IFN-γ receptor signaling mediates spinal microglia activation driving neuropathic pain

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出版情報：Proceedings of the National Academy of Sciences. 106 (19), pp.8032-8037-, 2009-05-12. National Academy of Sciences
バージョン：
権利関係：

Title page

MS#: 2008-10420

Biological Sciences: Neuroscience

IFN-γ receptor signaling mediates spinal microglia activation driving neuropathic pain

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25 text pages (including references and figure legends) and 5 figures

183 words in the abstract and 45,666 characters (all text including spaces, 38,214; the number of characters displaced by figures, 7,452) in the paper.

Abbreviations footnote: IFN-γR, interferon-γ receptor; Iba1, ionized calcium-binding adapter molecule-1; STAT1, signal transducer and activator of transcription 1; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; NeuN, neuronal nuclei; PWT, paw withdrawal threshold; BrdU, bromodeoxyuridine; SFKs, Src-family kinases; $P2X_4R$, $P2X_4$ receptor; GABA, γ-aminobutyric acid; ANOVA, analysis of variance; ISH, *in situ* hybridization; PBS, phosphate-buffered saline

Abstract

Neuropathic pain, a highly debilitating pain condition that commonly occurs after nerve damage, is a reflection of the aberrant excitability of dorsal horn neurons. This pathologically altered neurotransmission requires a communication with spinal microglia activated by nerve injury. However, how normal 'resting' microglia become activated remains unknown. Here we show that in naive animals, spinal microglia express a receptor for the cytokine interferon-γ (IFN-γR) in a cell-type specific manner and that stimulating this receptor converts microglia into activated cells and produces a long-lasting pain hypersensitivity evoked by innocuous stimuli (tactile allodynia – a hallmark symptom of neuropathic pain). Conversely, ablating IFN-γR severely impairs nerve injury-evoked microglia activation and tactile allodynia without affecting microglia in the contralateral dorsal horn or basal pain sensitivity. We also find that IFN-γ-stimulated spinal microglia show upregulation of Lyn tyrosine kinase and purinergic $P2X_4$ receptor, crucial events for neuropathic pain, and genetic approaches provide evidence linking these to IFN-γR-dependent microglial and behavioral alterations. These results imply that IFN-γR is a key element in the molecular machinery through which 'resting' spinal microglia transform into an activated state that drives neuropathic pain.

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Introduction

Neuropathic pain is a chronic pain condition that occurs after nerve damage, such as that induced by bone compression in cancer, diabetes, infection, autoimmune disease or physical injury (1). One troublesome hallmark symptom of neuropathic pain is pain hypersensitivity to normally innocuous stimuli, a phenomenon known as tactile allodynia, which is refractory to currently available treatments such as non-steroidal anti-inflammatories and opioids (2, 3). Accumulating evidence from diverse animal models of neuropathic pain has suggested that neuropathic pain might involve aberrant excitability of the nervous system, notably at the levels of the primary sensory ganglia and the dorsal horn of the spinal cord, resulting from multiple functional and anatomical alterations following peripheral nerve injury (3, 4). While it has long been considered that these alterations might occur mainly in neurons, emerging lines of evidence have revealed that they also occur in spinal microglia, a group of immune cells (5-9). After injury to peripheral nerve, microglia in the normal state (traditionally called 'resting' microglia) in the spinal dorsal horn are converted to an activated state through a series of cellular and molecular changes. Activated spinal microglia show hypertrophied soma, thickened and retracted processes, and increased proliferation activity (6, 9, 10). Furthermore, these microglia induce or enhance expression of various genes including neurotransmitter receptors (11) (for example, $P2X_4R$: a subtype of ATP-gated cation channels (12)) and intracellular signaling kinases (for example, mitogen-activated protein kinases (13-16) and Lyn tyrosine kinase (17)). By responding to extracellular stimuli such as ATP, the activated microglia evoke various cellular responses such as production and release of bioactive factors including cytokines and neurotrophic factors

(18, 19). Importantly, pharmacological, molecular and genetic manipulations of the function or expression of these microglial molecules have been shown to substantially influence nerve injury-induced pain behaviors (12-16, 20-22) and hyperexcitability of dorsal horn pain pathway (23, 24). Therefore, activated spinal microglia after nerve injury critically contribute to the pathologically enhanced pain processing in the dorsal horn that underlies neuropathic pain (5-9). Thus understanding how 'resting' spinal microglia are transformed into activated cells after nerve injury may be an important step in unraveling the pathogenesis of neuropathic pain, yet it remains obscured.

In the present study, we investigated this issue, focusing on the proinflammatory cytokine interferon-γ (IFN-γ). IFN-γ is among the biologically active signaling molecules that have been reported to activate primary cultured microglial cells (25). A recent study has indicated that IFN-γ levels are increased in the spinal cord after nerve injury (20), leading to speculation that it has a role in neuropathic pain. However, there is no direct evidence indicating that IFN-γ signaling contributes to microglia activation in the dorsal horn and tactile allodynia under neuropathic pain conditions. Here, we report that the receptor for IFN-γ (IFN-γR) which is constitutively expressed in normal 'resting' microglia in the dorsal horn is a key component in the molecular machinery through which peripheral nerve injury converts spinal microglia from the resting state to the activated state that underlies the pathogenesis of neuropathic pain.

Results

Stimulating IFN-γRs in spinal microglia under normal conditions induces activation of microglia and long-lasting allodynia

We first investigated expression of IFN-γRs by *in situ* hybridization for IFN-γR mRNA on sections of the L5 dorsal spinal cord of naive rats. Signals for IFN-γR mRNA were readily detected in the dorsal horn (Fig. 1*A*, intense violet dots indicated by arrowheads); these were not observed in sections hybridized with a corresponding sense probe (Fig. 1*A*). Similar results were obtained with another set of cRNA probes for IFN-γR (data not shown). To identify the type of cells expressing IFN-γRs, we performed *in situ* hybridization combined with immunohistochemistry for ionized calcium-binding adapter molecule-1 (Iba1), a marker of microglia, we showed that the IFN-γR mRNA signals were restricted to cells labeled with Iba1 (Fig. 1*B*, arrowheads). In addition, IFN-γR protein was also detected in homogenates from the spinal cord of naive rats and microglial cells in culture in Western blot analysis (data not shown). To determine whether IFN-γRs are expressed as functional receptors in spinal microglia, we spinally administered recombinant IFN-γ to naive rats and immunohistochemically examined the level of activated signal transducer and activator of transcription 1 (phosho-STAT1), a molecule downstream of IFN-γR (26). At 15 min after intrathecal IFN-γ administration (1000 U), immunofluorescence of phosho-STAT1 was increased in the dorsal horn (Fig. 1*C*). Consistent with microglia-restricted localization of IFN-γR, STAT1 phosphorylation evoked by IFN-γ also occurred specifically in cells that were double-labeled with OX-42, a microglial marker, but not with glial fibrillary acidic protein (GFAP; an astrocyte marker), microtubule-associated protein 2 (MAP2) or neuronal nuclei (NeuN) (neuronal markers) (Fig. 1*D*). STAT1 phosphorylation was also induced in cultured spinal microglial cells stimulated directly with IFN-γ (data not shown). These findings imply that under normal conditions, IFN-γRs are expressed as functional receptors in 'resting' microglia in the dorsal horn.

To examine the *in vivo* responses evoked by IFN-γ, we spinally administered IFN-γ to naive rats and subsequently employed a behavioral assay for tactile allodynia. We found that a single intrathecal administration of IFN-γ (1000 U) produced marked and long-lasting tactile allodynia: the paw withdrawal threshold (PWT) to mechanical stimulation applied to the hindpaw progressively decreased over the first 2 days, peaking between days 2 and 3 ($P<0.01$), and the decreased PWT persisted for at least 10 days after the administration ($P<0.05$, Fig. 2*A*). The IFN-γ-induced allodynia was dose-dependent (10 U: $P<0.05$, 100 and 1000 U: $P<0.01$, on day 3, Fig. 2*B*). While a similar allodynic behavior was produced in wild-type C57BL/6J mice injected intrathecally with IFN-γ (10 U, $P<0.01$, Fig. 2*C*), IFN-γ receptor (IFN-γR)-deficient (*ifngr*$^{-/-}$) mice failed to produce this response. Interestingly, we found that *ifngr*$^{-/-}$ mice that had been intrathecally infused with primary cultured microglia taken from wild-type C57BL/6J mice show a decrease in the paw withdrawal threshold after IFN-γ administration without any change in threshold in *ifngr*$^{-/-}$ mice infused with *ifngr*$^{-/-}$ microglia (Fig. 2*D*). This implies that a deficiency of IFN-γ-evoked allodynia in *ifngr*$^{-/-}$ mice is rescued by infusing IFN-γR-expressing microglia. In addition, the paw withdrawal threshold was not affected by infusion of microglia either from wild-type C57BL/6J or *ifngr*$^{-/-}$ mice alone (data not shown). These results indicate that stimulating IFN-γRs in spinal microglia produces persistent tactile allodynia in otherwise naive animals.

These results then prompted us to investigate the status of microglia in the dorsal horn after IFN-γR stimulation, and we performed immunohistochemical analyses on sections of L5 dorsal spinal cord. On day 3 after IFN-γ administration, microglial cells in the dorsal horn had enhanced Iba1 labeling, hypertrophic cell bodies and thickened and shortened processes (Fig. 2*E*). By contrast, immunofluorescence of ED-1 (a

macrophage marker) was not enhanced by IFN-γ and neither morphology nor number of ED-1$^+$ cells was not changed [supporting information (*SI*), Fig. S1]. In addition, there were only very few circulating ED-1$^+$ macrophages that labelled by intravenously injecting PKH26-PCL (Fig. S2), an inert fluorescent dye that selectively label cells with phagocytic capabilities (27), implying that intrathecal administration of IFN-γ did not cause macrophage infiltration. Next, to test whether IFN-γ-stimulated microglia undergo proliferation, we visualized proliferating cells by administering intraperitoneally with a single dose of bromodeoxyuridine (BrdU), a marker of the S-phase of cell cycle. Intrathecal administration of IFN-γ drastically increased the number of BrdU$^+$Iba1$^+$ cells in the dorsal horn on day 1 (P<0.001, Fig. 2*F*). In addition, we observed a few BrdU$^+$Iba1$^-$ cells in the dorsal horn, the number of which was not altered by IFN-γ (vehicle: 3.0 ± 1.5 cells; IFN-γ: 4.7 ± 2.2 cells). The proliferation of spinal microglia is strongly supported by our further immunohistochemical analyses demonstrating that the number of OX-42$^+$ microglia positive to Ki-67, a nuclear protein expressed in all phases of the cell cycle except the resting phase, was also increased in the dorsal horn of IFN-γ-administered rats (Fig. S3A). In addition, there were also only very few Ki-67$^+$ED-1$^+$ cells in the dorsal horn (Fig. S3A). By counting microglial cells within the dorsal horn on day 3, we observed that the total number of Iba1$^+$ microglia increased in IFN-γ-administered rats compared with vehicle-administered rats (P<0.01, Fig. 2*G*). IFN-γ did not increase the number of OX-42$^+$ P2Y$_{12}$R$^-$ cells in the dorsal horn, and almost all OX-42$^+$ cells are double-labeled with an antibody of P2Y$_{12}$ purinoceptor (P2Y$_{12}$R) (Fig. S3), a G-protein-coupled receptor that is expressed in microglia but not in macrophages (29, 30). These changes in the morphology and number of microglia in IFN-γ-administered rats are all consistent with immunohistochemical criteria for

activated microglia *in vivo* (28), and are observed in the dorsal horn after nerve injury (6).

We next tested the effect of minocycline, which inhibits microglia activation *in vivo* (31), on IFN-γ-induced tactile allodynia. Minocycline (40 mg/kg) suppressed the decrease in the PWT at all time points of testing ($P<0.001$, Fig. 2*H*) as well as microglia activation in the dorsal horn on day 3 after intrathecal administration of IFN-γ (Fig. 2*H*). These findings together indicate that stimulation of spinal IFN-γRs expressed selectively in microglia under normal conditions causes tactile allodynia by directly activating spinal microglia.

Lack of IFN-γR impairs activation of microglia and tactile allodynia after nerve injury

To next investigate the role of IFN-γR in microglia activation and tactile allodynia in the dorsal horn under neuropathic pain conditions, we injured the fifth lumbar spinal nerve of wild-type C57BL/6J and *ifngr*$^{-/-}$ mice, an animal model of neuropathic pain. Consistent with our previous studies (17, 32), in wild-type C57BL/6J mice, a marked activation of microglia was observed on the ipsilateral side of the dorsal horn 14 days after nerve injury, as indicated by alterations in Iba1 immunofluorescence (Fig. 3*A*), morphology (Fig. 3*B*) and number ($P<0.001$, Fig. 3*C*). However, in *ifngr*$^{-/-}$ mice with nerve injury, these alterations in activated microglia were severely impaired (Figs. 3*A* and *B*), and the number of Iba1^{+} cells was much lower than that in wild-type C57BL/6J mice ($P<0.001$, Fig. 3*C*). The number of microglia in the contralateral dorsal horn was not different between these two genotypes (Fig. 3*C*). Behaviorally, wild-type C57BL/6J mice showed a marked decrease in PWT after nerve injury (day 1: $P<0.01$; day 3 to 14:

P<0.001, Fig. 3*D*). By contrast, the nerve injury-induced allodynic behavior was strikingly attenuated in *ifngr*$^{-/-}$ mice at all time points of testing (day 1 and 7: *P*<0.05, day 3 and 5: *P*<0.01, day 10 and 14: *P*<0.001, Fig. 3*D*). The loss of IFN-γR did not change either basal mechanical sensitivity or the PWT of the contralateral hindpaw after nerve injury (Fig. 3*D*). Nor did IFN-γR deficiency affect motor behaviors in the rotarod test (time on rotarod; wild-type C57BL/6J: 55.4 ± 4.6 sec, *ifngr*$^{-/-}$: 53.2 ± 6.8 sec). These findings indicate that IFN-γR-mediated signaling is required for switching spinal microglia to the activated phenotype in the spinal dorsal horn after nerve injury, and for producing the subsequent tactile allodynia.

Lyn tyrosine kinase is a critical intermediary in the IFN-γR-dependent activation of spinal microglia

To elucidate the molecular mechanism by which IFN-γ activates spinal microglia, we examined the role of Src-family kinases (SFKs), which have been implicated in cellular responses including the proliferation of many types of cells (33). Among five major SFKs (Src, Fyn, Lck, Yes and Lyn) known to be expressed in the CNS (34), expression of Lyn in spinal microglia is upregulated and activated by nerve injury, which is essential for tactile allodynia after nerve injury (17). In the spinal cord of animals administered IFN-γ intrathecally, expression of Lyn protein was increased 1 day after the administration (*P*<0.05, Fig. 4*A*), and this was observed selectively in microglia labeled by Iba1 (Fig. 4*B*). When cultured microglial cells were treated with IFN-γ, the upregulation of Lyn expression was also observed in a dose-dependent manner (Fig. 4*C*). We also found that immunofluorescence for the active form of SFKs including Lyn that were autophosphorylated in the kinase domain (phospho-SFK) increases

exclusively microglia labeled by OX-42 in dorsal horn after intrathecal administration of IFN-γ (Fig. 4*D*), suggesting that Lyn kinase may become activated in IFN-γ-stimulated spinal microglia. To determine the *in vivo* role of Lyn kinase, we spinally administered IFN-γ to wild-type C57BL/6J and Lyn-knockout (*lyn*$^{-/-}$) mice. In contrast to the changes in morphology (Fig. 4*E*) and number of microglia ($P<0.01$, Fig. 4*F*) in the dorsal horn of wild-type C57BL/6J mice following IFN-γ administration, dorsal horn microglia lacking Lyn showed less activated morphology (Fig. 4*E*) and a reduction in the size of the increase in their number ($P<0.05$, Fig. 4*F*). Furthermore, the loss of Lyn blunted the decrease in PWT following intrathecal administration of IFN-γ ($P<0.01$, Fig. 4*G*). Moreover, the nerve injury-induced increase in microglial cell number in the ipsilateral dorsal horn was lower in *lyn*$^{-/-}$ mice than wild-type C57BL/6J mice ($P<0.001$, Fig. 4*H*). These results indicate that Lyn tyrosine kinase is a critical intermediary in the activation of microglia caused by IFN-γ and nerve injury.

P2X$_4$ receptors upregulated in microglia are required for IFN-γ-induced allodynia

Following nerve injury, activated spinal microglia upregulate expression of P2X$_4$ receptors (P2X$_4$Rs), a principal subtype of ATP-gated ion channels crucial for neuropathic pain (12, 23). We therefore determined whether IFN-γR-induced allodynia involves P2X$_4$R. Applying IFN-γ directly to primary cultured microglial cells increased the level of P2X$_4$R protein (Fig. 5*A*). Furthermore, intrathecal administration of IFN-γ increased the expression of P2X$_4$R protein in the spinal cord of rats (Fig. 5*B*). In the dorsal horn, cells showing P2X$_4$R immunofluorescence were double-labeled with OX-42 (Fig. 5*C*). Using P2X$_4$R-deficient mice (*p2rx4*$^{-/-}$), we found that the marked decrease in PWT induced by intrathecal administration of IFN-γ in wild-type C57BL/6J mice

was significantly attenuated in *p2rx4*$^{-/-}$ mice ($P<0.01$, Fig. 5*D*). In contrast to the reduced behavior, IFN-γ-induced microglia activation in the dorsal horn was not different between the two genotypes (Fig. 5*E*). This is consistent with our previous findings demonstrating that an antisense knockdown of $P2X_4R$ in the spinal cord fails to affect microglia activation (12). These results indicate that IFN-γR-mediated tactile allodynia depends on microglial $P2X_4R$.

Discussion

Growing evidence has revealed several microglial molecules involved in neuropathic pain (5-9). While the expression levels or activities of these molecules are upregulated in activated spinal microglia after nerve injury, they remain at low levels in 'resting' microglia under normal conditions (9). By contrast, we show that in otherwise naive animals, spinal microglia express IFN-γRs in a cell-type specific manner and that acute stimulation of these receptors alone induces a conversion of these cells into the activated state and produces long-lasting tactile allodynia. Our genetic approach revealed that IFN-γR deficiency results in a marked attenuation in spinal microglia activation and tactile allodynia in a model of neuropathic pain, in which IFN-γ levels have been reported to be increased in the spinal cord (20). The attenuated microglia activation observed in IFN-γR-deficient mice seems to be in stark contrast to the phenotype observed in mice lacking $P2Y_{12}R$. $P2Y_{12}R$ is among the molecules expressed in microglia under normal conditions (30) and is implicated in neuropathic pain (32, 35), however, the loss of $P2Y_{12}R$ fails to affect nerve injury-induced morphological and numerical alterations of spinal microglia (32). Therefore, these results imply that the IFN-γ/IFN-γR system is a critical device in 'resting' spinal microglia to transform these

cells into the activated state linking them to tactile allodynia. This is emphasized by our further evidence demonstrating that, in addition to cellular alterations, stimulation of resting spinal microglia by IFN-γ causes a change in their molecular profile (increased expression of Iba1, Lyn and $P2X_4R$), which have also been shown to occur in animal models of neuropathic pain (6-8, 12, 17). However, the fact that activation of spinal microglia after nerve injury was not completely eliminated in IFN-γR-deficient mice suggests that IFN-γR-mediated signaling, although important, is not the only mechanism underlying microglia activation and that there may be independent and/or cooperative mechanisms involving other signals (8).

Among numerous genes whose expression levels have been reported to be altered in microglia stimulated by IFN-γ (36), the present study identified Lyn tyrosine kinase to be upregulated by IFN-γ and required for IFN-γR-dependent microglia activation. This kinase was shown to be upregulated exclusively in microglia in a model of neuropathic pain (17) and to be implicated in various cellular events in microglia (37-40). Previous work using Lyn-deficient mice also showed attenuated microglia activation evoked by β-amyloid peptide in the brain (39), which supports our notion that Lyn is critically involved in the molecular machinery underlying microglia activation. Consistent with impaired neuropathic allodynia in mice lacking Lyn (17), these mice were also resistant to IFN-γ-induced tactile allodynia. We have previously indicated that Lyn kinase in microglia also controls expression of $P2X_4R$ (17), a receptor that is crucial for tactile allodynia (6-8, 12, 23, 41-43). Interestingly, we further revealed that IFN-γ-stimulated spinal microglia allowed upregulated expression of $P2X_4R$ and that the deletion of $P2X_4R$ blunted tactile allodynia, implying that IFN-γR-dependent tactile allodynia involves the $P2X_4R$. As shown previously(23), activation of $P2X_4R$ upregulated in

activated spinal microglia may lead to release of bioactive factors such as brain-derived neurotrophic factor, which causes hyperexcitability of dorsal horn neurons by reducing inhibition and converting γ-aminobutyric acid$_A$ ($GABA_A$) receptor-mediated inhibition to excitation (23) and, in turn, produces tactile allodynia (12, 23). It is of particular interest to note that intrathecal delivery of IFN-γ to normal rats enhances the excitability of dorsal horn neurons evoked by innocuous stimulation *in vivo* (44, 45), which might involve a reduction of GABAergic inhibitory control (45). Although such effects of IFN-γ have been considered a neuronally-mediated phenomenon (44, 46, 47), our present findings indicating that spinal microglia are stimulated directly by IFN-γ administered spinally and contribute to IFN-γR-dependent pain hypersensitivity, together with recent evidence (6-9, 12, 23), propose a new mechanism that spinal microglia are key intermediaries for modulation of spinal pain processing by IFN-γ.

Our present study demonstrates that IFN-γR is a key element in the molecular machinery through which 'resting' spinal microglia transform into the activated state that underlies the pathogenesis of neuropathic pain. On the other hand, the loss of IFN-γR did not change microglia morphology and number under normal conditions, despite the expression of IFN-γR in resting microglia. The lack of a phenotype in resting microglia has also been reported in the brains of IFN-γ-deficient mice (48). It is thus conceivable that this receptor may not affect the development and localization of microglia in the spinal cord and may not be activated under normal conditions. No obvious activation of STAT1 in the normal spinal cord supports this notion. These findings together suggest the possibility that the attenuating effects of IFN-γR deficiency on spinal microglia activation in a model of neuropathic pain may be due to activation of this receptor by IFN-γ, which is elevated in the spinal cord after nerve

injury (20). Because the loss of IFN-γR did not change basal pain sensitivity, our results also implicate that interfering with IFN-γR-mediated signaling in spinal microglia may be a novel approach to treating neuropathic pain without affecting physiological acute pain.

Materials and Methods

Detailed methods are presented in SI Methods.

Behavioral studies. All experimental procedures were performed under the guidelines of Kyushu University. The PWT was measured using calibrated von Frey filaments in rats (Wistar) or mice [*ifngr*$^{-/-}$ (B6.129S7-*Ifngr1*^{tm1Agt}/J, The Jackson Laboratory), *lyn*$^{-/-}$ (49), *p2rx4*$^{-/-}$ (50) and their background wild-type control C57BL/6J]. The three knockout mouse lines were backcrossed to C57BL/6J mice for more than 10 generations. Minocycline was administered intraperitoneally once a day from day 0 to day 7.

***In situ* hybridization.** A digoxigenin-labeled antisense probe for the rat IFN-γR (NM_053783, sequence position 997-1713) was used.

Immunohistochemistry. Transverse L5 spinal cord sections (30 μm) were cut and processed for immunohistochemistry as described previously (12, 17). To visualize proliferating cells, BrdU (75 mg/kg, i.p.) was injected 22 hr after intrathecal administration of IFN-γ; 2 hr later, BrdU-treated rats were fixed by paraformaldehyde.

Microglial culture. Rat primary cultured microglia were prepared in accordance with a method described previously (12, 51).

Western blotting. Western blot analysis of Lyn and $P2X_4R$ expression in the membrane fractions from spinal cord segments L4-L6 and in whole-cell lysates of

cultured microglial cells were performed in accordance with methods described previously (17).

Statistics. Statistical analyses of the results were made with Student's *t* test, Student's paired *t* test or one-way ANOVA with a post hoc test (Dunnett's multiple comparison test).

ACKNOWLEDGMENTS

We thank Dr. Tadashi Yamamoto (The University of Tokyo) and Dr. Joji Ando (The University of Tokyo) for providing Lyn- and $P2X_4R$-knockout mice, respectively. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to M.T., K.I.). T.M. is a research fellow of the Japan Society for the Promotion of Science.

COMPETING INTERESTS STATEMENT

The authors declare no conflict of interest.

References

1. Baron R (2006) Mechanisms of disease: neuropathic pain--a clinical perspective. *Nat Clin Pract Neurol* 2:95-106.
2. Woolf CJ & Mannion RJ (1999) Neuropathic pain: aetiology, symptoms, mechanisms, and management. *Lancet* 353:1959-1964.
3. Scholz J & Woolf CJ (2002) Can we conquer pain? *Nat Neurosci* 5 Suppl:1062-1067.
4. Woolf CJ & Salter MW (2000) Neuronal plasticity: increasing the gain in pain. *Science* 288:1765-1769.
5. Watkins LR, Milligan ED, & Maier SF (2001) Glial activation: a driving force for pathological pain. *Trends Neurosci* 24:450-455.
6. Tsuda M, Inoue K, & Salter MW (2005) Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. *Trends Neurosci* 28:101-107.
7. Marchand F, Perretti M, & McMahon SB (2005) Role of the immune system in chronic pain. *Nat Rev Neurosci* 6:521-532.
8. Scholz J & Woolf CJ (2007) The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci* 10:1361-1368.
9. Suter MR, Wen YR, Decosterd I, & Ji RR (2007) Do glial cells control pain? *Neuron Glia Biol* 3:255-268.
10. Echeverry S, Shi XQ, & Zhang J (2008) Characterization of cell proliferation in rat spinal cord following peripheral nerve injury and the relationship with neuropathic pain. *Pain* 135:37-47.
11. Pocock JM & Kettenmann H (2007) Neurotransmitter receptors on microglia. *Trends Neurosci* 30:527-535.
12. Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, *et al.* (2003) P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* 424:778-783.
13. Jin SX, Zhuang ZY, Woolf CJ, & Ji RR (2003) p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. *J Neurosci* 23:4017-4022.
14. Tsuda M, Mizokoshi A, Shigemoto-Mogami Y, Koizumi S, & Inoue K (2004) Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to pain hypersensitivity following peripheral nerve injury. *Glia* 45:89-95.
15. Zhuang ZY, Gerner P, Woolf CJ, & Ji RR (2005) ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain* 114:149-159.
16. Obata K, Katsura H, Mizushima T, Sakurai J, Kobayashi K, *et al.* (2007) Roles of extracellular signal-regulated protein kinases 5 in spinal microglia and primary sensory neurons for neuropathic pain. *J Neurochem* 102:1569-1584.
17. Tsuda M, Tozaki-Saitoh H, Masuda T, Toyomitsu E, Tezuka T, *et al.* (2008) Lyn tyrosine kinase is required for P2X(4) receptor upregulation and neuropathic pain after peripheral nerve injury. *Glia* 56:50-58.
18. Inoue K (2006) The function of microglia through purinergic receptors: Neuropathic pain and cytokine release. *Pharmacol Ther* 109:210-226.

19. Farber K & Kettenmann H (2006) Purinergic signaling and microglia. *Pflugers Arch* 452:615-621.
20. Tanga FY, Nutile-McMenemy N, & DeLeo JA (2005) The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proc Natl Acad Sci U S A* 102:5856-5861.
21. Clark AK, Gentry C, Bradbury EJ, McMahon SB, & Malcangio M (2007) Role of spinal microglia in rat models of peripheral nerve injury and inflammation. *Eur J Pain* 11:223-230.
22. Griffin RS, Costigan M, Brenner GJ, Ma CH, Scholz J, *et al.* (2007) Complement induction in spinal cord microglia results in anaphylatoxin C5a-mediated pain hypersensitivity. *J Neurosci* 27:8699-8708.
23. Coull JA, Beggs S, Boudreau D, Boivin D, Tsuda M, *et al.* (2005) BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature* 438:1017-1021.
24. Keller AF, Beggs S, Salter MW, & De Koninck Y (2007) Transformation of the output of spinal lamina I neurons after nerve injury and microglia stimulation underlying neuropathic pain. *Mol Pain* 3:27.
25. Hanisch UK & Kettenmann H (2007) Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* 10:1387-1394.
26. Bach EA, Aguet M, & Schreiber RD (1997) The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol* 15:563-591.
27. Maus U, Herold S, Muth H, Maus R, Ermert L, *et al.* (2001) Monocytes recruited into the alveolar air space of mice show a monocytic phenotype but upregulate CD14. *Am J Physiol Lung Cell Mol Physiol* 280:L58-68.
28. Raivich G, Bohatschek M, Kloss C, Werner A, Jones LL, *et al.* (1999) Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Res Brain Res Rev* 30:77-105.
29. Sasaki Y, Hoshi M, Akazawa C, Nakamura Y, Tsuzuki H, *et al.* (2003) Selective expression of Gi/o-coupled ATP receptor P2Y12 in microglia in rat brain. *Glia* 44:242-250.
30. Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, *et al.* (2006) The P2Y(12) receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci* 9:1512-1519.
31. Raghavendra V, Tanga F, & DeLeo JA (2003) Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *J Pharmacol Exp Ther* 306:624-630.
32. Tozaki-Saitoh H, Tsuda M, Miyata H, Ueda K, Kohsaka S, *et al.* (2008) P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. *J Neurosci* 28:4949-4956.
33. Thomas SM & Brugge JS (1997) Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol* 13:513-609.
34. Salter MW & Kalia LV (2004) Src kinases: a hub for NMDA receptor regulation. *Nat Rev Neurosci* 5:317-328.
35. Kobayashi K, Yamanaka H, Fukuoka T, Dai Y, Obata K, *et al.* (2008) P2Y12 receptor upregulation in activated microglia is a gateway of p38 signaling and neuropathic pain. *J Neurosci* 28:2892-2902.

36. Rock RB, Hu S, Deshpande A, Munir S, May BJ, *et al.* (2005) Transcriptional response of human microglial cells to interferon-gamma. *Genes Immun* 6:712-719.
37. Combs CK, Johnson DE, Cannady SB, Lehman TM, & Landreth GE (1999) Identification of microglial signal transduction pathways mediating a neurotoxic response to amyloidogenic fragments of beta-amyloid and prion proteins. *J Neurosci* 19:928-939.
38. Tan J, Town T, & Mullan M (2000) CD45 inhibits CD40L-induced microglial activation via negative regulation of the Src/p44/42 MAPK pathway. *J Biol Chem* 275:37224-37231.
39. Moore KJ, El Khoury J, Medeiros LA, Terada K, Geula C, *et al.* (2002) A CD36-initiated signaling cascade mediates inflammatory effects of beta-amyloid. *J Biol Chem* 277:47373-47379.
40. Hooper C, Taylor DL, & Pocock JM (2005) Pure albumin is a potent trigger of calcium signalling and proliferation in microglia but not macrophages or astrocytes. *J Neurochem* 92:1363-1376.
41. Nasu-Tada K, Koizumi S, Tsuda M, Kunifusa E, & Inoue K (2006) Possible involvement of increase in spinal fibronectin following peripheral nerve injury in upregulation of microglial P2X(4), a key molecule for mechanical allodynia. *Glia* 53:769-775.
42. Tsuda M, Toyomitsu E, Komatsu T, Masuda T, Kunifusa E, *et al.* (2008) Fibronectin/integrin system is involved in P2X(4) receptor upregulation in the spinal cord and neuropathic pain after nerve injury. *Glia* 56:579-585.
43. Zhang Z, Zhang ZY, Fauser U, & Schluesener HJ (2008) Mechanical allodynia and spinal up-regulation of P2X4 receptor in experimental autoimmune neuritis rats. *Neuroscience* 152:495-501.
44. Vikman KS, Siddall PJ, & Duggan AW (2005) Increased responsiveness of rat dorsal horn neurons in vivo following prolonged intrathecal exposure to interferon-gamma. *Neuroscience* 135:969-977.
45. Vikman KS, Duggan AW, & Siddall PJ (2007) Interferon-gamma induced disruption of GABAergic inhibition in the spinal dorsal horn in vivo. *Pain* 133:18-28.
46. Robertson B, Xu XJ, Hao JX, Wiesenfeld-Hallin Z, Mhlanga J, *et al.* (1997) Interferon-gamma receptors in nociceptive pathways: role in neuropathic pain-related behaviour. *Neuroreport* 8:1311-1316.
47. Vikman KS, Hill RH, Backstrom E, Robertson B, & Kristensson K (2003) Interferon-gamma induces characteristics of central sensitization in spinal dorsal horn neurons in vitro. *Pain* 106:241-251.
48. Mount MP, Lira A, Grimes D, Smith PD, Faucher S, *et al.* (2007) Involvement of interferon-gamma in microglial-mediated loss of dopaminergic neurons. *J Neurosci* 27:3328-3337.
49. Nishizumi H, Taniuchi I, Yamanashi Y, Kitamura D, Ilic D, *et al.* (1995) Impaired proliferation of peripheral B cells and indication of autoimmune disease in lyn-deficient mice. *Immunity* 3:549-560.
50. Yamamoto K, Sokabe T, Matsumoto T, Yoshimura K, Shibata M, *et al.* (2006) Impaired flow-dependent control of vascular tone and remodeling in P2X4-deficient mice. *Nat Med* 12:133-137.

51. Nakajima K, Shimojo M, Hamanoue M, Ishiura S, Sugita H, *et al.* (1992) Identification of elastase as a secretory protease from cultured rat microglia. *J Neurochem* 58:1401-1408.

Figure Legends

Fig. 1. IFN-γRs in the spinal cord are expressed in microglia under normal conditions. (*A*) *In situ* hybridization (ISH) analysis of the IFN-γ receptor (IFN-γR) mRNA in the dorsal horns of normal rats. Scale bar, 80 μm. (*B*) IFN-γR mRNA signals overlapped with immunoreactivity for Iba1. Scale bar, 80 μm. (*C*) Phospho-STAT1 immunofluorescence 15 min after IFN-γ injection. Scale bar, 20 μm. (*D*) Double immunofluorescence labeling for phospho-STAT1 (green) and cell-type markers (OX-42, a marker of microglia; GFAP, a marker of astrocytes; MAP2 and NeuN, markers of neurons). Scale bar, 20 μm.

Fig. 2. Intrathecal delivery of IFN-γ to normal animals induces long-lasting tactile allodynia and activation of spinal microglia. (*A*) Paw withdrawal threshold (PWT) after a single intrathecal administration of recombinant IFN-γ (1000 U) or vehicle (PBS) to naive rats (n=6, *P<0.05, **P<0.01). (*B*) Dose-dependent tactile allodynia on day 3 (n=5-6, *P<0.05, **P<0.01). (*C*) PWT in wild-type C57BL/6J and IFN-γR-deficient (*ifngr*$^{-/-}$) mice 3 days after IFN-γ (10 U) administration (n=6, **P<0.01 vs. pre-injection; $^{\#\#}P$<0.01 vs. wild-type C57BL/6J mice with IFN-γ). (*D*) Effects of intrathecal infusion of wild-type (C57BL/6J) and *ifngr*$^{-/-}$ primary cultured microglia to *ifngr*$^{-/-}$ mice on IFN-γ (10 U)-induced change in PWT (n=X, ***P<0.001 vs. pre-injection; $^{\#\#}P$<0.0X vs. wild-type C57BL/6J microglia with IFN-γ injection). (*E*) Immunofluorescence of Iba1 in the dorsal horn 3 days after IFN-γ (1000 U) administration. Scale bar, 200 μm. Insets are images at high-magnification. (*F*) Double immunofluorescence labeling of Iba1 (green) with BrdU (red) in the dorsal horn 1 day after IFN-γ administration. Scale bar, 80 μm. The number of BrdU^{+}Iba1^{+} cells in the

dorsal horn of rats (n=3, ***P<0.001). (*G*) The number of Iba1$^+$ microglia in the dorsal horn of vehicle- and IFN-γ-treated rats (**P<0.01). (*H*) Effect of minocycline (40 mg/kg, i.p.) on IFN-γ-induced microglia activation (photographs: immunofluorescence of Iba1 on day 3, scale bar, 200 μm) and tactile allodynia (n=5, ***P<0.001 vs. IFN-γ/PBS group). Data are mean ± SEM.

Fig. 3. Lack of IFN-γR impairs activation of microglia and tactile allodynia after nerve injury. (*A*) Photographs show immunofluorescence of Iba1 in the L5 dorsal spinal cord of wild-type C57BL/6J and *ifngr*$^{-/-}$ mice 14 days after nerve injury. Scale bar, 150 μm. (*B*) Morphological changes in the spinal microglia in the spinal dorsal horn of wild-type C57BL/6J or *ifngr*$^{-/-}$ mice. Scale bar, 20 μm. (*C*) The change in the number of Iba1-positive microglia cells in the dorsal horn of wild-type C57BL/6J and *ifngr*$^{-/-}$ 14 days after nerve injury (n=5, *P<0.05; ***P<0.001 vs. the contralateral side; $^{###}P$<0.001 vs. the ipsilateral side in wild-type C57BL/6J mice). (*D*) PWT of wild-type C57BL/6J and *ifngr*$^{-/-}$ mice before and after nerve injury (n=7, **P<0.01; ***P<0.001 vs. the contralateral side of wild-type C57BL/6J mice; $^{#}P$<0.05; $^{##}P$<0.01; $^{###}P$<0.001 vs. the ipsilateral side of wild-type C57BL/6J mice). Data are mean ± SEM.

Fig. 4. Lyn is a crucial kinase mediating both IFN-γ- and nerve injury-induced activation of spinal microglia. (*A*) Western blot analysis of Lyn in spinal cord homogenates from rats treated intrathecally with IFN-γ (1000 U). The relative values of Lyn protein were normalized to β-actin protein (n=3, *P<0.05). (*B*) Double immunofluorescence labeling for Lyn (green) and Iba1 (red) in the dorsal horn of IFN-γ-treated rats (merged: yellow). Scale bar, 30 μm. (*C*) Lyn and β-actin proteins in a

whole cell lysate from primary cultured microglial cells treated with IFN-γ (50-300 U/mL) for 24 h. (*D*) Immunofluorescence of phospho-SFK (green) and double immunofluorescence labeling with OX-42 (red) in the dorsal horn of IFN-γ-treated rats (merged: yellow). Scale bars, 80 μm (upper) and 30 μm (lower). (*E*) Iba1 immunofluorescence in the dorsal horn of wild-type C57BL/6J and Lyn-deficient mice ($lyn^{-/-}$) 3 days after intrathecal administration of IFN-γ (10 U). Scale bar, 80 μm. (*F*) The number of $Iba1^{+}$ microglia in the dorsal horn (n=5-6, $^{**}P$<0.01 vs. vehicle-treated wild-type C57BL/6J mice; $^{\#\#}P$<0.01 vs. IFN-γ-treated wild-type C57BL/6J mice). (*G*) PWT after intrathecal administration of IFN-γ in wild-type C57BL/6J and $lyn^{-/-}$ mice (n=6, $^{**}P$<0.01; $^{***}P$<0.001 vs. before IFN-γ injection; $^{\#\#}P$<0.01 vs. IFN-γ-treated wild-type C57BL/6J mice). (*H*) Photographs show Iba1 immunofluorescence in the dorsal horn of C57BL/6J or $lyn^{-/-}$ mice 10 days after nerve injury. Scale bar, 80 μm. The number of $Iba1^{+}$ microglia in the dorsal horns (n=5, $^{**}P$<0.01; $^{***}P$<0.001 vs. contralateral; $^{\#\#}P$<0.01 vs. ipsilateral side in wild-type C57BL/6J mice). Data are mean ± SEM.

Fig. 5. IFN-γ-stimulated microglia show upregulated expression of $P2X_4$ receptor, which is required for tactile allodynia. (*A*, *B*) Western blot analysis of $P2X_4$ receptor ($P2X_4R$) and β-actin proteins in a whole cell lysate from cultured microglial cells treated with IFN-γ and vehicle for 24 h (*A*) and in homogenates from the spinal cord of rats 1 days after intrathecal administration of IFN-γ (1000 U) or vehicle (*B*). (*C*) Double immunofluorescence labeling for $P2X_4R$ (green) and OX-42 (red) in the L5 dorsal spinal cord of IFN-γ-treated rats (merged: yellow). Scale bar, 30 μm. (*D*) PWT after intrathecal administration of IFN-γ (10 U) in wild-type C57BL/6J or $P2X_4R$-deficient

mice (*p2rx4*$^{-/-}$) (*n*=5-6, **P*<0.05; ***P*<0.01; ****P*<0.001 vs. before IFN-γ injection; $^{\#\#}$*P*<0.01 vs. IFN-γ-treated wild-type C57BL/6J mice). (*E*) Photographs show immunofluorescence of Iba1 in the dorsal horn of wild-type C57BL/6J or *p2rx4*$^{-/-}$ mice 3 days after intrathecal IFN-γ injection. Scale bar, 40 μm. Data are mean ± SEM.