 

# *Spinal Microglial Expression and Mechanical Hypersensitivity in a Postoperative Pain Model: Comparison with a Neuropathic Pain Model*

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***Background:*** **Postoperative pain control contributes to quality of life. Activation of spinal cord microglia after peripheral nerve injury contributes to mechanical hypersensitivity. The contribution of spinal cord microglia to hypersensitivity after surgery, however, is not well understood. Here, the authors evaluated whether inhibition of spinal microglia reduced postoperative mechanical hypersensitivity, and if so, whether the effect differed from that in a rat neuropathic pain model.**

***Methods:*** **Male Sprague-Dawley rats underwent either unilateral plantar hind paw incision (postoperative pain model) or L5 spinal nerve transection (neuropathic pain model), and the development of mechanical hypersensitivity was assessed using von Frey filaments. The microglial inhibitor minocycline was intraperitoneally administered daily for either 3 or 7 days. Spinal microglial activation was evaluated by OX42 immunohistochemistry. We also tested the effect of intrathecal administration of a p38 mitogen-activated protein kinase inhibitor, SB203580.**

***Results:*** **In the postoperative pain model, minocycline did not suppress mechanical hypersensitivity, but did inhibit an increase in spinal OX42 expression. In contrast, in the neuropathic pain model, minocycline reduced mechanical hypersensitivity in a dose-related manner and inhibited spinal OX42 expression. SB203580 attenuated hypersensitivity in the neuropathic pain model, but not in the postoperative pain model.**

***Conclusions:*** **The results of the present study suggest that spinal OX42 expression has a more important role in the development of neuropathic pain than in postoperative pain, and that an increase in spinal OX42 expression does not contribute to postoperative mechanical hypersensitivity.**

MICROGLIA are immune cells in the central nervous system. They are active and versatile cells, and adapt to different stimuli through a sequence of reactive profiles.[1] Spinal microglia contribute to the development and maintenance of mechanical hypersensitivity after a variety of peripheral nerve injuries, including sciatic inflammatory neuropathy,[2] chronic constriction nerve injury,[3] partial sciatic nerve ligation,[4] spinal nerve ligation,[5] spinal nerve transection,[6,7] and peripheral inflammation.[8,9]

Postoperative pain represents an unmet medical need. The behavioral characteristics of a postoperative pain model in rats produced by hind paw incision resemble postoperative hypersensitivity in humans.[10] Several lines of evidence suggest that the nature of hypersensitivity in this pain model is different from that of other sustained pain models, such as the neuropathic pain model.[11–14] Although markers for both microglia and astrocytes are upregulated in the spinal cord in a rat model of postoperative pain,[15,16] the role of spinal cord microglia in postoperative hypersensitivity is not well understood. Thus, the purpose of the present study was to determine whether microglia are important for the development and maintenance of postoperative hypersensitivity, similar to neuropathic hypersensitivity.

Minocycline inhibits microglial activation and also reduces nociception, including that resulting from tissue injury and inflammation-evoked pain,[17] spinal cord contusion injury,[18] and spinal nerve ligation.[19] In the present study, the role of spinal microglia in the rat model of postoperative pain induced by paw incision was evaluated using minocycline. The role of p38 mitogen-activated protein kinase (p38 MAPK) was also evaluated in the postoperative pain model. Pain hypersensitivity after nerve injury and paw incision depends on activation of the p38 MAPK signaling pathway in the dorsal horn of the spinal cord,[5,6,20] and p38 MAPK is exclusively activated in microglia, and not in the neurons or astrocytes in the dorsal horn after nerve injury.[5,6] We hypothesized that microglial p38 MAPK activation is equally important for postoperative mechanical hypersensitivity. Therefore, in the present study, the role of microglia and p38 MAPK in the spinal dorsal horn in hypersensitivity after paw incision was compared with that after peripheral nerve injury. Our findings suggest that spinal microglia have only a minor role in the postoperative pain model, as compared with neuropathic pain models.

## Materials and Methods

### *Animals*

Male Sprague-Dawley rats (200–270 g), housed under a 12-h light-dark cycle with free access to food and water, were used in the study. The study protocols and procedures were approved by the Animal Care and Experimentation Committee (Gunma University Graduate School of Medicine, Maebashi, Japan).

### *Surgical Preparations*

For hind paw incision (postoperative pain model), following the protocol outlined by Brennan *et al.*,[10] a 1-cm–long incision was made in the left hind paw, start-

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ing 0.5 cm from the heel under isoflurane anesthesia (2–3% in 100% oxygen). The plantaris muscles were lifted and incised longitudinally. The wound was closed with two 5.0 silk mattress sutures. For peripheral nerve injury (neuropathic pain model), we used the L5 spinal nerve transection (SNT) model under isoflurane anesthesia as previously described,[21] with some modifications. On the left side, the L5 spinal nerve of rats was tightly ligated with 6-0 silk sutures distal to the dorsal root ganglion, and cut just distal to the ligature under isoflurane anesthesia (2% in 100% oxygen).

Rats that underwent p38 MAPK studies were implanted with an intrathecal catheter as described previously,[22] with slight modifications. Indwelling catheters were constructed with 32-G polyurethane tubing connected to Tygon tubing (0.01 mm inner diameter; Saint-Gobain Performance Plastics, Paris, France) *via* a Tygon tubing cuff (0.02 mm inner diameter). All catheter joints were fused using cyclohexanone (Sigma Chemical Co., St. Louis, MO). Briefly, under isoflurane anesthesia, the 32-G polyurethane catheter containing a guide wire (ReCath Co. LLC, Allison Park, PA) was inserted through a puncture hole in the atlanto-occipital membrane and advanced caudally 7.5 cm so that the tip of the catheter was at the level of the lumbar enlargement. The animals were examined for neurologic deficits after surgery, and any animals exhibiting such deficits were immediately killed. The animals were housed individually after surgery and allowed to recover for 4 to 5 days before being used in the experiments.

*Behavioral Testing*

The rats were placed in individual plastic chambers with a plastic mesh floor, and allowed to acclimate to the environment for 30 min. The mechanical withdrawal threshold was determined using calibrated von Frey filaments (Stoelting, Wood Dale, IL). The filaments were applied vertically to an area adjacent to the wound for 5 to 6 s with gentle bending of the filament. The tactile stimulus producing a 50% likelihood of withdrawal threshold was determined using the up-down method, as previously described.[23] Changes in general behavior, including vocalization, repetitive movements, and activity level, were noted throughout the testing period. All studies were performed in a randomized, blinded manner.

## Experimental Procedures

Minocycline was used to examine the role of microglial activation after paw incision and SNT. In the postoperative pain model, rats (260–270 g) were randomly assigned to receive intraperitoneal injections of either minocycline or water. First, a single intraperitoneal administration of minocycline was performed 1 or 3 days after paw incision to determine the appropriate dose of minocycline. Withdrawal thresholds were determined before (preoperation) and 24 or 72 h after incision (0 min), then again 30, 60, and 90 min after the administration of minocycline (25 mg/kg or 50 mg/kg) or water. We also examined the effect of daily intraperitoneal injections of minocycline for 3 or 7 days in other groups of rats. Minocycline (25 mg/kg or 50 mg/kg) was injected 1 h before paw incision, and again 7 h after surgery. From Day 1, minocycline was injected twice a day (at 9:00 and 17:00) for either 2 or 6 days (50 mg $\cdot$ kg$^{-1}$ $\cdot$ day$^{-1}$ or 100 mg $\cdot$ kg$^{-1}$ $\cdot$ day$^{-1}$). Withdrawal thresholds were determined before paw incision, and on Days 1, 2, and 3 or Days 1, 3, 5, and 7 before the first minocycline injection. Upon completion of testing, the rats were perfused for immunohistochemical analysis, as described below.

In the SNT model, rats (200–220 g) were randomly assigned to receive either intraperitoneal injections of minocycline or water for 3 days, and after completion of testing, the rats were perfused for immunohistochemical analysis as described below.

To evaluate the role of p38 MAPK in the postoperative pain and neuropathic pain models, we injected a selective p38 MAPK inhibitor, SB203580 (10 $\mu$g), intrathecally 24 h and 72 h after surgery. Withdrawal thresholds were determined before surgery (preoperation) and before drug administration (0 min), every 30 min between 30 and 120 min, and then every 60 min up to 240 min after drug administration.

*Tissue Preparation*

Spinal cord tissue was obtained from rats at the indicated times after surgery. After being deeply anesthetized with sodium pentobarbital (100 mg/kg), the animals were perfused transcardially with 0.01 M phosphate-buffered saline containing 1% sodium nitrite, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). Sections of the lower lumbar spinal cord were removed and postfixed in 4% paraformaldehyde for 2 to 3 h. Tissue was then cryoprotected for 72 h at 4°C in 30% sucrose. Spinal cord sections (L4-L5) of 40 $\mu$m thickness were cut using a cryostat, mounted on glass slides, and stored at −80°C.

*Immunohistochemistry*

Immunohistochemistry was performed on free-floating spinal cord sections. The sections were washed 4 times in 0.01 M phosphate buffered saline + 0.3% Triton-×100 (PBST). Endogenous peroxidase activity was quenched with 15-min incubation in 0.3% hydrogen peroxide followed by 4 washes with PBST. Nonspecific binding was blocked with 1.5% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were incubated with an antibody for a microglial marker, OX42; complement receptor type 3/CD11b (mouse monoclonal; 1:200 in 1.5% normal goat serum;

Chemicon International, Temecula, CA), overnight at 4°C. Sections were washed twice in 0.01 M PBST and placed in biotinylated secondary antibodies (goat anti-mouse; 1:200; Vector Laboratories) for 1 h at room temperature. Sections were washed twice in PBST, placed in ABC complex (1:100, Vectastain ABC-Elite kit; Vector Laboratories) for 1 h, and washed twice in PBST. Antibodies were visualized using the enhanced glucose-nickel-diaminobenzidine method. Sections were mounted on glass slides, dehydrated through an ascending series of alcohols, cleared with xylene, and coverslipped.

Images were captured on an Olympus BX41 microscope (Olympus Co., Tokyo, Japan) using a QIMAGING MicroPublisher camera and QCapture software (VayTek, Inc., Fairfield, IA). For OX42 immunostaining, positively labeled areas in the whole dorsal horn of the spinal cord were identified for automated counting using SigmaScan Pro 5.0 (Jandel Scientific, Novato, CA) at a preset intensity threshold. Quantification of OX42 immunostaining was performed by calculating the percentages of immunostaining ([number of pixels of positively labeled objects within the fixed area]/[number of pixels within the same fixed area] $\times$ 100). Labeling was quantified in the whole dorsal horn of L4-L5 spinal cord slices stained with diaminobenzidine, with four slices quantified per animal.

### *Immunofluorescence*

Sections were washed in PBST and incubated in 1.5% normal goat serum for 1 h at room temperature, then incubated overnight at 4°C in the primary antibody, antiphospho-p38 MAPK (1:200; Cell Signaling, Beverly, MA) and OX42 (1:200). After incubation, tissue sections were washed in PBST and incubated for 2 h at room temperature in the secondary antibody solution (antirabbit immunoglobulin G-conjugated Alexa Fluor 488 or antimouse immunoglobulin G-conjugated Alexa Fluor546, 1:200; Molecular Probes, Eugene, OR). Sections were washed in PBST, mounted on slides, coverslipped with ProlongGold, and sealed with fingernail polish. The spinal cord sections were visualized using confocal laser scanning microscopy (LSM 510 META; Carl Zeiss, New York, NY). For quantification of phosphorylated (p-p38) MAPK immunostaining, the number of p-p38 MAPK immunopositive cells in the whole dorsal horn of L4-L5 spinal cord slices was counted using SigmaScan Pro 5.0. The person performing the data quantification was blinded to drug and dose.

### *Drugs and Administration*

Minocycline and SB203580 were purchased from Sigma Chemical Co. Minocycline was dissolved in sterilized distilled water (2.0 ml) and injected intraperitoneally (25 mg/kg or 50 mg/kg). SB203580 was dissolved in 100% dimethyl sulfoxide and diluted with sterile distilled water (final concentration of dimethyl sulfoxide, 2%), and injected intrathecally in a volume of 5 $\mu$l. Dimethyl sulfoxide (2%) diluted in distilled water was used as the vehicle. All drug injections were randomized.

### *Statistical Analysis*

We chose a sample size of at least 4 for behavioral studies and immunostaining based on a previous study.[14] Statistical analysis was conducted using Sigmastat (Version 3.1, Systat software Inc, San Jose, CA) or StatView (Version 5.0, Hulinks Co. Tokyo, Japan) software. For the behavioral studies, the results are presented as the mean $\pm$ SEM of the withdrawal thresholds. Time courses of the effects of minocycline and SB203580 were analyzed using two-way ANOVA. One-way ANOVA with Student-Newman-Keuls *post hoc* test was used for comparisons at each time point when significant differences were detected by two-way ANOVA.

Immunohistochemical data are presented as the mean percentages of the areas of immunostaining $\pm$ SEM and were analyzed using one-way ANOVA with Student-Newman-Keuls *post hoc* test. A $P$ value of less than 0.05 was considered statistically significant.

## Results

### *Effect of Minocycline on Mechanical Hypersensitivity after Paw Incision and SNT*

Animals demonstrated robust mechanical hypersensitivity at 24 h after paw incision. Withdrawal thresholds increased after intraperitoneal administration of minocycline when injected 24 h after paw incision ($n = 8$; $P = 0.001$ by two-way ANOVA; fig. 1A). We also injected minocycline (50 mg/kg) 72 h after paw incision, because a pilot study demonstrated that OX42 immunoreactivity increased 3 days after paw incision.[15] This treatment, however, did not increase withdrawal thresholds ($n = 6$; fig. 1B).

Intraperitoneal injection of minocycline twice daily for either 3 or 7 days also did not affect the withdrawal thresholds ($n = 8$, fig. 2). In contrast, intraperitoneal injection of minocycline twice daily for 3 days reduced mechanical hypersensitivity in rats with SNT in a dose-related manner ($n = 8$, $P < 0.001$ by two-way ANOVA, fig. 3).

### *Effect of Minocycline on OX42 Immunoreactivity after Paw Incision*

To examine whether microglia were activated in the dorsal horn of the spinal cord in rats that underwent paw incision, we performed immunohistochemical analysis with an antibody targeting OX42, a microglial surface antigen antibody. In the vehicle-treated group, microglia assessed by OX42 immunostaining were more hypertrophic on the side of the paw incision than on the contralateral side, especially in the medial parts of the dorsal

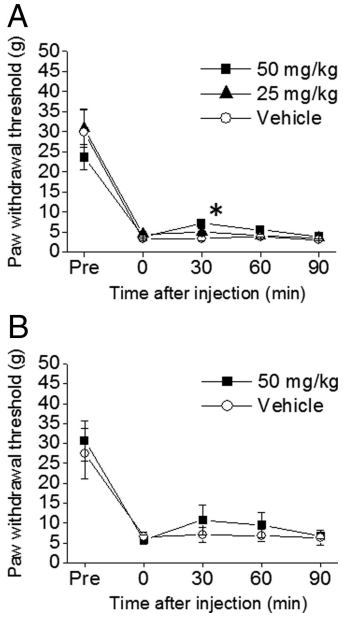


**Fig. 1. Effect of a single intraperitoneal injection of vehicle, 25 mg/kg or 50 mg/kg minocycline, on postoperative mechanical hypersensitivity. Withdrawal threshold increased after the administration of minocycline when injected 24 h after paw incision (*A*) ($P = 0.001$ by two-way ANOVA). The highest dose of minocycline, however, did not increase the withdrawal threshold when injected 72 h after paw incision (*B*). The hind paw withdrawal thresholds (g) are shown as mean ± SEM (n = 6–8 for each group). * $P < 0.05$ as compared with vehicle-treated group by one-way ANOVA.**

horn. Minocycline inhibited this increase in OX42 (fig. 4). Figure 5 shows representative images of OX42 immunoreactivity in the ipsilateral spinal dorsal horn after paw incision and L5 spinal nerve transection. The percentage of

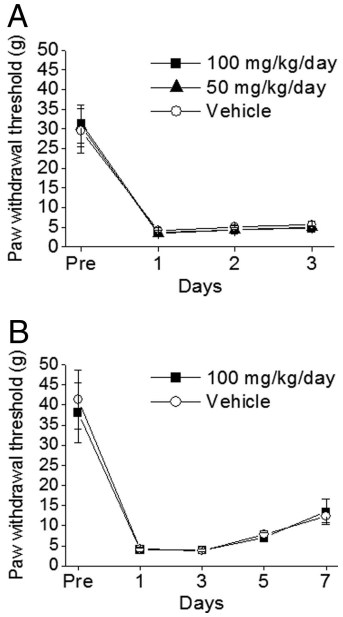


**Fig. 2. Effect of twice-daily intraperitoneal injection of vehicle, 50 mg · kg$^{-1}$ · day$^{-1}$ or 100 mg · kg$^{-1}$ · day$^{-1}$ minocycline for 3 days (*A*) and 7 days (*B*) in the postoperative pain model. Withdrawal thresholds were not altered after treatment. The hind paw withdrawal thresholds (g) are shown as mean ± SEM (n = 8 for each group).**

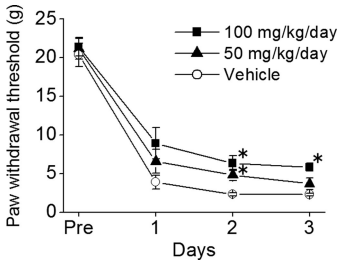


**Fig. 3. Effect of minocycline on L5 spinal nerve transection model. Minocycline reduced mechanical hypersensitivity after twice-daily intraperitoneal injection of vehicle, 50 mg · kg$^{-1}$ · day$^{-1}$ or 100 mg · kg$^{-1}$ · day$^{-1}$ minocycline for 3 days in a dose-related manner ($P < 0.001$ by two-way ANOVA). The hind paw withdrawal thresholds (g) are shown as mean ± SEM (n = 8 for each group). * $P < 0.05$ as compared with vehicle-treated group by one-way ANOVA.**

OX42 immunoreactive cells in the whole dorsal horn of the spinal cord is shown in figure 6. The administration of minocycline twice daily for 3 days reduced OX42 immunostaining in a dose-related manner (n = 4, $P < 0.001$ by one-way ANOVA, fig. 6A). The maximum dose of minocycline for 7 days also inhibited OX42 upregulation (n = 4, $P < 0.001$ by one-way ANOVA, fig. 6C).

### *Effect of Minocycline on OX42 Immunoreactivity after SNT*

In the vehicle-treated group, OX42 immunoreactivity increased in the whole dorsal horn of the spinal cord on the side of SNT, whereas this increase was suppressed by

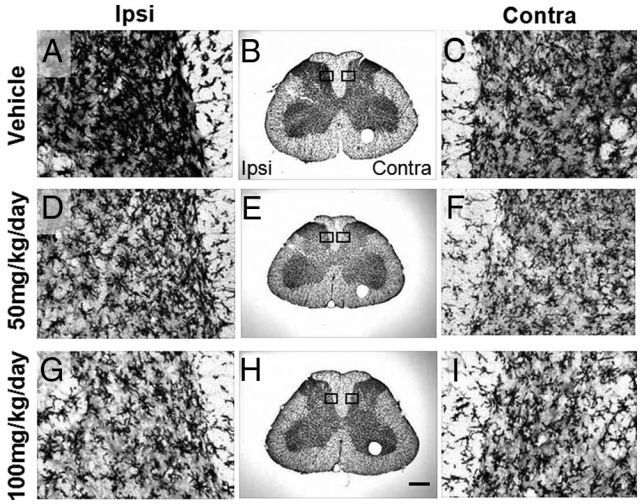


**Fig. 4. Representative images of microglia in the ipsilateral (ipsi) and contralateral (contra) spinal cord after paw incision. Microglia are represented by OX42 immunoreactivity after twice-daily intraperitoneal injection of vehicle (*A*, *B*, and *C*), 50 mg · kg$^{-1}$ · day$^{-1}$ (*D*, *E*, and *F*) and 100 mg · kg$^{-1}$ · day$^{-1}$ (*G*, *H*, and *I*) minocycline for 3 days. (*A*), (*D*) and (*G*) are high-magnification images of the squares in the ipsilateral spinal cord shown in (*B*), (*E*) and (*H*), respectively. Similarly, (*C*), (*F*), and (*I*) are high-magnification images of the squares in the contralateral spinal cord shown in (*B*), (*E*) and (*H*), respectively. Increased OX42 expression was observed in the medial parts of the ipsilateral dorsal horn of the spinal cord after injection of vehicle. Minocycline administration reduced the upregulation of OX42 immunoreactivity after paw incision. Scale bar = 400 μm.**

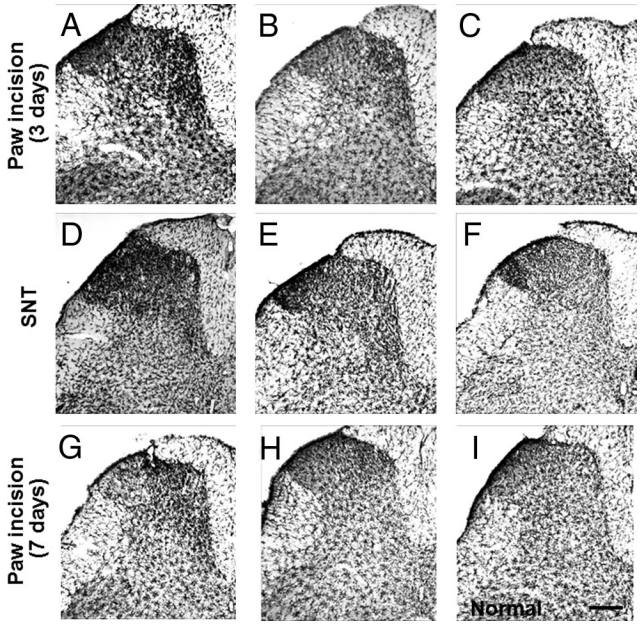


**Fig. 5. Representative images of microglia in the ipsilateral spinal dorsal horn after paw incision and L5 spinal nerve transection. After paw incision, microglia are represented by OX42 immunoreactivity after twice-daily intraperitoneal injection of vehicle (*A*), 50 mg · kg$^{-1}$ · day$^{-1}$ (*B*), or 100 mg · kg$^{-1}$ · day$^{-1}$ (*C*) minocycline for 3 days. After L5 spinal nerve transection, microglia are represented by OX42 immunoreactivity after twice-daily intraperitoneal injection of vehicle (*D*), 50 mg · kg$^{-1}$ · day$^{-1}$ (*E*), or 100 mg · kg$^{-1}$ · day$^{-1}$ (*F*) minocycline for 3 days. After paw incision, microglia are represented by OX42 immunoreactivity after twice-daily intraperitoneal injection of vehicle (*G*), or 100 mg · kg$^{-1}$ · day$^{-1}$ minocycline (*H*) for 7 days. (*I*) Images of microglia in the spinal dorsal horn of a normal rat. OX42 immunoreactivity in the whole dorsal horn was increased on the ipsilateral side of the spinal cord after paw incision and L5 spinal nerve transection. After twice-daily administration of minocycline, OX42 upregulation was decreased. Scale bar = 200 μm.**

minocycline administration (fig. 5, D–F). Minocycline reduced the area of immunostaining in a dose-related manner after twice-daily administration for 3 days (n = 4, $P < 0.001$ by one-way ANOVA, fig. 6B).

*Effect of p38 MAPK Inhibition in the Postoperative Pain and Neuropathic Pain Models*

Figure 7 shows the effect of p38 MAPK inhibition on hypersensitivity in the spinal cord. In rats with SNT, intrathecal administration of SB203580 (10 μg) inhibited mechanical hypersensitivity, and the effect continued for 2 to 3 h after injection when SB203580 was administered 24 h and 72 h after SNT (n = 4, $P < 0.001$ as compared with the vehicle-treated group by two-way ANOVA, fig. 7B). In contrast, SB203580 did not alter hypersensitivity after paw incision (n = 5, fig. 7A).

*Comparison of Phosphorylated p38 MAPK Expression between the Postoperative Pain and Neuropathic Pain Models*

As compared with normal rats (fig. 8A), p-p38 MAPK expression (green) was increased in the ipsilateral dorsal horn of the spinal cord 3 days after paw incision (fig. 8B) and SNT (fig. 8C). Phosphorylated-p38 MAPK expression was higher after SNT than after paw incision. The mean number of p-p38 MAPK immunopositive cells in SNT, paw incision, and normal rats in the ipsilateral whole dorsal horn of the spinal cord was 1,841, 925, and 409, respectively (n = 4). The increase in p-p38 MAPK in microglia was inferred based on the merged images (fig. 8, D and E) of OX42 (red) and p-p38 MAPK (green) staining. Most p-p38 MAPK staining occurred in the centers of the OX42-positive structures; the stained structures therefore likely represent the nuclei and cytosol/cell surface, respectively, of microglia.

## Discussion

In the present study, a single injection of minocycline 1 day after paw incision suppressed postoperative hypersensitivity. In contrast, twice-daily minocycline injection after paw incision did not attenuate hypersensitivity. Twice-daily minocycline injection, however, did

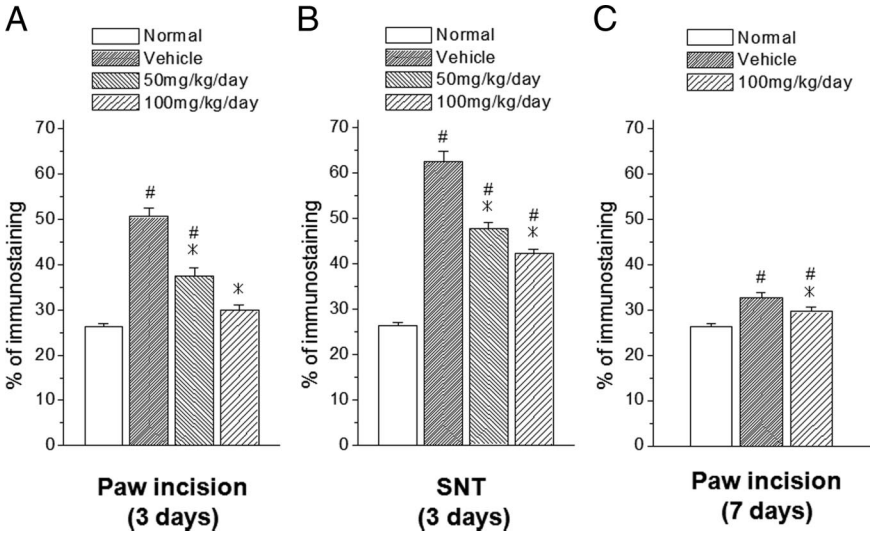


**Fig. 6. Quantification of microglial expression in the whole dorsal horn of the spinal cord after twice-daily injection of vehicle, 50 mg · kg$^{-1}$ · day$^{-1}$, or 100 mg · kg$^{-1}$ · day$^{-1}$ minocycline in the postoperative pain model for 3 days (*A*), 7 days (*C*) and the L5 spinal nerve transection (SNT) model for 3 days (*B*). Minocycline inhibited the area of OX42 immunostaining (microglia) after twice-daily injections in a dose-related manner. Percentages of immunostaining are expressed as mean ± SEM (n = 4 for each group). * $P < 0.05$ as compared with the vehicle-treated group by one-way ANOVA. # $P < 0.05$ as compared with the normal group by one-way ANOVA.**

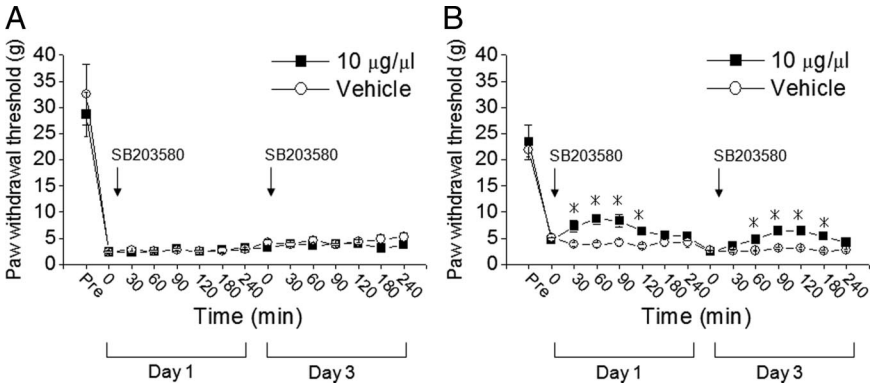


**Fig. 7. Effect of a p38 MAPK inhibitor (SB203580) after paw incision (*A*) and L5 spinal nerve transection model (*B*). There were no changes in the withdrawal thresholds in the postoperative pain model after the administration of SB203580. In the neuropathic pain model, withdrawal thresholds were increased by intrathecal administration of SB203580 (10 μg) when injected at 24 h and 72 h after the nerve transection. The mean hind paw withdrawal thresholds (g) are shown as mean ± SEM (n = 4 or 5). * $P < 0.05$ as compared with the vehicle-treated group by one-way ANOVA.**

reduce the expression of OX42, a microglial marker. Further, although p-p38 MAPK expression in the dorsal horn of the spinal cord increased after paw incision, intrathecal administration of the p38 MAPK inhibitor SB203580 did not attenuate hypersensitivity. After SNT, however, both minocycline and SB203580 effectively decreased hypersensitivity, and minocycline attenuated OX42 expression in the spinal dorsal horn. These findings suggest that the role of spinal microglia in postoperative pain differs from that in neuropathic pain.

Until recently, chronic pain has been considered to be a result of sensitization and reorganization of the central nervous system. Recent reports, however, indicate that neuroimmune alterations may also contribute to pain hypersensitivity. Microglia have a significant role in exaggerated pain states with inflammatory and neuropathic etiologies. Microglial activation results in upregulated receptor function and further changes in microglial morphology, proliferation, and function.[24] Although the role of microglia in postoperative pain has not been clarified, Obata *et al.* [15] suggested that upregulation of microglia and astrocytes in the spinal cord contributes to mechanical hypersensitivity after paw incision.

Minocycline is a second-generation tetracycline derivative that exerts antiinflammatory effects independent of its antimicrobial activity.[25] Minocycline prevents microglial activation and thus has neuroprotective effects in various models of neurologic disease, including cerebral ischemia,[26,27] Parkinson's disease,[28–30] multiple sclerosis,[31,32] intracerebral hemorrhage,[33] traumatic brain injury,[34] glutamate-induced neurotoxicity,[35,36] Huntington's disease,[37] and amyotrophic lateral sclerosis.[38,39] Minocycline also inhibits exaggerated pain induced by inflammation or nerve injury. Intrathecal minocycline reduces formalin-evoked second-phase flinching behavior,[8] prevents carrageenan-induced thermal hyperalgesia,[17] reverses spinal-cord injury-induced nociceptive behaviors,[18] prevents the development of nociceptive behaviors induced by L5/6 spinal-nerve ligation,[19] and attenuates the development of pain hypersensitivity in spinal immune activation by sciatic inflammatory neuropathy and intrathecal human immunodeficiency vi-

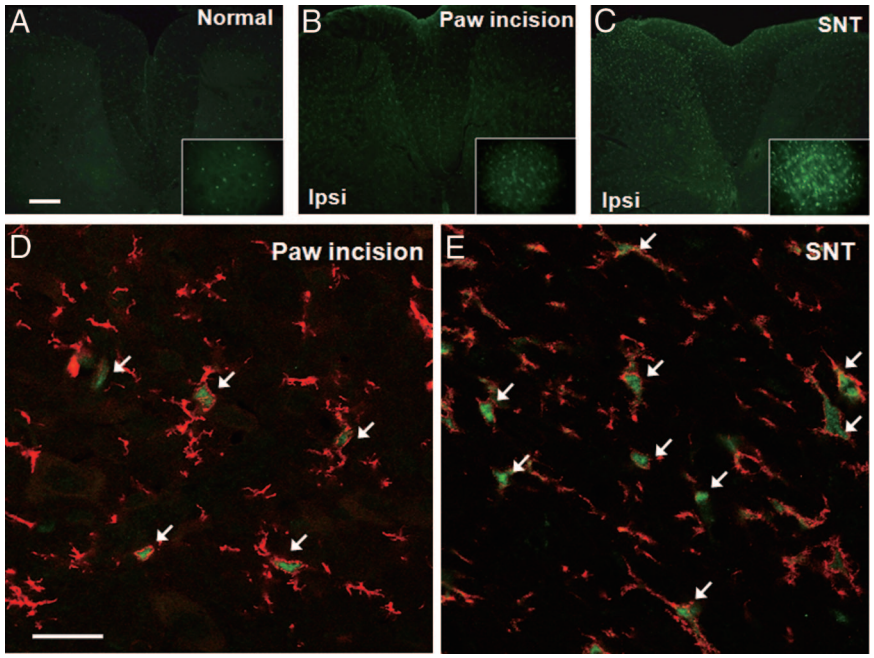


**Fig. 8. Representative images of phosphorylated p38 (p-p38) mitogen-activated protein kinase (MAPK) expression in the spinal dorsal horn in a normal rat (*A*), 3 days after paw incision (*B*), and 3 days after L5 spinal nerve transection (SNT; *C*) in the ipsilateral (ipsi) spinal dorsal horn. Highly magnified images of the ipsilateral spinal dorsal horn are shown in the square in the upper panels. In the L5 spinal nerve transection model, p-p38 MAPK immunoreactivity was higher than that in normal rat and the postoperative pain model. Merged double-labeled immunofluorescence images demonstrate colocalization (*arrows*) of OX 42 (red) and p-p38 MAPK (green) in the postoperative pain model (*D*) and the L5 spinal nerve transection model (*E*). Scale bar = 200 μm for (*A*), and 50 μm for (*D*).**

rus-1 gp120 administration.[2] Intraperitoneal injection of minocycline prevents the development of L5 SNT-induced mechanical hypersensitivity.[7]

In the present study, the withdrawal threshold increased after a single intraperitoneal administration of minocycline (25 or 50 mg/kg) 1 day after paw incision in the postoperative pain model. Based on this outcome, we determined the dose of minocycline for twice-daily administration. We expected that this treatment would suppress mechanical hypersensitivity after paw incision, but, surprisingly, there was no effect after twice-daily intraperitoneal minocycline injections for 3 days. A pilot study demonstrated that OX42 immunoreactivity begins to increase 3 days after paw incision and continues for up to 5 days.[15] Therefore, we tested the effect of twice-daily intraperitoneal injections of minocycline for 7 days, but this treatment also did not suppress mechanical hypersensitivity after paw incision. This finding suggests that minocycline has only a short-term effect in the postoperative pain model, and that this effect is not cumulative. Alternatively, microglia may have an active role only at specific time points. On the other hand, minocycline (100 mg $\cdot$ kg$^{-1}$ $\cdot$ day$^{-1}$ either for 3 or 7 days) inhibited the expression of OX42 after paw incision. These results suggest that microglia in the spinal cord have only a minor role in the postoperative pain model. This speculation is supported by the findings of a previous study,[15] which showed that the increased immunoreactivity of glial fibrillary acidic protein immunoreactivity, an astrocyte marker, was closely correlated with mechanical hypersensitivity in a postoperative pain model. OX42 immunoreactivity, however, did not parallel mechanical hypersensitivity. Another possible explanation for the inconsistency between the behavioral findings and OX42 expression is that this marker is not associated with microglial function. The surface marker complement receptor type 3/CD11 recognized by OX42 might reflect a morphologic change rather than a functional alteration of microglia. Romero-Sandoval *et al.*[40] reported that the expression of ionized calcium-binding adapter molecule 1, another microglia marker, in the spinal cord is associated with mechanical hypersensitivity after paw incision, and that the spontaneous resolution of postoperative pain parallels the spontaneous reduction in spinal calcium-binding adapter molecule 1 expression. Specific microglial markers may have different roles in different types of pain. Assessment using multiple markers may help to determine if there is a causative link between microglial activation and behavioral change.

In contrast, minocycline attenuated the mechanical hypersensitivity of neuropathic pain, similar to previous findings.[7] Twice-daily intraperitoneal injections of minocycline for 3 days produced a dose-related reduction in mechanical hypersensitivity after SNT in the present study. Furthermore, spinal microglial expression was inhibited by minocycline, therefore it is possible that spinal microglia strongly contribute to neuropathic pain. In the neuropathic pain model, microglial immunoreactivity on the ipsilateral side was more extensive than that in the postoperative pain model. These results suggest that the effect of noxious stimulation to increase OX42 expression in neuropathic pain is more robust, and microglia may have a more important role in the development of neuropathic pain than in postoperative pain. In the present study, the effect of minocycline on mechanical hypersensitivity in the SNT model was less than in a previous study by Raghavendra *et al.*[7] We used a minocycline dose of 100 mg $\cdot$ kg$^{-1}$ $\cdot$ day$^{-1}$ for 3 days and observed a 20% change, whereas in their study, 40 mg $\cdot$ kg$^{-1}$ $\cdot$ day$^{-1}$ minocycline produced a 50% reduction of hypersensitivity. Based on their findings that minocycline attenuated the development of hypersensitivity, but not existing hypersensitivity, after SNT, we injected minocycline beginning 1 h before nerve transection. Therefore, we speculate that this dissociation between the two studies is a result of the differences in the experimental methodology, such as behavioral testing with von Frey filaments. Raghavendra *et al.*[7] evaluated hypersensitivity by determining the number of paw withdrawals with 30 stimulations of the plantar surface of the hind paw using 2- and 12-g von Frey filaments. In contrast, we determined the withdrawal threshold using the up-down method.

Activation of p38 MAPK in spinal cord microglia plays a critical role in nerve-injury- and inflammation-induced spinal pain processing.[5,6,41–43] In neuropathic pain models, p38 MAPK is specifically activated in microglia in the spinal cord, and intrathecal administration of p38 inhibitors reduces neuropathic pain behavior.[5,6,44,45] These findings strongly suggest that p38 phosphorylation is a key intracellular signal in microglial activation. In the present study, the expression of p-p38 MAPK increased in the ipsilateral dorsal horn of the spinal cord after paw incision and SNT, as compared with normal rats. Previous studies indicate that p-p38 MAPK staining is not colocalized with that of either NeuN (neuronal marker) or glial fibrillary acidic protein, but is completely colocalized with OX42 in the spinal cord after nerve injury.[5,6] In the postoperative pain model, although p-p38 MAPK might be initially present in another cell type, it is exclusively expressed in microglia at 1 day after paw incision.[20] We also demonstrated with double immunofluorescence studies that p-p38MAPK expression is increased in microglia 3 days after paw incision.

To evaluate whether p38 inhibition has a role in existing hypersensitivity after paw incision and SNT, we administered a single intrathecal injection of SB203580 on Days 1 and 3 after surgery. This drug binds to the adenosine triphosphate pocket in p38 MAPK, and consequently inhibits its enzymatic activity without affecting the phosphorylation of p38 MAPK.[46] We determined the

dose of SB203580 (10 $\mu$g) based on a previous study[5] in which this dose effectively attenuated hypersensitivity after nerve injury. Our data were consistent with the findings of this previous study, because intrathecal administration of SB203580 reduced existing hypersensitivity in the neuropathic pain model. The same drug treatment, however, was not effective in the postoperative pain model. A recent study demonstrated that intrathecal pretreatment with another p38 inhibitor effectively prevented the development of hypersensitivity after paw incision.[20] In that study, the p38 inhibitor FR167653 blocked p38 phosphorylation and inhibited paw incision–induced hypersensitivity. This result suggests that inhibition of p38 phosphorylation is necessary to block postoperative pain. In the present study, p-p38 MAPK expression was greater after SNT than after paw incision. This difference in p-p38 MAPK expression may also explain the difference in efficacy of SB203580 and minocycline between SNT and paw incision.

In summary, daily intraperitoneal administration of minocycline did not attenuate mechanical hypersensitivity after paw incision, despite the decline in OX42 expression in the spinal cord. Although p-p38 MAPK expression was increased in the spinal cord, intrathecal injection of the p38 MAPK inhibitor SB203580 did not effectively attenuate pain in the postoperative pain model. In contrast, minocycline and SB203580 were efficacious in attenuating nerve injury–induced hypersensitivity, and minocycline attenuated OX42 expression in the spinal dorsal horn in the SNT model. The results of the present study suggest that the effect of noxious stimulation to increase OX42 expression in neuropathic pain is more robust, and that microglia may have a more important role in the development of neuropathic pain than in postoperative pain. An increase in spinal OX42 expression, however, may not contribute to postoperative pain.

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