JAK-STAT3 pathway regulates spinal astrocyte proliferation and neuropathic pain maintenance in rats

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Neuropathic pain, a debilitating pain condition, is a common consequence of damage to the nervous system. Optimal treatment of neuropathic pain is a major clinical challenge because the underlying mechanisms remain unclear and currently available treatments are frequently ineffective. Emerging lines of evidence indicate that peripheral nerve injury converts resting spinal cord glia into reactive cells that are required for the development and maintenance of neuropathic pain. However, the mechanisms underlying reactive astrogliosis after nerve injury are largely unknown. In the present study, we investigated cell proliferation, a critical process in reactive astrogliosis, and determined the temporally restricted proliferation of dorsal horn astrocytes in rats with spinal nerve injury, a well-known model of neuropathic pain. We found that nerve injury-induced astrocyte proliferation requires the Janus kinase-signal transducers and activators of transcription 3 signalling pathway. Nerve injury induced a marked signal transducers and activators of transcription 3 nuclear translocation, a primary index of signal transducers and transcription 3 activation, in dorsal horn astrocytes. Intrathecally administering inhibitors of Janus kinase-signal transducers and activators of transcription 3 activation, in dorsal horn astrocytes. Intrathecally administering inhibitors of Janus kinase-signal transducers and activators of transcription 3 signalling to rats with nerve injury reduced the number of proliferating dorsal horn astrocytes and produced a recovery from established tactile allodynia, a cardinal symptom of neuropathic pain that is characterized by pain hypersensitivity evoked by innocuous stimuli. Moreover, recovery from tactile allodynia was also produced by direct suppression of dividing astrocytes by intrathecal administration of the cell cycle inhibitor flavopiridol to nerve-injured rats. Together, these results imply that the Janus kinase-signal transducers and activators of transcription 3 signalling pathway are critical transducers of astrocyte proliferation and maintenance of tactile allodynia and may be a therapeutic target for neuropathic pain.
Introduction

Injury to the nervous system arising from bone compression in cancer, diabetes, infection, autoimmune disease or physical injury results in debilitating chronic pain states (referred to as neuropathic pain) (Baron, 2006). One troublesome hallmark symptom of neuropathic pain is tactile allodynia (pain hypersensitivity evoked by normally innocuous stimuli), which is refractory to currently available treatments, such as non-steroidal anti-inflammatory drugs and even opioids (Woolf and Mannion, 1999; Scholz and Woolf, 2002). Unravelling the molecular and cellular basis for the development and maintenance of pain hypersensitivity after nerve damage is therefore essential for the understanding of mechanisms underlying neuropathic pain and for the development of new therapeutic drugs.

Accumulating evidence from studies utilizing diverse animal models of neuropathic pain indicates that neuropathic pain is a reflection of the aberrant excitability of dorsal horn neurons evoked by peripheral sensory inputs (Woolf and Salter, 2000; Costigan et al., 2009). This hyperexcitability might result from multiple cellular and molecular alterations in the dorsal horn occurring after peripheral nerve injury. It has long been considered that there are relevant changes in neurons, but many recent studies provide compelling evidence indicating that spinal microglia, immune-like glial cells in the CNS, rapidly respond to peripheral nerve injury and become activated with changing morphology, increasing their number and expressing a variety of genes (Watkins et al., 2001; Tsuda et al., 2003, 2005; Scholz and Woolf, 2007; Costigan et al., 2009; Inoue and Tsuda, 2009; McMahon and Malcangio, 2009). Activated spinal microglia secrete various biologically active signalling molecules including proinflammatory cytokines (Inoue, 2006) and brain-derived neurotrophic factor (Trang et al., 2009), which produces hyperexcitability of dorsal horn neurons (Coull et al., 2005; McMahon and Malcangio, 2009).

Compared with rapid progress in our understanding of the microglial regulation of pain, relatively little is known about the role of astrocytes, an abundant cell type in the CNS. However, recent studies have identified signalling molecules in astrocytes that are upregulated by peripheral nerve injury such as extracellular signal-regulated protein kinase (Zhuang et al., 2005), c-jun N-terminal kinase (Zhuang et al., 2006), transforming growth factor-activated kinase 1 (Katsura et al., 2008), S100β (Tanga et al., 2006) and matrix metalloproteinase 2 (Kawasaki et al., 2008). Importantly, intrathecal administration of inhibitors for these molecules reduces both peripheral nerve injury-induced hyperexcitability of dorsal horn neurons and pain behaviours (Zhuang et al., 2005, 2006; Katsura et al., 2008; Kawasaki et al., 2008). Astrocytes receive signals from activated microglia via interleukin-18 and disrupting this interaction alleviates peripheral nerve injury-induced allodynia (Miyoshi et al., 2008). These molecules are all expressed in reactive astrocytes responding to peripheral nerve injury. In addition, dorsal root injury leads to upregulation of expression of an extracellular serine protease, tissue type plasminogen activator, in spinal reactive astrocytes and inhibiting the protease reduces root injury-induced mechanical hypersensitivity (Kozai et al., 2007). Therefore, it raises the possibility that the reactive process of dorsal horn astrocytes (reactive astrogliosis) may be crucial for neuropathic pain.

Although a variety of signal transduction pathways have been shown to be involved in the activation of astrocytes in vitro, very few signalling modules have been linked to induction of reactive astrogliosis in vivo (Sofroniew, 2009; Sofroniew and Vinters, 2010). Reactive astrogliosis is known as a finely graded continuum of progressive cellular and molecular changes in relation to the severity of injury and is characterized by cellular hypertrophy, hyperplasia, increased glial fibrillary acidic protein (GFAP), proliferation and, in severe cases such as spinal cord injury, scar formation (Sofroniew, 2009; Sofroniew and Vinters, 2010). Among them, proliferation is a critical process for generating numerous reactive astrocytes, which later may result in producing proinflammatory cytokines, thereby modulating dorsal horn pain processing. In contrast to cellular hypertrophy and GFAP upregulation in dorsal horn astrocytes that have been characterized in various animal models of neuropathic pain (Garrison et al., 1991; Coyle, 1998; Schwei et al., 1999; Tanga et al., 2004; Obata et al., 2006; Vega-Avelaira et al., 2007), the temporal profile and molecular mechanism of proliferation of dorsal horn astrocytes and more importantly, its role in the pathological process of neuropathic pain, remains to be determined.

In the present study, we addressed these issues using the spinal nerve injury model, a well-characterized model of neuropathic pain (Kim and Chung, 1992; Tsuda et al., 2003; Tanga et al., 2004; Zhuang et al., 2006; Kawasaki et al., 2008; Miyoshi et al., 2008). Here, we demonstrate for the first time that: (i) temporally restricted proliferation of dorsal horn astrocytes is induced after peripheral nerve injury; (ii) it involves the Janus kinase (JAK)-signal transducers and activators of transcription 3 (STAT3) signalling pathway; and (iii) intrathecal administration of reagents that inhibit astrocyte proliferation in rats with peripheral nerve injury produces a recovery from tactile allodynia. These results imply that astrocyte proliferation regulated by JAK-STAT3 signalling participates in the maintenance of peripheral nerve injury-induced allodynia.

Materials and methods

Animals

Male Wistar rats (250–270 g, n = 224, Japan SLC, Shizuoka, Japan) were used. Animals were housed in individual cages at a temperature...
of 22 ± 1°C with a 12-h light-dark cycle (light on 08:30–20:30), and fed food and water ad libitum. All animal experiments were conducted according to relevant national and international guidelines ‘Act on Welfare and Management of Animals’ (Ministry of Environment of Japan) and ‘Regulation of Laboratory Animals’ (Kyushu University) and under the protocols approved by the Institutional Animal Care and Use committee review panels at Kyushu University.

Neuropathic pain model

We used the spinal nerve injury model (Kim and Chung, 1992) with some modifications (Tsuda et al., 2003, 2009). Briefly, under isoflurane (2%) anaesthesia, a unilateral fifth lumbar spinal nerve of rats was tightly ligated by a 5-0 silk suture and cut just distal to the ligature.

Behavioural analysis

Rats were placed individually in a wire mesh cage and habituated for 30–60 min to allow acclimatization to the new environment (Tsuda et al., 2003, 2009). Calibrated von Frey filaments (0.4–15 g, Stoelting, IL, USA) were applied to the plantar surface of the rat hindpaw from below the mesh floor. The 50% paw withdrawal threshold was determined using the up-down method (Chaplan et al., 1994). Behavioural measurements were carried out before, 1, 2, 3, 5, 7, 10, 14, 17 or 21 days after peripheral nerve injury (Figs 6A and 7B). For experiment testing, a single administration of AG490 (an inhibitor of the STAT3 activator JAK) on the established allodynia was examined on Days 3 and 5 after peripheral nerve injury (Fig. 6A and E) and the behavioural test was performed immediately before, 1, 3 and 24 h after a single bolus injection of AG490 (Fig. 6D). Locomotor activity was measured using a photobeam activity monitoring system (Chronobiology Kit; Stanford Software Systems, Santa Cruz, CA, USA) (Shinohara et al., 2008), and activity counts (number of movements) were recorded for 1 h.

Drug administration to the intrathecal space

Under isoflurane (2%) anaesthesia, a 32-gauge intrathecal catheter (ReCathCo, PA, USA) was inserted through the atlanto-occipital membrane into the lumbar enlargement and externalized through the skin of the rat hindlimb. A unilateral fifth lumbar spinal nerve was ligated and cut distal to the ligature. The catheter was inserted 1 cm deep into the dura mater. Animals that failed to display paralysis by lidocaine were not included in the experiments. Two to 3 days after the ligature test, a unilateral fifth lumbar spinal nerve of rats was injured as described above. Rats were injected with AG490 (3 and 10 nmol/10 μl, Calbiochem, CA, USA), JAK Inhibitor I, 1-(2-(1-1-Dimethylhydrazo)-9-fluoro-3,6-dihydro-7-benz[h]-imidaz[4,5-f]isoquinolin-7-one, 2.5 nmol/10 μl, Calbiochem) or flavopiridol (5 nmol/10 μl, Santa Cruz Biotechnology, CA, USA) through the catheter once at 09:00 (AG490 and JAK Inhibitor I) or twice (once at 09:00 and once at 19:00; flavopiridol) a day from Days 3 to 5 (for immunohistochemical experiments of proliferating cells) or to Day 7 (for behavioural experiments) (Figs 5A, 6A and 7B). To examine the role of JAK-STAT3 signalling in the spinal cord and dorsal root ganglia, measurements were carried out between 13:00 and 14:00 (Figs 6A and 7B) (except experiments shown in Fig. 6D and E).

Immunohistochemistry

Rats were deeply anaesthetized by intraperitoneal injection of pentobarbital (100 mg/kg) and perfused transcardially with 100 ml of phosphate-buffered saline (composition in mM: NaCl 137, KCl 2.7, KH2PO4 1.5, NaH2PO4 8.1; pH 7.4), followed by 250 ml ice-cold 4% (w/v) parafomaldehyde/phosphate buffered saline (time-line: Figs 5A and 7B). The fifth lumbar segments of the spinal cord and dorsal root ganglion were removed, post-fixed in the same fixative for 3 h at 4°C, and placed in 30% (w/v) sucrose solution for 24 h at 4°C. Transverse spinal cord and dorsal root ganglion sections (30 μm) were incubated in blocking solution (3% v/v normal goat serum) for 2 h at room temperature and then incubated for 48 h at 4°C with primary antibodies: anti-Ki-67 (rabbit polyclonal, 1:5000, Novocasta, Newcastle, UK), anti-phosphorylated-histone H3 (Ser10) (p-His H3, rabbit polyclonal, 1:1000, Upstate/Millipore, MA, USA), anti-STAT3 (rabbit polyclonal, 1:1000, Cell Signalling, MA, USA), anti-GFAP (mouse monoclonal, 1:1000, Chemicon, CA, USA), anti-S100 (mouse monoclonal, 1:5000, Sigma-Aldrich), anti-OX-42 (mouse monoclonal, 1:1000, Serotec, Oxford, UK), anti-neuronal nuclei (mouse monoclonal, 1:200, Chemicon, CA, USA) and anti-cyclin D1 (rabbit polyclonal, 1:100, Transgenic, Kumamoto, Japan). Following incubation, tissue sections were washed and incubated for 3 h at room temperature in secondary antibody solution (Alexa FluorTM 488 and/or 546, 1:1000, Molecular Probes, OR, USA). The tissue sections were washed, slide-mounted and subsequently coverslipped with Vectashield hardmount with 4′,6-diamidino-2-phenylindole (DAPI; a cellular nuclear marker, 1.5 μg/ml) (Vector Laboratories, PA, USA). Three to five sections from the fifth lumbar spinal cord segments and the fifth lumbar dorsal root ganglia of each rat were randomly selected and were analysed using an LSM510 Imaging System (Zeiss, Oberkochen, Germany). Fluorescence intensities of STAT3 in ipsilateral and contralateral dorsal root ganglion sections were calculated. The numbers of GFAP+/S100β+ astrocytes and Iba1+ microglia with clear, visible cell bodies of and p-His H3+ nuclei with a small, rounded shape (diameter ~10 μm) and a signal to noise ratio of 3.0 or more were counted. Background levels were obtained from an area in the dorsal horn of the same section where immunoreactive cells are not contained.

Real-time quantitative polymerase chain reaction

Rats were deeply anaesthetized with pentobarbital, perfused transcardially with phosphate-buffered saline and the fourth to sixth lumbar spinal cord was removed immediately. Total RNA from the fourth to sixth lumbar spinal cord was extracted using Trisure (Bioline, Danwon-Gu, Korea), according to the manufacturer’s protocol, and purified using the RNeasy mini plus kit (QIAGEN, CA, USA). The amount of total RNA was quantified by measuring the optical density260 using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE). For reverse transcription, 200 ng of total RNA was transfected to the reaction with Prime Script reverse transcriptase (Takara, Kyoto, Japan) and random 6-mer primers. Quantitative polymerase chain reaction was carried out with Premix Ex Taq (Takara) using a 7500 real-time polymerase chain reaction system (Applied Biosystems, Foster City, CA) according to the manufacturer’s specifications, and the data were analysed by 7500 System SDS Software 1.3.1 (Applied Biosystems) using the standard curve method. All values were normalized with to GAPDH expression. TaqMan probe, forward primer and reverse primer used in this study were designed as follows: STAT3, probe, 5′-FAM-TGGCAGCCATCCGTGAGACCTACCTT-3′; 5′-TGGCAGCCATCCGTGAGACCTACCTT-TAMRA-3′;
forward primer, 5'-TTGTGATGCCTCCTTGATTGTC-3', and reverse primer, 5'-ATCCAGAGCTTAGAAGAGTTC-3'; GAPDH, probe, 5'-FAM-AC CACCAACGTGTTAGCCCCCCTG-TAMRA-3'; forward primer, 5'-TGCC CCCATGTTTTGATG-3'; reverse primer, 5'-GGCATGGACTGTGGTCA ATGA-3'.

Statistics
Statistical analyses of results were evaluated using the unpaired Student’s t-test, the Mann–Whitney U-test or the Freedman test. Analysis of the time-course of peripheral nerve injury-induced tactile allodynia between vehicle- and drug-treated groups was performed by two factors (group and times) repeated measures analysis of variance (ANOVA). Values were considered significantly different at P < 0.05.

Results
Proliferation activity of dorsal horn astrocytes after peripheral nerve injury

To visualize proliferating cells in the fifth lumbar dorsal horn after peripheral nerve injury induced by injury of the fifth lumbar spinal nerve, we performed immunohistochemical experiments using Ki-67, a nuclear protein expressed in all phases of the cell cycle except the resting phase (Taupin, 2007). In contrast to normal rats that only had very few Ki-67-positive (Ki-67+) cells in the dorsal horn, 2 and 5 days after peripheral nerve injury a number of strong Ki-67+ cells were observed in the dorsal horn ipsilateral to the injury (Fig. 1). Ki-67+ cells were still observed 10 days post-peripheral nerve injury, but the number of Ki-67+ cells markedly decreased (Fig. 1). To identify the type of cells positive for Ki-67, we performed double-immunolabelling for Ki-67 and for cell type-specific markers. Almost all Ki-67+ cells in the dorsal horn 2 days post-injury were double-labelled with OX-42 (a microglia marker), but not with GFAP (an astrocyte marker) or neuronal nuclei (a neuronal marker) (Fig. 1B). This result is consistent with our (Inoue and Tsuda, 2009) and other previous reports (Suter et al., 2007; Echeverry et al., 2008) showing microglial proliferation around 2 days after peripheral nerve injury. Unexpectedly, on Day 5, we found that cells with Ki-67 immunoreactivity were not double-labelled for OX-42 or neuronal nuclei. Instead, almost all Ki-67+ cells were double-labelled with GFAP (Fig. 1B), indicating proliferation of astrocytes in the dorsal horn after peripheral nerve injury.

To determine the mitotic phase of cycling astrocytes, we immunostained for p-HisH3, a marker for the G2/M phase of the cell cycle (Hendzel et al., 1997; Taupin, 2007). On postoperative Day 5, strong p-HisH3+ cells were seen in the dorsal horn ipsilateral to the peripheral nerve injury but not in the contralateral dorsal horn (Fig. 2A) or in the dorsal horn of control rats (data not shown). Double-immunolabelling experiments using cell markers revealed that GFAP+ cells expressed p-HisH3 immunofluorescence (Fig. 2B and C). By counting p-HisH3+ cells in the grey matter of the dorsal horn, we found a marked increase in the number of p-HisH3+ cells on Day 5 as well as Day 2 post-nerve injury (P < 0.05, Fig. 2D). Consistent with our data using Ki-67, on Day 2, 86% of total p-HisH3+ cells were positive for the microglial marker OX-42, and, conversely, on Day 5 a substantial proportion of p-HisH3+ dividing cells were identified as astrocytes following double-labelling with GFAP (86% of total p-HisH3+ cells) (Fig. 2D). The number of cells that showed p-HisH3+/OX-42+ and p-HisH3+/GFAP+ were significantly different (Day 2, P < 0.05; Day 5, P < 0.01). We further quantified the number of p-HisH3+/GFAP+ cells in the dorsal horn at 12-h intervals from postoperative Day 2. A marked increase in p-HisH3+/GFAP+ dividing cells started from 4 days after peripheral nerve injury, peaked on Day 5 and then returned to the pre-injured level over the next 5 days (10 days post-injury) (Fig. 2E). No significant

Figure 1
Immunohistochemical characterization of proliferating cells in the dorsal horn after peripheral nerve injury in rats. (A) Immunofluorescence of Ki-67, a nuclear protein expressed in all cell cycle phases except the resting phase, in fifth lumbar dorsal horn sections on Day 0, 2, 5 and 10 after peripheral nerve injury. (B) Double immunofluorescence labelling for Ki-67 (green) and cell-type markers (magenta: OX-42, microglia; GFAP, astrocytes; neuronal nuclei, neurons) in fifth lumbar dorsal horn sections on Day 2 (upper three panels) and Day 5 (lower three panels) post-peripheral nerve injury. Scale bar = 200 μm (A), 50 μm (B).
change in the number of p-HisH3⁺/GFAP⁺ cells was observed at Days 10 and 14 (Fig. 2E). In addition, we confirmed a significant increase in the number of astrocytes (GFAP⁺/S100β⁺) in the ipsilateral dorsal horn on postoperative Day 7 (see below) and Days 14 and 21 (Supplementary Fig. 1). These results imply a shift of actively cycling cells from microglia to astrocytes in the dorsal horn after peripheral nerve injury, and we identified astrocytes as the principal type of dividing cell in the dorsal horn from Day 4–7 post-peripheral nerve injury.

Role of STAT3 in astrocyte proliferation in the dorsal horn after peripheral nerve injury

To explore the mechanisms regulating astrocyte proliferation, we investigated the role of STAT3 signalling. STAT3 is a principal mediator in a variety of biological processes, including cell proliferation (Levy and Lee, 2002), and there is evidence that JAK-STAT3 signalling regulates proliferation of cultured astrocytes in vitro (Washburn and Neary, 2006; Sarafian et al., 2010). Quantitative polymerase chain reaction analysis demonstrated that the level of STAT3 messenger RNA in the spinal cord was significantly increased in the ipsilateral side on Day 5 (P < 0.05, Fig. 3A). By immunostaining fifth lumbar dorsal horn sections with a STAT3 antibody, we observed punctate STAT3 immunofluorescence dotted in the grey matter of the dorsal horn 5 days after peripheral nerve injury compared with the contralateral side (Fig. 3B and C). The STAT3 immunofluorescence was evident from post-nerve injury Day 3 (Fig. 3C). In contrast, STAT3 expression in the dorsal root ganglion was significantly decreased in the ipsilateral dorsal root ganglion on Day 5 post-nerve injury (P < 0.001, Fig. 3D and E).

To define the cells expressing punctate STAT3 immunofluorescence, we performed double-immunolabelling with cell type markers and found that almost all STAT3⁺ cells were double-labelled with GFAP (Fig. 4A) and S100β (Fig. 4B), both of which are markers of astrocytes, but not with OX-42 and neuronal nuclei (Fig. 4C and D). STAT3 immunofluorescence accumulated in the nuclear region that was stained by DAPI (Fig. 4E). Active STAT3 is known to translocate to the nucleus (Reich and Liu, 2006), suggesting that dorsal horn astrocytes express activated STAT3 after peripheral nerve injury.

To investigate the role of STAT3 in astrocyte proliferation in vivo, we administered AG490, an inhibitor of the STAT3 activator JAK, intrathecally once a day for 2 days from Day 3 after peripheral nerve injury (Fig. 5A). AG490 reduced the nerve injury-induced STAT3 translocation in GFAP⁺ astrocytes in the dorsal horn (Fig. 5B). On Day 5, the number of p-HisH3⁺/GFAP⁺ cells in the dorsal horn was significantly lower in AG490-treated rats than in vehicle-treated rats (P < 0.01, Fig. 5C and D). A similar result was obtained from rats with nerve injury treated with a JAK inhibitor (JAK Inhibitor I) (P < 0.05, Fig. 5D). In addition, AG490 or JAK Inhibitor I treatment resulted in a decrease in GFAP immunofluorescence in the dorsal horn 7 days after peripheral nerve injury (Fig. 5E and F).
Effect of STAT3 inhibition on nerve injury-induced tactile allodynia

To examine the role of astrocyte proliferation in nerve injury-induced tactile allodynia, we measured paw withdrawal threshold to mechanical stimulation. Rats with nerve injury (treated with vehicle) displayed a marked decrease in the paw withdrawal threshold of the ipsilateral side \( (F(6,139) = 6.165, P < 0.001) \) but not the contralateral side (Fig. 6B). Rats with nerve injury treated with AG490 (10 nmol) from Days 3 to 7 (Fig. 6A), a regimen based on the time course of astrocyte cycling (Fig. 2E), showed a significant recovery in the decreased paw withdrawal threshold \( (F(6,143) = 12.747, P < 0.001; \text{Day 5}, P < 0.05; \text{Day 7}, P < 0.01; \text{Fig. 6B}) \). A similar recovery from allodynia on Day 7 was observed in rats with peripheral nerve injury treated with either AG490 (3 nmol; \( P < 0.01 \)) or JAK Inhibitor I (2.5 nmol; \( P < 0.05 \)) (Fig. 6C). After the last administration of AG490 on Day 7, paw withdrawal threshold still remained elevated on Day 10 (\( P < 0.001, \text{Fig. 6B} \)).

We tested the effect of acute inhibition of STAT3 signalling by a single bolus intrathecal injection of AG490 (10 nmol) on postoperative Day 3 or 5. AG490 did not produce any effect on paw withdrawal threshold over a period of 24 h (Fig. 6D and E). Notably, neither motor abnormality nor sedative effects were observed in either AG490- or JAK Inhibitor I-treated rats on Day 7 [locomotor activity (counts for 1 h): vehicle, 604.3 ± 83.8 (\( n = 7 \)); AG490, 596.3 ± 203.5 (\( n = 4 \)); JAK Inhibitor I, 774.3 ± 105.7 (\( n = 3 \))]. These results indicate that inhibiting the JAK-STAT3 signalling pathway suppresses both proliferation of dorsal horn astrocytes and the maintenance of tactile allodynia. We also examined the role of JAK-STAT3 signalling after astrocyte proliferation had finished. Intrathecal administration of AG490 for 5 days from Day 10 post-nerve injury (Fig. 6A) also significantly reduced the established tactile allodynia \( (F(4,15) = 2.794, P < 0.05; \text{Days 12 and 14}, P < 0.05, \text{Fig. 6F}) \).

The anti-allodynic effect of AG490 (Fig. 6F) was weaker than that of AG490 administered from Day 3 post-nerve injury (Fig. 6B),
and after the last administration of AG490 on Day 14, the paw withdrawal threshold on Day 17 returned to the pre-injection level (Fig. 6F).

Effects of the cyclin-dependent kinase inhibitor flavopiridol on astrocyte proliferation and tactile allodynia

If astrocyte proliferation contributes to neuropathic pain, then interfering with astrocyte cycling should alleviate allodynia. To test this hypothesis, we conducted an independent test using the cell cycle inhibitor flavopiridol that inhibits astrocyte proliferation in vitro and in vivo (Di Giovanni et al., 2005; Byrnes et al., 2007). Flavopiridol inhibits cyclin-dependent kinases, leads to a reduction in cyclin D1 expression, and arrests cells in G1 or at the G2/M transition (Swanton, 2004). Because cyclin D1 is essential for astrocyte proliferation (Zhu et al., 2007), we first examined cyclin D1 expression in the dorsal horn. The expression of cyclin D1 was induced in the dorsal horn 5 days post-injury (Fig. 7A), and almost all cyclin D1+ cells were double-labelled with GFAP (98.6% of total cyclin D1+ cells; Fig. 7A). We administered flavopiridol (5 nmol) intrathecally to rats with nerve injury twice a day for 2 days post-operative Day 3 (Fig. 7B). The number of p-HisH3+/GFAP+ cells in the dorsal horn on Day 5 was significantly lower in flavopiridol-treated rats than vehicle-treated rats (P < 0.01, Fig. 7C and D). Furthermore, flavopiridol also significantly reduced the nerve injury-induced increase in the number of GFAP+/S100β+ astrocytes in the dorsal horn on Day 7 (P < 0.01, Fig. 7E) without affecting that of Iba1+ microglia (Fig. 7F).

Behaviourally, rats with nerve injury treated with flavopiridol (5 nmol) from Days 3 to 7 showed a significant recovery in the decreased paw withdrawal threshold after the injury [F(6,44) = 3.19, P < 0.01; Day 5, P < 0.01; Day 7, P < 0.001; Fig. 7G]. After the last administration of flavopiridol on Day 7, the significant attenuation of decreased paw withdrawal threshold remained on Day 10 (P < 0.05, Fig. 7G).

Discussion

A rapidly growing body of evidence has indicated that reactive spinal astrocytes, as well as microglia, are critical components for maintaining neuropathic pain (Watkins et al., 2001; Marchand et al., 2005; Tsuda et al., 2005; Scholz and Woolf, 2007; Suter et al., 2007; Costigan et al., 2009; Hald, 2009; Inoue and Tsuda, 2009; McMahon and Malcangio, 2009; Milligan and Watkins, 2009). Despite such recent progress, very little is known about the reactive process of astrocytes in the dorsal horn after peripheral nerve injury, in particular cell proliferation, a critical process in reactive astrogliosis. In the present study, our detailed immunohistochemical analyses utilizing three independent markers of the cell cycle (Ki-67, p-HisH3 and cyclin D1) now provide compelling evidence that dorsal horn astrocytes undergo proliferation after peripheral nerve injury in rats. Consistent with previous observations using 5-bromo-2′-deoxyuridine (Narita et al., 2006; Suter et al., 2007; Echeverry et al., 2008), these three markers also successfully detected proliferating microglia 2 days post-peripheral nerve injury, confirming the specificity of these markers. Apparently, our findings are not in line with a prevailing view that dorsal

Figure 4 STAT3 translocation to the nucleus of dorsal horn astrocytes after peripheral nerve injury in rats. (A–D) Double immunofluorescence labelling for STAT3 (green) and cell-type markers (magenta: A, GFAP; B, S100β; C, OX-42; D, neuronal nuclei) in fifth lumbar dorsal horn sections on Day 5 post-peripheral nerve injury. (E) Representative confocal z-stack digital images of a single cell triple labelled with STAT3 (green), GFAP (magenta) and DAPI (blue) shown in double channels (left, STAT3/GFAP; middle, DAPI/GFAP) and as a merged image (right, STAT3/GFAP/DAPI) from grey matter of the fifth lumbar dorsal horn 5 days after peripheral nerve injury. Scale bar = 50 μm (A–D), 10 μm (E).
horn astrocytes do not proliferate after peripheral nerve injury (Gehrmann and Banati, 1995; Suter et al., 2007; Echeverry et al., 2008; Hald, 2009). However, this might be explained by the following reasons. First, these previous studies did not test proliferation activity around Day 5 post-peripheral nerve injury. Second, only the S-phase marker 5-bromo-2'-deoxyuridine, whose half-life is very short (Taupin, 2007), was used for detecting proliferating cells. Third, the present study used Ki-67 that
labels cells in all phases of the cell cycle except the resting phase (Taupin, 2007). Furthermore, by detecting p-HisH3+/GFAP+ astrocytes at 12-h intervals, we could determine the temporally restricted proliferation activity of dorsal horn astrocytes after peripheral nerve injury. To our knowledge, this is the first report determining the time course of astrocyte proliferation in the dorsal horn in a model of neuropathic pain and provides evidence for changing the prevailing view.

By showing STAT3 nuclear translocation (a primary index of activation) restricted to astrocytes in the dorsal horn after peripheral nerve injury and the suppression of dividing astrocytes by JAK inhibitors, our findings further demonstrate that peripheral nerve injury-induced astrogliosis in the rat dorsal horn is regulated by the JAK-STAT3 signalling pathway. Astrocytic STAT3 activation has also been observed under other neuropathological conditions accompanied by reactive astrogliosis, such as spinal cord injury (Okada et al., 2006; Herrmann et al., 2008), brain ischaemia (Choi et al., 2003), dopamine neuron damage in the striatum (Sriram et al., 2004) and facial nerve axotomy (Schwaiger et al., 2000). However, there is conflicting reports that immunofluorescence of phosphorylated STAT3 increases in spinal microglia 24 h after peripheral nerve injury and, to a much lesser extent,
Figure 7  Effects of the cell cycle inhibitor flavopiridol on astrocyte proliferation and tactile allodynia after peripheral nerve injury. (A) Double immunofluorescence labelling for cyclin D1 (green) and GFAP (magenta) shown as a merged image from grey matter of the fifth lumbar dorsal horn 5 days after peripheral nerve injury. The numbers of cyclin D1+ cells (open and closed columns), cyclin D1+/GFAP+ cells (green column) and cyclin D1+/GFAP− cells (dark green column) in fifth lumbar dorsal horn sections from rats 5 days after peripheral nerve injury. Values represent the number of cells (per section) (n=4 rats; *P < 0.05). (B) Schematic time-line for intrathecal administration, fixation and behavioural tests. (C) p-HisH3 immunofluorescence in representative images of fifth lumbar dorsal horn sections from vehicle- and flavopiridol-treated rats on Day 5 post-peripheral nerve injury. Rats with peripheral nerve injury were injected intrathecally with flavopiridol (5 nmol/10 μl) or vehicle (10 μl) twice a day for 2 days from Day 3. (D) The numbers of p-HisH3+/GFAP+ cells in the fifth lumbar dorsal horn ipsilateral and contralateral to peripheral nerve injury from vehicle- or flavopiridol-treated rats on Day 5 post-peripheral nerve injury. Values represent the number of p-HisH3+/GFAP+ cells (per section) (n=5 rats; ***P < 0.001 versus contralateral side of vehicle group; **P < 0.01 versus ipsilateral side of vehicle group). (E and F) The numbers of GFAP+/S100β+ (E) and Iba1+ (F) cells in the fifth lumbar dorsal horn ipsilateral and contralateral to peripheral nerve injury from vehicle- or flavopiridol-treated rats on Day 7 post-peripheral nerve injury. Values represent the number of GFAP+/S100β+ (E) and Iba1+ (F) cells (per section) (n=4 rats; ***P < 0.001 versus contralateral side of vehicle group; **P < 0.01 versus ipsilateral side of vehicle group). (G) Paw withdrawal threshold (PWT) to mechanical stimulation by von Frey filaments was measured before (Day 0), and 1, 3, 5, 7 and 10 days after peripheral nerve injury. Rats with peripheral nerve injury were injected intrathecally with flavopiridol (5 nmol/10 μl) or vehicle (10 μl) twice a day for 5 days from Days 3 to 7. Values represent the threshold (g) to elicit paw withdrawal behaviour (n=5 rats; *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle group at corresponding time point). Scale bar = 50 μm (A), 200 μm (C). Data are mean ± SEM. IHC = immunohistochemistry.
7 days after peripheral nerve injury (Dominguez et al., 2008, 2010). A striking difference is the subcellular localization of STAT3. In their studies, immunofluorescence of phospho-STAT3 is not localized in the nucleus but is merged with OX-42 immunofluorescence, a cell surface marker of microglia. In contrast, our data clearly show the nuclear translocation of STAT3 in an astrocyte-specific manner, which is demonstrated by using two astrocyte markers and the nuclear marker DAPI. Although it is uncertain whether phosphorylated STAT3 in the cytoplasm of microglia functions as a transcription factor, STAT3’s function in dorsal horn astrocytes would be different in microglia. The present study shows that proliferating dorsal horn astrocytes are suppressed by spinal administration of the JAK inhibitors AG490 and JAK inhibitor I during the period when astrocyte-restricted proliferation and STAT3 activation occur. Thus, it is reasonable to conclude that inhibiting JAK-STAT3 signalling in dorsal horn astrocytes results in suppression of their proliferation. However, we cannot exclude the possible involvement of STAT3 in the dorsal root ganglion to which drugs administered intrathecally can reach (Zhuang et al., 2006). Nevertheless, STAT3 expression markedly decreased in the injured dorsal root ganglion, and, importantly, recent studies have demonstrated impaired proliferation activity of either AG490-treated or STAT3-deficient cultured astrocytes (Washburn and Neary, 2006; Sarafian et al., 2010). These results provide direct evidence for a crucial role of astrocytic JAK-STAT3 signalling in their proliferation and strongly support our conclusion. It should also be noted that dorsal horn astrocyte proliferation after peripheral nerve injury was not completely abolished by AG490 and JAK Inhibitor I, suggesting that there may be independent and/or cooperative mechanisms involving other signals. Extracellular signal-regulated protein kinase and c-jun N-terminal kinase are known to regulate astrocyte proliferation in vitro (Neary et al., 1999; Gadea et al., 2008). After peripheral nerve injury, extracellular signal-regulated protein kinase and c-jun N-terminal kinase are activated in dorsal horn astrocytes (Zhuang et al., 2005, 2006). However, astrocytic extracellular signal-regulated protein kinase activation occurs from 10 days after peripheral nerve injury (Zhuang et al., 2005). Astrocytic c-jun N-terminal kinase is gradually activated from Day 3 post-peripheral nerve injury, but a c-jun N-terminal kinase inhibitor fails to suppress the number of GFAP* dorsal horn astrocytes (Zhuang et al., 2006). Thus, it is unlikely that these kinases contribute to the proliferation of dorsal horn astrocytes after peripheral nerve injury in vivo.

The mechanisms underlying STAT3-mediated proliferation of dorsal horn astrocytes remain unknown. STAT3 nuclear translocation may induce transcriptional changes in astrocytes, leading to the proliferation of these cells. There are a number of genes whose expression is controlled by STAT3 in cultured astrocytes with an estimation of over 1200 (Sarafian et al., 2010). Among genes encoding cell cycle proteins, the well-known cell cycle driver cyclin D1 is increased by STAT3 (Sarafian et al., 2010). Our findings demonstrate that the expression of cyclin D1 protein in the dorsal horn is induced specifically in astrocytes after peripheral nerve injury, and interestingly, cyclin D1-deficient mice have been reported to display impaired proliferation activity of astrocytes in vivo (Zhu et al., 2007). Thus, it is possible that cyclin D1 may participate in STAT3-dependent dorsal horn astrocyte proliferation within a specific time window after peripheral nerve injury.

As very few extracellular signalling molecules have been linked to reactive astrogliosis in the dorsal horn after peripheral nerve injury in vivo, our findings demonstrating JAK-STAT3 signalling as a critical pathway of dorsal horn astrocyte proliferation would aid future investigations to identify molecule(s) required for peripheral nerve injury-induced reactive astrogliosis. It has been reported that peripheral nerve injury results in upregulation of signalling molecules, which can activate STAT3, in the dorsal root ganglion and spinal cord, including interleukin-6 (Murphy et al., 1999) and fibroblast growth factor-2 (Madai et al., 2005; Ji et al., 2006). Activator for κ-opioid receptor may also be involved (Xu et al., 2007). It is also interesting to note that proliferation of dorsal horn astrocytes after peripheral nerve injury occurs after that of microglia. Identification of factors that are responsible for STAT3-dependent astrocyte proliferation and their cellular source is an important issue that needs to be investigated in future studies.

Another important finding in the present study was that suppression of astrocyte proliferation by inhibitors of JAK-STAT3 signalling leads to a recovery of tactile allodynia, a major feature of peripheral nerve injury-induced neuropathic pain. Although the anti-allodynic effect of AG490 when administered before peripheral nerve injury has previously been reported (Dominguez et al., 2008; Maeda et al., 2009), our findings reveal the first evidence that AG490 and JAK inhibitor I effectively alleviate tactile allodynia even by post-treatment with the inhibitors after peripheral nerve injury. Inhibition of tactile allodynia by intrathecal administration of AG490 and JAK inhibitor I from Day 3–7, a time window corresponding with astrocyte proliferation, implies that recovery from allodynia is associated with the reduction of proliferating astrocytes. In support of this, recovery from tactile allodynia was mimicked by direct suppression of dividing astrocytes by the cell cycle inhibitor flavopiridol. Although astrocyte proliferation was inhibited equally by JAK inhibitors and flavopiridol, it appears that JAK inhibitors were more effective than flavopiridol in attenuating allodynia. Besides proliferation, STAT3 also regulates reactive astrogliosis such as cellular hypertrophy and GFAP expression (Sofroniew, 2009). Moreover, STAT3 controls transcription of matrix metalloproteinase 2 (Xie et al., 2004) and monocyte chemoattractant protein-1 (Potula et al., 2009), critical molecules that have been shown to be upregulated in reactive astrocytes and implicated in the genesis of neuropathic pain (Kawasaki et al., 2008; Gao et al., 2009). Indeed, AG490 administered after astrocyte proliferation had finished produced a partial recovery of tactile allodynia. Thus, it is likely that inhibition of JAK-STAT3 signalling may globally repress reactive astrogliosis by inhibiting peripheral nerve injury-elicited astrocyte proliferation and gene expression in reactive astrocytes in the dorsal horn.

In summary, our findings here support the idea that reactive astrocytes contribute to maintaining neuropathic pain. In addition, we show that astrocytic JAK-STAT3 signalling is important for regulating astrocyte proliferation and that disrupting this proliferative process alleviates neuropathic pain after peripheral nerve injury. The levels of STAT3 immunofluorescence accumulated in the nuclear region were much lower in the dorsal horn of naive
rasts and in the contralateral dorsal horn of rats with peripheral nerve injury as compared with the ipsilateral dorsal horn of rats with peripheral nerve injury. The JAK-STAT3 signalling pathway serves as a target for pharmacological modification of reactive astrogliosis in the dorsal horn responding to peripheral nerve injury and for treating neuropathic pain. In addition, allodynia is also known as a severe side effect after neural stem cell transplantation, which is associated with differentiation to astrocytes (Hofstetter et al., 2005). Interestingly, STAT3 signalling is implicated in astrogliogenesis from neural stem cells (Bonacci et al., 1997). Thus, targeting JAK-STAT3 signalling may also prevent the side effects of neural stem cell therapy and thereby improve its efficacy.

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Supplementary material
Supplementary material is available at Brain online.

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