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Local infusion of nerve growth factor attenuates myelinated nerve fiber sprouting into lamina II of the spinal dorsal horn and reduces the increased responsiveness to mechanical stimuli in rats with chronic constriction nerve injury

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Abstract

Purpose. To clarify the relationship between allodynia and the sprouting of myelinated fibers, we examined whether the administration of nerve growth factor (NGF) affected the paw withdrawal response to non-noxious mechanical stimuli and the sprouting of myelinated fibers into lamina II of the spinal dorsal horn, using a chronic constriction injury model of the sciatic nerve.

Methods. Mechanical allodynia was determined as the threshold of the withdrawal response stimulated by von Frey filaments. Sprouting was examined using horseradish peroxidase conjugated to the B fragment of cholera toxin (B-HRP). NGF was continuously infused into the site of nerve injury for 14 days after nerve ligation.

Results. With vehicle infusion, significantly increased responsiveness to mechanical stimuli was observed on postoperative days (PODs) 5, 7, and 14 after ligation, compared with before surgery, and B-HRP-positive fibers were newly localized in lamina II on PODs 7 and 14. Infusion of NGF reduced the responsiveness to mechanical stimuli on 5, 7, and 14 PODs and B-HRP-positive fibers in lamina II on PODs 7 and 14.

Conclusion. We propose that the suppression of the increased responsiveness to mechanical stimuli produced by NGF could be related to the disappearance of B-HRP-positive fibers in lamina II.

Key words Chronic nerve constriction · Mechanical allodynia · Nerve growth factor · Sprouting of myelinated fiber · Neuropathic pain

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Introduction

In the dorsal horn of the spinal cord, somatic nociceptive afferents normally terminate in laminae I/II, whereas non-nociceptive fibers terminate in laminae III/IV. When peripheral nerve injury occurs, the central terminals of axotomized myelinated afferents, including the large Aβ fibers, sprout into lamina II of the spinal dorsal horn [1,2]. Such structural reorganization of the adult central nervous system may contribute to pain, such as allodynia, evoked by touch.

Nerve growth factor (NGF), a neurotrophic factor, is essential for the differentiation of neurons, as well as the survival of tyrosine kinase receptor-A (Trk-A)-expressing neurons such as sympathetic or thin sensory fibers [3]. NGF also regulates the production of neuropeptides, such as substance P and calcitonin gene-related peptide, in adult sensory neurons [4,5], and affects the connectivity of afferent neurons in the adult spinal cord [6]. In adult animals, it has been shown that NGF can be continuously synthesized in peripheral tissue and retrogradely transported to the cell bodies of sensory neurons [3]. Nerve injury disturbs the transport of NGF [7]. The decrease in NGF may evoke degeneration of neurons. According to a recent report [8], NGF suppresses the development of thermal hyperalgesia in a neuropathic pain model. However, in intact animals [9], NGF evokes thermal hyperalgesia. Thus, the functional role of the contribution of NGF to pain behavior also remains to be elucidated. Furthermore, there is little available information on the causal relationship between allodynia and the sprouting of myelinated fibers.

A chronic constriction nerve injury (CCI) model [10], with loose ligation of the sciatic nerve, produces increased responsiveness to mechanical stimuli. Thus, in contrast to the axotomy model, nociceptive and non-nociceptive stimuli are transmitted to the central ner-

vous system in this model. In the present study, using the CCI model in rats, we aimed to clarify the relationship between allodynia and the sprouting of myelinated fibers. We designed the study to determine whether the administration of NGF affected anatomical changes, such as the sprouting of myelinated fibers into lamina II of the spinal dorsal horn, and also whether the NGF affected the increased responsiveness to mechanical stimuli. Elucidation of the effects of NGF may be useful for the treatment of neuropathic pain.

Methods

Experimental protocol

The experiment was conducted in accordance with the guidelines of the International Association for the Study of Pain. In this study, surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of Miyazaki Medical College. Young adult male Sprague-Dawley rats, weighing 150–220 g at the beginning of the experimental procedure, were housed in a clear plastic cage at the Experimental Animal Center of Miyazaki Medical College. The rats were individually housed in a temperature- and humidity-controlled environment with a 12-h light-dark cycle, and permitted free access to food and water.

To assess the sprouting of myelinated fibers, we examined fibers positive for horseradish peroxidase conjugated to the B fragment of cholera toxin (B-HRP), which labels the fibers of myelinated afferents [1], in the dorsal horn in two groups of rats: those with loose ligation of the right sciatic nerve (ligation group) (examined on postoperative days [PODs] 5, 7, 14, 28, and 42) and in sham-operated rats without ligation (examined on POD 14). Three rats were examined at each time point.

To assess the effect of NGF, three groups of rats were prepared for a behavioral study for 42 days after surgery and histochemical studies at 7 and 14 days after surgery. In the ligation + vehicle group, the right sciatic nerve was loosely ligated and each animal received infusion of vehicle alone ($n = 12$ for assessment of pain-related behavior, and $n = 3$ each on PODs 7 and 14 for histochemistry). In the ligation + NGF group, the right sciatic nerve was loosely ligated and NGF was infused ($n = 12$ for assessment of pain behavior, and $n = 3$ each on PODs 7 and 14 for histochemistry). In the sham-operated group, the right sciatic nerve was exposed without ligation ($n = 3$ for assessment of pain behavior, and $n = 3$ each on PODs 7 and 14 for histochemistry).

Surgical procedures

The animals received an intraperitoneal injection of sodium pentobarbital at a dose of 50 mg per kg. In the ligation, ligation + vehicle and ligation + NGF groups, the right sciatic nerve was exposed at the level of the mid-thigh by blunt dissection and separated from adhering tissue in a region just proximal to its trifurcation. Four ligatures were placed around the sciatic nerve at intervals of 1–2 mm, using 4-0 chromic gut suture material according to the method of Bennett and Xie [10]. An osmotic minipump (delivering 0.5 μl per h for 14 days, Alzet Model; Alza, CA, USA) was implanted subcutaneously in the back, and was connected to a silicon tube. The other end of the tube (Silascon; Kaneka Medix, Osaka, Japan) was placed along the proximal side of the right sciatic nerve ligation site. Human recombinant β nerve growth factor (NGF-β) was purchased from Sigma Chemical (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS; pH 7.4) containing 1% bovine serum albumin. In the ligation + NGF group, NGF-β was infused for 14 days at 1 μg per day into the ligation site via the silicon tube. In the ligation + vehicle group, the same amount of PBS, containing 1% bovine serum albumin, was infused at the same rate. Finally, the incision was closed and sutured in layers, and the rats were allowed to recover from anesthesia before being housed.

von Frey filaments test

The withdrawal response to mechanical stimuli was used to test for the presence of abnormal pain-related behavior following nerve injury. The test was performed before ligation and on PODs 5, 7, 14, 28, and 42 in the ligation + vehicle, the ligation + NGF, and the sham-operated groups. The mechanical threshold was determined by measuring the paw withdrawal response after the skin was touched with a series of calibrated Semmes-Weinstein set of von Frey filaments (Stoelting, Wood Dale, IL, USA). The test was performed after the rat was placed on a raised platform in a plexiglas box with a mesh floor to permit touching of the ventral surface of the hindpaws with von Frey filaments. von Frey filaments, ranging from 0.08–76 g, were applied to produce stimulation of variable intensity. The test was repeated five times at 10- to 20-s intervals on the right paw, and the withdrawal threshold was determined by increasing the stimulus strength until paw withdrawal occurred. The lowest filament in the series that evoked at least one response was assigned as the threshold. This procedure was performed according to the method described by Tal and Bennett [11], in which there is negligible stress on animals. The mechanical thresholds in the three groups were compared.

Within each group, the results of repeated measurements were analyzed by analysis of variance for repeated measures, followed, where appropriate, by Fisher's test. Comparisons among the three groups were

made by using one way analysis of variance, followed by Fisher's test. Data values are presented as means ± SEM. *P* values less than 0.05 were considered statistically significant.

Enzyme histochemistry

Animals used for histochemical staining of myelinated fibers were anesthetized with an intraperitoneal injection of sodium pentobarbital at a dose of 50 mg/kg, and the ligated sciatic nerve was exposed under a light microscope. Two μl of 1.5% B-HRP (List Biological Laboratories, Campbell, CA, USA) entrapped in a 5% polyacrylamide gel solution was injected at adequate pressure into the nerve proximal to the site of ligation with a fine glass micropipette connected to an electric microinjector (Model IM-30; Narishige, Tokyo, Japan) on PODs 5, 7, 14, 28, and 42 in the ligation group, and on PODs 7 and 14 in the ligation + vehicle and ligation + NGF groups. After injection, the incisional wound was closed and sutured.

The rats were re-anesthetized deeply with sodium pentobarbital 48–72 h after the injection of B-HRP, and perfused transcardially with 300 ml of heparinized saline, followed by 500 ml of 1% paraformaldehyde and 1.25% glutaraldehyde, in 0.1 mol per l phosphate buffer for 30 min. The lumbar spinal cord was removed en bloc and then cryopreserved in 20% phosphate-buffered sucrose solution for at least 12 h. Transverse frozen sections (50 μm) from the L3-5 segments were serially collected in PBS. Alternate sections were stained immediately for B-HRP according to the tetramethyl benzidine protocol of Mesulam [12]. Sections were mounted on gelatin-coated glass slides and coverslipped after penetration with xylene. The sections were evaluated under bright-field microscopy.

Results

The withdrawal threshold to the force applied to the hindpaws, using von Frey filaments before surgery, was similar in the three groups (Fig. 1). There were no significant differences in withdrawal responses among groups. After surgery, the withdrawal thresholds of the ligation + vehicle group were significantly lower than those of the sham-operated group on PODs 5, 7, and 14. The withdrawal thresholds of the ligation + NGF group on PODs 5, 7, and 14 were significantly higher than those of the ligation + vehicle group. However, no significant differences were found among these groups on PODs 28 and 42. The withdrawal thresholds of the ligation + vehicle group decreased significantly until 14 days after surgery, and then increased slightly, compared with control values before surgery. There were no significant differences between the sham-operated and the ligation + NGF groups during the course of this experiment.

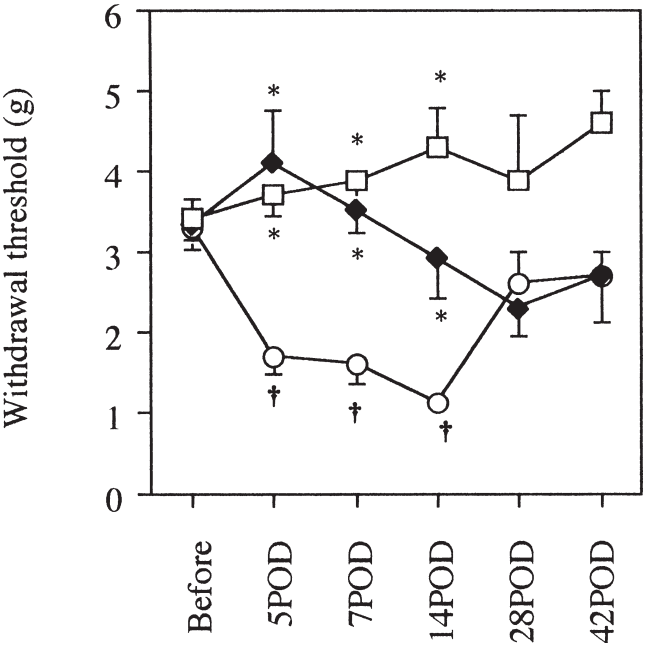

Fig. 1. Changes in the mean threshold of the paw withdrawal response with von Frey filaments in the three groups: ligation + vehicle group (*circles*), ligation + nerve growth factor (NGF) group (*diamonds*), and sham-operated group (*squares*). Data values are presented as means ± SEM. $*P < 0.05$ compared with the ligation + vehicle group; $^{\dagger}P < 0.05$ compared with control values before surgery. *POD*, Postoperative day

For the histochemical studies, representative results from one animal in each group are shown in Figs. 2 and 3. B-HRP-positive fibers in the central terminals of the sciatic nerve were apparent in laminae I and III/IV, but absent in lamina II of the spinal dorsal horn in the sham-operated (no ligation) group on PODs 7 and 14 (Figs. 2A and 3E, 3F). As shown in Fig. 2, in the ligation group, however, B-HRP-positive fibers were observed in lamina II, as in laminae III/IV, on PODs 5, 7, and 14 (Fig. 2B,C,D). On POD 42, B-HRP-positive fibers had disappeared in the medial dorsal horn, but were still present in the lateral dorsal horn in the ligation group. Furthermore, B-HRP-positive fibers were more dense in the lateral part on POD 42 than in the medial part on PODs 7 and 14. As compared with the ligation + vehicle group on PODs 7 and 14 (Fig. 3A,B), B-HRP-positive-fibers in lamina II were reduced in density in the ligation + NGF group (Fig. 3C,D).

Discussion

Using the CCI model in rats, we demonstrated that the withdrawal threshold to a non-nociceptive mechanical

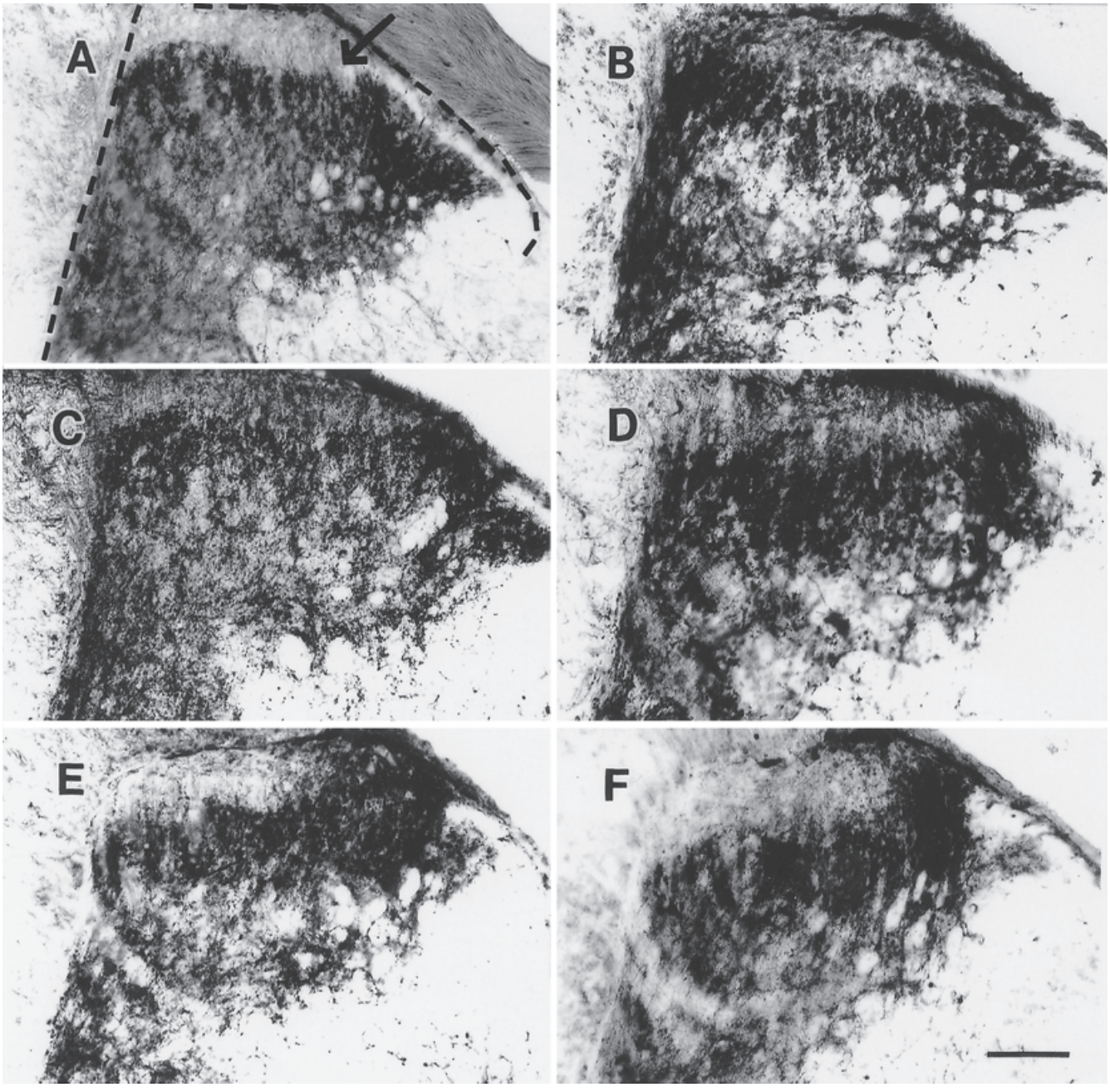

Fig. 2A–F. Bright-field photomicrographs of 50-μm transverse sections of the spinal dorsal horn, showing fibers positive for horseradish peroxidase conjugated to the B fragment of cholena toxin (B-HRP) in the central terminals of the sciatic nerve. Lamina II in the sham-operated rat was apparently devoid of B-HRP-positive fibers (**A**). In the rat with chronic constriction nerve injury (CCI), B-HRP-positive fibers were localized in lamina II of the right dorsal horn on POD 5 (**B**), POD 7 (**C**), POD 14 (**D**), POD 28 (**E**), and POD 42 (**F**). *Dotted lines* represent boundaries of the dorsal horn (**A**). *Scale bar*, 100 μm

stimulus decreased significantly in the animals infused with vehicle solution. The response began on POD 5 and the maximum was reached on POD 14. The reduction of the threshold indicated the presence of mechanical allodynia. Interestingly, the reduced threshold was recovered by continuous local infusion of NGF, suggesting that the administration of NGF attenuated the development of allodynia in this model. Furthermore, in this study using B-HRP staining, there were large numbers of B-HRP-positive fibers in lamina II of the spinal dorsal horn in the CCI rats, and continuous local infusion of NGF reduced the density of B-HRP-positive fibers in lamina II on PODs 7 and 14. These results suggested that the suppression of the increased respon-

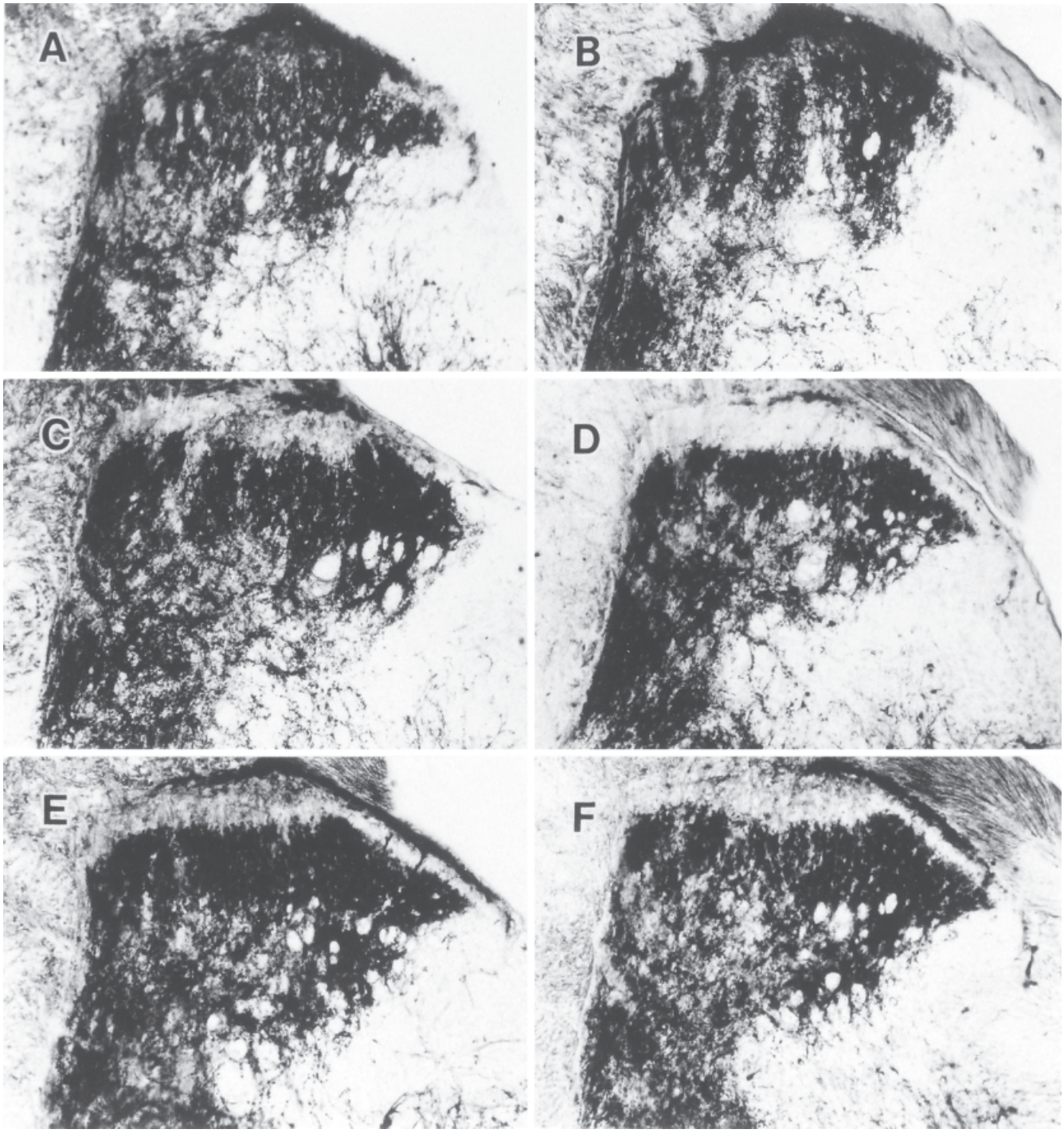

Fig. 3A–F. Bright-field photomicrographs of 50-μm transverse sections of the spinal dorsal horn showing B-HRP-positive fibers in the central terminals of the sciatic nerve. B-HRP-positive fibers were observed on POD 7 (**A**) and POD 14 (**B**) in the ligation + vehicle group, but were reduced in density on POD 7 (**C**) and POD 14 (**D**) in the ligation + NGF group; and these fibers were not observed on POD 7 (**E**) and POD 14 (**F**) in the sham-operated group. *Scale bar*, 100 μm

siveness to mechanical stimuli produced by the administration of NGF could be related to the disappearance of B-HRP-positive fibers in lamina II.

As compared with the sprouting and pain-related behavior in the CCI rats, the period of reduction of the withdrawal threshold from PODs 5 to 14 (the early stage) coincided with the appearance of B-HRP-positive fibers in lamina II. Although B-HRP-positive fibers were found in the lateral part of lamina II in the late stage (PODs 28 and 42), this period was coincident

with recovery of the withdrawal threshold. The medial part of lamina II in the spinal dorsal horn in the fourth and fifth lumbar segments represents the projected region of the sciatic nerve that innervates the plantar aspect of the paw. Thus, sprouting to the medial part of lamina II could be related to the mechanical allodynia of the plantar aspect of the paw in the CCI rats, although we could not demonstrate a direct relationship between the mechanical allodynia and the sprouting of myelinated fibers.

Based on these results, NGF was administered immediately after ligation, continuously, and to the site of nerve injury. The administration of NGF at the site of nerve injury attenuated the increased responsiveness to mechanical stimuli. Physiologically, nerve injury interrupts the retrograde transport of NGF and induces central hyperexcitability caused by the loss of endogenous neurotrophic signals [13]. C-fibers that express Trk-A upregulate brain-derived neurotrophic factor (BDNF) after nerve injury [14], and BDNF is transported to the central terminal of C-fibers in the superficial layer of the spinal dorsal horn. Reduction of the NGF level in the dorsal root ganglion after nerve injury decreases Trk-A, but cannot fully trigger a signal transduction cascade [13]. The administration of NGF at the site of nerve injury provides a substitute source of NGF, and may facilitate the recovery of Trk-A receptors [13,15] and the function of C-fibers. The local administration of NGF to the damaged nerve would play the same role as endogenous NGF. As mentioned above, in this study, infusion of NGF in the early stage of nerve injury was effective in preventing the sprouting of myelinated fibers. In axotomy model rats, the administration of NGF has been shown to prevent the sprouting of myelinated fibers into lamina II [16]. In our study, NGF was injected at the site of nerve injury, although in a previous study, it was administered intrathecally [16]. Compared with intrathecal or systemic administration, local administration would be easier and preferable in clinical practice.

B-HRP-positive fibers originate mainly from Aβ fibers [1,2,17]. It has been reported that the small dorsal root ganglion neurons are labelled for the B fragment of cholera toxin; that is, unmyelinated cells may take up the B fragment of cholera toxin [18]. In contrast, there are no significant differences in the cell size distribution of neurons labelled for B-HRP among intact models, axotomy models, and axotomy models with administration of NGF [16].

In conclusion, we showed that the continuous infusion of NGF at the site of sciatic nerve constriction attenuated pain-related behavior and prevented the sprouting of myelinated nerve fibers in the dorsal horn of the spinal cord. Our results support the hypothesis that such sprouting fibers play an important role in the development and/or maintenance of neuropathic pain.

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