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 (Madiai 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

rats 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 (Bonni *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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Neuronal CCL21 up-regulates microglia P2X4 expression and initiates neuropathic pain development

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Up-regulation of P2X4 receptors in spinal cord microglia is crucial for tactile allodynia, an untreatable pathological pain reaction occurring after peripheral nerve injury. How nerve injury in the periphery leads to this microglia reaction in the dorsal horn of the spinal cord is not yet understood. It is shown here that CCL21 was rapidly expressed in injured small-sized primary sensory neurons and transported to their central terminals in the dorsal horn. Intrathecal administration of a CCL21-blocking antibody diminished tactile allodynia development in wild-type animals. Mice deficient for CCL21 did not develop any signs of tactile allodynia and failed to up-regulate microglial P2X4 receptor expression. Microglia P2X4 expression was enhanced by CCL21 application *in vitro* and *in vivo*. A single intrathecal injection of CCL21 to nerve-injured CCL21-deficient mice induced long-lasting allodynia that was undistinguishable from the wild-type response. This effect of CCL21 injection was strictly dependent on P2X4 receptor function. Since neuronal CCL21 is the earliest yet identified factor in the cascade leading to tactile allodynia, these findings may lead to a preventive therapy in neuropathic pain.

Introduction

Neuropathic pain is a pathological pain condition that often develops in response to peripheral nerve injury. Diabetes,

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cancer, infections with herpes zoster, nerve compression or trauma as well as autoimmune diseases are pathological conditions that may lead to the development of neuropathic pain (Campbell and Meyer, 2006). Tactile allodynia is the most distinctive symptom of neuropathic pain, whereby non-painful innoxiously stimuli, like light touch, become painful. Since neuropathic pain is an extremely intractable problem from the therapeutic point of view, new insights into the development of neuropathic pain may open new therapeutic windows.

Neuropathic pain is a result of abnormal neuronal activity, occurring throughout the neuraxis (Campbell and Meyer, 2006; White *et al*, 2007) and is underlined by an increased neuronal expression and/or activation of various ion channels and receptors that are responsible for the abnormal generation of action potentials and synaptic transmission in the pain pathways (White *et al*, 2007). Although originally thought to be a disease solely related to neurons, it is now increasingly evident that glial cells, in particular microglia, are actively involved in the development of neuropathic pain (Abbadie, 2005; Tsuda *et al*, 2005; Hains and Waxman, 2006; Scholz and Woolf, 2007; Saab and Haines, 2009). Rapidly after the first description that dorsal horn microglia activation correlates to the onset of tactile allodynia (Coyle, 1998) it was recognized that these cells are of crucial importance to the development of the disease (Watkins *et al*, 2001). An inhibition of microglia activity (i.e. with the antibiotic minocycline) attenuated predominately the development of tactile allodynia in animals (Raghavendra *et al*, 2003). More specifically, it is known now that up-regulation of ionotropic purinoceptors P2X4 in spinal cord microglia and subsequent release of brain-derived neurotrophic factor from these cells are crucial for the initiation and maintenance of tactile allodynia (Tsuda *et al*, 2003, 2005; Coull *et al*, 2005; Ulmann *et al*, 2008). However, it is not yet clear how nerve injury in the periphery leads to microglia activation in the dorsal horn of the spinal cord (Abbadie, 2005; Tsuda *et al*, 2005; Hains and Waxman, 2006; Scholz and Woolf, 2007; Saab and Haines, 2009). Since in peripheral nerve lesion, the activation of dorsal horn microglia depends on an intact dorsal root (Colburn *et al*, 1999), it was suggested that dorsal horn microglia activation is initiated by a factor derived from injured peripheral nerves (Tsuda *et al*, 2005). This factor, however, remained to be identified.

Neurons are important elements in the control of microglia activity under physiological circumstances and pathological conditions (Biber *et al*, 2007). Accordingly, microglia express numerous receptors for the various families of neuronal signalling molecules and respond to these stimuli with great specificity (Biber *et al*, 2007; Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009). Chemokines recently gained much attention in the field of neuron-microglia communication (Biber *et al*, 2008; Miller *et al*, 2008). The chemokine CCL21 functions in the CNS as microglia-activating factor that is exclusively

expressed in endangered or mechanically injured neurons (Biber *et al*, 2001; de Jong *et al*, 2005). Since neuronal CCL21 is sorted into large-dense core vesicles and specifically transported into the axons of endangered neurons (de Jong *et al*, 2008), we here investigated the question whether CCL21 is involved in neuron-microglia signalling and tactile allodynia in response to peripheral nerve injury.

Results

Peripheral nerve injury induced CCL21 expression in DRG neurons and in the dorsal horn

Spinal nerve injury induced by the transection of the L5 spinal nerve (Chung model) (Kim and Chung, 1992) induced rapid expression of CCL21 in ipsilateral L5 DRG

cells (Figure 1A and B). No or very little CCL21 expression was found in contralateral L5 DRG neurons (Figure 1A) and L4 DRG neurons. Induction of CCL21 was prominent after 12 h, peaked at 24 h where almost 10% of the DRG neurons displayed CCL21 expression and slightly declined thereafter (Figure 1B). CCL21 was specifically found in small-sized C-fibres (determined by the cell size-frequency distribution of DRG neurons with CCL21 expression and double staining with TRPV1, IB4, CGRP or NF200) (Guseva and Chelyshev, 2006) (Figure 1C and D). Higher magnification revealed that neuronal CCL21 expression was found in vesicles located in the axonal hillock of C-fibres (Figure 1E) and within axons of the dorsal root (Figure 1F). The primary afferents of DRG neurons in the dorsal horn of the spinal cord showed no (naive) or little CCL21 (12 h after injury) expression, whereas
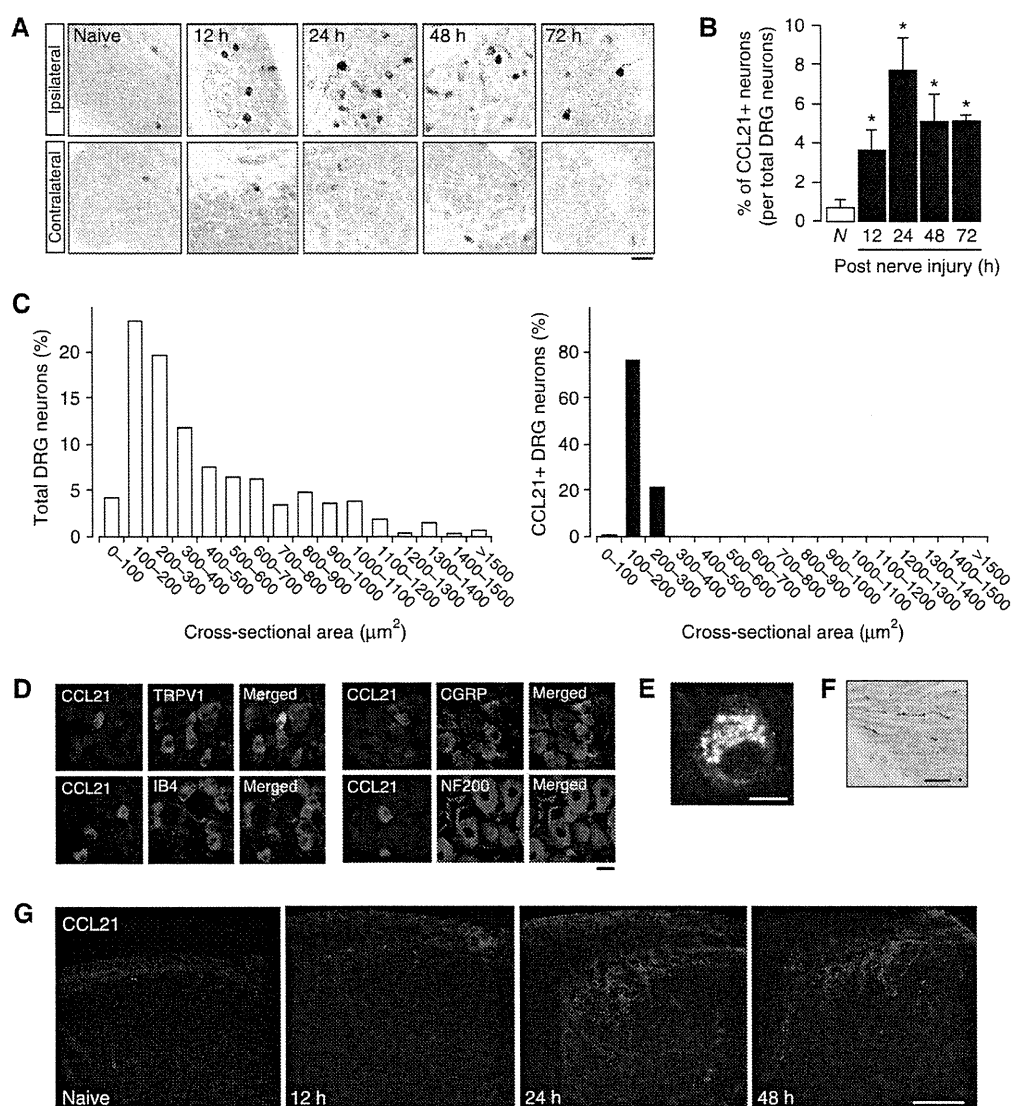
Figure 1 Expression of CCL21 in DRG neurons and spinal cord. (A) Rapid induction of CCL21 expression after spinal nerve injury in ipsilateral DRG. (B) Expression of CCL21 peaked at 24 h after spinal nerve injury with ~8% positive neurons in the DRG. (C) Cell size-frequency histogram illustrating the distribution of the cross-sectional areas of total (left panel) and CCL21-positive (right panel) DRG neurons 24 h after spinal nerve injury in wild-type mice. (D) Double-staining experiments at 24 h after spinal nerve injury showed the exclusive expression of CCL21 (green fluorescence) in C-fibres positive to TRPV1 (red fluorescence) and negative to IB4, CGRP and NF200 (red fluorescence) (12). (E, F) CCL21 expression in vesicles at the axonal hillock (E) and in axons between the DRG and the spinal cord (F). (G) The primary afferents in the dorsal horn of the spinal cord showed CCL21 expression at 1 and 2 days after spinal nerve injury. The immunohistochemical staining showed similar results in at least four independent experiments. Data shown in (B) are derived from three animals. *Significantly different from control animals, $P < 0.05$, Scale bars: (A) 50 µm; (D, F) 20 µm; (E) 10 µm; (G) 100 µm.

1 and 2 days after nerve injury expression of CCL21 was observed (Figure 1G). The staining for CCL21 in the dorsal horn of the spinal cord was only found in nerve terminals of the primary afferents and not in microglia (please refer to Supplementary Figure S1 for higher magnification) or in astrocytes.

No development of tactile allodynia in plt animals after spinal nerve injury
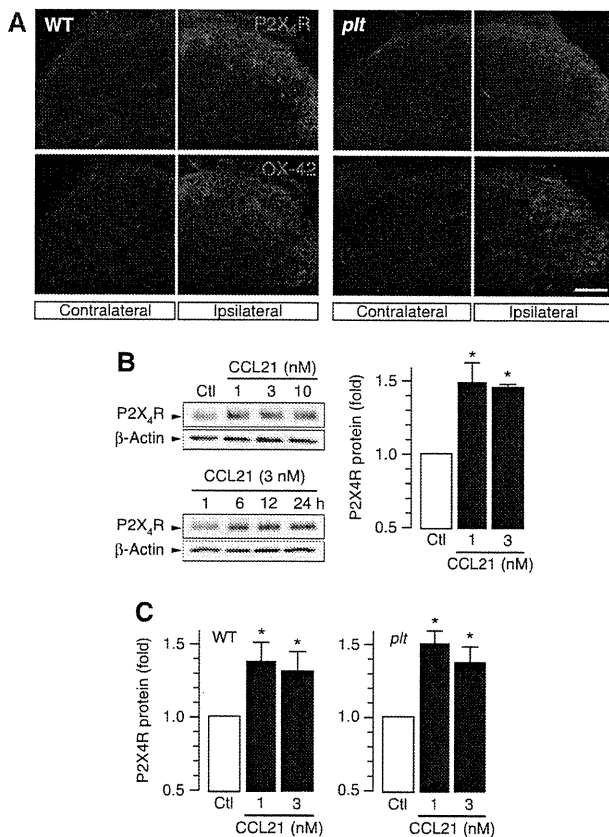
To determine the functional relevance of CCL21, we compared wild-type animals with mice carrying the paucity of lymphoid T cells (*plt*) mutation, which lack the genes for CCL19 and CCL21-Ser (Nakano and Gunn, 2001), the CCL21 isoform that is expressed in lymphoid organs (Mori *et al*, 2001) and in neurons (Rappert *et al*, 2004). Immunohistochemistry confirmed the absence of CCL21 in lymph nodes and DRG neurons in *plt* animals (Supplementary Figure S2) and verified the specificity of the used antibody for CCL21. Since *plt* animals also lack the gene for CCL19, we investigated whether CCL19 is expressed in nervous structures. Neither RT–PCR experiments (unpublished data) nor immunohistochemical staining revealed expression of CCL19 in DRG neurons or spinal cord tissue (Supplementary Figure S2), confirming earlier findings that CCL19

is not expressed in neuronal tissue (Biber *et al*, 2001; Rappert *et al*, 2002).

Spinal nerve injury induced a significant drop of the paw withdrawal threshold (PWT) in wild-type animals from 1.4 ± 0.3 g ($n = 6$ animals) before the injury to 0.4 ± 0.09 g ($n = 6$ animals) at day 3 ($P = 0.001$) with no significant recovery of the PWT within 14 days after nerve injury (Figure 2A). In contrast, *plt* mice did not develop any sign of tactile allodynia in response to spinal nerve injury, PWT stayed at control levels 1.42 ± 0.1 g throughout the experiment (Figure 2A). On the other hand, spinal nerve injury also enhanced the sensitivity to a heat stimulus as measured by the hot-plate test, but the thermal hypersensitivity of the operated hindpaw did not differ between wild-type and *plt* animals (Figure 2B). Moreover, acute pain responses were normal in *plt* mice as measured by tail-flick and paw-flick tests (Figure 2C), neither was the paw withdrawal frequency after mechanical stimulation (2.0 g) different between *plt* and wild-type animals (Figure 2D), indicating that *plt* animals had no general pain detection deficit. Intrathecally administration of 0.3 μg CCL21 neutralizing antibody to wild-type mice before and for the first 3 days after spinal nerve injury significantly attenuated tactile allodynia throughout the 14-day experiment (Figure 2E).
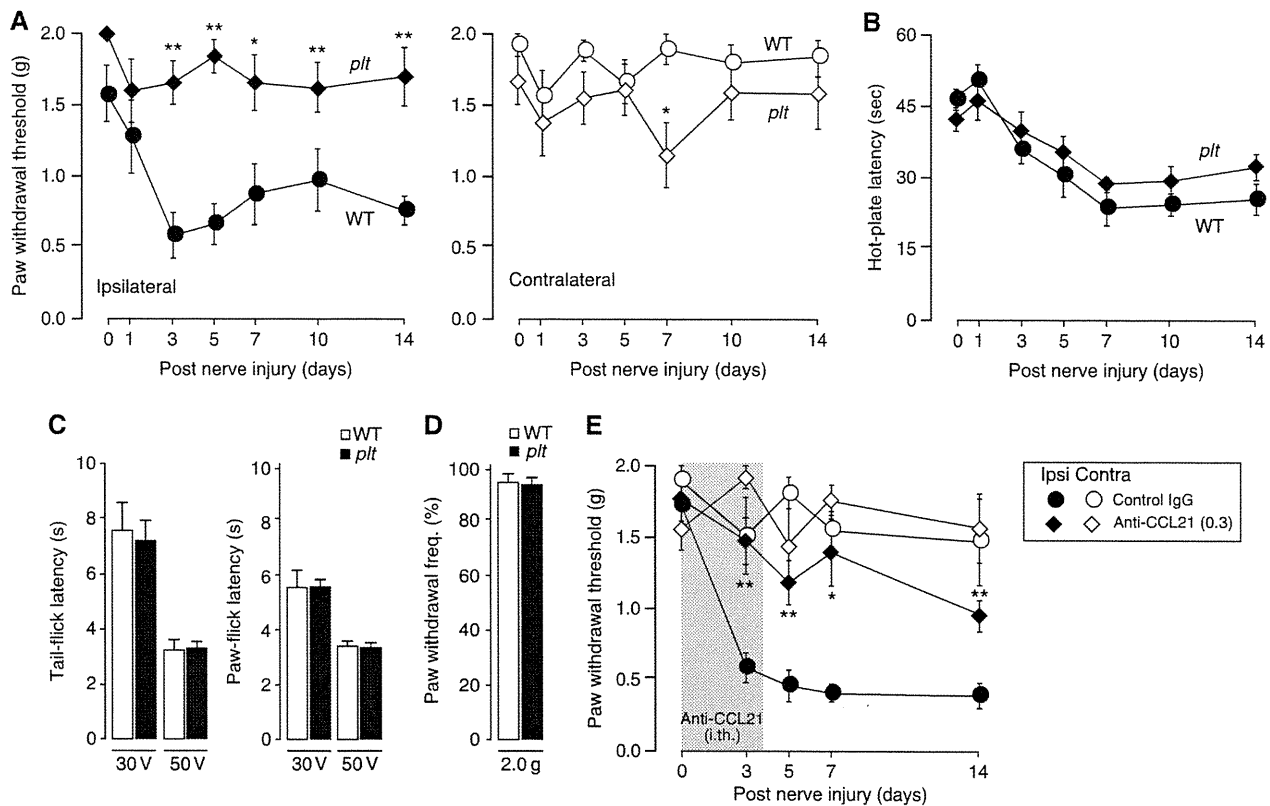
Figure 2 Spinal nerve injury does not lead to tactile allodynia in *plt* mice. (A) The withdrawal threshold to tactile was examined at the ipsilateral (left panel, black symbols) and contralateral hindpaw (right panel, open symbols). Whereas spinal nerve injury induced within 2 days a significant drop in the paw withdrawal threshold (PWT) of the ipsilateral hindpaw in wild-type animals (circles), no such response was observed in *plt* animals (diamonds). No significant change in PWT after spinal nerve injury was observed at the contralateral hindpaw in both mouse strains. (B) Thermal sensitivity after spinal nerve injury was assessed by hot-plate test and revealed no difference of the ispilateral paw between wild-type (circles) and *plt* animals (diamonds). (C) Tail-flick and paw-flick tests revealed no differences between wild-type mice and *plt* mice in acute pain reception. (D) The paw withdrawal frequency after mechanical stimulation (2.0 g) did not differ between wild-type and *plt* animals. (E) Intrathecal injection of blocking antibodies (0.3 μg) for CCL21 twice a day from before and post-operative day 3 significantly attenuated the development of tactile allodynia. Data are presented in mean ± s.e.m. from $n = 6$ animals for (A, B), from five animals in (C, D) and from four to six animals for (E). *,**Statistically significant difference from control IgG-injected animals, $P < 0.05$ and $P < 0.01$, respectively.

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CCL21 up-regulates P2X4 expression in microglia in vitro and in vivo

Since P2X4 receptor up-regulation in activated microglia is essential for the development of tactile allodynia after peripheral nerve lesion (Tsuda *et al*, 2003, 2009a; Ulmann *et al*, 2008), the possible effect of CCL21 on microglial P2X4 expression was assessed. Whereas in wild-type mice, spinal nerve injury induced a pronounced up-regulation of P2X4 receptor expression in dorsal horn microglia within 7 days (Figure 3A), this was attenuated in *plt* animals. Here, microglial P2X4 expression was barely detectable although OX-42 staining indicated a spinal nerve injury-dependent activation of microglia also in *plt* animals (Figure 3A). Moreover, direct stimulation of cultured rat microglia with CCL21 induced a rapid (<6 h) and significant up-regulation of P2X4 protein expression at concentrations as low as 1 nM (Figure 3B). Similar results have been obtained in microglia cultured from wild-type and *plt* mice (Figure 3C).

Activation of spinal cord microglia

The OX-42 staining shown in Figure 3A revealed that in *plt* animals, the morphological activation of microglia did not differ from the microglia reaction in wild-type animals. We therefore compared several microglial activation markers in wild-type and *plt* animals at different time points (2, 7 and 14 days) after spinal nerve injury. Microglia proliferation (assessed by phosphorylated-histone H3 (p-HisH3) staining, shown for 2 days after spinal nerve injury, Figure 4A and B) was not prominent confirming earlier findings in rat (Tsuda *et al*, 2011) and did not differ between wild-type and *plt* animals. The proliferation of microglia was an early event, since p-HisH3-positive cells were not found at day 7 or 14 after spinal nerve injury (Supplementary Figure S3), whereas Iba1-positive microglia were observed at all time points investigated (Supplementary Figure S3). However, we did not observe differences in microglia morphology (determined by Iba1 staining, shown for 7 days after spinal nerve injury, Figure 4C and D) at any time point after spinal nerve injury between lesioned wild-type and *plt* animals. Moreover, we could not find a major difference in spinal nerve injury-dependent expression of microglial Lyn kinase. Although Lyn kinase was slightly less expressed in *plt* microglia 2
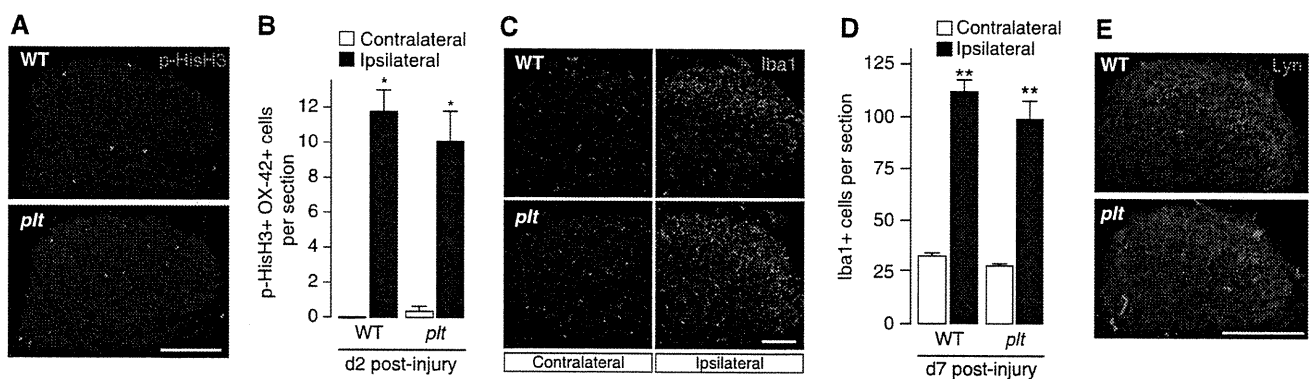
Figure 3 CCL21 is required for microglial P2X4 receptor induction *in vitro* and *in vivo*, but not for morphological microglia activation in response to spinal nerve injury. (**A**) Immunohistochemical staining showed a spinal nerve injury-dependent induction of microglial P2X4 expression (green) and activation determined by OX-42 (red) expression at the ipsilateral side of the dorsal horn only. Whereas microglia OX-42 staining was unchanged in *plt* animals, the induction of P2X4 expression was strongly attenuated in *plt* mice. The immunohistochemical staining showed similar results in three independent experiments. (**B**) Western blot analysis showed a time and concentration-dependent induction of P2X4 receptor expression in cultured rat microglia by CCL21. *Significant difference of P2X4 receptor expression in CCL21 stimulated microglia compared with non-stimulated controls. (**C**) Western blot analysis showed a concentration-dependent induction of P2X4 receptor expression in cultured mouse wild-type and *plt* microglia by CCL21. *Significant difference of P2X4 receptor expression in CCL21 stimulated microglia. Data presented are mean ± s.e.m. from five independent experiments. Scale bar, 100 µm.

Figure 4 Comparison of various aspects of microglia activation after spinal nerve injury between wild-type and *plt* animals. (**A**) Phosphorylated-histone H3 (p-HisH3) staining in the spinal cord 2 days after spinal nerve injury as a measure for microglia proliferation. (**B**) Quantification of p-HisH3-positive microglia did not reveal a difference between wild-type and *plt* animals. (**C**) Iba1 staining in the spinal cord 7 days after spinal nerve injury revealed the morphology of microglia. (**D**) Quantification of Iba1-positive cells did not show a difference between wild-type and *plt* animals. (**E**) Lyn kinase staining 7 days after spinal nerve injury showed no major difference between wild-type and *plt* animals. Data presented are mean ± s.e.m. from four to five independent experiments. Scale bar, 100 µm. Statistically significant different from contralateral side, $*P<0.05$; $**P<0.01$.
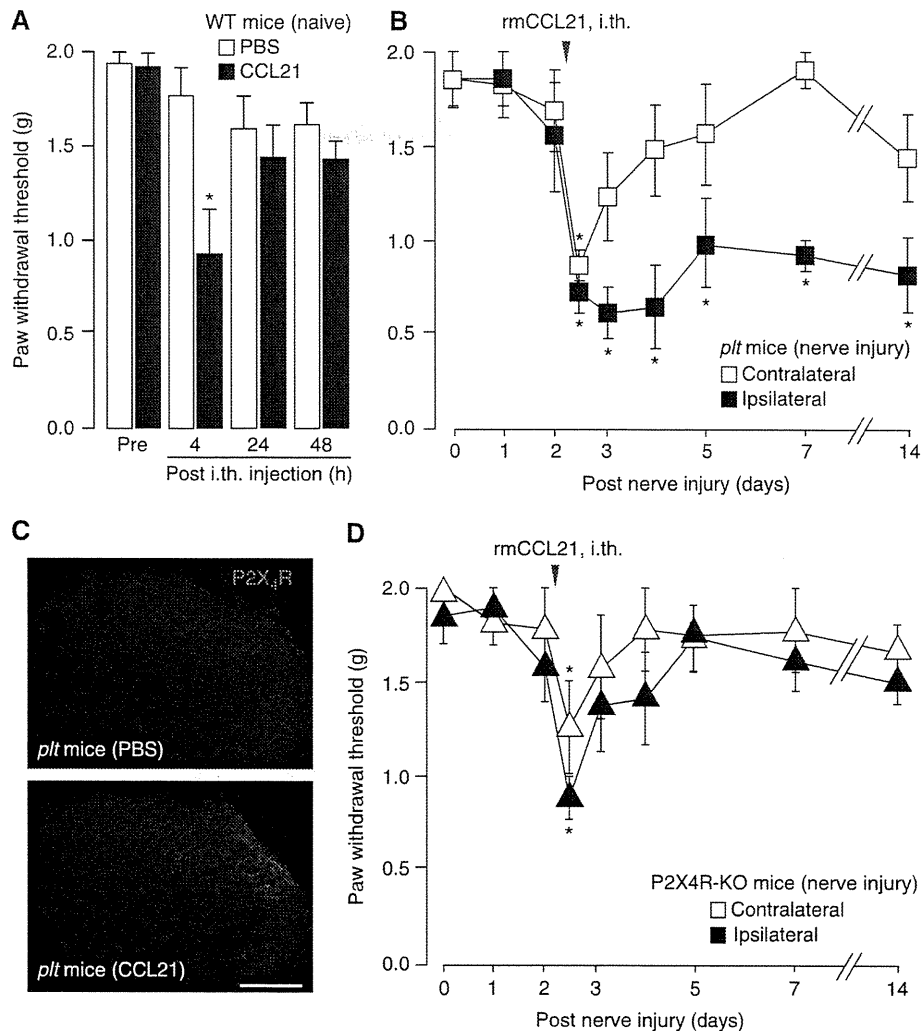
Figure 5 Intrathecal injection of CCL21 rescues normal tactile allodynia development after spinal nerve injury in *plt* animals but not in P2X4 receptor-deficient mice. (A) Intrathecal injection of CCL21 (0.06 µg) in naive wild-type animals resulted in a temporary (<24 h) drop of the PWT. (B) Intrathecal injection of CCL21 (0.06 µg) into *plt* animals 2 days after L5 spinal nerve lesion induced a long-lasting formation of tactile allodynia of the ipsilateral hindpaw (■), whereas the PWT of the contralateral hindpaw was temporary (□). (C) Intrathecal injection of CCL21 (0.06 µg) enhanced the expression of P2X4 receptor in *plt* animals whereas control injections with PBS had no effect. (D) Intrathecal injection of CCL21 (0.06 µg) in P2X4 receptor-knockout mice caused a transient (<24 h) drop of the PWT of both hindpaws. Data are given as mean ± s.e.m. from five to eight animals. Statistically significant difference from control values, *P<0.05.

days after nerve injury (Supplementary Figure S3) this small difference between lesioned wild-type and *plt* animals vanished at 7 and 14 days after spinal nerve injury (Figure 4E; Supplementary Figure S3).

Since *plt* animals have a changed peripheral immune system and a changed T-cell response (Mori et al, 2001), the possible role of peripheral T cells in the spinal cord was determined with the pan T-cell marker CD3. In general, very few T cells where found in the ipsilateral spinal cord at 7 days after spinal nerve injury with no differences between wild-type controls and *plt* animals (Supplementary Figure S4).

CCL21 requires P2X4 function to induce tactile allodynia
Next CCL21 was injected (0.06 µg/mouse) intrathecally into naive wild-type mice. After 4 h, a significant (P=0.036) but transient drop of the PWT to 0.93 ± 0.25 g was observed, that returned to control values 1.44 ± 0.10 g within 48 h (Figure 5A). Similar findings were observed in naive *plt* mice (unpublished data). CCL21 was furthermore injected into *plt* mice 2 days after spinal nerve injury (Figure 5B).

The PWT of the ipsilateral hindpaw dropped rapidly to 0.721 ± 0.11 g (P=0.003) and did not show any recovery throughout the experiment (Figure 5B). The PWT of the contralateral hindpaw showed a rapid (4 h) but transient decrease (0.865 ± 0.08 g/P=0.014) (Figure 5B) similar to the response seen in naive animals after CCL21 injection.

The intrathecally injection of CCL21 (0.3 µg) caused up-regulation of microglial P2X4 receptor expression (Figure 5C), confirming our findings in cultured microglia (see Figure 3)

In order to further investigate the function of P2X4 for the allodynia-inducing effects of CCL21, P2X4 receptor-deficient animals were used. Intrathecal injection of CCL21 2 days after spinal nerve injury caused a transient drop (ipsi: 0.878 ± 0.11 g/P<0.001; contra: 1.258 ± 0.24 g/P=0.024) in PWT of both hindpaws (Figure 5D). However, unlike *plt* mice (Figure 5B), the CCL21-induced decreased PWT in the ipsilateral side of P2X4-deficient mice was not maintained, and the PWT recovered to control values within 48 h after the injection (Figure 5D).
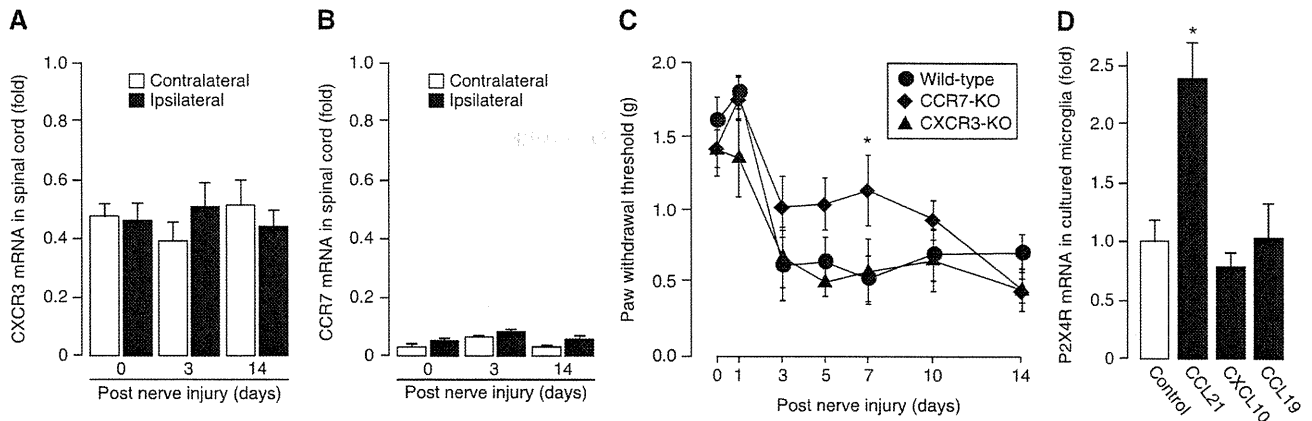
Figure 6 Chemokine receptor expression in mouse spinal cord tissue and the potential role of a yet unidentified chemokine receptor in neuropathic pain development. (**A**) Real-time PCR analysis of CXCR3 expression in mouse spinal cord. Compared with the mRNA expression levels in lymph nodes (reference point 1.0) less CXCR3 mRNA was detected in mouse spinal cord tissue, which was not affected by spinal nerve injury. (**B**) Real-time PCR analysis of CCR7 expression in mouse spinal cord. Compared with the mRNA expression levels in lymph nodes (reference point 1.0) CCR7 mRNA was close to detection limit in mouse spinal cord tissue and was not affected by spinal nerve injury. Data in (**A, B**) are given as mean ± s.e.m. from three to five animals. (**C**) Spinal nerve injury induced development of neuropathic pain in CXCR3- and CCR7-deficient mice. There was no difference in the PWT of the ipsilateral hindpaw between wild-type (circles) and CXCR3 −/− animals (triangles) in response to spinal nerve injury. The development of neuropathic pain was significantly attenuated in CCR7 −/− animals (diamonds) at day 7 after spinal nerve injury. At earlier or later time points, no difference between CCR7 −/− and wild-type mice were observed. Data are given as mean ± s.e.m. from five to eight animals. *Statistically significant from wild-type values. Only data from the ipsilateral paw are shown. (**D**) Real-time PCR analysis of P2X4 receptor mRNA expression in cultured microglia after 6 h stimulation with 100 nM of CCL21, CXCL10 (CXCR3 ligand) and CCL19 (CCR7 ligand). Data are given as mean ± s.e.m. from six to nine experiments. Statistically significant difference from control values, *$P < 0.05$.

Neither CCR7 nor CXCR3 are solely involved in CCL21-dependent induction of neuropathic pain

Two different receptors for CCL21 (CXCR3 and CCR7) have been described in mice, both of which can be found in microglia (Biber *et al*, 2001; Rappert *et al*, 2002; Dijkstra *et al*, 2006). Whereas CXCR3 is constitutively expressed in these cells, microglial CCR7 expression can be induced by activation (Dijkstra *et al*, 2006). To determine which of those receptors is mediating the neuropathic pain inducing effect of CCL21, we first determined the mRNA expression levels of these chemokine receptors in spinal cord tissue of mice. As shown in Figure 6, CXCR3 mRNA was detected in non-injured spinal cord tissue of the mouse (albeit at lower levels than in lymph node control tissue) (Figure 6A), whereas the signal for CCR7 mRNA was close to detection limit (Figure 6B), thereby confirming earlier findings that CCR7 mRNA is not expressed in healthy nervous tissue (Biber *et al*, 2001; Rappert *et al*, 2002, Dijkstra *et al*, 2006). Spinal nerve injury did not change the mRNA expression levels for CXCR3 or CCR7 at 3 and 14 days after the nerve injury (Figure 6A and B). Furthermore, chemokine receptor-deficient animals were subjected to spinal nerve injury and the PWT was analysed. As depicted in Figure 6C, there was no difference in the PWT of the ipsilateral paw between CXCR3 −/− and wild-type animals. The development of neuropathic pain in CCR7-deficient mice in the first week appeared somewhat less severe than in wild-type mice (statistically significant from wild-type controls at the 7-day time point only) but reached wild-type levels thereafter (Figure 6C). Cultured microglia were furthermore stimulated with different chemokine ligands (10 nM for 6 h) and P2X4 mRNA expression was assessed. Only CCL21 caused a significant up-regulation of P2X4 receptor mRNA expression, whereas neither the specific CXCR3 ligand CXCL10, nor the specific CCR7 ligand CCL19 had such an effect (Figure 6D).

Discussion

It was recognized in the last couple of years that microglia activity in the dorsal horn is crucial for the initiation and maintenance of tactile allodynia (Tsuda *et al*, 2005). It is therefore of interest to discover the signal(s) that control microglia activation in response to spinal nerve injury.

Neuronal CCL21 is crucial for the development of tactile allodynia

We here provide evidence for the neuronal chemokine CCL21 as such a signal. CCL21 is a microglia-activating chemokine that in nervous tissue is exclusively found in damaged neurons (Biber *et al*, 2001; de Jong *et al*, 2005, 2008). Using live cell imaging we showed that neuronal CCL21 is sorted into large-dense core vesicles and transported anterogradely along the axons to presynaptic terminals, indicating its function in directed neuron-microglia signalling (de Jong *et al*, 2005, 2008). In injured DRG neurons, CCL21 expression was detected within 12 h, specifically in vesicles of small-sized C-fibres. Since CCL21 expression was at later time points (24 h after injury) furthermore observed in axons of the dorsal root and in the primary afferents in the dorsal horn of the spinal cord, a vesicle-based, anterograde transport of CCL21 is suggested in small-sized C-fibres.

The importance of CCL21 for the development of tactile allodynia became evident in *plt* animals that lack neuronal CCL21. Spinal nerve injury in these animals did not induce any signs of tactile allodynia. However, the development of tactile allodynia was initiated in *plt* animals when spinal nerve injury was followed by an intrathecally injection of CCL21. Although CCL21 was injected only once, it induced a pain reaction that lasted until the end of the experiment (day 14) and was undistinguishable from the pain reaction in wild-type animals. An inhibition of CCL21 function by antibody

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treatment before and after spinal nerve injury prominently reduced the development of tactile allodynia in wild-type animals throughout the 14-day experiment. Furthermore, whereas wild-type mice up-regulated P2X4 receptor expression in microglia after spinal nerve injury (Tsuda *et al*, 2003; Ulmann *et al*, 2008), this response was reduced in *plt* animals. Stimulation with recombinant CCL21 induced a rapid and concentration-dependent increase in P2X4 protein expression in cultured microglia, indicating a direct CCL21 effect in these cells. This was further corroborated by intrathecally injections of CCL21 in *plt* animals, which resulted in up-regulation of P2X4 expression. It is therefore suggested that CCL21 *in vivo* is crucial for the induction of microglial P2X4 expression. This assumption is corroborated by the findings that intrathecal injection of CCL21 in P2X4-deficient animals did not induce long-lasting tactile allodynia, showing that CCL21 function is upstream of P2X4 up-regulation in the cascade leading to tactile allodynia.

CCL21 was not required for the development of thermal hypersensitivity after spinal nerve lesion as *plt* animals did not show a difference compared with wild-type animals. Since it is known that tactile allodynia and thermal hypersensitivity in the spinal nerve lesion model show differences in morphine response and disease course (Kim and Chung, 1992; Wegert *et al*, 1997; Lashbrook *et al*, 1999), our data about the selective involvement of CCL21 add up to the concept that tactile allodynia and thermal hypersensitivity are due to different mechanisms.

Taken together, these data show that the expression of neuronal CCL21 is an early event after spinal nerve injury, which is required and sufficient for the development of tactile allodynia specifically.

Temporary versus prolonged pain reaction

An intrathecal injection of inflammatory factors (cytokines and chemokines) often causes a temporary drop in PWT in rodents (Watkins *et al*, 2001; Abbadie, 2005), suggesting that this reflects a temporary hypersensitivity caused by local inflammation. In line with this it is shown here that in naive wild-type, *plt* and P2X4-deficient mice, injection of CCL21 induced a temporary drop of the PWT. This indicates that the general responsiveness to CCL21 and the overall inflammatory reaction in the spinal cord induced by CCL21 was comparable in all animal strains used in this study. Interestingly, injection of CCL21 only in combination with spinal nerve injury induced the development of long-lasting tactile allodynia in *plt* mice. This indicates not only that temporary hypersensitivity and development are most likely due to different processes but furthermore shows that more than one signal is needed for the development of tactile allodynia.

Microglia morphology is not a sufficient readout of their function

Based on morphological changes has microglia activation long been considered a stereotypic response; this view has been challenged recently. New notions concerning microglia activity suggest that these cells respond in quite distinct ways to different pathological situations, thereby integrating various inputs and responding appropriately with a variety of different reactions (Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009). Although the arrival of CCL21

in the primary afferents coincides timely with the first morphological signs of microglia activation, our data show that this chemokine is not required for the morphological microglia activation or proliferation. Numerous factors, such as cytokines, chemokines or ATP can induce morphological microglia activity (Davalos *et al*, 2005; Cardona *et al*, 2006; Haynes *et al*, 2006; Koizumi *et al*, 2007), are expressed and most likely released in the spinal cord after spinal nerve injury and may thus account for the morphological activation of microglia (Coull *et al*, 2005; Tsuda *et al*, 2005; White *et al*, 2007). Although spinal nerve injury leads to an accumulation of activated microglia in lamina I and II of the dorsal horn at day 7 after the nerve injury, at earlier time points there is a more equal distribution of activated cells. It is at the moment neither clear which factors are responsible for the activation of microglia nor is it understood whether the accumulation of activated microglia in lamina I and II is due to migration of microglia into these areas or a deactivation of microglia in other layers at day 7 after nerve injury. These questions are at the moment under investigation in our laboratory. However, our findings clearly show that morphologically activated microglia did not cause the development of tactile allodynia in the absence of CCL21 and subsequent P2X4 receptor up-regulation. The data presented here thus add up to that concept that microglia are able to integrate various inputs and that the lack of one factor significantly affects their reaction.

Potential role of a yet unknown CCL21 receptor in the induction of neuropathic pain

There are two known receptors for CCL21 in mice: CCR7 and CXCR3, both of which can be detected in microglia (Biber *et al*, 2001; Rappert *et al*, 2002; Dijkstra *et al*, 2006). Cultured microglia from CXCR3-deficient animals lose their chemotactic response to CCL21 stimulation (Rappert *et al*, 2002) and CXCR3-deficient animals display markedly reduced microglia activation after neuronal injury in the entorhinal cortex lesion model (Rappert *et al*, 2004), indicating a prominent role of CXCR3 in detecting CCL21 in the nervous system. However, the development of neuropathic pain was not affected in CXCR3-deficient animals, showing the involvement of a different receptor here. CCR7-deficient animals displayed a somewhat milder disease course, especially in the first days after spinal nerve injury. This delay in allodynia development might point to an induction of CCR7 expression in activated dorsal horn microglia, similar to what was found in a mouse model of multiple sclerosis (Dijkstra *et al*, 2006). However, in agreement with earlier studies we were not able to detect any CCR7 mRNA in the spinal cord, neither was CCR7 mRNA induced by the nerve lesion. Given this lack of CCR7 in spinal cord tissue, the slightly milder disease development after spinal nerve injury in CCR7-deficient animals is most likely due to a yet not understood effect in the periphery. The fact that only CCL21, but not the specific CXCR3 ligand CXCL10 or the specific CCR7 ligand CCL19 were able to induce P2X4 mRNA expression in cultured mouse microglia might point to another CCL21 receptor in these cells. Indeed, we have recently provided functional evidence for a third, yet not identified, CCL21 receptor in mouse astrocytes (van Weering *et al*, 2010), indicating that the question of CCL21 receptors in glia cells is more complex than originally anticipated. Taken together, the responsible receptor for the CCL21-dependent

development of neuropathic pain after spinal nerve injury remains to be established.

CCL21 as potential drug target to prevent neuropathic pain

Whether or not blocking of CCL21 would be of therapeutic relevance to stop already ongoing neuropathic pain is unknown. Since CCL21 expression peaked very early after nerve injury and diminished thereafter and since the stop of CCL21-blocking antibody treatment did not lead to increased pain states for > 10 subsequent days, our data might not argue for a role of CCL21 in the maintenance of neuropathic pain. The data presented in this study strongly indicate that inhibition of CCL21 expression in DRG neurons or attenuation of its function in the dorsal horn would prevent the development of neuropathic pain, which would be a welcomed therapeutic intervention for patients with acute nerve injury. Anatomical considerations might support the possibility that similar processes are also occurring in humans. In rodents it is known that microglia activity and subsequent development of neuropathic pain depends on an intact dorsal root, indicating the transport of activating molecules (Colburn *et al*, 1999; Obata *et al*, 2006a, b). In humans cutting of dorsal roots (e.g. to treat spasticity or for tumour removal) does not lead to neuropathic pain, in contrast to lesions distal from the DRG (Campbell and Meyer, 2006), which might suggest a transport of microglia-activating molecules through the dorsal root also in humans. Whether or not CCL21 is also expressed in human DRG neurons in response to nerve injury, however, remains to be established.

Taken together, we provide evidence that neuronal CCL21 in mice is a rapid signal that up-regulates P2X4 receptor expression in spinal cord microglia, identifying CCL21 as the neuronal signal that is required and sufficient for the induction of tactile allodynia after spinal nerve injury.

Materials and methods

Animals

plt (Mori *et al*, 2001; Nakano and Gunn, 2001) and wild-type C57BL/6 mice (Clea Japan) were used. Animals were housed in groups of two to three per cage at a temperature of $22 \pm 1\,°C$ with a 12-h light–dark cycle (light on from 08:30 to 20:30 hours), 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.

Spinal nerve injury

We used the spinal nerve injury model (Kim and Chung, 1992) with some modifications (Tozaki-Saitoh *et al*, 2008; Tsuda *et al*, 2009b) in 9- to 12-week-old mice. Under isoflurane (2%) anaesthesia, a small left-side incision at ~L4–S2 was made. Paraspinal muscle and fat were removed from the L6 traverse process, and the part of this traverse process was removed to expose the parallel-lying L4 and L5 spinal nerves. The L5 nerve was isolated and cut. The wound was sutured with 3-0 silk. The surrounding skin was pulled together and sutured with 3-0 silk.

Behavioural studies

To assess the tactile allodynia, mice were placed individually in an opaque plastic cylinder, which was placed on a wire mesh and habituated for 1 h to allow acclimatization to the new environment. After that, calibrated von Frey filaments (0.02–2.0 g, Stoelting) were applied to the plantar surface of the hindpaw from below the mesh floor, and the 50% PWT was determined using the up–down method (Chaplan *et al*, 1994). The PWT was measured before and after the injury of the L5 spinal nerve (an animal model of neuropathic pain) or intrathecal administration of recombinant CCL21 (0.06 µg/mouse, R&D Systems). Intrathecal administration to mice was performed according to a procedure described previously (Tsuda *et al*, 2008a) using a 25-µl Hamilton syringe with 30-gauge needle. A neutralizing antibody for CCL21 (0.3 µg/mouse, Peprotech) was administered intrathecally twice a day from day 0 (15 min before nerve injury) to day 3. Nerve injury-induced thermal hyperalgesia was assessed using hot-plate test. Mice were placed on a metal surface maintained at either 49 °C within a 25-cm-high Plexiglas box (25 cm²). The latency to either lick or shake the hindpaw was measured as a nocifensive end point. Noxious heat-evoked hindpaw withdrawal responses were detected by the application of radiant heat (Ugo Basile, Italy) to the tail and the plantar surface of hindpaw, respectively (Tsuda *et al*, 2007). The intensity of the heat stimulus was adjusted to 30 or 50 V, and the latency of the paw withdrawal response (seconds) was measured (Tsuda *et al*, 2007). The sensitivity to noxious mechanical stimulus was assessed by measuring the frequency of withdrawal responses to application of a von Frey filament (2.0 g) 10 times. Motor coordination was assessed using the rotarod performance test (Tsuda *et al*, 2007).

Immunohistochemistry

According to a procedure described previously (Nakajima *et al*, 1992), anaesthetized mice were perfused transcardially with PBS, followed by 4% paraformaldehyde. The fifth lumbar (L5) segments of the DRG, dorsal root and spinal cord were removed, post-fixed in the same fixative for 3 h at 4 °C, and placed in 30% sucrose solution for 24 h at 4 °C. Sections (30 µm) were cut and processed for immunohistochemistry with antibodies for CCL21 (1:500, Peprotech), TRPV1 (1:1000, Santa Cruz), isolectin B4 (1:200, Sigma), CGRP (1:20 000, Peninsula Laboratories), NF200 (1:400, Sigma), OX-42 (1:1000, Serotec), Iba1 (1:2000, Wako), Lyn (1:200, Wako), phosphorylated-histone H3 (Ser10) (p-HisH3, 1:1000, Upstate/Millipore), CD3 (1:100, eBioscience) and P2X4 (1:1000, kindly provided by Dr Francois Rassendren) (Ulmann *et al*, 2008). Following incubation, the sections were incubated with secondary antibodies conjugated to Alexa Fluor™ 488 or 546 (1:1000, Molecular Probes). The sections were analysed using an LSM510 Imaging System (Zeiss). For the cell size-frequency analyses, the cross-sectional areas of total and CCL21-positive DRG neurons with clearly visible nuclei were measured.

Microglial culture

Primary cultured microglia were prepared in accordance with a method described previously (Nakajima *et al*, 1992). In brief, a mixed glial culture was prepared from neonatal Wistar rats, C57BL/6 and *plt* mice and maintained for 10–16 days in DMEM with 10% fetal bovine serum. Immediately before experiments, microglia were collected by a gentle shake as the floating cells over the mixed glial culture. The microglia were transferred to coverslips for subsequent experiments. The cultures were of >99% purity, determining by immunostaining for OX-42 and Iba1.

Western blotting

Whole-cell lysates of cultured microglial cells were prepared in accordance with the methods described previously (Tsuda *et al*, 2008b). Aliquots (4 µg) were subjected to a 10% polyacrylamide gel electrophoresis (Bio-Rad), and proteins were transferred electrophoretically to nitrocellulose membranes. After blocking, the membranes were incubated with anti-P2X4R rabbit polyclonal antibody (1:1000, Alomone) or anti-β-actin (1:2000, Sigma) then incubated with HRP-conjugated secondary antibody (1:1000). The blots were detected using ECL western blotting detection system (GE Healthcare) and an LAS-3000 imaging system (Fujifilm). Bands were quantified using the software NIH Image J 1.36.

Real-time PCR

Real-time amplification, using an iCycler (Bio-Rad) and iQ SYBR Green supermix (Bio-Rad), was performed on 4 ng cDNA derived from microglia culture or spinal cord tissue. RNA preparation and cDNA synthesis was performed as described

recently (Dijkstra *et al*, 2006). The primers used for real-time PCR were designed using Primer Designer (Scientific and Educational Software, Version 3.0) and have been published earlier (Dijkstra *et al*, 2006). PCR amplification with CCR7 and CXCR3 primers was run in parallel with primers for the housekeeping gene hydroxymethylbilane synthase and quantified as described recently (Dijkstra *et al*, 2006). Primers used for P2X4 were (F): 5'-TGG CGGACTATGTGATTCCA-3', primer (B): 5'-GGTTCACGGTGACGATC ATG-3'. Lymph node tissue served as positive control and reference for CCR7 and CXCR3 expression.

Statistics

Statistical analyses of the results were made with Student's *t*-test, Mann–Whitney *U*-test or two-way repeated measures ANOVA (*post hoc* test using Tukey).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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Ⅴ．参考資料

脊髄障害性疼痛症候群

1．概要

脊髄障害性疼痛症候群は脊髄障害に起因すると考えられる多彩な痛みであり、通常痛みを起こさない刺激が痛みを引き起こすアロデニアや障害脊髄高位以下の締め付けられるような自発痛などを生ずる。脊髄の障害が引き起こされる後縦靱帯骨化症や脊髄空洞症や脊髄損傷後などに発症し、一部の薬剤が奏功する症例も存在するが一般に治療抵抗性であるため患者の日常生活を極めて悪化させる要因となる。

2．疫学

地域研究の結果では手足にしびれや痛みを生ずるものは人口の 20%ほど存在するが本症候群に至るケースは人口の 0.15%程度であることが判ってきている。

多彩な痛みを呈するが、全国の本症候群の患者（１６８２名）を対象とした調査では脊髄障害高位にアロデニアを呈したものが 43%、脊髄障害よりも下位にしびれ痛みを呈したものが 68%、筋性疼痛：46%であった。

3．原因

脊髄損傷だけでなく、頚椎症性脊髄症や後縦靱帯骨化症などの圧迫性脊髄症において脊髄が直接傷害されることで症状が出現する。

圧迫の解除などによっては改善しないことも多く、MRI においては脊髄病変が観察されるがその発症のメカニズムについては不明な部分が多い。

4．症状

脊髄の障害が引き起こされた部位以下の自発痛、しびれ、感覚鈍麻。

障害高位のアロデニア（異痛症）：通常痛みを引き起こさない触覚刺激あるい冷温刺激で痛みを生ずることがある。痛覚過敏：侵害刺激（機械的痛み刺激あるいは温熱痛刺激）によって強い痛みを生じることがある。MRI による画像診断では障害部位に圧迫性病変、脊髄空洞症、脊髄内高輝度変化などを認めることが多い。

5．合併症

脊髄性の運動障害

運動障害に起因する関節拘縮、廃用症候群など

自律神経障害

膀胱直腸障害

反応性うつ状態

6．治療法

抗てんかん剤、抗うつ剤などの薬物療法が一部有効なことがある。抗てんかん剤はナトリウムチャネルやカルシウムチャネルを阻害することで神経の興奮性を抑制することで鎮痛効果が得られると推察される。抗うつ剤の本症候群に対する作用機序は不明であるが、下行性鎮痛抑制系の賦活作用あるいは抗うつ作用によると考えられる。カウンセリングや痛みに対する生活の指導も一部有効である。

7．研究班

脊髄障害性疼痛症候群の実態の把握と病態の解明に関する研究班

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