressing the relative contribution of resident and infiltrating cells has been difficult. Using a model in which healthy bone marrow is transplanted into lethally irradiated recipients, a previous study concluded that circulating monocytes infiltrate into the CNS and contribute to the expansion of the microglia population[19]. On the other hand, using chimeric mice generated by parabiosis, another group concluded that the microglia expansion in the facial nucleus after VIIth (facial) nerve injury or in the spinal cord in an ALS mouse model derives exclusively from self-renewal of resident microglia[20]. Regardless of the source of the proliferation, neither the identity nor the cellular origin of the factor(s) by which injured neurons trigger microglia proliferation *in vivo* is known.

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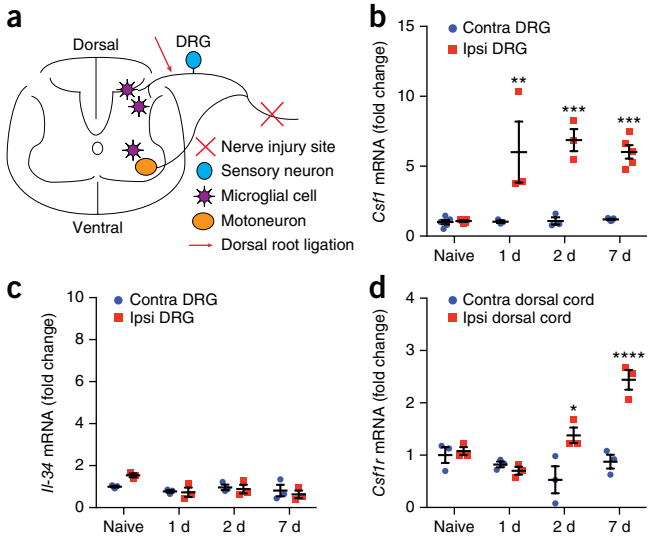

Figure 1 *Csf1* and *Csf1r* are induced in the DRG and dorsal spinal cord, respectively, ipsilateral to the peripheral nerve injury. (**a**) Schematic illustrating relevant neuroanatomy. (**b**) qRT-PCR illustrated *Csf1* induction in the DRG ipsilateral to the peripheral nerve injury compared with the contralateral side. (**c**) qRT-PCR revealed that there was no induction of *Il-34*. (**d**) qRT-PCR illustrated *Csf1r* induction in the dorsal cord ipsilateral to the nerve injury compared with the contralateral side. $n = 3$ mice per time point. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$. Data are presented as mean ± s.e.m.

To address these questions, we performed RNA-Seq and recorded a significant induction of CSF1 (namely, macrophage colony stimulating factor, MCSF) in the injured DRG. The nerve injury–induced upregulation of CSF1 occurred not only in injured DRG sensory neurons, but also in ventral horn motoneurons. Using Cre-mediated selective deletion of *Csf1* from sensory neurons, we found that sensory neuron–derived CSF1 was required for the development of the neuropathic pain phenotype, as well as for microglia proliferation in the dorsal horn. Finally, we identified a critical downstream pathway in microglia that includes the membrane adaptor protein DAP12 in the generation of nerve injury and CSF1-induced neuropathic pain. However, nerve injury– and CSF1-induced microglial proliferation were DAP12 independent.

RESULTS

De novo induction of CSF1 in injured sensory neurons

To identify the genes that are upregulated in DRG and dorsal horn after nerve injury and the signals through which injured sensory neurons interact with microglia to produce pain, we first performed an RNA-Seq analysis after nerve injury (**Fig. 1a**). Many studies have reported transcriptional changes after nerve injury, but few examined both DRG and spinal cord and most were performed using microarray[21,22]. We found a marked upregulation of *Csf1* in the ipsilateral DRG and of *Csf1r* in the ipsilateral dorsal cord after nerve injury (**Supplementary Table 1**). This finding is particularly important, as CSF1 is an essential factor added to culture medium to expand microglia *in vitro*[23], and CSF1R is required *in vivo* for microglia development[24]. In fact, *Csf1r* is among the earliest genes expressed in microglia progenitors in yolk sac during microglia development[24,25]. Notably, the expression of IL-34, another CSF1R ligand[26], did not change (**Supplementary Table 1**). Quantitative RT-PCR (qRT-PCR) confirmed our finding that *Csf1*, but not *Il-34*, was induced in the DRG (**Fig. 1b**,**c**), and that *Csf1r* was induced in the dorsal spinal cord (**Fig. 1d**) after nerve injury.

Subsequent *in situ* hybridization for *Csf1* mRNA (**Fig. 2a**) and immunostaining for CSF1 protein (**Fig. 2b**) revealed that CSF1 was induced in DRG neurons that coexpressed ATF3, a marker of cells with damaged peripheral axons[27]. In fact, 1 d after nerve injury, all CSF1+ DRG neurons coexpressed ATF3 and ~80% of ATF3+ neurons coexpressed CSF1 (**Fig. 2b** and **Supplementary Fig. 1**). The CSF1 induction occurred in both small- and large-diameter DRG neurons (**Fig. 2b**) within 18 h of the nerve injury and persisted for at least 3 weeks (**Supplementary Fig. 1**). As we could not detect CSF1 in DRG neurons in the absence of injury (**Fig. 2b**), we conclude that nerve injury induces *de novo* CSF1 expression in the injured sensory neurons. We observed some CSF1 immunoreactivity in satellite cells of the DRG, but there was no change after nerve injury (**Fig. 2b**).

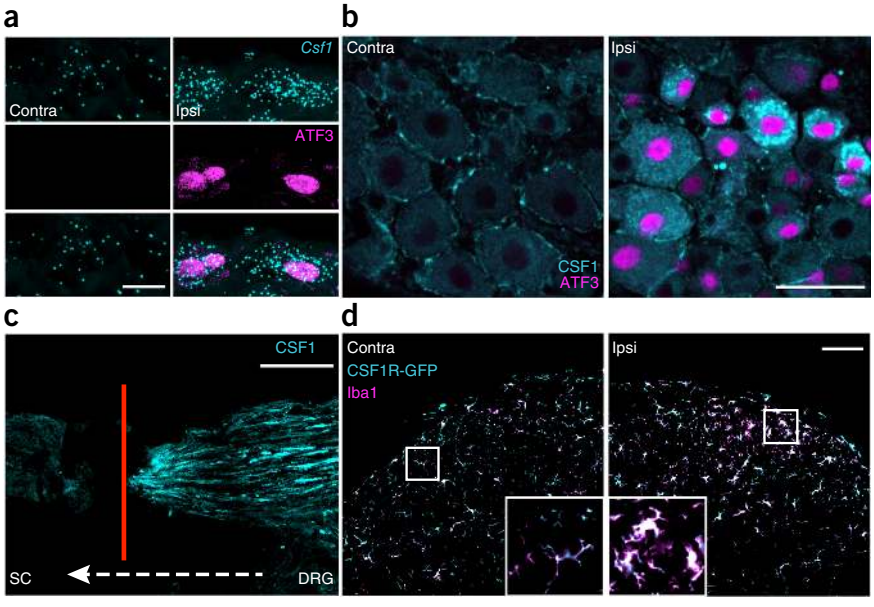

Figure 2 CSF1 is *de novo* induced in injured sensory neurons and transported to the spinal cord, where CSF1R is expressed exclusively in microglia. (**a**) Coexpression of *Csf1* mRNA (*in situ* hybridization) and ATF3 (immunostaining) in injured DRG neurons (1 d post injury), compared with contralateral side. Scale bar represents 10 µm. (**b**) Compared with the contralateral side, we found *de novo* CSF1 (immunostaining) in axotomized, ATF3+ DRG neurons (1 d post injury). Note that there was mild CSF1 immunoreactivity in satellite cells. Scale bar represents 50 µm. (**c**) Concurrent L4 and L5 dorsal root ligation and peripheral nerve injury results in the accumulation of CSF1 protein (immunoreactivity) at the dorsal root ligature (4 d post surgery). Red line denotes ligature site (see **Fig. 1a**). Scale bar represents 200 µm. (**d**) Complete overlap of the microglial markers, Iba1 and GFP in the dorsal horn of a CSF1R-GFP reporter mouse. Both markers were present at higher levels in the dorsal horn ipsilateral to the nerve injury (3 d post injury) compared with the contralateral side. Inset, control (left) and activated (right) microglia. Note the amoeboid morphology of activated microglia. Scale bar represents 100 µm. Inset, maximum projection of *z*-stack images.

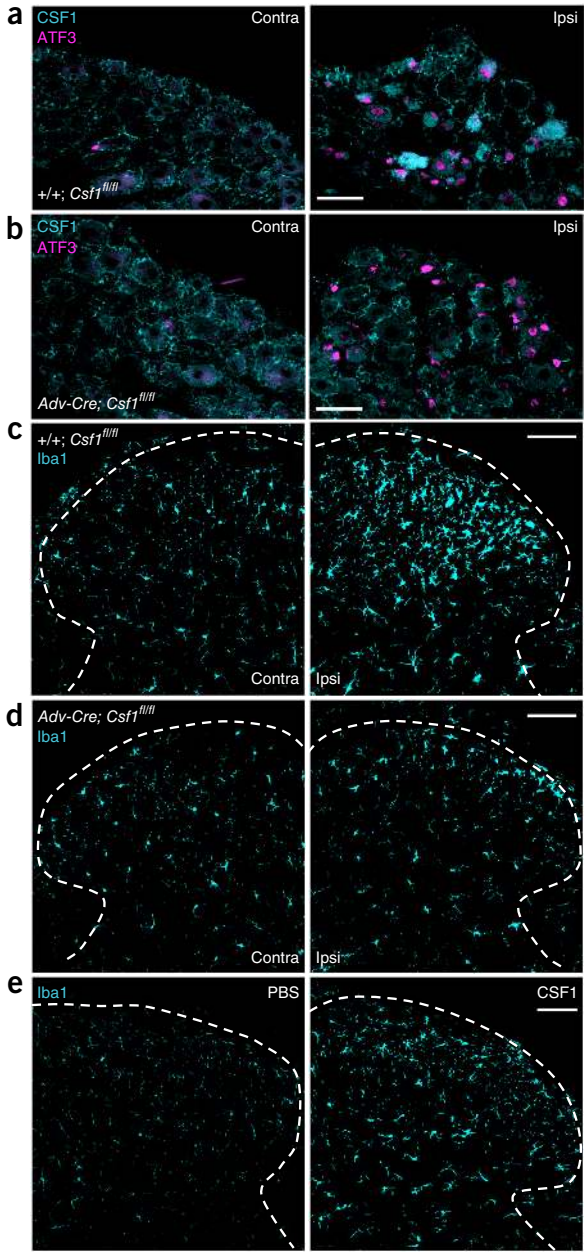

Figure 3 Sensory neuron–derived CSF1 is necessary, and CSF1 by itself is sufficient for nerve injury–induced microglia activation in the dorsal horn. (**a**) Injury-induced CSF1 and ATF3 in ipsilateral DRG neurons of a control mouse (+/+; *Csf1*$^{fl/fl}$) (3 d post injury). Scale bar represents 50 µm. (**b**) Despite a complete loss of CSF1 induction in injured DRG neurons in *Adv-Cre; Csf1*$^{fl/fl}$ mice (3 d post injury), ATF3 expression persisted. Note that the CSF1 immunoreactivity in satellite cells of the DRG was intact in the mutant mice. Scale bar represents 50 µm. (**c**) Peripheral nerve injury–induced microglia activation (increased Iba1 expression) in the ipsilateral dorsal horn (3 d post injury) in control animal (+/+; *Csf1*$^{fl/fl}$). Scale bar represents 100 µm. (**d**) *Csf1* deletion from sensory neurons (*Adv-Cre; Csf1*$^{fl/fl}$) reduced nerve injury–induced dorsal horn microglia activation. Note that the density and morphology of microglia in the spinal cord contralateral to the nerve injury was comparable between control (+/+; *Csf1*$^{fl/fl}$) and mutant (*Adv-Cre; Csf1*$^{fl/fl}$) mice. Scale bar represents 100 µm. (**e**) Compared to PBS, intrathecal CSF1 activates microglia (increased Iba1 expression) in the dorsal horn. Scale bar: 100 µm. $n = 3$ mice/condition.

CSF1 is transported to the spinal cord after nerve injury

To determine whether sensory neuron–derived *de novo* CSF1 is transported to the spinal cord, we ligated the L4 and L5 dorsal roots (between the DRG and spinal cord; **Fig. 1a**) after peripheral nerve injury and found accumulation of CSF1 at the ligatures (**Fig. 2c**). Coexpression in DRG neurons and at the ligature site of CSF1 and NPY (**Supplementary Fig. 2a**,**b**), a peptide that is upregulated in injured sensory neurons[28], confirmed the intra-axonal transport of CSF1.

In spinal cord, CSF1R is expressed only in microglia

Next, using a CSF1R-GFP reporter mouse[29] and immunostaining for CSF1R, we found that CSF1R is expressed exclusively in spinal cord microglia and is indeed upregulated after nerve injury (**Fig. 2d** and **Supplementary Fig. 2c–f**). The fact that we did not observe a corresponding CSF1 increase in the dorsal horn suggests that CSF1 is rapidly released after its transport to the cord.

CSF1 is necessary and sufficient for microglia activation

To investigate the functional relevance of CSF1 upregulation in injured sensory neurons, we deleted *Csf1* selectively from sensory neurons (**Fig. 3a**,**b**) by crossing a floxed *Csf1* mouse (*Csf1*$^{fl/fl}$)[30] with an Advillin-Cre (Adv-Cre) mouse in which Cre recombinase is only expressed in DRG sensory neurons[31]. Neither the morphology nor the density of spinal cord microglia contralateral to the nerve injury differed from that of wild-type mice (**Fig. 3c**,**d**), indicating that microglial development is not compromised in these mice. However, nerve injury–induced microglia activation in the ipsilateral dorsal horn, as demonstrated by increased Iba1 expression, was substantially reduced in these mice (**Fig. 3c**,**d** and **Supplementary Fig. 3a**), even though the ATF3 induction in the injured sensory neurons was preserved (**Fig. 3b**). To test whether CSF1 by itself triggers microglial activation *in vivo*, we injected CSF1 intrathecally, once per day for 3 d and observed a marked activation of dorsal horn microglia, which manifested as enhanced Iba1 expression (**Fig. 3e** and **Supplementary Fig. 3b**). Together, these results suggest that CSF1 induction in injured sensory neurons is necessary and that CSF1 by itself is sufficient for nerve injury–induced microglia activation in the spinal cord dorsal horn.

CSF1 is necessary and sufficient for neuropathic pain

Next we asked whether sensory neuron–derived CSF1 also contributes to the neuropathic pain produced by nerve injury. Adv-Cre–mediated *Csf1* deletion from sensory neurons completely prevented nerve injury–induced mechanical hypersensitivity, the hallmark of neuropathic pain[32] (**Fig. 4a** and **Supplementary Fig. 4a**). In these mutant mice, body weight (**Supplementary Fig. 4b**), motor activity (**Supplementary Fig. 4c**), response to acute noxious heat stimulation (**Supplementary Fig. 4d**,**e**), hindpaw formalin (inflammation)-induced nocifensive behaviors (**Supplementary Fig. 4f**), and numbers and neurochemical subpopulations of DRG neurons (**Supplementary Fig. 4g**,**h**) did not differ from wild-type mice. Consistent with a sufficiency of the CSF1 contribution to the neuropathic pain phenotype, intrathecal injection of CSF1 provoked substantial mechanical hypersensitivity in both wild–type animals (**Fig. 4b**) and in the mice in which *Csf1* was deleted from DRG neurons (**Fig. 4c**). We also recorded morphological changes in dorsal horn microglia 2 h after intrathecal CSF1 (**Supplementary Fig. 5a**), as well as a small, but substantial, increase in Iba1 expression (**Supplementary Fig. 5b**). Consistent with these findings, the microglia inhibitor minocycline prevented the hypersensitivity produced by intrathecal CSF1 (**Supplementary Fig. 5c**). Notably, although

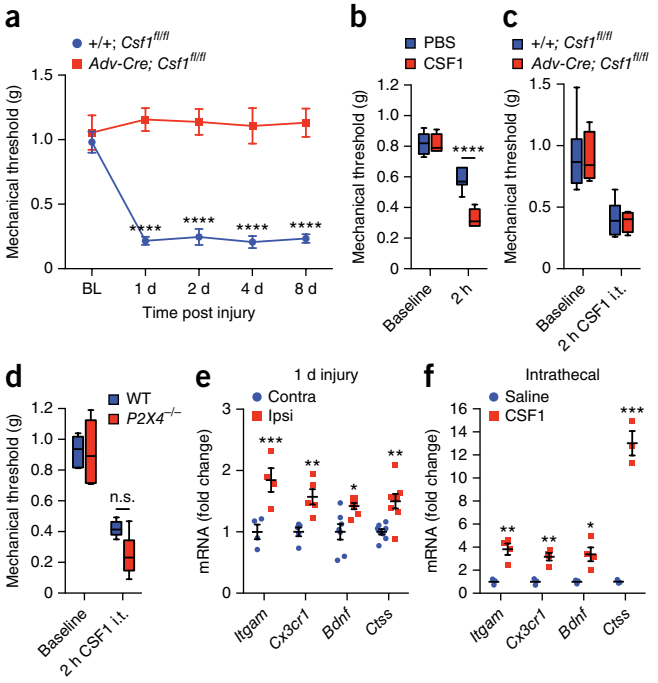

Figure 4 Sensory neuron–derived CSF1 is necessary, and CSF1 by itself is sufficient for nerve injury–induced neuropathic pain (mechanical hypersensitivity). (**a**) Adv-Cre–mediated *Csf1* deletion from sensory neurons prevented the development of nerve injury–induced mechanical hypersensitivity ($n = 5$–6 mice per group). (**b**) The mechanical hypersensitivity produced by intrathecal (i.t.) CSF1 was significantly greater than that induced by PBS ($n = 7$ mice per group) and comparable to that produced by nerve injury. (**c**) Adv-Cre–mediated *Csf1* deletion from sensory neurons had no effect on intrathecal CSF1-induced (2 h CSF1 i.t.) mechanical hypersensitivity ($n = 4$–6 mice per group). (**d**) Intrathecal CSF1 induced comparable mechanical hypersensitivity in wild-type and *P2X4*$^{-/-}$ mice ($n = 6$ mice per group). (**e**) Neuropathic pain–related microglial genes were upregulated in the spinal cord 1 d post injury ($n = 4$ mice per group). (**f**) Upregulation of the same set of microglial genes occurs in the dorsal horn after intrathecal injection of CSF1 ($n = 3$–4 mice per group). In the box plots, the box limits show the first and third quartile, the center line is the median and the whiskers represent the minimum and maximum values. Other data are presented as mean ± s.e.m. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$; n.s., not significant ($P = 0.1332$).

the P2X4 receptor is considered to be critical to the hypersensitivity following nerve injury[6], intrathecal CSF1–induced mechanical hypersensitivity persisted in P2X4 knockout mice (**Fig. 4d**).

Finally, intrathecal CSF1 substantially upregulated several microglial genes (**Fig. 4f**), including *Itgam* (encoding CD11b), *Cx3cr1*, *Bdnf* (encoding brain-derived neurotrophic factor) and *Ctss* (encoding CatS). Many of these genes have been implicated in the development of neuropathic pain[8,33]. Notably, the same microglia genes were upregulated in the dorsal cord 1 d after nerve injury (**Fig. 4e**). We conclude that *de novo* induction of CSF1 in injured sensory neurons triggers the expression of neuropathic pain–relevant microglial genes in the dorsal spinal cord, as well as the ensuing neuropathic pain condition.

DAP12 mediates microglial gene upregulation and pain

We next addressed the signal transduction pathway downstream of the microglial CSF1R. Our RNA-Seq analysis of the dorsal spinal cord ipsilateral to the nerve injury revealed a substantial upregulation of *Tyrobp*, the gene that encodes DAP12 (**Supplementary Table 1**). We focused on DAP12 as it is central to adult microglial functionality[5,34] and is induced in microglia in the XIIth nucleus after hypoglossal nerve injury[35]. qRT-PCR showed that the *Tyrobp* induction was substantial within 1 d of injury (**Fig. 5a**) and lasted for at least 7 d (**Supplementary Fig. 6a**). Intrathecal CSF1 also induced *Tyrobp* (**Fig. 5b**), and, notably, *Tyrobp* deletion[36] completely prevented nerve injury and intrathecal CSF1–induced mechanical hypersensitivity (**Fig. 5c**,**d**), as well as the microglial gene upregulation (**Fig. 5e**,**f**), without affecting the *de novo* induction of CSF1 in sensory neurons (**Supplementary Fig. 6b**). The mild hypersensitivity induced by CSF1 in the *Tyrobp*$^{-/-}$ mice (**Fig. 5d**) was comparable to that produced by phosphate-buffered saline (PBS) vehicle in wild-type mice (**Fig. 4b**). Motor activity and response to acute noxious heat stimulation in the *Tyrobp*$^{-/-}$ mice did not differ from those of wild-type mice (**Supplementary Fig. 6c–e**). We conclude that DAP12 lies downstream of CSF1R and is necessary for the CSF1-CSFR1–triggered upregulation of pain-related microglial genes and of the consequent neuropathic pain condition. Notably, DAP12 is also required for hypoglossal nerve injury–induced expression of pro-inflammatory cytokines, including M1-phenotype markers[35]. Finally, in the rat, we found that DAP12 mechanisms also contribute to ongoing neuropathic pain. Autotomy (self-mutilation of a denervated limb) is presumed to arise from a persistent pain comparable to phantom limb pain. We found that basal levels of spinal cord *Tyrobp* mRNA were substantially higher in a strain of rats with high autotomy (HA)

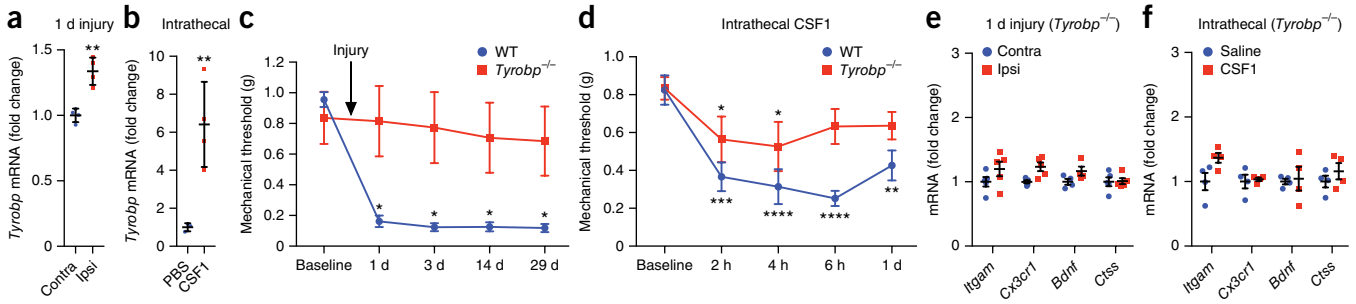

Figure 5 DAP12 is required for nerve injury-induced microglia gene upregulation and neuropathic pain (mechanical hypersensitivity). (**a**) Upregulation of *Tyrobp* mRNA (qRT-PCR) in the dorsal cord ipsilateral to nerve injury (1 d). (**b**) Upregulation of *Tyrobp* mRNA (qRT-PCR) in the spinal cord after intrathecal CSF1. (**c**) *Tyrobp*$^{-/-}$ mice did not develop mechanical hypersensitivity after nerve injury ($n = 5$–6 per group). (**d**) Intrathecal CSF1 did not provoke mechanical hypersensitivity in *Tyrobp*$^{-/-}$ mice ($n = 5$ per group). The mild hypersensitivity observed in the *Tyrobp*$^{-/-}$ mice was comparable to that produced by PBS in wild-type mice (**Fig. 4b**). (**e**) *Tyrobp*$^{-/-}$ prevented nerve injury–induced upregulation of neuropathic pain–related microglial genes (1 d post injury, $n = 4$–5 mice per group). (**f**) *Tyrobp*$^{-/-}$ also prevented intrathecal CSF1-induced microglial gene induction ($n = 4$–5 mice per group). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$. Data are presented as mean ± s.e.m.

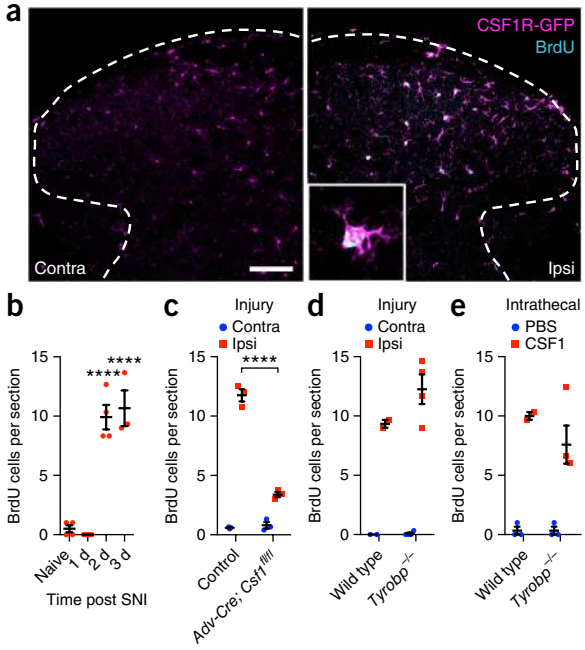

Figure 6 Sensory neuron–derived CSF1 is necessary and sufficient for nerve injury–induced microglia proliferation in the dorsal horn. (**a**) Double-labeling for BrdU (to label proliferating microglia) and GFP in the dorsal horn of a CSF1R-GFP mouse after nerve injury (3 d). Note that all the BrdU+ cells expressed CSF1R. Inset, BrdU incorporation was limited to CSF1R-expressing microglia. Scale bar represents 100 μm. Inset, maximum projection of *z*-stack images. (**b**) Microglia proliferation (BrdU incorporation) began 2 d after injury ($n = 3$–4 mice per group). (**c**) Adv-Cre–mediated deletion of *Csf1* from sensory neurons significantly decreased injury-induced dorsal horn microglia proliferation (3 d post injury, $n = 3$ mice per group). (**d**) Comparable nerve injury–induced microglia proliferation in wild-type and *Tyrobp*$^{-/-}$ mice (3 d post injury, $n = 3$–4 mice per group). (**e**) Intrathecal CSF1 induced dorsal horn microglia proliferation in wild-type and this proliferation was preserved in *Tyrobp*$^{-/-}$ mice ($n = 3$–4 mice per group, 3 d post injury). Data are presented as mean ± s.e.m. $^{****}P \leq 0.0001$.

scores[37] than *Tyrobp* levels in rats that rarely develop this condition (low autotomy, LA). These *Tyrobp* differences were present both before and after nerve injury (**Supplementary Fig. 7**).

Nerve injury induces microglia self-renewal in spinal cord

In addition to establishing the neuropathic pain condition, peripheral nerve injury expands the spinal cord microglia population. Whether this expansion results from the infiltration of circulating monocytes or by self-renewal from local microglia remains controversial. Despite the comparable gene profile of microglia and monocytes, some genes (*Csf1r* and *Cx3cr1*) are expressed at higher levels in microglia; others (*Trem1* and *Trem3*) are expressed exclusively in monocytes[38]. Our RNA-Seq analysis showed that, although the microglia-enriched genes were upregulated, the monocyte-specific genes remained undetectable after nerve injury (**Supplementary Table 1**). We confirmed these RNA-Seq findings by qRT-PCR (**Supplementary Fig. 8**) and conclude, in agreement with a previous study[20], that monocytes do not substantially infiltrate the spinal cord after nerve injury. Rather, microglia expansion after nerve injury involves self-renewal of resident microglia.

CSF1 is necessary and sufficient for microglia self-renewal

We next asked whether the *de novo* expression of CSF1 in injured sensory neurons is also required for nerve injury–induced microglia self-renewal *in vivo*. We first confirmed a previous report[18] that nerve injury triggers dorsal horn microglia proliferation, as demonstrated by incorporation of the thymidine analog BrdU into CSF1R-expressing microglia (**Fig. 6a**). All dorsal horn BrdU+ cells expressed CSF1R 3 d after nerve injury (**Fig. 6a**), indicating that these proliferating cells originate from resident microglia, that is, the proliferation reflects microglial self-renewal. Note that the microglia proliferation occurred after the CSF1 was induced. We detected no microglia proliferation in the dorsal horn at 1 d post injury (**Fig. 6b**), when CSF1 induction

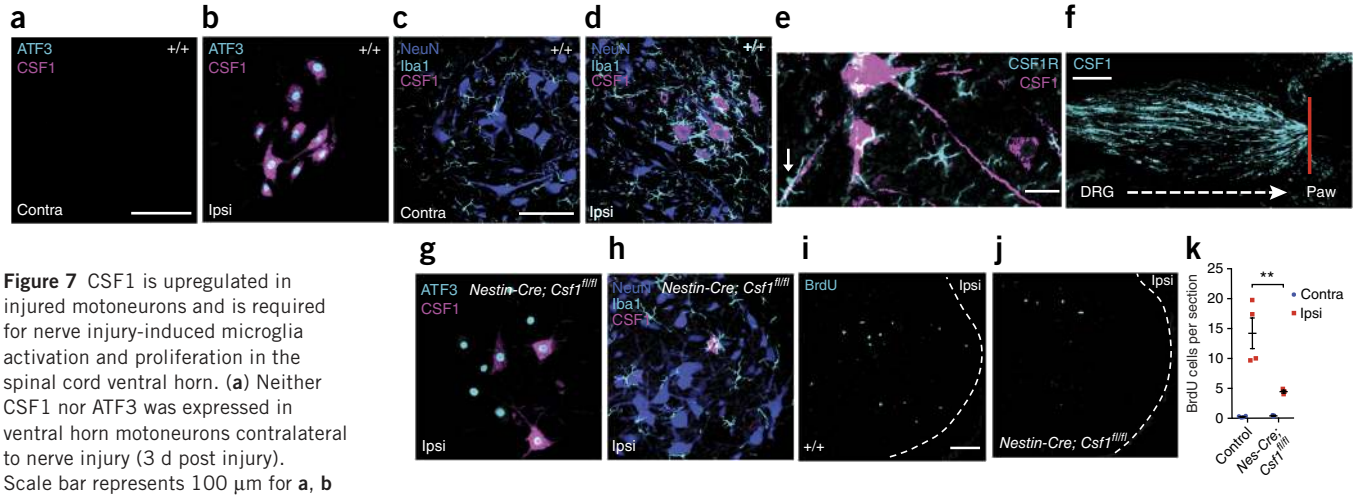

Figure 7 CSF1 is upregulated in injured motoneurons and is required for nerve injury-induced microglia activation and proliferation in the spinal cord ventral horn. (**a**) Neither CSF1 nor ATF3 was expressed in ventral horn motoneurons contralateral to nerve injury (3 d post injury). Scale bar represents 100 μm for **a**, **b** and **g**. (**b**) Coexpression of CSF1 and ATF3 (immunostaining) in axotomized ventral horn motoneurons (3 d post injury). (**c**) No microglia activation in contralateral ventral horn (8 d post injury). Scale bar represents 100 μm for **c**, **d** and **h**. (**d**) Activated ventral horn microglia (enhanced Iba1 expression) surrounded CSF1-expressing motoneurons (8 d post injury). (**e**) Motoneuron axons transported CSF1. Note the apposition of CSF1R-expressing microglia and a CSF1+ motoneuron dendrite (arrow). Scale bar represents 25 μm. (**f**) CSF1 accumulation at the peripheral nerve injury site (4 d post injury). Red line denotes ligature site. Scale bar represents 100 μm. (**g**) CSF1 upregulation was significantly reduced in *Nestin-Cre; Csf1*$^{fl/fl}$ mice, despite the persistent motoneuronal ATF3 expression. Given that ~30% of motoneurons continued to express CSF1 after injury, Nestin-Cre was likely not expressed in all motoneurons. (**h**) *Csf1* deletion from the majority of CNS neurons (*Nestin-Cre; Csf1*$^{fl/fl}$) reduced ventral horn microglia activation after injury. (**i**,**j**) Peripheral nerve injury (3 d) induced microglia proliferation in the ventral horn in wild-type (**i**), and this was greatly attenuated when *Csf1* was deleted from CNS neurons (*Nestin-Cre; Csf1*$^{fl/fl}$) (**j**). Scale bar represents 100 μm for **i** and **j**. (**k**) Quantification of **i** and **j** ($n = 3$–4 mice per group). $^{**}P \leq 0.01$. Data are presented as mean ± s.e.m.

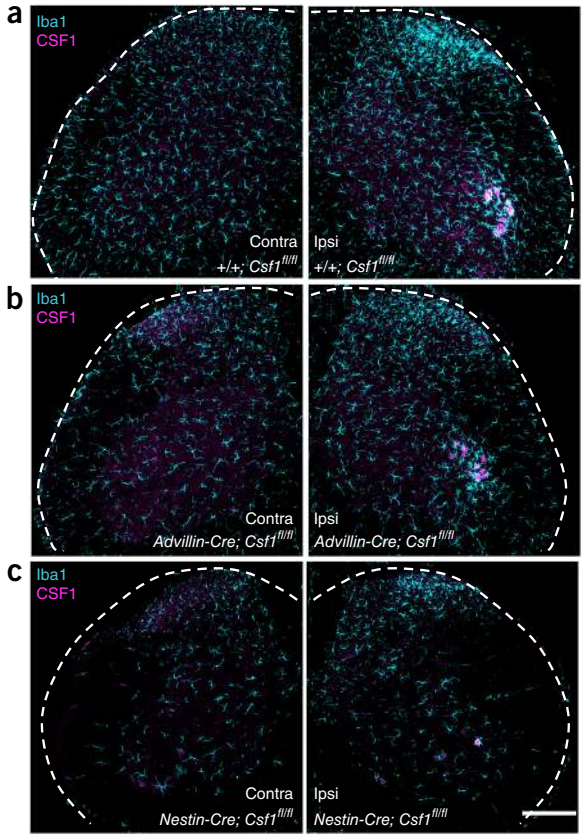

Figure 8 Cre-mediated neuronal *Csf1* deletion reveals topographic distribution of microglia activation after nerve injury. (**a**) Peripheral nerve injury induced microglia activation (increased Iba1 expression) in the ipsilateral dorsal and ventral horn, and upregulation of CSF1 in ventral horn motoneurons (3 d post injury) in a control animal (+/+; *Csf1*$^{fl/fl}$). (**b**) *Csf1* deletion from sensory neurons (*Adv-Cre; Csf1*$^{fl/fl}$) reduces injury-induced dorsal horn microglia activation. There was no effect on ventral horn microglia activation or on motoneuronal CSF1 induction. (**c**) *Csf1* deletion from the majority of CNS neurons (*Nestin-Cre; Csf1*$^{fl/fl}$) reduced ventral horn microglia activation after injury (see also **Fig. 7h**). Note that the density of microglia in the spinal cord contralateral to the nerve injury was reduced in the mutant mice. Despite this overall reduction, microglia were still activated in the dorsal horn ipsilateral to the nerve injury in the mutant mice. Scale bar represents 200 μm.

in sensory neurons is readily observed (**Figs. 1b** and **2a**,**b**). Notably, Adv-Cre–mediated deletion of *Csf1* from DRG neurons largely eliminated the nerve injury–induced dorsal horn microglia proliferation (**Fig. 6c** and **Supplementary Fig. 9a**). Finally, intrathecal injection of CSF1 also induced microglia proliferation in the dorsal horn (**Fig. 6e** and **Supplementary Fig. 9c**) comparable to that provoked by nerve injury (**Fig. 6a**,**b**). We conclude that sensory neuron–derived CSF1 is necessary and that CSF1 by itself is sufficient for microglia proliferation/self-renewal in the dorsal horn.

DAP12 is not required for microglia proliferation *in vivo*

Given that DAP12 is required for CSF1-induced proliferation of bone marrow–derived macrophages *in vitro*[39] and that it lies downstream of spinal cord microglial CSF1R in regulating pain-related microglial gene expression *in vivo* (**Fig. 5e**,**f**), we expected that DAP12 also mediates microglia proliferation *in vivo*. To our surprise, however, *Tyrobp* deletion altered neither nerve injury nor intrathecal CSF1–induced dorsal horn microglial proliferation (**Fig. 6d**,**e** and **Supplementary Fig. 9b**,**d**). Thus, although nerve injury– and CSF1-induced microglia gene induction and the consequent neuropathic pain condition are DAP12 dependent, nerve injury– and CSF1-induced microglia proliferation/self-renewal involves a DAP12-independent pathway.

CSF1 is induced in injured motoneurons

As the sciatic nerve contains sensory and motor axons, its transection damages both DRG sensory neurons and ventral horn motoneurons[27] (**Fig. 1a**). As for sensory neurons, we observed marked CSF1 induction in axotomized (ATF3 expressing) motoneurons (**Fig. 7a**,**b**). The motoneuronal CSF1 induction occurred within 18 h of the injury and persisted for at least 3 weeks (**Supplementary Fig. 10**). Virtually all ATF3-expressing motoneurons coexpressed CSF1, even 3 weeks after nerve injury (**Fig. 7b** and **Supplementary Fig. 10**). These results differ greatly from previous reports that found either no change in CSF1 (ref. 40) or an induction of CSF1 in microglia, not neurons[41], in the facial motor nucleus after VIIth nerve injury. Importantly, we found that the nerve injury–induced ventral horn microglia activation (enhanced Iba1 immunostaining) and microglial engulfment of motoneurons occurred only around motoneuron cell bodies and dendrites in which CSF1 expression increased (**Fig. 7d**,**e**). And just as sensory neuron–derived CSF1 is intraxonally transported (**Fig. 2c** and **Supplementary Fig. 2b**), so the induced motoneuronal CSF1 is also transported in axons that exit the spinal cord (**Fig. 7e**). Indeed, we observed accumulation of CSF1 at the peripheral nerve injury site (**Fig. 7f**). Thus, CSF1 is induced in both injured sensory and motoneurons and is axonally transported to the dorsal horn and to the periphery, respectively.

Ventral horn microglia proliferation is CSF1 dependent

To investigate the consequence of CSF1 induction in injured motoneurons, we crossed *Csf1*$^{fl/fl}$ mouse with a Nestin-Cre mouse, in which Cre recombinase is expressed in most CNS neurons[42]. Nerve injury–induced ATF3 expression in axotomized motoneurons was not affected in these mice, but the CSF1 upregulation in motoneurons was substantially reduced (**Fig. 7g**). Only ~30% of ATF3+ motoneurons expressed CSF1 (**Fig. 7g**), compared with 100% of ATF3+ motoneurons in wild-type mice (**Fig. 7b**). The residual expression of CSF1 in motoneurons presumably reflects incomplete Nestin-Cre–mediated recombination in motoneurons. Notably, preventing CSF1 upregulation in motoneurons largely eliminated the nerve injury–induced microglia activation (**Fig. 7h**) and proliferation (**Fig. 7i–k**) in the ventral horn.

The topographic consequences of neuronal deletion of *Csf1* were impressive. Deletion of *Csf1* from sensory neurons (Adv-Cre; **Fig. 3b**) altered neither motoneuronal CSF1 induction nor ventral horn microglial activation after nerve injury (**Fig. 8a**,**b**). Rather, the reduced nerve injury–induced microglia activation was limited to the dorsal horn, in the terminal field of the injured afferents (**Fig. 8a**,**b**). In contrast, deletion of *Csf1* from CNS neurons (Nestin-Cre; **Fig. 7g**) markedly reduced nerve injury–induced microglia activation in the ventral horn (**Fig. 8c**). Note that baseline microglial density was also reduced in these mice (**Fig. 8a**,**c**). Despite this overall reduction, the nerve injury–induced CSF1 induction was preserved in sensory neurons in these mice (**Supplementary Fig. 11**), as was the dorsal horn microglial activation (**Fig. 8c**).

DISCUSSION

Although there is general agreement that microglia are important contributors to the neuropathic pain following peripheral nerve injury, the manner in which injured sensory neurons communicate with

and activate microglia to produce this pain condition is not known. We found that injured sensory neurons *de novo* expressed CSF1 and transported it to the spinal cord, where it engaged microglia via an interaction with microglial CSF1R. Via a DAP12-dependent microglial pathway, CSF1 in turn upregulated microglial genes that have been implicated in the neuropathic pain phenotype. Injured neuron–derived CSF1 also triggered a DAP12-independent microglia proliferation/self-renewal in the spinal cord.

Injured neuron–derived CSF1, microglia activation and pain

Although CSF1 is known for its *in vitro* colony-stimulating effect on cultured microglia[23], its *in vivo* role is much less understood, largely because of limitations of the available *in vivo* animal models, notably the *Csf1* point mutation op/op mouse[43]. Given that this mouse has a substantial deficit in microglia development[24], it is not ideal for the study of adult microglia functionality[4]. Moreover, because of the global *Csf1* mutation in these mice, the contribution of CSF1 from a specific cell type cannot be assessed. Indeed, although it has been reported that nerve injury–induced microglial activation in the facial nucleus is attenuated in op/op mice[44], another study concluded that the source of the CSF1 triggering the microglial response was microglia[41], not neurons. Very recent studies used systemic administration of CSF1R inhibitors[45]. However, as the CSF1R has two ligands, CSF1 and IL-34 (ref. 26), the action of the inhibitor cannot be unequivocally attributed to CSF1 blockade. In addition, the source of relevant CSF1 cannot be determined with this approach.

We found that CSF1 was markedly and selectively induced in injured (ATF3+) sensory neurons after nerve injury and was transported to the spinal cord, where CSF1R was also upregulated. Notably, selective deletion of *Csf1* from sensory neurons substantially reduced nerve injury–induced dorsal horn microglial activation and completely prevented the neuropathic pain behavioral phenotype. Furthermore, intrathecal injection of CSF1 produced both mechanical hypersensitivity and microglia activation. Taken together, these results provide, to the best of our knowledge, the first evidence that upregulation of CSF1 in injured neurons is the critical contributor to nerve injury–induced microglia activation and neuropathic pain.

An important basis for our conclusion as to the essential contribution of sensory neuron–derived CSF1 came from our concurrent demonstration of spinal cord upregulation of microglial CSF1R. In sharp contrast, although CCL21 is reportedly upregulated in the DRG after nerve injury[13], none of the CCL21 receptors, namely CCR7 and CXCR3 (ref. 46), was expressed in the dorsal cord, even after peripheral nerve injury (**Supplementary Table 1**). In fact, our RNA-Seq analysis could not even confirm the upregulation of CCL21 in the DRG (**Supplementary Table 1**). Finally, although it has been suggested that neurons express several membrane proteins (for example, CD200) that inhibit microglia activity and that microglia activation results from downregulation of these inhibitory proteins[47], we found no change in the expression of their corresponding genes in the DRG after nerve injury (**Supplementary Table 1**).

A microglial CSF1R-DAP12 pathway mediates neuropathic pain

There are many microglial genes that have been implicated in neuropathic pain, but the microglial signaling pathways through which these genes are induced after nerve injury have yet to be fully defined. We found that the *de novo* expression of CSF1 in sensory neurons engages a DAP12-dependent pathway. Notably, we found that this CSF1-CSF1R-DAP12 pathway lies upstream of microglial genes that are critical to neuropathic pain development[6,48,49], including CatS, CX3CR1 (**Figs. 4e**,**f** and **5e**,**f**), P2X4, Irf8 and Irf5 (**Supplementary Fig. 12**). Given that DAP12 lies upstream of these genes, it follows that targeting DAP12 should be considered in the pharmacotherapy of neuropathic pain. Notably, although the CSF1-CSF1R-DAP12 pathway lies upstream of P2X4 gene induction, intrathecal CSF1 induced equivalent mechanical hypersensitivity in P2X4 knockout and wild-type mice (**Fig. 4d**). We conclude that the initial microglial signaling via CSF1R is P2X4 independent.

Nerve injury induces CSF1-dependent microglia self-renewal

Our finding that microglial-specific, rather than monocyte-specific, genes are upregulated in the spinal cord after nerve injury is consistent with a previous report of no significant monocyte infiltration after nerve injury[20]. Although it has been reported that Nestin-expressing microglia progenitor cells contribute to microglia repopulation after pharmacological depletion of microglia[45], we did not observe Nestin expression in any of the proliferating BrdU+ cells in our model (data not shown). In fact, all of the BrdU+ cells expressed CSF1R (**Fig. 6a**), suggesting that expansion of the spinal cord microglia population after nerve injury results from self-renewal of resident microglia.

To the best of our knowledge, however, little is known about the signal that triggers microglia self-renewal *in vivo,* after peripheral nerve injury or indeed in any neurological condition. We found that CSF1 induced in injured sensory and motoneurons is, in fact, the *in vivo* signal that transforms the microglia from a resident, homeostatic state into a highly proliferative one. As CSF1R signaling is required for microglia embryonic development[25], our findings indicate that injury-induced adult microglia proliferation and self-renewal recapitulates the CSF1R-mediated pathway that is active in embryonic stem cells. However, although DAP12 is required for CSF1-induced proliferation of bone marrow–derived macrophages *in vitro*[39], we found that nerve injury– and CSF1-induced microglia proliferation *in vivo* are DAP12 independent. Clearly, the signal transduction pathway that operates in adult microglia *in vivo* differs greatly from the pathway that *in vitro* studies identified from bone marrow–derived macrophages.

CSF1-induced microglial activation and proliferation

Microglia activation after nerve injury is typically concluded from enhanced expression of specific microglia markers, notably CD11b or Iba1, but self-renewal–based proliferation of microglia is also a major manifestation of their activation. We distinguished two biomarkers of microglia activation (**Supplementary Fig. 13**). Within 1 d of nerve injury, before microglia proliferation (BrdU incorporation) occurred, we documented a DAP12-dependent induction of microglial genes, including many implicated in neuropathic pain. Among these genes were *Ctss*, which encodes CatS, the protease that is released by activated microglia to cleave fractalkine from neuronal membrane[8], and *Bdnf*, which is reported to be released from microglia, resulting in a shift of the anion gradient of pain transmission neurons[33]. This shift reduces GABAergic inhibitory control, which contributes to the ensuing hyperexcitability[50]. Only at 2 d after nerve injury did we detect the BrdU marker of microglial proliferation. Unlike microglial gene upregulation, however, neither nerve injury– nor CSF1-induced microglia proliferation is DAP12 dependent. We conclude that the *de novo* expression of CSF1 after injury engages two distinct microglial processes, a DAP12-dependent pathway for microglia gene upregulation and the consequent neuropathic pain, and a DAP12-independent pathway for microglia proliferation/self-renewal. Given that the DAP12-dependent pathway is presumably also engaged by the newly generated microglia, we suggest that interfering with this pathway will reduce both the resident and proliferating microglia contribution to the neuropathic pain phenotype.

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

Z.G. and A.I.B. designed the experiments and with J.A.K., wrote the manuscript. Z.G. performed and organized experiments to which J.A.K., X.W., C.S., S.V., A.K.G., Z.E.-R. and J.B. contributed. J.A.K. completed many of the neuroanatomical studies. B.C. performed RNA-Seq analysis. M.D. provided spinal cord tissue from HA and LA rats. S.L.A.-W. provided *Csf1*$^{fl/fl}$ mice. L.L.L. provided *Tyrobp*$^{-/-}$ mice. L.L.L. and S.L. contributed to experimental design and interpretation of results.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Animal lines. Animal experiments were approved by UCSF Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory animals. Wild type and CSF1R–EGFP mice were purchased from Jackson Laboratory. *Tyrobp*$^{-/-}$[36], *Csf1*$^{fl/fl}$ (ref. 30), Advillin-Cre[31], Nestin-Cre[42] and *P2X4*$^{-/-}$ (ref. 51) mice, and HA and LH rats[37] were described previously. All experiments were performed in male animals.

Surgeries, injections and behavioral analysis. We performed either sciatic nerve ligation and transection (SNL, for DRG RNA-Seq and ventral horn microglia proliferation) or combined sciatic and femoral nerve transection (dorsal cord RNA-Seq) and the SNI model of neuropathic pain[32] for all other experiments. For SNI, we ligated and transected the sural and superficial peroneal branches of the sciatic nerve, leaving the tibial nerve intact. To analyze CSF1 transport from the DRG to the spinal cord, we ligated the ipsilateral L4 and L5 dorsal roots immediately after SNI. Intrathecal injections were made as previously described[52]. To study CSF1-induced microglia proliferation, we injected 10 µl of 3 ng µl^{-1} CSF1 (total of 30 ng) daily for 3 d. To study CSF1-induced microglial gene induction, we injected CSF1 twice within 24 h, with 17 h between the two injections. Spinal cord tissue was collected 24 h after the first injection. Minocycline (40 mg per kg of body weight) was intraperitoneally injected twice daily for 3 d and 1 h before CSF1 i.t. (intratheal) injection. All behavioral experiments were performed as previously reported in a blinded manner during the light cycle[53,54].

RNA-Seq. Ipsilateral and contralateral DRGs and the dorsal quadrant of the spinal cords were collected 7 d after nerve injury. RNA was purified with QIAgen RNeasy Mini Kit with DNase I digestion. RNA-Seq libraries were built with Epicentre ScriptSeq mRNA-Seq Library Preparation Kit and were sequenced by Illumina HiSeq 2000. Differential expression testing was performed using Cuffdiff 1.3.0 using default parameters. Resulting significant gene lists were filtered for genes with an absolute fold change greater than 2.

Immunohistochemistry. We immunostained tissue as previously described[53], with antibodies to GFP (1:2,000, Abcam #ab13970), CSF1R (1:15,000, Millipore #06-174), CD11b (1:1,000, Abcam #ab8878), ATF3 (1:2,000, Santa Cruz #sc-188), CSF1 (1:500 to 1:1,000, R&D #AF416), NPY (1:5,000, gift from C.J. Allen, University of Minnesota), Iba1 (1:2,000, Wako #019-19741), NeuN (1:2,000, Millipore #MAB377) and BrdU (1:400, Abcam #ab6326), and fluorophore-coupled secondary antibodies (1:1,000, Alexa Fluor 488, 555, 594, 647, Invitrogen). To localize CSF1 in DRG neurons and in their processes, we used antibody to goat biotin IgG (Vector Laboratories) and streptavidin coupled to an Alexa Fluor 488 or 594 (Invitrogen). Images were collected with a Carl Zeiss LSM 700 microscope or a Zeiss Axio Image M2 (DRG overview images only) and were processed with Fiji/ImageJ (NIH). Corresponding images (for example, ipsilateral versus contralateral; CSF1 versus PBS; wild type versus mutant) were processed in an identical manner. Each experiment was performed in at least three animals.

Extended imaging methods. All images except DRG overview images were taken on a lsm 700 confocal microscope (Zeiss) equipped with 405-nm (5-mW fiber output), 488-nm (10-mW fiber output), 555-nm (10-mW fiber output) and 639-nm (5-mW fiber output) diode lasers, a main dichroic beam splitter URGB and a gradient secondary beam splitter for lsm 700 using a 10× EC Plan-Neofluar (10×/0.3) or a 20× Plan-Apochromat (20×/0.8) objective (Zeiss). Image acquisition was done with ZEN 2010 (Zeiss), and image dimensions were 1,024 × 1,024 or 2,048 × 2,048 pixels with an image depth of 8, 12 or 16 bit. Two times averaging was applied during image acquisition. Laser power and gain were adjusted to avoid saturation of single pixels. Adjustment of brightness/contrast, changing of artificial colors (LUT), and maximum projections of Z-stack images were done in Fiji/ImageJ.

Cell counting. For cell counting of DRG neurons, we collected 14-µm cryosections of the L5 DRG from three animals per group. The sections were directly mounted on Superfrost microslides. To avoid double counting of the same cell, we mounted, immunostained, and counted neurons in every fourth section of each ganglion. With this approach, at least 150 neurons were counted for each marker. To quantify the percentage of ATF3-immunoreactive DRG neurons that coexpress CSF1, we counted at least 150 ATF3+ neurons for each mouse, at each time point, and calculated the percentage of double-labeled ATF3/CSF1 immunoreactive neurons. To analyze BrdU incorporation in spinal cord microglia, we counted BrdU+ cells in the dorsal spinal cord from 3–4 mice per group in the three lumbar spinal cord sections containing the highest number of BrdU+ cells. Microglia identity was verified by double labeling with a microglia marker (Iba1, CD11b or CSF1R-GFP). The individual analyzing the images was blinded to the groups.

Image quantification. For the quantification of signal intensities of CSF1R, CSF1R-GFP and Iba1 in dorsal horn microglia, we collected 30-µm cryosection of the lumbar enlargement from 3–4 mice per group. Confocal images were taken from the 3 sections showing the highest microglia signals in each animal. The border of the dorsal horn was outlined, all microglia cells were identified using an independent microglia marker (Iba1 or CD11b), and signal intensities within this mask were analyzed using Fiji/ImageJ.

Microglia proliferation. Mice were injected with BrdU (100 µg per kg body weight, intraperitoneal) 2 h prior to perfusion. Tissue sections were pretreated with 1 M HCl (10 min, on ice), 2 M HCl (10 min, 18–20 °C), 2 M HCl (20 min, 37 °C), and five times in PBS before BrdU immunostaining.

qRT-PCR. We performed qRT-PCR as previously described[53]. In mice with a peripheral nerve injury, we collected ipsilateral and contralateral L4-6 DRGs and dorsal spinal cord. For the mice that received an intrathecal CSF1 injection, we collected the entire lumbar spinal cord. All primers were designed using the NCBI Primer-BLAST program. β-actin was used as the internal control for all the DRG samples, and Snap25 was used as the internal control for all spinal cord samples. The primer pair used are: Csf1 TGCTAAGTGCTCTAGCCGAG/CCCCCAACAGTCAGCAAGAC, Il34 ACGTACAGCGGAGCCTCAT/CATGACCCGGAAGCAGTTGT, Csf1r ACAC GCACGGCCACCATGAA/GCATGGACCGTGAGGATGAGGC, Tyrobp CCGA GGTCAAGGGACAGCGGA/TGCCTCTGTGTGTTGAGGTCACTGT, Cx3cr1 GCCTCTGGTGGAGTCTGCGTG/CGCCCAAATAACAGGCCTCAGCA, Itgam GAGTCTGCCTCCGTGTCCGC/TACGTGAGCGGCCAGGGTCT, CatS GGGGGCATAGAGGCAGACGCT/GGGCATCCTCGTCACCAAACGG, Bdnf CAGGTTCGAGAGGTCTGACG/AAGTGTACAAGTCCGCGTCC, P2X4 CGACTATGTGGTCCCAGCTC/GCGTCTGAATCGCAAATGCT, Irf8 GGGCAGCGTGGGAACC/GCTTCCAGGGGATACGGAAC, Irf5 TGGG GACAACACCATCTTCA/CTGGAAGTCACGGCTTTTGT, Trem1 ACTGC TGTGCGTGTTCTTTG/GCCTTCTGGCTGTTGGCATA, Trem3 CAAGATGT GGGGCTGTACCA/AAGCCACACGTCAGAACGAT, β-actin CCACACCC GCCACCAGTTCG/TACAGCCCGGGGAGCATCGT, Snap25 AGCGGACAGC ATCCTCCGGAG/GTCTGCGTCTTCGGCCATGGT, Tyrobp (rat) AAACA GCACATGGCTGAGAC/GCATAGGGTGGGTTCATCTGT, Snap25 (rat) ACCACTGACTTGCTGGCCCCG/CGACGGGTGCTTTCCAGGGAC.

***In situ* hybridization.** *In situ* hybridization (ISH) was performed using the Panomics' QuantiGene ViewRNA tissue assay (Affymetrix/Panomics) as previously described[55], with a probe set designed to cover all three variants of the mouse *Csf1* coding sequence. The signal was detected using an alkaline phosphatase reaction with a fluorescent Fast Red substrate. For combine ISH with immunostaining of ATF3, 12 µm cryostat sections on glass slides were immersed in 10% (vol/vol) formalin in PBS for 10 min and then processed according to the manufacturer's ISH protocol, with protease treatment for 12 min. The slides were then blocked in 5% (vol/vol) normal goat serum in PBS (without Triton X-100) for 1 h and then processed for immunohistochemistry.

Statistical analysis. Student's *t* test was used to compare means of two groups, and two-way ANOVA tests were used for multiple comparisons. The tests were two-sided, except for **Supplementary Figure 7**, which is one-sided, and all our data met the assumptions of the tests. Data are presented as mean ± s.e. No statistical methods were used to predetermine sample sizes, but our sample sizes are comparable to those reported in previous publications[53,54]. The data distribution

was assumed to be normal and variances were assumed to be equal across groups, but this was not formally tested. In all the behavior studies, the animals were randomly assigned to test cylinders, with the person who performed the behavioral test blind to the animal assignment.

A **Supplementary Methods Checklist** is available.

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