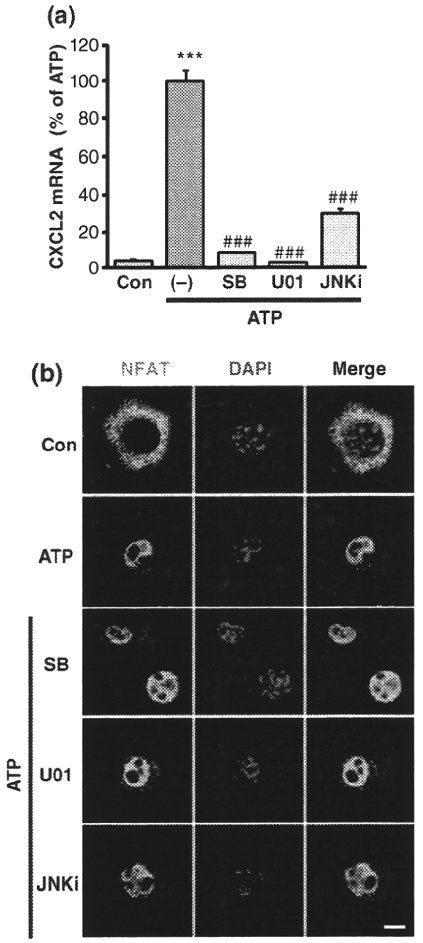

Fig. 5 Signaling pathways of p38, MEK/ERK and JNK are involved in ATP-induced CXCL2 expression but not modulate ATP-induced nuclear translocation of NFAT. (a) Effects of SB203580 (SB), U0126 (U01), and JNK inhibitor II (JNKi) on ATP-induced CXCL2 expression in BV-2 microglial cells. Cells were pre-treated with these inhibitors for 10 min before ATP treatment. ATP treatment was for 1 h. Results are expressed as the mean ± SEM of at least two independent experiments performed in triplicate. ***, $p < 0.001$ compared with control; ###, $p < 0.001$ compared with ATP. (b) Effects of MAPK inhibitors on ATP-induced nuclear translocation of NFAT. BV-2 microglial cells were pre-treated with these inhibitors for 10 min before 30 min ATP treatment. At least two independent experiments were performed and similar results were obtained. Scale bar, 10 μm.

possible that there are direct or indirect interactions between NFAT and MAPKs. Previous reports have suggested that MAPKs regulate the activation of NFAT in Jurkat T cells and HeLa cells (Genot *et al.* 1996; Ortega-Perez *et al.* 2005), however, in microglia, it remains unclear if MAPKs regulate activation of NFAT. Thus, to investigate this, we observed ATP-induced nuclear localization of NFAT using MAPK inhibitors. Pre-treatment of cells with any MAPK inhibitors did not affect ATP-induced nuclear localization of NFAT (Fig. 5b). These results indicate that activation of MAPKs do not regulate the ATP-induced nuclear localization of NFAT.

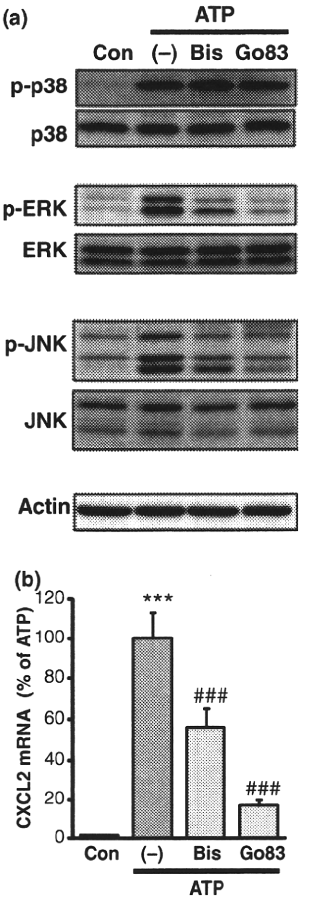

Fig. 6 PKC-MAPK pathways are involved in ATP-induced CXCL2 expression. (a) Effects of PKC inhibitors such as Bis and Gö 83 (Go 83, a) on ATP-induced p38, ERK and JNK phosphorylation. BV-2 microglial cells were pre-treated with PKC inhibitors for 10 min before ATP treatment. ATP treatment was for 15 min. At least two independent experiments were performed and similar results were obtained. (b) Effects of PKC inhibitors on ATP-induced CXCL2 expression in BV-2 microglial cells. Cells were pre-treated with PKC inhibitors for 10 min before ATP treatment. ATP treatment was for 1 h. Results are expressed as the mean ± SEM in triplicate. At least two independent experiments performed and similar results are obtained. ***, $p < 0.001$ compared with control; ###, $p < 0.001$ compared with ATP.

PKC-MAPK pathways are involved in ATP-induced CXCL2 expression

It has been suggested that certain subtypes of PKC act as upstream regulators of MAPKs (Belcheva *et al.* 2005; Geraldes *et al.* 2009); it is also suggested that many subtypes of PKC are expressed in microglia (Slepko *et al.* 1999; Kang *et al.* 2001). Thus, we investigated whether PKC acts as an upstream regulator of MAPKs in BV-2 cells. We assessed ATP-induced phosphorylation of MAPK using broad-spectrum PKC inhibitors, namely, bisindolylmaleimide I (Bis) and Gö 6983 (Gö 83). Pre-treatment of BV-2 cells with Bis and Gö 83 prevented ATP-induced phosphorylation of ERK and

JNK; however, phosphorylation of p38 was insensitive to pre-treatment with Bis or Gö 83 (Fig. 6a). Next, we investigated whether these pathways are involved in ATP-induced CXCL2 expression. Pre-treatment of cells with Bis and Gö 83 inhibited ATP-induced CXCL2 expression (Fig. 6b). Bis and Gö 83 also inhibited ATP-induced CXCL2 expression in rat primary microglia (Figure S1f). These results indicate that it is highly possible that certain subtypes of PKC act as upstream regulators of ERK and JNK, and are involved in ATP-induced mRNA expression of CXCL2 in microglia.

Discussion

In the present study, we found that new pathways for production of CXCL2, namely a calcineurin-NFAT pathway and a MAPK pathway downstream of P2X7R. ATP plays a crucial role in the CNS as an extracellular messenger through interaction with P2 receptors. P2 receptors are involved in many neurodegenerative pathologies such as traumatic injury (Wang *et al.* 2004), Alzheimer's disease (Parvathenani *et al.* 2003; McLarnon *et al.* 2006), and neuropathic pain (Tsuda *et al.* 2003; Tozaki-Saitoh *et al.* 2008). ATP is released via several mechanisms including co-release with other neurotransmitters from neurons (Bodin and Burnstock 2001), release from non-neuronal cells through membrane channels or gap junctions (Bodin and Burnstock 2001; Anselmi *et al.* 2008), and release from damaged cells surrounding lesions. Recently, it was reported that amyloid β induces ATP release from microglia (Sanz *et al.* 2009). These reports support the possibility that ATP is an important modulator of glial cell activities in the CNS. It is now clear that the ATP concentration in the extracellular space under normal conditions is in the low nanomolar range, but that, at sites of inflammation, ischemia, tissue trauma, or intensive cell stimulation, ATP levels can reach the high micromolar range owing to an increase in ATP release and a decrease in ectonucleotidase activity (Braun *et al.* 1998; Igarashi *et al.* 2003; Wang *et al.* 2004). Furthermore, it is suggested that actual ATP levels in the vicinity of the plasma membrane can be much higher (Beigi *et al.* 1999; Pellegatti *et al.* 2005, 2008). These reports suggest that in many pathological conditions, ATP concentrations are sufficient to activate even low affinity P2 receptors such as P2X7R. It is widely acknowledged that P2X7R is involved in various CNS pathologies by regulating the expression and release of cytokines and inflammatory mediators in microglia including IL-1β, tumor necrosis factor-α, CCL3 (Ferrari *et al.* 1997b; Hide *et al.* 2000; Kataoka *et al.* 2009). Our finding that activation of P2X7R induces mRNA expression and release of CXCL2 from microglia suggests that in microglia, P2X7R is important in regulating the expression and release of not only proinflammatory cytokines but also chemokines. Moreover, because BBG treatment reduces neutrophil infiltration to the spinal cord after spinal cord injury (Peng *et al.* 2009), it is suggested that release of chemokines including CXCL2, via P2X7R, plays an important role in neutrophil infiltration in CNS pathologies. CXCL2, a CXC chemokine family member, interacts with a chemokine receptor, CXCR2. CXCR2 is involved in Alzheimer's disease, ischemia, traumatic injury, and multiple sclerosis (Popivanova *et al.* 2003; Valles *et al.* 2006; Bakshi *et al.* 2009; Kerstetter *et al.* 2009). Most of these pathologies are affected by P2X7R activity. Our findings also suggest that microglia is a candidate of source of CXCL2 as a ligand for CXCR2 in these pathologies. A previous report showed that CXCL2 up-regulates expression of other chemokines such as monocyte chemoattractant protein 1 (MCP-1, CCL2), 10 kDa interferon gamma-induced protein (IP-10, CXCL10), and CCL5 in astrocyte (Luo *et al.* 2000). It is possible that CXCL2 from microglia is also involved in this event.

To date, it has been suggested that Toll-like receptor-mediated nuclear factor-κB (NF-κB) activation is a major pathway leading to expression of CXCL2 (Kim *et al.* 2003; Negishi *et al.* 2005). In N9 microglial cells, it was reported that ATP also activates NF-κB at 3 h after 3 mM ATP stimulation (Ferrari *et al.* 1997a). Our results showed that ATP-induced mRNA expression of CXCL2 peaked at 1 h after ATP treatment (Fig. 1e). In addition, ATP-induced mRNA expression of CXCL2 was markedly inhibited by an NFAT inhibitor (Fig. 3b). These results suggest that ATP-induced mRNA expression of CXCL2 is mainly regulated by NFAT in BV-2 cells. Furthermore, we demonstrated that ATP could induce the transcriptional activity of NFAT response element in BV-2 cells (Figure S2) and also found putative NFAT-binding site at −43 to −38 upstream of the potential transcription start site (Widmer *et al.* 1993) using TESS (Transcription Element Search System, http://www.cbil.upenn.edu/tess). Therefore, it is highly possible that NFAT is involved in ATP-induced CXCL2 expression via direct binding to this NFAT-binding site. However, it is also suggested that NFAT works as transcriptional coactivator without direct binding to DNA (Youn *et al.* 2000). Recent studies have suggested that MAPK pathways modulate NFAT activation. The MEK-ERK pathway enhances NFAT activation in T lymphocytes (Genot *et al.* 1996). On the other hand, there are several reports suggesting that each MAPK directly phosphorylates NFAT and negatively regulate nuclear localization of NFAT (Chow *et al.* 1997; Porter *et al.* 2000; Yang *et al.* 2002). We demonstrated that inhibitors of each MAPK pathway did not affect ATP-induced nuclear localization of NFAT. These results suggest that these MAPK pathways do not modulate nuclear localization of NFAT (Fig. 5b), but there still remains a possibility that MAPK pathways have an effect on the transcriptional activity of NFAT. It is well-known that NFAT interacts with other transcription factor in the nucleus, including activator protein 1 (AP-1) (Rao *et al.* 1997), C/EBP (Yang and Chow 2003), and Maf (Ho *et al.* 1996).

MAPKs act upstream of some of these molecules. JNK and ERK regulate activation of c-*jun* and transcription of c-*fos*, respectively. These two molecules are components of AP-1 (Karin *et al.* 1997). P38 phosphorylates Maf proteins and regulates their activities (Sii-Felice *et al.* 2005). It is possible that each MAPK acts as an upstream regulator of certain transcription factors that interact with NFAT. It is also suggested that MAPK is involved in activation of histone acetyltransferases such as CBP/p300 (Youn *et al.* 2000).

We also found that certain PKC subtypes act as upstream regulators of ERK and JNK in ATP-treated BV-2 cells using the PKC inhibitors Bis and Gö 83. These inhibitors inhibit many subtypes of PKC; however, they show partial subtype specificity (Martiny-Baron *et al.* 1993; Gschwendt *et al.* 1996). Among several subtypes of PKC expressed in BV-2 cells (Kang *et al.* 2001) and rat primary microglia (Slepko *et al.* 1999), it is suggested that PKC δ is the most potent candidate subtype for acting as an upstream regulator of ERK and JNK. PKC δ is inhibited by Bis (Martiny-Baron *et al.* 1993) and Gö 83 (Gschwendt *et al.* 1996), and activation of P2X7R increases the tyrosine phosphorylation of PKC δ in parotid acinar cells (Bradford and Soltoff 2002). Moreover, PKC δ is involved in substance P-induced chemokine synthesis acting as an upstream regulator of MEK1, ERK, JNK, NF-κB, and AP-1 in pancreatic acinar cells (Ramnath *et al.* 2008). Because phosphorylation of p38 was insensitive to pre-treatment with Bis or Gö 83, it is suggested that an upstream regulator of p38 is different from those of ERK and JNK.

In this study, we used rat primary microglia in addition to BV-2 cells. We acquired largely similar results from both cells, however, there are some differences between BV-2 cells and rat primary microglia.

From difference of BBG effect, it is suggested that in rat primary microglia, not only P2X7 receptor but other P2 receptors are involved in ATP-induced CXCL2 release. However, given that BzATP, a potent agonist of P2X7 receptor, strongly induced CXCL2 release, it is highly possible that P2X7 receptor is a major component that is involved in ATP-induced CXCL2 release. From this view, it is also suggested that sensitivity to BBG is different between BV-2 cells and rat primary microglia. Considering experiments with JNK inhibitor, it is also suggested that there are differences between both cells about sensitivities to some kinds of regents. Previous reports showed that the several kinds of mRNAs have the AU-rich elements (AREs) in their 3′-untranslated region. In general, AREs involved in regulation of mRNA stability and exist in mRNA of cytokines (Khabar 2005). Several recent studies indicated that AREs confer stabilization of mRNA by p-38 pathways (Dean *et al.* 2004). Furthermore, Numahata *et al.*, revealed that rat CXCL2 mRNA also has 3′-untranslated region which contains AREs (Numahata *et al.* 2003). Therefore, it is suggested that in rat primary microglia, p-38 pathways are slightly active and involved in the stability of mRNA of CXCL2 in the control state.

In conclusion, we found that ATP induces mRNA expression and release of CXCL2 via P2X7R in microglia, and also that P2X7R-mediated activation of the calcineurin–NFAT and PKC/MAPK pathways are required for ATP-induced mRNA expression of CXCL2. These findings suggest that P2X7R, NFAT and MAPK are important in inducing expression of chemokines in microglia and as therapeutic targets for many neurodegenerative disorders. Moreover, it is also suggested that microglia play a crucial role in various pathologies producing chemokines including CXCL2.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. ATP induces expression and release of CXCL2 in primary microglia.

Figure S2. NFAT can induce the transcriptional activity with direct binding to their binding site in ATP-stimulated BV-2 cells.

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Nerve Injury-Activated Microglia Engulf Myelinated Axons in a P2Y12 Signaling-Dependent Manner in the Dorsal Horn

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KEY WORDS

microglia; P2Y12; peripheral nerve; nerve injury; neuropathic pain; rat

ABSTRACT

The mechanisms underlying neuropathic pain are poorly understood. However, several studies have implied a role for reactive microglia located in the dorsal horn in neuropathic pain. To clarify the roles of activated microglia in neuropathic pain, we investigated the interactions among microglia and other neural components in the dorsal horn using electron microscopy. Microglia were more abundantly localized in layers II–III of the dorsal horn than in other areas, and some of them adhered to and engulfed both injured and uninjured myelinated axons. This microglial engulfment was rarely observed in the normal dorsal horn, and the number of microglia attached to myelinated axons was markedly increased on postoperative day 7 on the operated side. However, after blocking the P2Y12 ATP receptor in microglia by intrathecal administration of its antagonist, AR-C69931MX, the increase in the number of microglia attached to myelinated axons, as well as the development of tactile allodynia, were markedly suppressed, although the number of activated microglia did not change remarkably. These results indicate that engulfment of myelinated axons by activated microglia via P2Y12 signaling in the dorsal horn may be a critical event in the pathogenesis of neuropathic pain.

INTRODUCTION

Peripheral nerve injury possibly leads to chronic neuropathic pain, which includes increased sensitivity to noxious stimuli (hyperalgesia), perception of innocuous mechanical stimuli as painful (allodynia), and spontaneous pain (stimulus independent). Neuropathic pain is relatively common, and largely resistant to treatment (Harden and Cohen, 2003). Although the underlying mechanisms are still poorly understood, recent studies have revealed the participation of activated microglia in chronic neuropathic pain (Marchand et al., 2005; Tsuda et al., 2005; Watkins and Maier, 2003). Under normal conditions, resting microglia localize relatively evenly in the CNS and have a ramified morphology with small cytoplasm and thin and branched processes. However, under pathological conditions, including ischemia, trauma, infection, and neurological disease, microglia change their morphology from a resting ramified shape into an active amoeboid shape, increase their number, migrate to lesion sites, and phagocytose the debris (Kreutzberg, 1996). Recently ATP, which is released from neurons and astrocytes following some stimuli, and which reacts with ATP receptors on microglia, has been highlighted as one of the major factors mediating the activation of microglia as well as the changes in their morphology (Apasov et al., 1995; Brake and Julius, 1996; Guthrie et al., 1999; Inoue, 2006; Neary et al., 1996). A recent study revealed that microglia express ionotropic (P2X4 and P2X7) and metabotropic (P2Y12) ATP receptors after peripheral nerve injury, and that released ATP contributes to the activation, movement and phagocytic activity of microglia (Koizumi et al., 2007). A previous report indicated that augmented microglial P2X4 receptors (P2X4Rs) in the spinal dorsal horn after peripheral nerve injury induced tactile allodynia (Tsuda et al., 2003). Blocking P2X4Rs suppressed the onset and expression of neuropathic pain (Coull et al., 2005; Tsuda et al., 2003). Significant inhibition of neuropathic pain behaviors was reported following P2X7 deletion or antagonism (Chessell et al., 2005; McGaraughty et al., 2007). More recently, the participation of P2Y12 receptor (P2Y12R) in spinal microglia in neuropathic pain after peripheral nerve injury was reported. An increase in the levels of P2Y12R expression was observed in activated microglia in connection with the development of allodynia. Blockade of P2Y12R activation by its antagonists prevented the development of tactile

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allodynia (Tozaki-Saitoh et al., 2008). Furthermore, mice lacking *p2ry12(p2ry12 −/−)* displayed impaired tactile allodynia (Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008). Thus, the involvement of ATP-activated microglia in allodynia became undoubted; however, the behaviors of activated microglia and the relationships between activated microglia and other neural components have remained obscure. Therefore, in the present study, we examined the interactions of microglia with other neural components using electron microscopy and a tracing method using biotin-dextran (BD) injection from a peripheral nerve. We show that activated microglia attach to and engulf myelinated axons in the dorsal horn after peripheral nerve injury, and that inhibition of P2Y12R suppresses the microglial engulfment of myelinated axons.

MATERIALS AND METHODS

Animals and Surgical Procedure for Inducing Neuropathic Pain

Ten-week-old male Wistar rats (Japan SLC) were used in this study. Animals were housed with a 12-h light/dark cycle (8:00/20:00) at a constant room temperature (RT) of 23 ± 2°C and a humidity of 45–65%. After induction of anesthesia by intraperitoneal (i.p.) injection of chloral hydrate (35 mg/kg), the left L5 spinal nerve at the level of 2 mm distal to the L5 DRG was tightly ligated with silk and cut just distal to the ligature as previously described (Tozaki-Saitoh et al., 2008). After this procedure, the wound was closed and rats were allowed to recover. To assess tactile allodynia, calibrated von Frey filaments (0.4–15 g, Stoelting) were applied to the plantar surfaces of the hindpaws. The 50% paw withdrawal threshold (PWT) was determined by the up-down method (Chaplan et al., 1994). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka City Medical School and Kyushu University.

Specific Labeling of the Injured L5 Spinal Nerve of the L5 Spinal Cord

Some of the transected spinal nerve was anterogradely labeled by application of biotin-dextran (BD) to the transected peripheral stump of the L5 spinal nerve (n = 20). At the time of operations to induce allodynia, 20 μL of a 10% solution of BD was applied to the transected stump.

Immunofluorescence Histochemistry Light Microscopy

Seven or 14 days after nerve injury, animals were perfused with 4% paraformaldehyde, 2.1% picric acid in 0.1 M phosphate buffer (PB), for 30 min, and the spinal cords were removed and post-fixed at 4°C for 5 h; then, a series of coronal sections of the spinal cord at the L5 level were cut with a microslicer at a thickness of 30 μm and stored until used for immunohistochemical staining. Floating transverse sections were blocked in solution containing 10% normal goat serum for 1 h at RT. Then, the sections were incubated for 24 h at 4°C with primary antibodies against ionized calcium-binding adapter molecule-1 (Iba1, rabbit polyclonal anti-Iba1, 1:500, Wako). After washing, the sections were incubated with a fluorescent-conjugated secondary antibody (Alexa 488 1:500, Molecular Probes) for 5 h at RT. Fluorescence images were obtained using a confocal microscope and analyzed using Zeiss LSM Image Brower. Control tissue sections, in which the primary antibody was omitted, showed no specific staining. The numbers of immunolabeled microglia on the operated and contralateral sides were counted in different 6,000-μm^2 areas of L5 spinal cord, including layers II–III, V, and X, and the dorsal, lateral and ventral horns, from each of four animals.

Immunohistochemistry for Electron Microscopy

For immunohistochemistry of anterogradely labeled spinal cord from transected spinal nerve by the application of biotin-dextran (BD) and non-labeled spinal cord without BD application, operated animals (n = 5 for each time point), were killed 7 or 14 days after peripheral spinal nerve transection at the L5 DRG level. The intact contralateral side was taken as a control. Immunolabeling was performed on floating tissue sections from nonoperated and operated animals using avidin-biotin horseradish peroxidase complex-conjugated 3,3′-diaminobenzidine tetrahydrochloride (DAB). Rats were perfused with fixative solution consisting of 4% paraformaldehyde and 0.1% glutaraldehyde in PB (pH 7.4). Following perfusion, the spinal cords were removed and immersed in the same fixative at 4°C overnight (O/N). Horizontal sections of spinal cord, including the L5 level, were cut with a microslicer at a thickness of 50 μm. Sections from nonlabeled animals without BD application from transected spinal nerves were incubated O/N at 4°C with a rabbit polyclonal antibody against Iba-1 (1:500; Wako), after preincubation with PBS containing 10% normal goat serum (NGS) for 30 min at RT, for immunostaining of microglia. Sections were incubated with goat biotinylated anti-rabbit IgG (1:200; Vector Labs) for 30 min at RT and incubated with avidin-biotin horseradish peroxidase complex (Vector Labs) in PBS for 60 min at RT. Sections were then stained in Tris-HCl containing DAB (0.2 mg/mL) and 0.01% hydrogen peroxide. In animals in which BD was applied from transected spinal nerves, sections were incubated overnight in Vector ABC solution in 0.1 M PBS at 4°C and washed for 1 h in PBS. Reaction products were detected using the glucose oxidase-nickel diaminobenzidine method (Shu et al., 1988). Then, sections were post-fixed with 1% OsO_4 for 1 h, dehydrated in ethanol, and flat-embedded on siliconized glass slides in Durcupan ACM resin (Fluka).

Ultrathin sections were cut, mounted on Formvar-coated single slot grids, and contrasted with uranyl acetate and lead citrate. Electron micrographs were taken at 80 kV on a JEM-1200EX electron microscope. For qualitative and quantitative analysis at the electron microscopic level, cell counting was carried out in the middle sections of the respective sets through the spinal L5 level (five parts of each layer/five sections from each animal/four animals for each time course). In each of the four animals, the numbers of immunolabeled microglia on the operated and control sides were counted. Also, the amount of adherence of microglia to the surface of each myelin membrane was counted.

Inhibition of P2Y12R In Vivo

Intrathecal drug administration for blocking spinal P2Y12 was performed as described in a previous report (Tozaki-Saitoh et al., 2008). Under isoflurane (2%) anesthesia, rats were implanted with a 32-gauge intrathecal catheter (ReCathCo) in the lumbar enlargement (close to the L4-L5 segments) of the spinal cord (Tsuda et al., 2003). After peripheral nerve injury, rats were administered PBS intrathecally (5 μL, as a vehicle control) or AR-C69931MX (50 nmol in 5 μL of PBS) twice a day (9 a.m. and 7 p.m.) from day 0 to day 7. Measurement of PWT was performed just before evening drug administration (at least 9 h after the first daily administration).

Data Analysis

Data on immunofluorescent light-microscopic sections from rats that had survived 7, 14 days after the operation (n = 6) were analyzed. Eight Iba1-immunostained sections from the L5 layer were selected randomly from each animal. In layers I, II, V, and X, the anterior column and lateral column, the numbers of Iba1-immunoreactive microglia on the operated and control sides were counted in a 6,000-μm^2 area in the L5 spinal cord of each animal selected. Data are expressed as means ± SEM. The statistical significances of differences between values were determined by paired t-tests. A P value of <0.05 was considered to be statistically significant.

RESULTS

On postoperative day 7, when the allodynia behavior was apparent, the number of microglia identified by the microglial marker Iba1 was dramatically increased in the ipsilateral side of spinal cord (see Fig. 1). However, the increase pattern varied among areas. To assess regional differences in the intensity of the response, microglial cell counts were performed in cytoarchitecturally distinct laminae of the dorsal horn. The most prominent increase in the number of microglia was observed in layers II–III (Figs. 1 and 2), whereas such increases were not dramatic in layer X and the white matters, except for layer IX, where most of the spinal motor neurons were surrounded by number of microglia. We therefore examined the fine structural changes of the increased microglia in layers II–III using an electron microscope in conjunction with the immunoelectron microscopy for Iba1 immunoreactivity.

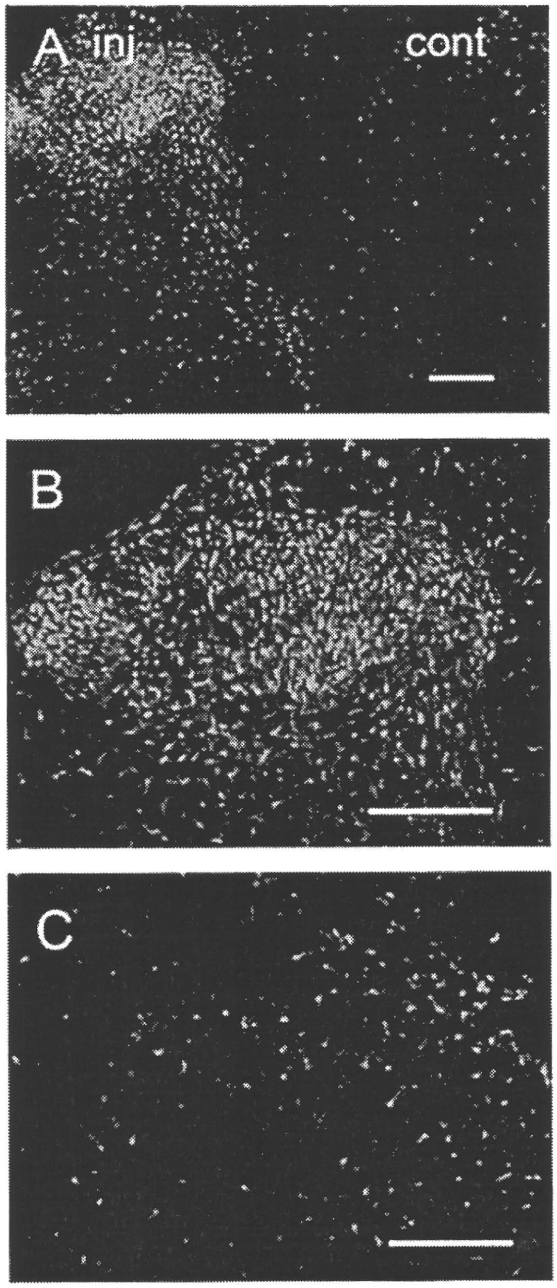

Fig. 1. The number of microglia is increased in the dorsal horn after nerve injury. Increase in the number of Iba1-positive microglia in the dorsal horn of the L5 spinal cord seven days after unilateral nerve injury (A). The left-hand side is the injured side (inj), and the right-hand side is the non-injured side (cont). Higher power magnification of the dorsal horn of injured side (B) and control side (C). Scale bar = 200 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Iba1-immunopositive microglia on the ipsilateral side were scattered in the neuropil with amoeboid features, rod-shaped components, and with long and thick

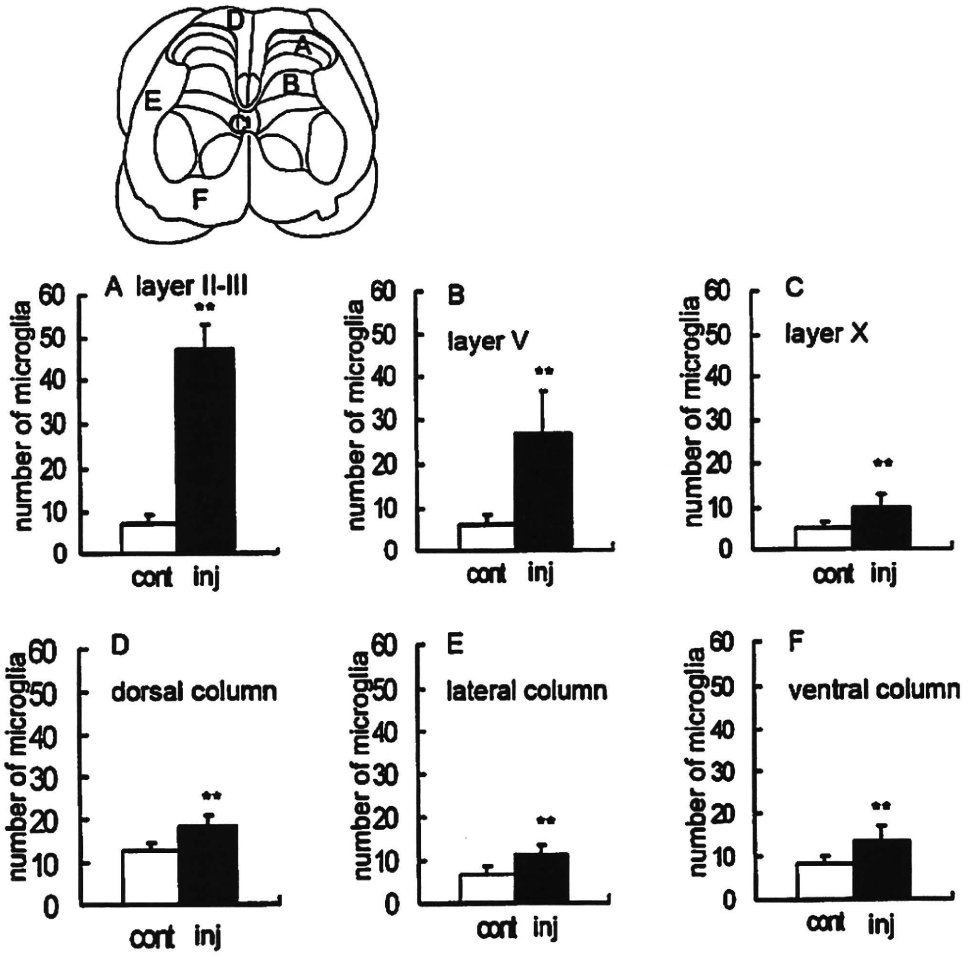

Fig. 2. Microglia is the most abundant in layers II–III of the dorsal horn. The increases in the numbers of microglia were counted in six regions of the L5 spinal cord seven days after nerve injury. The regions assessed were: (A) layer II-III, (B) layer V, (C) layer X, (D) dorsal column, (E) lateral column, and (F) ventral column. The numbers were compared between the injured (inj) and noninjured (cont) sides. On post-operative day 7, the mean numbers of cells per 6,000-μm^2 area of ipsilateral spinal cord were 47 ± 5.8 (A: layers II–III), 27 ± 9.6 (B: layer V), 10 ± 2.9 (C: layer X), 18 ± 2.7 (D: dorsal column), 11 ± 2.1 (E: lateral column), and 14 ± 3.3 (F: ventral column). The corresponding values on the contralateral sides were 7 ± 2.0 (A: layers II–III), 6 ± 2.4 (B: layer V), 5 ± 1.3 (C: layer X), 13 ± 1.3 (D: dorsal column), 7 ± 2.0 (E: lateral column), and 8 ± 1.7 (F: ventral column). The increase in every region is significant (**$P < 0.01$).

branched process, suggesting activation of microglia. Tight adhesion of microglia to the myelin sheaths of myelinated axons was frequently observed (see Fig. 3), although such adhesion was rarely observed in normal animals. As a criterion of adhesion, we counted the Iba1 immuno-positive microglia whose attachment length was more than 20% of encirclement of myelinated axon. Percent of adhesive and engulfing microglia were markedly higher in layers II–III on the operated side compared with other areas (mean ± SEM: cont 0%, layer I: 11.8 ± 2.2%, layer II–III: 36.3 ± 2.5%, layer V: 18.1 ± 3.5%) (Fig. 3D). Some myelinated axons were completely engulfed in the microglial cytoplasm (~2% of microglia per measured area) (see Fig. 4). Although many of these attached axons appeared normal, some axons engulfed by microglia showed degenerative changes, including decreased numbers of organelle, and an increase in the electron-density (see Fig. 4). The unmyelinated axons, which were engulfed by microglia, were rarely observed after nerve injury.

Next, we examined whether the axons engulfed or adhered to by microglia originated from the DRG neurons whose peripheral processes (L5) were injured. To evaluate this, we used a biotin-dextran (BD) tracer from the peripheral cut stamp. BD was taken up by the injured peripheral nerve tips and transported to the central processes (axons) of the DRG neurons, which mainly project to the dorsal horn. In addition, most motor neurons were labeled by this tracer (Fig. 5A). Under the electron microscope, BD immunoreactivity was observed as a highly electron-dense deposit in both nonmyelinated and myelinated fibers originating from the injured DRG neurons (see Fig. 5). Activated microglia attached to or engulfed both BD-positive axons (injured axon) and BD-negative axons (uninjured axon) (Fig. 5B,C). The ratio of BD-positive and BD-negative axon, which was attached by microglia, were approximately 63.2 ± 6.9% and 36.8 ± 6.0%, respectively (Fig. 5D), suggesting the activated microglia did not seem to have any certain preference for the myelinated axons, either injured or

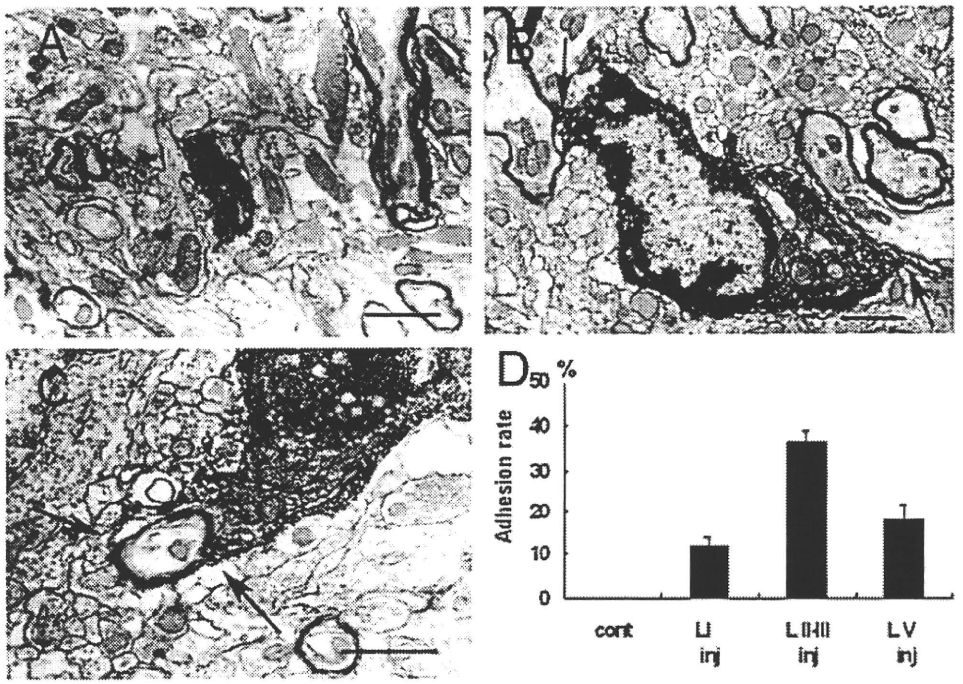

Fig. 3. Microglia adhere to myelinated axons in response to nerve injury. Immunoelectron microscopic observation showing that, in layer II of the L5 spinal cord on postoperative day 7, Iba1-immunopositive microglia do not adhere to myelinated axons on the noninjured side (A); however, microglial adhesions are frequently observed on the injured side (B–C; arrows). Scale bar = 1 μm. (D) Rate of microglia adhering to myelinated axons per unit area (6,000 μm^2). LI, layer I; L II–III, layers II–III; L V, layer V. The rate of microglia adhering to myelin is the highest in layers II–III on the injured side.

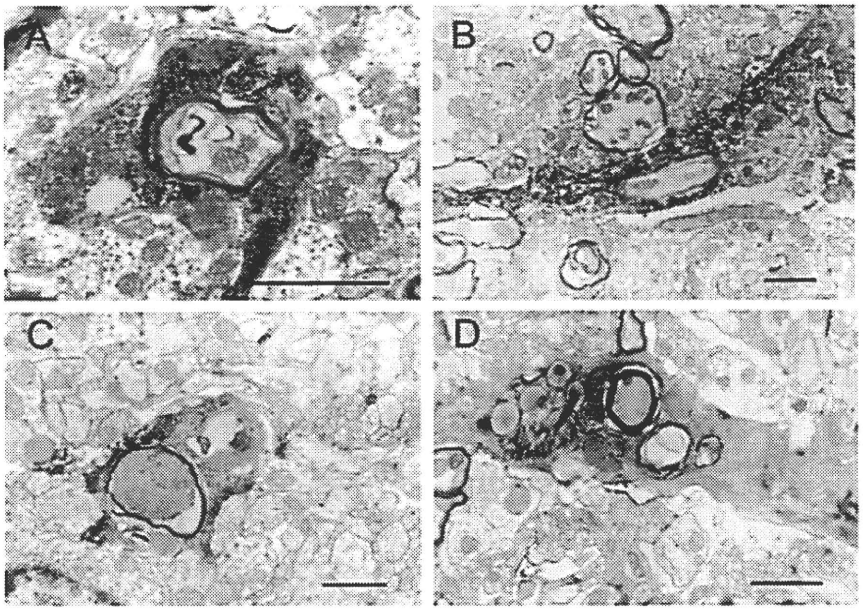

Fig. 4. Microglia engulf myelinated axons in response to nerve injury. Immunoelectron microscopic observation showing Iba1-immunopositive microglia engulfing a myelinated axon in layer II of the L5 spinal cord on postoperative day 7. (A–D) Some myelinated axons are completely engulfed by the Iba1-immunopositive microglia. Some axons demonstrate degenerative features (C and D). Scale bar = 1 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

uninjured. BD positive unmyelinated axons were also observed in the upper layers of the dorsal horn; however, the microglial adhesion or engulfment of those BD positive unmyelinated axons were rarely observed.

Next, to clarify relationship between microglial behavior and its activation by ATP, we focused on P2Y12R-mediated activation of microglia. We applied a P2Y12R inhibitor, AR-C69931MX, by intrathecal injection accord-

ing to the previous paper (Tozaki-Saitoh et al., 2008). In the peripheral nerve-injured group, after intrathecal injection of AR-C69931MX for seven days, we examined the suppression of allodynia behavior (see Fig. 6), and then performed morphological experiments. The P2Y12R inhibitor did not affect the proliferation of microglia (see Fig. 7). The numbers of Iba1-immunopositive microglia were increased markedly, as seen in the vehicle control group. In both groups, the morphology of microglia, including the round cytoplasm and thick processes, appeared the same (see Fig. 7). However, under immunoelectron microscopic observation, the number of microglia adhering to the myelinated axons was significantly decreased by 50% (see Fig. 8), although there was no difference in the total number of microglia in the dorsal horn. In addition, we could not find microglia engulfing myelinated axons in the inhibitor treated dorsal horn. This suggests that P2Y12R-mediated signal is highly associated with the characteristic behavior of the microglia after peripheral nerve injury.

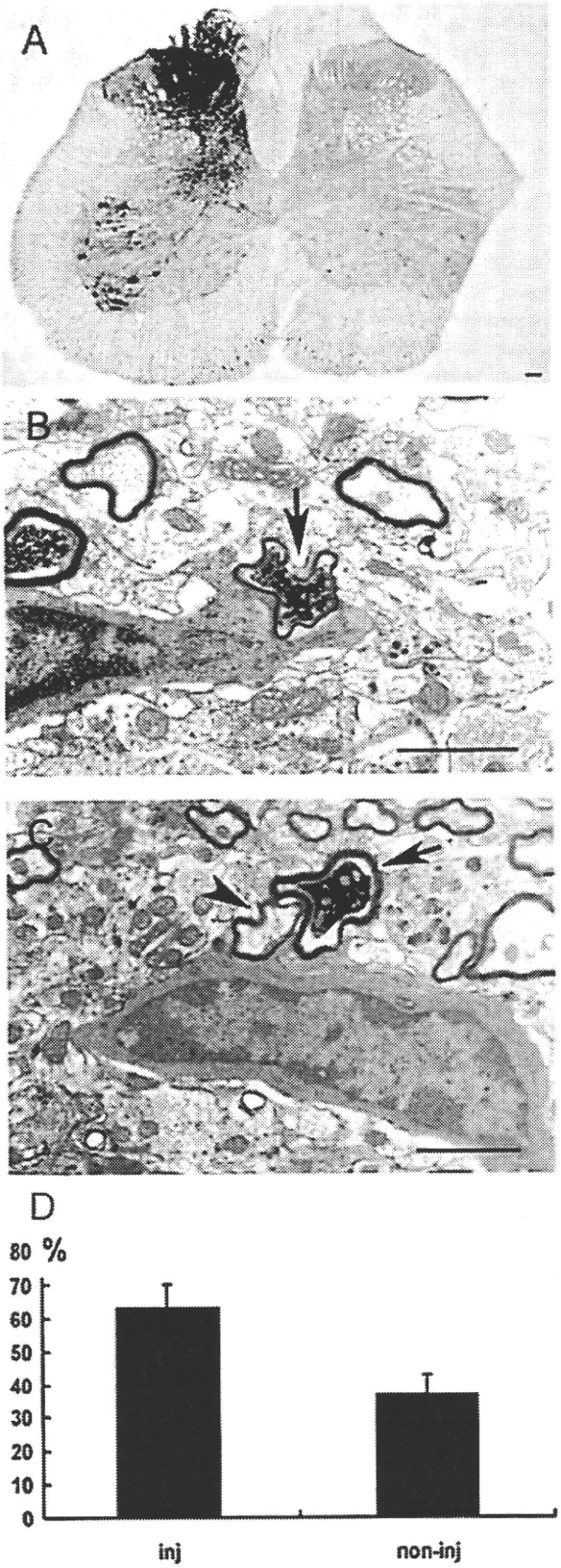

Fig. 5. Nerve injury-activated microglia adhered to both injured and non-injured myelinated axons. To identify axons originating from nerve-injured DRG neurons, BD was injected from injured nerve tips. (A) BD-immunopositive fibers located in the dorsal horn and BD-labeled motor neurons in the ventral horn are observed. Scale bar = 100 μm. (B and C) Immunoelectron microscopic observation showing BD-positive structures in layer II of the nerve-injured side. Activated microglia adhere to BD-positive myelinated axons (arrows in B and C) and BD-negative myelinated axons (arrowhead in C). (D) The ratio of BD-positive (injured axon) and BD-negative axon (uninjured axon), which was attached by microglia, were ~63.2 ± 6.9% and 36.8 ± 6.0%, respectively. Scale bar in A–C = 1 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

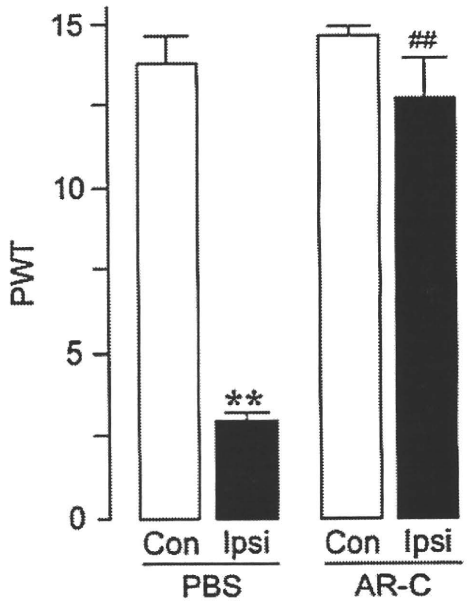

Fig. 6. Effects of the inhibition of P2Y12R on neuropathic pain behaviors. Inhibition of P2Y12R prevented the development of tactile allodynia. The PWT of the ipsilateral hindpaw in response to tactile stimulation in rats were examined seven days after nerve injury. Rats were subjected to intrathecal administration of AR-C69931MX (AR-C, 50 nmol, $n = 9$ each) or PBS ($n = 8$), twice a day from day 0 for 7 days, after peripheral nerve injury. Con, control side; Ipsi, injured side. Each column represents the mean ± SEM of PWT (**$P < 0.001$ vs. contralateral side of PBS-treated group; ##$P < 0.005$ vs. ipsilateral side of PBS-treated group by Mann-Whitney U-test.

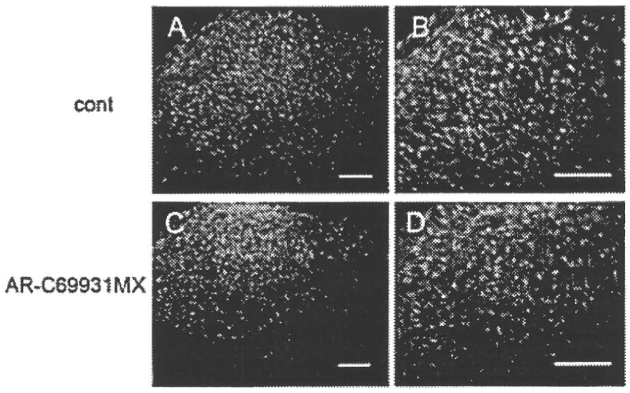

Fig. 7. P2Y12R blocker treatment failed to suppress the proliferation of microglia. Intrathecal administration of a P2Y12R blocker, AR-C69931MX, failed to suppress the increase in the number of Iba1-immunopositive microglia. With or without AR-C69931MX administration, Iba1-immunopositive microglia are abundantly observed in the dorsal horn following nerve injury. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

The increase of microglia and appearance of degenerating neural elements in CNS after peripheral nerve injury have been well known. Arvidsson et al. observed transganglionic degeneration in trigeminal primary sensory neurons suggesting some amount of central branches of primary sensory neurons were degenerated, and also indicated that microglia phagocytosed degenerating neural elements in sensory trigeminal nuclei following lesions of peripheral trigeminal nerve branches (Arvidsson, 1979, 1986). Cova et al. indicated increase of microglial cell numbers in the rat spinal cord dorsal horn laminae I, II following brachial plexus transection. (Cova et al., 1988). However, the interaction between the morphological changes and its consequences in neuropathic pain have not yet been elucidated. In this study, we showed the evidence that microglia engulfed myelinated axons in the dorsal horns of rats with tactile allodynia. We examined the fine structure of the reactive microglia in the dorsal horn of the spinal cord after peripheral nerve injury, and revealed that characteristic microglial behavior, such as an increase in the degree of adhesion to and engulfment of myelinated axons. Intriguingly, the interactions of reactive microglia with myelinated axons were not specific to axons originating from injured DRG neurons, and concomitantly, uninjured axons whose origins were unknown were targeted by reactive microglia in the dorsal horn after nerve injury. Furthermore we demonstrated that this characteristic microglial behavior was elicited in a P2Y12R activation-dependent manner.

In the dorsal horn, the activated microglia had rather thick and short processes (moderately activated form), but did not assume the fully active amoeboid shape seen in ischemic and traumatic brain injuries. This moderate activation of microglia in the dorsal horn together with the prominent increase in the numbers of microglia in layers II/III was assumed to give rise to the development of hyperalgesia and allodynia (Ledeboer et al., 2005; Raghavendra et al., 2003). Although the precise mechanism underlying the activation of microglia and neuropathic pain remains debated, the remarkable changes in microglial number and morphology suggest significant participation of microglia in the development

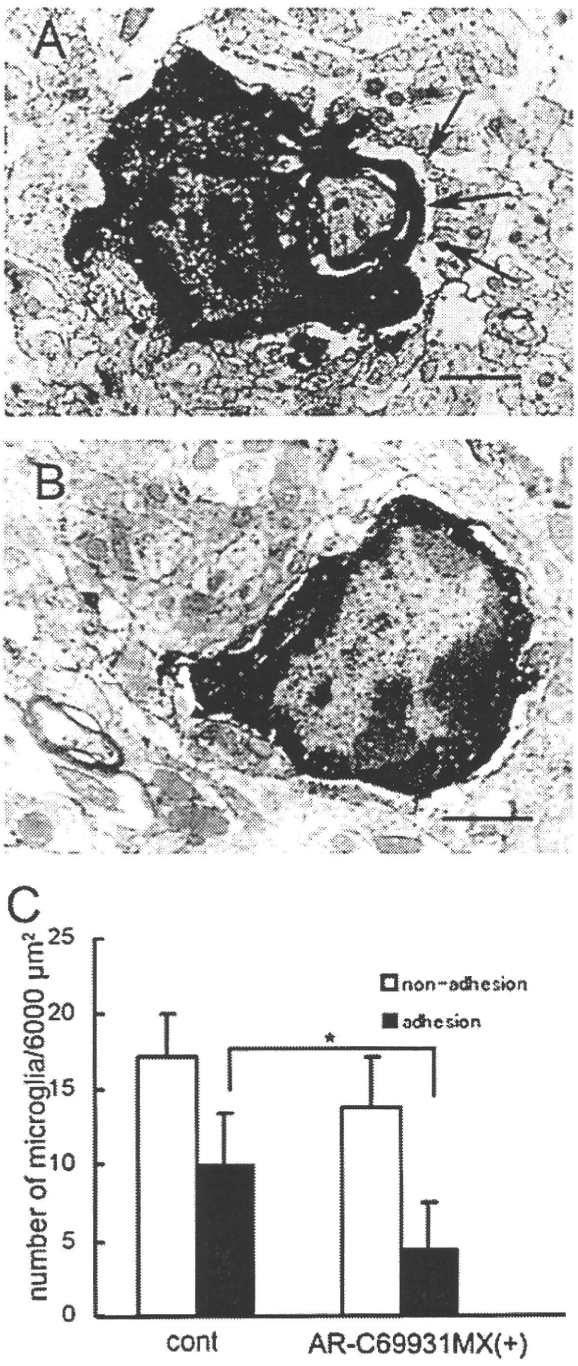

Fig. 8. P2Y12R blocker treatment suppresses the adhesion of microglia to myelinated axons. Electron microscopic observation showing Iba1-immunopositive microglia in layer II of the dorsal spinal cord of an animal administered vehicle (A) or AR-C69931MX (B). Scale bar = 1 μm. (C) Numbers of microglia that adhere or do not adhere to myelin in layer II of AR-C69931MX-treated (+) or a vehicle-treated (cont) animals $^*P < 0.05$.

of neuropathic pain. In this experiment, the most prominent observation was the engulfment of myelinated axons by microglia and the engulfment sometimes resulted in their demyelination and degeneration. These were especially observed in layers II–III of the dorsal horn 7–14 days after peripheral nerve injury, when the allodynia behavior occurred. Along with the development of tactile allodynia, many reactive microglia in the dorsal horn attached to myelinated axons and appeared to affect on the myelinated axons because of the occurrence of degenerating axons. Furthermore, some microglia engulfed a whole myelinated axon, and this engulfed axon sometimes showed degenerative features. Interestingly this microglial behavior, such as the adhesion and engulfment of the myelinated axon, occurred not only against axons originating from injured DRG neurons, but also against axons from uninjured neurons. Although the microglial adhesion to injured axon exceeded about twice as much as the adhesion to the noninjured, significant numbers of noninjured myelinated axons were adhered by microglia. This may suggest that the activated microglia in the dorsal horn react to myelinated axons nonselectively, and this spatially restricted but target unspecific behavior of microglia may lead to abnormal sensations such as allodynia. In this study the engulfed unmyelinated axon was rare under EM observation. The rate of engulfment of unmyelinated axon by microglia was less than 1%. Although we could not address the reason, this observation may suggest that the unmyelinated axons are almost unaffected, or shrinkage or retraction of unmyelinated axons occurs immediately after a contact by microglia.

The involvement of macrophages and microglia in demyelination after inflammatory stimuli has been well examined in a model of allergic encephalomyelitis (EAE). In the EAE model, after astrocytic edema formation, infiltration of polymorphonuclear cells, T lymphocytes, and macrophages are initially observed (Platten and Steinman, 2005). Infiltration of inflammatory cells, as well as activation of resident immune cells in response to nervous system damage, leads to subsequent production and secretion of various inflammatory mediators, such as cytokines and NO, and these might trigger the demyelination and activations of macrophages and resident microglia (Platten and Steinman, 2005). However, in this study, only Iba1-immunopositive microglia attached to and engulfing myelinated axons were found, while other macrophages or lymphocytes were not observed throughout the experimental period, at least for 14 days after the operation. This suggests that the microglial activation leading to the engulfment of myelinated axons is not mediated by infiltrating cells such as T-lymphocytes and macrophages. Rather, the primary afferent axons (and also dorsal horn astrocytes), which are capable of releasing ATP (Fields and Burnstock, 2006), are likely to be involved in the activation. This suggests that the consequence caused by the adhesion between activated microglia and myelinated axons observed in the present allodynia model would be distinct from those seen in ordinary EAE models. Although we could not prove the linkage between the microglial engulfment of myelinated axons and its consequence in axon function, activated microglia might release cytotoxins and chemokines that could affect axons as well as oligodendrocytes (Hanisch, 2002; Watkins and Maier, 2003). This may cause axonal disorder leading to allodynia. The present study also may suggest that the activated microglia gain a function nonspecifically affecting uninjured axons as well following nerve injury, without being activated by infiltrating cells such as T-lymphocytes and macrophages.

Among purinergic P2 receptors expressed in spinal microglia, P2Y12R is a critical regulator of microglial movement (Inoue, 2002; Sasaki et al., 2003). Expression of P2Y12R was also reported around the axotomized facial nerve nuclei in the rat, and in spinal microglia in a neuropathic pain model (Kobayashi et al., 2008; Sasaki et al., 2003; Tozaki-Saitoh et al., 2008). However, consistent with the previous report (Tozaki-Saitoh et al., 2008), the P2Y12R antagonist AR-C69931MX failed to suppress total number of activated microglia in the dorsal horn, despite a prevention of tactile allodynia in rats treated with P2Y12R antagonists such as AR-C69931MX and in mice lacking *p2ry12*(Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008). These findings suggested that the initial activation of microglia, such as proliferation and migration, and morphological changes, would be distinct from the mechanisms underlying allodynia (Haynes et al., 2006). In this study, the electron microscopic observations demonstrated that the P2Y12R blocker strikingly suppressed microglial adhesion to myelinated axons and engulfment of myelinated axons in the dorsal horn. Therefore, P2Y12R-mediated signaling in microglia might contribute to changing the fine microglial behavior against myelinated axons, and this might be one of the causes of tactile allodynia. The mechanism by which P2Y12R-mediated signaling changes fine microglial behavior is unknown. It has been shown that microglia in *p2ry12−/−* mice showed significantly diminished directional branch extension toward sites of cortical damage in the living mouse in an ATP-dependent manner (Haynes et al., 2006). Ohsawa et al. (2007) demonstrated that P2Y12R-mediated signaling evoked by ATP induces membrane ruffling structure in cultured microglia. From evidence indicating that primary sensory axons firing action potentials can cause a release of ATP (Fields and Burnstock, 2006), the P2Y12R-mediated movement of microglial processes and changes in membrane structure may be a crucial step for the fine morphological changes of microglia against myelinated axons observed under EM. A physical direct contact or extremely closer distance between axon and microglia, which might be elicited by P2Y12R-mediated signal, may be crucial for an effective interaction between to induce pathogenesis of abnormal pain. While there is no report showing that P2Y12R participates in engulfing behaviors of microglia, we have recently demonstrated that activating P2Y6Rs in activated microglia enhances phagocytotic activity (Koizumi et al., 2007). Furthermore, in our preliminary data, P2Y6R mRNA expression

is markedly upregulated in the dorsal horn after nerve injury (data not shown). Thus, it is possible that engulfment of myelinated axons by microglia whose fine processes are closed to axons in a P2Y12R-dependent manner may involve P2Y6R or other microglial molecules. This possibility need to be further clarified in future studies.

Conclusively, the local behavior of microglia processes, which we were able to identify at the EM level, could be a crucial focus for the understanding of the mechanisms underlying allodynia after peripheral nerve injury.

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