Original Paper

Early Postoperative Nociceptive Threshold and Production of Brain-Derived Neurotrophic Factor Induced by Plantar Incision Are Not Influenced with Minocycline in a Rat: Role of Spinal Microglia

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Key Words

Early postoperative pain • Microglia • Brain-derived neurotrophic factor • Spinal cord

Abstract

Background: Brain-derived neurotrophic factor (BDNF) from spinal microglia is crucial for aberrant nociceptive signaling in several pathological pain conditions, including postoperative pain. We assess the contribution of spinal microglial activation and associated BDNF overexpression to the early post-incisional nociceptive threshold. **Methods:** Male Sprague-Dawley rats were implanted with an intrathecal catheter. A postoperative pain model was established by plantar incision. Thermal and mechanical nociceptive responses were assessed by infrared radiant heat and von Frey filaments before and after plantar incision. Rats were injected intrathecally the microglial activation inhibitor minocycline before incision, 24 h after incision, or both. Other groups were subjected to the same treatments and the L4-L5 spinal cord segment removed for immunohistochemical analysis of microglia activation and BDNF expression. **Results:** Plantar incision reduced both thermal latency and mechanical threshold, indicating thermal hypersensitivity and mechanical allodynia. Minocycline temporally reduced thermal withdrawal latency but had no effect on mechanical withdrawal threshold, spinal microglial activity, or dorsal horn BDNF overexpression during the early post-incision period. **Conclusion:** These results suggest that spinal microglia does not contribute substantially to post-incisional nociceptive threshold. The BDNF overexpression response that may contribute to postoperative hyperalgesia and allodynia is likely derived from other sources.

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Introduction

Surgical incisions induce postoperative pain responses, including spontaneous pain, allodynia, and hyperalgesia. Postoperative pain is mediated by multiple processes depending on the extent of tissue damage, and may include inflammatory, neuropathic, and neurogenic components. Even in the very early postoperative period hours after incision, sensory afferent neurons exhibit elevated spontaneous and stimulus-evoked activity that mediate hyperalgesia and allodynia [1], indicating the existence of pain with neuropathic component due to surgical incision.

Many chemical modulators are involved in the initiation and maintenance of abnormal sensory responses induced by nerve injury. Induction of brain-derived neurotrophic factor (BDNF) overexpression and activation of spinal microglia are crucial for aberrant nociceptive signaling in several pathological pain conditions [2], including postoperative pain [3]. Following peripheral nerve injury, release of BDNF from spinal microglia contributes to impaired GABAergic inhibition of spinal neurons [4] and BDNF from microglia is suggested to be a molecular substrate for neuropathic pain [5]. On the other hand, BDNF expression and activation of microglia with cancer pain are controversial. One study demonstrates a bone cancer pain increases BDNF production in the spinal cord from activated microglia [6] while another study shows no hyperactive microglia with the cancer pain [7]. In postoperative pain model, BDNF overexpression was observed in both DRG neurons and spinal cord nerve terminals [3], although activation of microglia in spinal cord and subsequent BDNF production with surgical incision are obscure especially in the early post-incision period [8-10]. Thus, the role of microglia and BDNF are seem to be different in neuropathic, cancer and postoperative pain. In addition, the sources of BDNF after surgical incisions, especially in the early postoperative periods, could be diverse and dominant sites are not well recognized.

The purpose of this study was to examine the role of microglial activation and concomitant BDNF overexpression in nociceptive threshold during the early period following plantar incision in rats, a model of postoperative pain. This study was planned because 1) it remain unknown whether surgical incision activates the microglia in spinal cord to release BDNF in the early postoperative period, 2) the postoperative pain is involved in the pain with neuropathic component which is convincingly induced by BDNF from the microglia, therefore, it is possible that the early postoperative pain is partially modified by BDNF from the microglia. We hypothesize that intrathecal (IT) injection of the microglial activation inhibitor minocycline would inhibit spinal BDNF overexpression as in other neuropathic [8] and cancer [6] pain models, leading to suppression of heat hypersensitivity and mechanical allodynia.

Materials and Methods

Ethics Statements

This study was approved by the Animal Care Committee of Tohoku University (Permit Number 2015DnA-030) and all animal procedures were in concordance with the Guiding Principles for the Care and Use of the Field of Physiological Science of the Physiological Society of Japan.

Animals

Male Sprague-Dawley rats (250–300 g) were housed individually in standard animal cages under a 12-h light/dark cycle and provided free access to water and food. Animals were acclimated to these conditions for 6 days before experimentation. A total of 136 rats were subjected to plantar incision to establish a postoperative pain model and implanted with IT catheters for injection of minocycline. Seventy five rats (15 rats per group) were tested for thermal and mechanical nociceptive responses and 30 rats (6 rats per group) were used for immunohistochemistry. Moreover, 31 rats were only subjected to implantation of IT catheters. Twenty five rats (5 rats per group) were tested the nociceptive threshold after injection

of minocycline without planter incision and 6 rats were used for immunohistochemistry as sham surgery group (anesthesia only no incision).

IT catheterization

For IT administration of minocycline, IT catheters were implanted in rats under sevoflurane anesthesia (3.0%) in oxygen according to the methods of Yaksh and Rudy [11] with minor modifications. Rats were secured in a stereotaxic frame in a head flexed forward position. After shaving and skin sterilization with povidone iodine, midline skin and fascia incisions were made and the atlanto-occipital membrane was exposed. The membrane was carefully punctured using a 22-gauge needle and a single-lumen polyethylene-5 catheter (outer diameter, 0.36 mm), heat connected to a polyethylene-10 catheter (outer diameter, 0.61 mm), was passed into the IT space at the L4-L5 spinal cord segment. Animals were monitored for neurologic deficits after catheterization, and any animals exhibiting deficits were removed from the study.

Plantar incision

Six days after IT catheterization, a 1-cm longitudinal incision was made through the skin and fascia of the plantar aspect of the left hind limb starting 0.5 cm from the end of heel by the method of Brennan et al. [12] under sevoflurane anesthesia (3.0%) in oxygen. The underlying muscle was exposed and a single longitudinal incision made. The skin was closed with two mattress sutures of 5-0 nylon.

Drugs

Minocycline hydrochloride (NICHII-KO, Toyama, Japan) was dissolved in 0.9% normal saline for IT administration (100 or 200 µg/rat). The dose of minocycline chosen was based on previous studies [6, 13]. All injections were followed by a flush of 10 µl normal saline.

Experimental design for pain analysis

Sixty rats were randomized into 4 groups of 15: Pre-incision, Post-incision, Pre + Post, and Saline (control) groups (Fig. 1). Minocycline (100 µg/rat) was administered 1 h before plantar incision (Pre group), 24 h after plantar incision (Post group), or both (Pre + Post group). Alternatively, normal saline

Fig. 1. Experimental design. Minocycline was injected intrathecally (IT) 1 h before plantar incision (Pre group), 24 h after the plantar incision (Post group), or both (Pre + Post group). Normal saline was injected instead of minocycline pre-, post-, and both pre- and post -incision in the Post, Pre and Saline (control) group, respectively. The pain responses were analyzed (a) before plantar incision (after pre-operative administration of minocycline), (b) 1 day post-incision (immediately before administration of minocycline), (c) 1 day plus 30 min post-incision (30 min after administration of minocycline), (d) 1 day plus 120 min post-incision (120 min after administration of minocycline), and 2, 3, and 7 days after plantar incision. Spinal cords for immunohistochemical studies of BDNF expression and microglia activation were obtained 1 day plus 120 min post-incision.

was injected instead of minocycline preoperatively, postoperatively, and both pre- and postoperatively in the Post, Pre, and Saline group, respectively. The additional 15 rats were administered IT minocycline (200 µg/rat) 24 h after plantar incision. The five rats per group were catheterized and injected as described but subjected to sham surgery (anesthesia only no incision).

Pain response analysis

Rats were tested for thermal hypersensitivity and mechanical allodynia of the hind paw before plantar incision but after preoperative administration of minocycline or saline (baseline); 1 day after incision just before postoperative administration of minocycline; and 1 day plus 30 min, 1 day plus 120 min, 2 days, 3 days, and 7 days after plantar incision (Fig. 1). Heat hypersensitivity was evaluated by a plantar test apparatus (model 37370; Ugo Basile Biochemical Instruments, Comerio, Italy). An infrared radiant heat source under a heat-acrylic plastic floor was focused on the middle of the hind paw incision. Time to withdrawal was automatically measured. To avoid thermal injury, a cut-off was set at 20 s. The withdrawal latencies were averaged from three trials separated by 5-min intervals.

Mechanical nociceptive thresholds were evaluated using an automated von Frey-type system (dynamic plantar aesthesiometer, model 37450; Ugo Basile, Comerio, Italy). To measure mechanical thresholds, each rat was placed in a plastic cage with a wire mesh floor. The tip of a filament was applied to the middle of the plantar surface of the hind paw with increasing force until the paw was withdrawn or a cut-off of 50 g was reached. The force required to elicit reflex withdrawal of the hind paw was automatically recorded. The lowest force required from three trials separated by 5-min intervals was considered the withdrawal threshold.

Immunohistochemistry

A separate cohort of 24 rats subjected to plantar incision and IT catheter insertion were divided into the same 4 groups of 6 (Pre, Post, Pre + Post, and Saline) to assess the effects of IT minocycline (100 µg/rat) on incision-induced microglial activation and BDNF expression in the spinal cord. The 6 rats were administered IT minocycline (200 µg/rat) 24 h after plantar incision. The additional 6 rats were catheterized and injected saline but subjected to sham surgery (anesthesia only no incision). At 1 day plus 120 min after plantar incision (Fig. 1), rats were perfused intracardially with normal saline under deep urethane anesthesia (1 g/kg) and then perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). The L4-L5 spinal cord segment was removed, post-fixed in the same fixative for 4 h, and cryoprotected with 30% sucrose in PBS overnight. Transverse sections were cut at 30 µm using a cryostat. Freely floating sections were incubated in 0.3% H₂O₂ for 15 min to quench endogenous peroxidase activity and then blocked in 1.5% normal goat serum or 2.5% normal horse serum in 0.1 M PBS for 1 h at room temperature (RT), followed by incubation in either anti-BDNF antibody (rabbit polyclonal, 1:200 in 1.5% normal goat serum; Abcam, Tokyo, Japan) or an antibody for the microglial marker OX-42 (mouse monoclonal, 1:250 in 2.5 % normal horse serum; Abcam) overnight at 4 °C. Sections were incubated with a corresponding secondary antibody, either biotinylated goat anti-rabbit IgG or horse anti-mouse IgG (1:200 in PBS; Vector Laboratories, Burlingame, CA, USA), for 1 h at RT and then in avidin-biotin complex solution (1:100 in PBS; Vector Laboratories) for 1 h at RT. Bound antibodies were visualized by reaction with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, MO, USA). Sections were mounted on 3-aminopropyltriethoxysilane (APTS)-coated slides, dried, dehydrated, and coverslipped with Permount (Fisher Scientific, Waltham, MA, USA). Sections from the L4-L5 segment were randomly selected and five non-adjacent squares (100 × 100 µm) in each section were chosen within lamina I-II for measurement of the immunopositive area (n=6, each group) using ImageJ software (NIH, Bethesda, MD, USA). All quantitative analyses were conducted by an experimenter blind to minocycline treatment history.

Statistical analysis

Statistical analyses were performed using Prism software, version 5.02 (GraphPad, San Diego, CA, USA). Pain response results are presented as the mean ± SEM. Group means were compared by two-way ANOVA followed by Bonferroni post hoc tests for pair-wise comparisons. Immunohistochemical data are presented as mean ± SD. Group means were compared using unpaired *t*-tests. A *P* < 0.05 was considered statistically significant.

Results

Effect of minocycline on pain responses after plantar incision

Plantar incision produced hypersensitivity and allodynia against both heat and mechanical stimuli as indicated by reduced hind paw withdrawal latency to infrared radiant heat and reduced withdrawal threshold to mechanical stimulation 1 day post-surgery (Fig. 2). In the sham surgery group (anesthesia only no incision), both withdrawal latency and threshold remained stable, indicating no thermal hyperalgesia or mechanical allodynia (data not shown). Preoperative IT administration of minocycline did not change thermal withdrawal latency or mechanical withdrawal threshold at any test point (1 day plus 30 min, 1 day plus 120 min, 2 days, 3 days, and 7 days post-incision). In contrast, post-incision IT minocycline injection (Post group) significantly increased thermal withdrawal latency at 1 day plus 30 min post-incision compared to saline-injected controls (Saline: 4.7 ± 0.17 s, Post: 9.6 ± 1.1 s, $P < 0.05$), but this antinociceptive effect disappeared at 1 day plus 120 min post-incision and all test points thereafter. This brief post-incision antinociceptive effect of minocycline was not observed for mechanical stimulation (no change in threshold). Injection of minocycline both pre- and post-incision (Pre + Post group) also produced an antinociceptive effect against noxious heat stimulation at the first post-incision time point but the effect was not greater than that evoked by post-incision injection alone. The increased dose of minocycline (200 μ g/rat) did not change the nociceptive responses observed in the lower dose (100 μ g/rat) in both thermal withdrawal latency and mechanical threshold (Fig. 3). Minocycline did not affect the nociceptive responses in sham-operated rats (data not shown).

Effect of minocycline on OX-42 immunoreactivity after plantar incision

In the saline group, the expression of OX-42, a protein induced in microglia by activation, was same as that of the sham group and did not differ between the ipsilateral and contralateral spinal cord dorsal horns 1 day after plantar incision (Fig. 4, Table 1). Minocycline (both 100 and 200 μ g) had no effect on OX-42 expression compared to saline injection (Fig. 4, Table 1).

Fig. 2. The effects of IT minocycline on response to noxious heat (A) and mechanical stimuli (B). Incision markedly reduced both heat withdrawal latency (A) and mechanical withdrawal threshold (B), indicating successful establishment of a post-operative pain model. The pain responses were analyzed at the times indicated in Fig. 1. Post-incision minocycline (alone or plus pre-incision administration) reduced heat hypersensitivity only at 30 min post-injection (1 day plus 30 min post-incision) and had no effect on mechanical hypersensitivity. * $p < 0.05$ vs. saline, Data are expressed as mean $\pm$ SEM ($n = 15$ rats per group).

Fig. 3. The effects of increased dose of IT minocycline on response to noxious heat (A) and mechanical stimuli (B). Minocycline was injected intrathecally (IT) 24 h after the plantar incision and the pain responses were analyzed at 1 day plus 30 min post-incision (30 min after administration of minocycline). Post-incision minocycline reduced heat hypersensitivity and had no effect on mechanical hypersensitivity. * $p < 0.05$ vs. saline, Data are expressed as mean $\pm$ SEM (n=15 rats per group).

Fig. 4. Representative images of activated microglia in the L4-L5 spinal cord one day after plantar incision. Five non-adjacent squares (100 $\times$ 100 μ m) in section from the L4-L5 segment were chosen within lamina I-II for measurement of the immunopositive area. Scale bar = 500 μ m.

Effect of minocycline on BDNF immunoreactivity after plantar incision

In all groups, plantar incision resulted in a significant increase in BDNF expression in the ipsilateral spinal cord lamina I-II at the L4-L5 level compared to that in sham group (Fig. 5, Table 2). This response was not reduced by IT minocycline injection, whether administration was pre-incision, post-incision, or both. IT minocycline 200 µg did not suppress the BDNF expression after plantar incision (Fig. 5, Table 2).

Fig. 5. Segmental upregulation of spinal BDNF after plantar incision. Representative images of BDNF immunostaining density in the L4-L5 spinal cord after plantar incision. Five non-adjacent squares (100 × 100 μm) in section from the L4-L5 segment were chosen within lamina I-II for measurement of the immunopositive area. Scale bar = 500 μm.

Table 1. OX-42 immunopositive area in dorsal horn (%)

	ipsilateral	contralateral
Sham	14.6 ± 0.7	14.3 ± 0.6
Saline	14.6 ± 0.8	14.3 ± 0.7
Pre (100 μg)	15.5 ± 1.4	15.4 ± 1.6
Post (100 μg)	14.7 ± 1.1	16.2 ± 1.4
Post (200 μg)	15.8 ± 1.1	16.2 ± 0.8
Pre (100 μg) + Post (100 μg)	15.9 ± 0.2	16.2 ± 0.9

The OX-42 immunopositive area is expressed as mean ± SEM (%) (n=6 rats per group). There were no differences between groups

Table 2. BDNF immunopositive area in dorsal horn (%)

	ipsilateral	contralateral
Sham	43.3 ± 2.1	41.0 ± 5.1
Saline	54.4 ± 5.8*†	40.0 ± 8.6
Pre (100 μg)	56.5 ± 6.4*†	44.4 ± 4.7
Post (100 μg)	54.5 ± 6.6*†	40.0 ± 7.7
Post (200 μg)	54.6 ± 3.3*†	41.0 ± 5.8
Pre (100 μg) + Post (100 μg)	56.9 ± 3.7*†	43.3 ± 2.3

The BDNF immunopositive area is expressed as mean ± SEM (%) (n=6 rats per group). *p<0.05 vs Sham, †p<0.05 vs the contralateral side

Discussion

Contrary to our initial hypothesis, intrathecal administration of minocycline had no substantial antinociceptive effects in a rat postoperative pain model. The major findings of this study are as follows: (1) one day after plantar incision, there was no significant increase in spinal microglia activity as indicated by stable OX-42 expression in both the presence and absence of IT minocycline; (2) plantar incision did induce BDNF overexpression in the ipsilateral spinal cord dorsal horn during the early postoperative period, but this response was not inhibited by IT minocycline; and (3) IT administration of minocycline reduced thermal hypersensitivity (as measured by increased withdrawal latency to heat stimulation) only for a short time (30 min) after injection and did not alter post-incision mechanical allodynia. These results suggest that spinal microglia do not participate in the development of early postoperative nociceptive threshold. Furthermore, although BDNF was regionally overexpressed in the ipsilateral dorsal horn following incision, this overexpression was not reduced by minocycline. Thus, while BDNF may contribute to the decrease in post-incisional nociceptive threshold, the major source is not spinal microglia.

The activation of microglia in the postoperative pain model are reported in previous studies. Marked upregulation of p38 phosphorylation was observed in spinal microglia as early as 1 h after plantar incision and IT administration of a p38 inhibitor attenuated incision-induced mechanical allodynia, while in contrast, no upregulation of the microglial surface marker OX-42 was observed in the spinal cord until 3 days after incision [10]. Ito et al. have also demonstrated microglial activation with OX-42 3days after plantar incision without inhibitory effects of minocycline on mechanical hypersensitivity [8]. Those results are inconsistent with ours in terms of the activation of microglia by surgical incision, however, in both studies, OX-42 immunostaining 1day after the incision was not detected in spinal cord like our result. Moreover, BDNF expression was not examined in both studies. Although it is unclear whether the inhibition of spinal microglia with a p38 inhibitor soon after the incision could attenuate BDNF production, a p38 inhibitor may modulate other pathways to exert anti-allodynic effects rather than that of BDNF synthesis. No efficacy of minocycline on the decrease in post-incisional nociceptive threshold 1 day after the incision in the present study along with the failure of inhibitory effects of minocycline on mechanical hypersensitivity 3 days after the incision in the study of Ito et. al. [8] indicates no substantial role of microglia in the intense pain in early post-incisional phase. In fact, the proliferation of microglia in spinal cord was not detected 1day after spinal nerve injury [14]. Furthermore, while changes in p38 phosphorylation and/or expression of ionized calcium-binding adapter 1 may identify microglia activation earlier than OX-42, a potent inhibitor of microglial activation (minocycline) still had no effect on post-incisional pain. The most parsimonious conclusion is that microglia does not contribute substantially to post-incisional pain for the first few days.

Plantar incision produced a significant increase in BDNF expression in the spinal cord together with decrease in nociceptive threshold to heat and mechanical stimulation. Minocycline did not change either BDNF overexpression or the threshold, suggesting that the early postoperative release of BDNF, which could be responsible for the decrease in nociceptive threshold, is mediate by other spinal cell types. Although microglia are the major resource of spinal BDNF in several pathologic pain conditions, it has been demonstrated that DRG cells and spinal astrocytes can also secrete BDNF, resulting in pain hypersensitivity. The injection of complete Freund's adjuvant into the rat hind paw increased BDNF mRNA levels in the DRG one day after injection [15]. In a joint loading pain model, painful joint distraction significantly increased BDNF expression in the DRG, and IT administration BDNF-sequestering molecule reduced pain hypersensitivity [16]. Moreover, both BDNF overexpression and mechanical pain hypersensitivity induced by spinal nerve ligation were attenuated by IT injection of the astrocyte activation inhibitor fluorocitrate [17]. In our postoperative pain model, BDNF was upregulated in ipsilateral large-sized DRG neurons and primary nerve terminals in the spinal cord 1 h after hind paw incision, a response accompanied by mechanical allodynia [3]. Intrathecal minocycline failed to suppress both the early postoperative pain hypersensitivity and BDNF overexpression in the spinal cord, possibly because most of the BDNF is secreted by DRG cells and /or astrocytes, cells more resistant to the metabolic effects of minocycline.

After peripheral nerve injury and inflammation by surgical incision, a variety of factors such as tumor necrosis factor- α (TNF- α), interleukin 6 (IL-6), and prostaglandins are released not only at the site of injury but in the spinal cord as well [18], and these factors have been shown to contribute to the development of postoperative pain. In addition to inhibiting microglia activation, minocycline is also known to have anti-inflammatory actions. Minocycline was reported to inhibit the production of TNF- α , IL-6 [19], and prostaglandin E2 [20] in lipopolysaccharide-treated cells. The transient antinociceptive effects of minocycline observed in the present study may be due to these inhibitory effects on inflammatory mediators before substantial microglial activation and BDNF overexpression in the spinal cord. Indeed, transient antinociceptive effects of minocycline have been demonstrated prior to microglial activation following formalin-induced inflammatory pain in rats [21]. Moreover,

minocycline is known to exert its effects not only on microglia but other cell types including neurons. For example, minocycline inhibited Na^+ currents in DRG neurons, resulting antinociceptive effects [22]. It is possible that the inhibitory effects of minocycline on DRG neurons could contribute the transient antinociceptive effects of minocycline observed in the present study.

There are some limitations to this study. First, the doses of minocycline used in the present study might be insufficient to inhibit the microglia, resulting the negative effects of minocycline on BDNF production and the decrease in nociceptive threshold. In our postoperative pain model, the activity of microglia by surgical incision could not be identified with OX-42. It is probable that the minocycline could not inhibit the microglia because it was not activated. In previous studies, IT minocycline did reveal inhibitory effects of microglia which was activated in cancer [6] and neuropathic [13] pain models with the same doses used in the present study, suggesting the dose of minocycline used in the present study could be enough if microglia was activated. However, further studies are necessary to resolve this uncertainty. Second, we investigated microglia activity and BDNF production at only one post-incisional time point, one day after incision. Examination of microglia activity and BDNF expression at several time points post-incision could clarify the contributions of these microglial responses to postoperative pain. However, immunohistological analysis was conducted when pain responses are known to be most intense. Third, microglial activation may be measured with greater sensitivity by p38 phosphorylation status or ionized calcium-binding adapter 1 expression. Nonetheless, the ineffectiveness of minocycline still suggests that spinal microglia does not contribute to early postoperative decrease in nociceptive threshold in rats.

In summary, the microglia activation was not identified in postoperative pain model and, therefore, IT minocycline did not inhibit BDNF production or the decrease in the nociceptive threshold in the early post-incisional period, suggesting that microglia play only a minor if any role in early post-incisional dorsal horn BDNF overexpression and the decrease in nociceptive threshold. An alternative approach may be necessary to alleviate early postoperative pain, for example, regulation of BDNF secretion from DRG cells and astrocytes.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to E.M. (No. 26463056) and K.K. (15K11304).

Disclosure Statement

The authors do not have any competing interests.

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