A Critical Role of Toll-like Receptor 2 in Nerve Injury-induced Spinal Cord Glial Cell Activation and Pain Hypersensitivity

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The activation of spinal cord glial cells has been implicated in the development of neuropathic pain upon peripheral nerve injury. The molecular mechanisms underlying glial cell activation, however, have not been clearly elucidated. In this study, we found that damaged sensory neurons induce the expression of tumor necrosis factor-α, interleukin-1β, interleukin-6, and inducible nitric-oxide synthase genes in spinal cord glial cells, which is implicated in the development of neuropathic pain. Studies using primary glial cells isolated from toll-like receptor 2 knock-out mice indicate that damaged sensory neurons activate glial cells via toll-like receptor 2. In addition, behavioral studies using toll-like receptor 2 knock-out mice demonstrate that the expression of toll-like receptor 2 is required for the induction of mechanical allodynia and thermal hyperalgesia due to spinal nerve axotomy. The nerve injury-induced spinal cord microglia and astrocyte activation is reduced in the toll-like receptor 2 knock-out mice. Similarly, the nerve injury-induced pro-inflammatory gene expression in the spinal cord is also reduced in the toll-like receptor 2 knock-out mice. These data demonstrate that toll-like receptor 2 contributes to the nerve injury-induced spinal cord glial cell activation and subsequent pain hypersensitivity.

Traditionally, pain has been thought to be mediated solely by neurons. Recent discoveries on the role of spinal cord glial cells, however, provide support for a different model of pathological pain hypersensitivity. Increasing evidence indicates that glial cell activation in the spinal cord plays a critical role in the initiation and/or maintenance of pathological pain with various etiologies (1). In a neuropathic pain model with fifth lumbar (L5) spinal nerve ligation, microglia and astrocyte activation is manifested in the dorsal horn ipsilateral to the injury (2, 3). Inhibition of glial cell activation using chemical inhibitors such as minocycline suggested that microglia activation is required for the induction, but not for the maintenance, of nerve injury-mediated neuropathic pain (4). Upon activation, glial cells express various inflammatory mediators such as TNF-α, IL-1β, IL-6, iNOS, and COX-2 (5–9). It is suggested that the expression of these mediators in the spinal cord contributes to the development of pain hypersensitivity, although the mechanisms have not been clearly elucidated yet (10–13).

Thus far, several candidate molecules have been suggested to activate spinal cord glial cells upon peripheral nerve injury. For instance, fractalkine, a member of the chemokine family, was suggested to transmit neuron-derived signal to microglia. In addition, it was reported that the expression of P2X4, an ATP receptor, in microglia is required for the development of neuropathic pain (14), suggesting that ATP is a microglial activator. However, P2X4 is rarely expressed on resting microglia but induced only after nerve injury or inflammatory stimulation (14, 15), suggesting an additional spinal cord glial cell activator that induces P2X4 expression on microglia. Given these previous reports, it is still inconclusive how spinal cord glial cells are activated upon peripheral nerve injury. In this regard, it is of interest that damaged sensory neurons or peripheral nerves can induce inflammatory Schwann cell activation through toll-like receptors (TLRs) (16, 17). These reports suggest that spinal cord glial cells may also be activated by damaged sensory neuron-derived molecules.

TLRs are type I transmembrane-signaling proteins that are expressed in the cells of the innate immune system (18). Each TLR recognizes pathogen-associated molecular patterns, which are structures expressed by microorganisms. Several pathogen-associated molecular patterns have been identified, including lipopolysaccharides from Gram-negative bacteria,
Toll-like Receptor 2 Contributes to Neuropathic Pain

peptidoglycan, and lipoteichoic acid from Gram-positive bacteria, bacterial flagellin, double-stranded viral RNA, and bacterial DNA. Thus far, more than 10 different TLRs with distinct ligand specificities have been identified (19–22). In the central nervous system, microglia and astrocytes were shown to express various TLRs, including TLR2 and TLR4, which suggests their role as innate immune cells in the central nervous system (23). Interestingly, the expression of TLRs in glial cells is implicated in the development of neurological disorders. In the rat neuropathic pain model, TLR4 transcripts are up-regulated in the spinal cord upon spinal nerve injury (24). It has also been reported that TLR4 is required for the maximum induction of pain hypersensitivity upon spinal nerve transection (25). However, it is not known how TLR4 contributes to the degree of pain hypersensitivity. Neither, the involvement of other members of TLR in the development of neuropathic pain has been investigated.

EXPERIMENTAL PROCEDURES

All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Seoul National University. The animal treatments were performed according to the guidelines of the International Association for the Study of Pain.

Animals and Surgery—TLR2 knock-out mice (26) of C57BL/6 background and C57BL/6 mice were housed in an animal facility with a specific pathogen-free barrier at a temperature of 23 ± 2 °C. They experienced a 12-h light-dark cycle and were fed food and water ad libitum. All mice were aged 8 weeks at the time of surgery, and efforts were made to limit distress to the animals. Mice were anesthetized with halothane (2–4%) in O2 during surgery via a nose cone. The L5 spinal nerves were transected as previously described (25, 27) with the following minor modification. Briefly, following surgical preparation, the left paraspinal muscles were separated by an incision from spinal processes at L4 to S2 levels. The L6 transverse process was partially removed, and the L4 and L5 spinal nerves were identified. Then, the L5 spinal nerve was separated and transected. The wound was irrigated with saline and closed in two layers with surgical skin staples.

Behavioral Testing—Mechanical allodynia was assessed by measuring foot withdrawal thresholds in response to mechanical stimuli to the hind paw. The 50% withdrawal threshold was determined using the up-down method (28) with a set of von Frey filaments (0.02–6 gauge, Stoelting, Wood Dale, IL). Heat hyperalgesia was determined by using the plantar test (Ugo Basile, Italy) following a modified method of Hargreaves et al. (29). Two different intensities for heat hyperalgesia are employed: low intensity (IR 30) and high intensity (IR 60). Animals were habituated to the apparatus that consisted of three individual Perspex boxes on an elevated glass table. A mobile radiant heat source was located under the table and focused onto the hind paw. Paw withdrawal latencies were recorded in seconds. The heat intensity was adjusted to obtain average paw withdrawal latencies of 7–8 s and 4–5 s for the application of low intensity (IR 30) and high intensity (IR 60), respectively, and the cut-off time was set at 15 s to prevent tissue damage. The heat stimulation was repeated three times at an interval of 5–10 min for each paw, and the mean was calculated. The behavioral tests were performed blinded.

Cell Culture—Primary rat spinal cord glial cells were prepared from 7-day-old Sprague-Dawley rats as previously described (30) with the following minor modification. Briefly, after anesthetized, spines were cut out from 0.5 cm above the tail, a syringe with phosphate-buffered saline was inserted, and the spinal cord was pushed out by hydraulic pressure. After removal of the meninges, the spinal cord was triturated by repetitive pipetting and seeded in 75-cm² flasks. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 1 mM HEPES, 10% fetal bovine serum, 2 mM glutamine, and 1× antibiotic/antimycotic. After 2 weeks, the cells were seeded in 6-well plates and used for experiments. The cell composition of the spinal cord mixed glial cell culture was routinely monitored by immunostaining using anti-GFAP (DAKO, Denmark) and anti-CD11b (Serotec, Oxford, UK) antibodies. With a similar procedure, primary spinal cord glial cells were prepared from C57BL/6 and TLR2 knock-out mice. DRG explant cultures were prepared as previously described (31). Briefly, DRGs were dissected from embryonic day 15 Sprague-Dawley rats. After being stripped of nerve roots and connective tissues, five DRGs were plated on a glass slide in a 35-mm tissue culture dish containing 200 µl of culture medium (Neurobasal medium (Invitrogen, Carlsbad, CA) with 100 ng/ml nerve growth factor) and incubated at 37 °C in an atmosphere containing 5% CO2. The glass slides were coated with poly-l-lysine (Sigma) and laminin (Invitrogen). To eliminate non-neuronal cell growth, 10 µM 5-fluoro-2′-deoxyuridine (Sigma) and 1× antibiotic/antimycotic. After 3 days, the cultures were used. Cells were used from 7 to 10 days after plating. F11 cells (a hybrid cell line of mouse N18TG2 neuroblastoma and rat DRG sensory neuron) were maintained as described elsewhere (32).

Preparation of Supernatant of Damaged Sensory Neurons (SDSN)—To obtain SDSN, F11 were washed with phosphate-buffered saline and resuspended in a glial cell culture media at 10⁶ cells/ml. F11 cell damage was induced by four cycles of freezing on liquid nitrogen followed by thawing in water bath at 37 °C. Such treatment generally resulted in 95% lysis of the cells as judged by trypan blue inclusion. The cells were spun down, and the supernatant was used for the following studies. Prior to its use in the experiments, the SDSN was tested for endotoxin contamination using the kinetic colorimetric limulus amebocyte lysate assay (QCL-1000, Cambrex Bioscience, Walkersville, MD).

Real-time RT-PCR—Real-time RT-PCR was performed using the Sybr Green reagents kit and an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). The sequences of the primers used in this study are presented in Table 1. The level of each gene was normalized to the levels of the rat GAPDH or mouse GAPDH gene and represented as a -fold induction. The -fold induction was calculated using the 2−ΔΔCt method as previously described (33). All real-time RT-PCR experiments were performed at least three times,
and the mean ± S.E. values have been unless otherwise noted.

**Western Blot Analysis**—Primary rat spinal cord glial cells stimulated with SDSN for various periods of time were harvested with lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, and protease inhibitor mixture). A total of 50 μg of cell lysate from each sample was resolved by electrophoresis on a 10% SDS-PAGE. The proteins were then transferred to nitrocellulose membranes and blocked with 5% nonfat dry milk in Tween 20-containing Tris-buffered saline (TBST, 20 mM Tris, pH 7.4) on days 0, 4, and 7 after surgery. The L5 segment was measured using a Bio-Rad (Hercules, CA) protein assay kit (manufacturer’s instructions). Briefly, equal volumes of the culture supernatants and the Griess reagent were mixed, and the absorbance was measured at 540 nm after 15 min. The nitrite concentration in each sample was calculated from a standard curve and normalized to the cell number in each sample.

**Immunohistochemistry**—TLR2 knock-out mice and C57BL/6 wild-type mice were anesthetized and perfused transcardially with fresh 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) on days 0, 4, and 7 after surgery. The L5 segment of the spinal cord was removed by laminectomy and post-fixed at 4 °C overnight and then transferred to 30% sucrose in phosphate-buffered saline for 48 h. Transverse sections (20-μm thick) of L5 spinal cord were prepared using a cryocut microtome (Leica CM3050S). The floating sections were incubated with 3% H2O2 for 30 min and blocked in solution containing 5% normal goat/horse serum, 5% bovine serum albumin, and 0.1% Triton X-100 for 2 h at room temperature. Then, the sections were incubated overnight at 4 °C with monoclonal antibody for CD11b (1:500), GFAP (1:10,000), or phospho-p38 (1:500). After washing, the sections were then incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 1 h at room temperature, followed by incubation with avidin and biotinylated horseradish peroxidase complex (Vector Laboratories) at 1:200 for 1 h at room temperature, and then visualized with 3,3′-diaminobenzidine (Sigma). For double immunofluorescent staining, floating sections were incubated overnight at 4 °C with a mixture of mouse anti-TNF-α (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-phospho-p38 antibodies, or mouse anti-TNF-α and rabbit anti-ibA-1 antibodies (1:2000, Wako, Japan). The sections were then incubated for 1 h at room temperature with a mixture of fluorescein isothiocyanate-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG antibodies (1:200, Jackson ImmunoResearch). The sections were mounted with Vectashield (Vector Laboratories). Fluorescent images were obtained with a confocal microscope (LSM 5 Pascal, Carl Zeiss).

**Statistical Analysis**—All data are presented as means ± S.E. The statistical significance of differences between the values was determined by Student t test or analysis of variance with post hoc test. A p value of <0.05 was considered to be statistically significant.

**RESULTS**

**Damaged Sensory Neurons Induce Spinal Cord Glial Cell Activation**—To investigate the mechanisms of spinal cord glial cell activation due to peripheral nerve injury, we prepared the primary mixed glial cells from rat spinal cord and stimulated them with a supernatant of a damage-sensory neuronal cell line (F11). The addition of the SDN to the spinal cord mixed glial cell culture strongly induced the transcripts of the various pain-mediating inflammatory genes. It up-regulated the transcripts of TNF-α, IL-1β, IL-6, and iNOS genes by 160-, 280-, 940-, and 2500-fold, respectively (Fig. 1A). In addition, it induced COX-2
mRNA, another important gene involved in pain hypersensitivity (34), expression by 80-fold (data not shown). The supernatant of undamaged F11 culture, however, did not significantly induce expression of the above mentioned pain-mediating genes, indicating that the majority of the putative glial cell-activating molecules are released only after neuron damage has occurred. Similarly, the SDSN stimulation induced the secretion of TNF-α and IL-1β, IL-6, and the production of NO in the spinal cord glial cells (Fig. 1B). Interestingly, SDSN up-regulated the intracellular IL-1β level 30-fold (Fig. 1B), although it did not induce the secretion of the protein (data not shown). The SDSN activated the glial cells in a dose-dependent manner. Maximum induction of TNF-α gene expression was observed when SDSN was added into glial cells at a 1 \times 10^5 cells/ml concentration (Fig. 1C). Studies of the intracellular signaling pathways showed that SDSN stimulation induced the activation of p38, ERK, and JNK MAPKs. The activation of ERK and JNK MAPKs peaked after 45 min of stimulation with SDSN, whereas p38 activation peaked at 30 min. Similarly, NF-κB, which was measured by IκB degradation, was activated after 15 min of SDSN stimulation (Fig. 2A). Our study using pharmacological inhibitors revealed that the activation of NF-κB, ERK, and JNK is required for SDSN-mediated TNF-α, IL-1β, IL-6, and iNOS gene expression, whereas JNK activation is required for IL-6 gene expression (Fig. 2B). In an independent experiment, we stimulated the spinal cord glial cells with degenerating neurites of DRG. The data show that, in the absence of a neuronal cell
body, the DRG neurites alone are able to induce the expression of the aforementioned cytokines, iNOS and COX-2, in the spinal cord glial cells (Fig. 3). Taken together, these data demonstrate that damaged sensory neurons release strong glial cell-activating factor(s), which might be involved in the activation of spinal cord glial cells upon peripheral nerve damage.

**TLR2 Is Required for the SDSN-induced Pro-inflammatory Cytokine and iNOS Gene Expression**—Recently, it was reported that nectrocyt cells activate innate immune cells via a specific transmembrane receptor called TLR2 (18). To test whether TLR2 is involved in the SDSN-mediated spinal cord glial cell activation, we prepared spinal cord glial cells from TLR2 knock-out mice as well as wild-type control mice. Upon stimulation with SDSN, the mRNA expression of TNF-α, IL-1β, IL-6, and iNOS genes was up-regulated by 180-, 1010-, 80-, and 150-fold, respectively. The gene induction by SDSN, however, was almost completely abrogated in the cells from the TLR2 knock-out mice (Fig. 4). These data demonstrate that TLR2 is required for the expression of pro-inflammatory cytokines and iNOS in the spinal cord glial cells.

**TLR2 Contributes to the Induction of Pain Hypersensitivity after L5 Spinal Nerve Transection**—We then tested the susceptibility of the TLR2 knock-out mice to nerve injury-induced pain hypersensitivity. Upon L5 spinal nerve axotomy, wild-type mice showed increased sensitivity to both mechanical and thermal stimuli (Fig. 5). The paw withdrawal threshold to the mechanical stimuli decreased 1 day after nerve axotomy, and, after 7 days, was reduced to 0.05 ± 0.01 g, compared with the original threshold before transection (0.84 ± 0.08 g). The threshold remained below 0.1 g on day 14. The paw withdrawal threshold of the TLR2 knock-out mice, however, reduced only to 0.50 ± 0.12 g (Fig. 5A), compared with the original threshold (1.01 ± 0.14 g). Likewise, the threshold to the thermal stimuli in the wild-type mice decreased by >55% after 7 days, whereas, in TLR2 knock-out mice, it decreased <40% (Fig. 5B). Pain hypersensitivity was not observed in the sham-operated wild-type or TLR2 mice. These data indicate that TLR2 is required for the maximum induction of mechanical allodynia and thermal hyperalgesia upon L5 spinal nerve injury.

**TLR2 Is Required for the Nerve Injury-induced Spinal Cord Glial Cell Activation**—It is well known that peripheral nerve injury induces glial cell activation in the spinal cord (35–37). The activation of microglia and astrocytes in the nerve-damaged mice was tested by immunostaining with anti-CD11b and anti-GFAP antibodies, respectively. Upon L5 spinal nerve injury, strong activation of microglia and astrocytes is observed in the ipsilateral dorsal horn on day 4 and day 7, respectively (Fig. 6A, panels b and e). However, the activation was significantly decreased in the TLR2 knock-out mice (panels c and f). The activation level of spinal cord microglia and astrocytes upon spinal nerve injury was also quantified by measuring the transcript levels of CD11b and GFAP, respectively (Fig. 6, B and C). Four days after spinal nerve injury, the CD11b transcript level was increased >9-fold in the ipsilateral dorsal horn of control mice, whereas it was up-regulated by only 4-fold in the TLR2 knock-out mice. Similarly, the spinal nerve injury-induced GFAP expression in the dorsal horn was up-regulated by 3-fold. However, in TLR2 knock-out mice, it was up-regulated only by 1.6-fold (Fig. 6C). These data demonstrate that TLR2 is required for spinal nerve injury-induced glial cell activation.

**TLR2 Is Required for the Nerve Injury-induced Pro-inflammatory Cytokine and COX-2 Gene Expression**—The induction of pain-mediating gene expression in the spinal cord was also measured by real-time RT-PCR upon L5 spinal nerve axotomy. The transcript of TNF-α, IL-1β, IL-6, and COX-2 was up-regulated in the dorsal horn of nerve-damaged mice by 5-, 10-, 6-,
and 5-fold, respectively (Fig. 7). As expected, the induction levels of these genes were reduced in the TLR2 knock-out mice by 40–70% depending on the target gene. We did not detect induction of any of the above-mentioned pain-mediating genes in the sham-operated wild-type mice, indicating that the gene expression in the spinal cord dorsal horn was due directly to the nerve injury. Taken together, these data show that TLR2 is required for the L5 spinal nerve axotomy-induced pain-mediating inflammatory gene expression in the spinal cord in vivo.

The expression of TNF-α was further confirmed by immunohistochemistry (Fig. 8A). TNF-α expression was induced in the ipsilateral dorsal horn of wild-type mice on 3 days after spinal nerve transection (Fig. 8A, panel e). However, the expression was significantly decreased in the TLR2 knock-out mice (Fig. 8A, panel f). By double-immunostaining with a microglia-specific marker, most of the TNF-α-immunoreactive (IR) cells were identified as microglia (Fig. 8A, panel h). Similarly, iNOS expression was detected after nerve injury in the dorsal horn microglia of wild-type mice but not of TLR2 knock-out mice (supplemental Fig. S1). These data demonstrate that spinal nerve injury induces TNF-α and iNOS expression in spinal cord microglial cells via TLR2. In addition, it was reported that p38 MAPK is activated in spinal cord microglia upon nerve injury, which is critical for the induction of pain hypersensitivity (38). We also detected phospho-p38 in the spinal cord of nerve-injured wild-type mice, whereas the level of p38 phosphorylation was reduced in the TLR2 knock-out mice (Fig. 8B, panels b and
Toll-like Receptor 2 Contributes to Neuropathic Pain

FIGURE 7. TLR2 is required for the expression of pro-inflammatory cytokines and COX-2 genes upon L5 spinal nerve transection. Total mRNA was isolated from L5 lumbar spinal cord tissue of uninjured mice (n = 4), sham-operated wild-type (Sham, n = 4) mice, and injured-wild-type (WT-injured, n = 4) and TLR2 knock-out (TLR2 KO-injured, n = 4) mice at 4, 7, and 14 days after surgery. The mRNA expression of TNF-α (A), IL-1β (B), IL-6 (C), and COX-2 (D) genes was measured by real-time RT-PCR. The mRNA of each gene was normalized to the mouse GAPDH gene and represented as a fold induction compared with the values of uninjured mice (Pre) (*, p < 0.05; **, p < 0.01).

c). The expression of phospho-p38 localized in the nucleus of the TNF-α-IR microglial cells (panels e and h). Taken together, these data demonstrate that TLR2 plays an important role in the activation of nerve injury-induced p38 and the expression of TNF-α and iNOS in spinal cord microglial cells.

DISCUSSION

In this study, we found that damaged sensory neurons induced pain-mediating pro-inflammatory gene expression in spinal cord glial cells. From previous studies, several molecules have been implicated in the activation of spinal cord glial cells upon peripheral nerve injury, including ATP, fractalkine, glutamate, substance P, and LPA (39–43). In our study, however, none of these molecules, either by themselves or in combination, induced pro-inflammatory cytokines in the mixed spinal cord glial cells (data not shown). Thus, it is unlikely that the glial cell-activating factors in the SDSN were the above molecule(s). Indeed, our data using TLR2-deficient glial cells showed that SDSN activated spinal cord glial cells via TLR2. TLRs are conventionally thought to bind to pathogen-specific structural motifs and thereby induce inflammatory responses in the innate immune cells upon bacterial/viral infection (44). However, it was later suggested that TLRs also function as receptors for endogenous molecules. For example, TLR2 and TLR4 have been shown to recognize various endogenous molecules, including heat shock proteins (hsp), hyaluronan, and high mobility group box 1 to induce pro-inflammatory gene expression (45–49). Similarly, TLR3 binds to not only virus-derived double-stranded RNA but also mRNA molecules that are released from necrotic cells (50). It seems that different members of the TLR family recognize endogenous molecules that are exposed during tissue damage and thereby induce inflammatory responses and subsequent tissue healing. Thus far, the identity of the SDSN-derived molecule(s) that bind to TLR2 has not been elucidated. Although hsp was suggested as a ligand for TLR2 (46, 47), the depletion of rat hsp70 and hsp60 using anti-hsp60 and anti-hsp70 antibodies, respectively, did not alter the glia-activating effects of SDSN (supplemental Fig. S2). In addition, protein denaturation by boiling or protein degradation by treatment with proteinase K did not inhibit the SDSN-mediated glial cell activation (data not shown). This implies that proteins are not likely to be agonists of TLR2. Thus far, we did not identify the molecules that activate spinal cord glial cells, which need to be studied in the future.

The involvement of TLR2 in glial cell activation by SDSN suggests the possibility that TLR2 may also be involved in the spinal cord glial cell activation due to peripheral nerve injury. Thus far, the involvement of neuronal cell death in neuropathic pain is controversial. Although it has been reported that DRG neuronal cells undergo cell death upon peripheral nerve damage (51–53), others failed to detect neuronal cell death (54). In another study, it was suggested that inhibitory interneurons in the spinal dorsal horn die upon peripheral nerve injury (55, 56). Although, the induction of neuronal cell death upon peripheral nerve injury is inconclusive, it can be reasoned that glial cells will become activated due to death of interneurons or DRG neurons, once it occurs. Of note, in our study, the addition of damaged neurites alone induced pro-inflammatory gene expression in glial cells. This implies the possibility that spinal cord glial cells are activated by injured axons even in the absence of neuronal cell death. Therefore, it is also conceivable that peripheral nerve injury may induce degeneration of central axons (57, 58) and thereby induces activation of spinal cord glial cells via TLR2. During revision of the manuscript for this report it was reported that TLR2 is required for the hippocampal microglial activation due to axotomy of the entorhinal cortex (59). This report is in accordance with our data and supports the contention that degenerating axons may induce microglial activation via TLR2. Our in vivo studies using TLR2 knock-out mice clearly demonstrated that TLR2 contributes to the nerve
injury-induced spinal cord glial cell activation and pain hypersensitivity. Although, we cannot exclude the possibility, at this point, that the glial cells are activated indirectly by other TLR2-expressing cells, our in vivo data along with the in vitro data suggest it a putative mechanism of nerve injury-induced glial cell activation that TLR2 activation on spinal cord glial cells induce glial cell activation and subsequent pain hypersensitivity.

Of note, the behavioral pain hypersensitivity and the pro-inflammatory gene expression due to spinal nerve axotomy was partially inhibited but not completely reversed in the TLR2 knock-out mice. This suggests that there is an additional mechanism for the spinal cord glial cell activation upon spinal nerve injury that is independent of TLR2. In this regard, it is important to note that it has recently been reported that pain hypersensitivity is inhibited in TLR4 mutant mice (25). Considering that endogenous ligands such as hsp and mRNA can, respectively, activate TLR4 and TLR3 (45, 50), it is possible that some of the glia-activating effects of SDSN may be mediated by TLR4 or TLR3. Such a mechanism would explain the decreased glial cell activation in the TLR4 mutant mice upon spinal nerve injury. In this regard, it will be interesting to test if the pain hypersensitivity due to peripheral nerve injury is completely inhibited in the TLR2/4 or TLR2/3 double knock-out mice.

In our study, we determined glial cell activation by measuring the induction of pro-inflammatory genes (TNF-α, IL-1β, IL-6, iNOS, and COX-2). Among them, TNF-α was expressed in microglia of the dorsal horn and colocalized with the activated form of p38. These data, in concert with our in vitro data using the pharmacological inhibitor of p38, argue that spinal nerve injury activates p38 in the spinal cord microglia to induce expression of TNF-α. The significance of these pro-inflammatory gene expression patterns in the spinal cord in the development of neuropathic pain has been noted in several previous reports (8, 10–13). However, it is not clear how the expression of these genes leads to the induction of pain hypersensitivity. This will need to be elucidated in the future studies.

In conclusion, our study shows that TLR2 contribute to the development of spinal nerve injury-induced spinal cord glial cell activation and subsequent pain hypersensitivity. Our data...
suggest that TLR2 can be a target gene for the development of drugs or therapeutic treatment for neuropathic pain.

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