Effects of decompression on neuropathic pain behaviors and skin reinnervation in chronic constriction injury

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Abstract

Decompression is an important therapeutic strategy to relieve neuropathic pain clinically; there is, however, lack of animal models to study its temporal course of neuropathic pain behaviors and its influence on nerve regeneration to sensory targets. To address these issues, we established a model of decompression on rats with chronic constriction injury (CCI) and investigated the effect on skin reinnervation. Animals were divided into a decompression group, in which the ligatures were removed, and a CCI group, in which the ligatures remained at postoperative week 4 (POW 4). At this time point, the skin innervation indexes of protein gene product 9.5 (PGP 9.5), substance P (SP), and calcitonin gene-related peptide (CGRP) were reduced in both groups to similar degrees. Beginning from POW 6, the decompression group exhibited significant reductions of thermal hyperalgesia and mechanical allodynia compared to the CCI group (p<0.001). At POW 8, neuropathic pain behaviors had completely disappeared in the decompression group, and the decompression group had a higher skin innervation index of SP than the CCI group (0.45±0.05 vs. 0.16±0.03, p<0.001). These indexes were similar in both groups for PGP 9.5 (0.32±0.09 vs. 0.14±0.04, p=0.11) and CGRP (0.38±0.06 vs. 0.21±0.07, p=0.09). These findings demonstrate the temporal changes in the disappearance of neuropathic pain behaviors after decompression and suggest that decompression causes different patterns of skin reinnervation for different markers of skin innervation.

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Introduction

Nerve injury from compression is the foundation for establishing animal models of neuropathic pain, including chronic constriction injury (CCI), partial sciatic nerve ligation, and spinal nerve ligation (Bennett and Xie, 1988; Kim and Chung, 1992; Seltzer et al., 1990). Surgical decompression is frequently used in clinical practice to relieve symptoms of neuropathic pain, e.g., carpal tunnel syndrome (Steinberg, 2002; Thoma et al., 2004). Theoretically, several potential mechanisms underlie the disappearance or reduction of neuropathic pain after surgical decompression; these include regeneration of nerve fibers to reestablish contacts with the targets of cutaneous nerves, changes in the local environment of the previously injured nerves, and synaptic reorganization in the central nervous system, particularly, the dorsal horn of the spinal cord (George et al., 2000; Suzuki and Dickenson, 2005; Woolf et al., 1998; Woolf, 2000; Woolf, 2004; Woolf and Salter, 2006).

The assessment of skin innervation by examining sensory nerve terminals in the epidermis is a well-established approach for investigating the integrity of nociceptive nerves in both humans and animals (Hsieh et al., 2000; Kennedy, 2004; McCarthy et al., 1995). Several groups including our own have previously demonstrated that partial denervation of the skin in the territory of the injured nerve is a prerequisite for establishing animal models of neuropathic pain and painful neuropathies in humans (Chiang et al., 2005; Lin et al., 2001; Lindenlaub and Sommer, 2002; Ma and Bisby, 2000; Periquet et al., 1999). Traditionally, skin innervation is evaluated by immunohisto-
chemistry with a general neuronal marker, protein gene product 9.5 (PGP 9.5) (Kennedy, 2004; McCarthy et al., 1995). Some epidermal nerves are also immunoreactive for sensory peptides: calcitonin gene-related peptide (CGRP) and substance P (SP) (Chiang et al., 2005; Ma and Bisby, 2000). Epidermal nerves of PGP 9.5 are the most abundant ones compared with those of other phenotypes (Chiang et al., 2005). It is not clear whether the reduction of epidermal nerves is different among different markers in neuropathic pain. These findings also raise several issues regarding the relationship between neuropathic pain and skin reinnervation. For example, does the epidermis become fully reinnervated upon decompression, does the skin reinnervation parallel the disappearance of neuropathic pain, are the patterns of skin reinnervation similar for epidermal nerves of different phenotypes after decompression, and which phenotype of epidermal nerves reflects the states of neuropathic pain?

To address these issues, we established a model of decompression by removing all ligatures of CCI 4 weeks after neuropathic pain behaviors had well developed. Specifically, we investigated the temporal changes in neuropathic pain behaviors and the pattern of skin reinnervation after decompression.

Materials and methods

Study design and surgical procedures

Adult male Sprague–Dawley rats, weighing 250–300 g, were used in these experiments. Three animals were housed together in plastic cages with soft sawdust as bedding to avoid mechanical damage to the hindpaw skin. These animals were placed in a temperature- and humidity-controlled room with a 12-h light/dark cycle. Food and water were available ad libitum. All procedures were conducted in accordance with ethical guidelines set up by the International Association for the Study of Pain (IASP) on the use of laboratory animals in experimental research (IASP Committee, 1980; Zimmermann, 1983).

CCI was induced in animals following established surgical procedures (Bennett and Xie, 1988; Lin et al., 2001). Briefly, under chloral hydrate anesthesia (400 mg/kg, i.p., Sigma, St. Louis, MO), the right sciatic nerve was exposed at the mid-thigh level by freeing the adhering fascia between the gluteus and biceps femoris muscles. Four ligatures (of 4/0 chromic gut) were loosely tied around the sciatic nerve at 1-mm intervals above the nerve’s trifurcation. Ligatures constricted only about 1/3–1/4 of the diameter of the nerve and produced a small, brief twitch in the muscle around the exposure. The circulation through the superficial epineural vasculature was blocked between the ligatures. This side was defined as the operated side; the contralateral side was used for comparison to normalize individual variations of different animals.

To examine the effect of decompression on neuropathic pain and skin reinnervation, animals were randomly assigned to two groups. In one group, all four ligatures were carefully removed without destroying the surrounding vessels at postoperative week 4 (POW 4); this group was designated the decompression group hereafter. The other group was designated the CCI group, in which ligatures remained throughout the experimental period. Examiners were blinded to the grouping information, and this information was only decoded during the final analyses.

Thermal hyperalgesia

We evaluated thermal hyperalgesia with a Hargreaves-type algalesiometer (Ugo Basile, Comerio–Varese, Italy) by measuring the paw withdrawal latency upon heat stimulation. Rats were individually placed in one of three separate Plexiglas containers (22 × 17 × 14 cm) located on an elevated floor of a clear glass plate (3 mm thick) and allowed 30 min to habituate to the apparatus. A radiant heat source was placed directly beneath the plantar surface of the hindpaw. The withdrawal latency was automatically measured as the time elapsed from the onset of radiant heat stimulation to the withdrawal of the hindpaw. A maximal time of 20 s for the thermal stimulus was imposed to avoid possible tissue damage. Each hindpaw was alternatively tested seven times with a minimal interval of 5 min between measurements, and readings were recorded to the nearest 0.1 s. Values of the last five consecutive measurements were used for the analysis (Chiang et al., 2005).

Mechanical alldynia

Mechanosensitivity was determined by measuring the withdrawal thresholds to a series of calibrated von Frey filaments (Somedic, Sweden) according to the up-and-down method (Chiang et al., 2005). Rats were individually placed in one of three separate Plexiglas containers on a wire mesh floor and allowed to acclimate for 10 min. The examiner touched the plantar surface of the hindpaw with a filament until a brisk withdrawal or paw flinching was noted, which was considered a positive response. Five stimuli using the selected hair were applied at 5-s intervals. If there was no withdrawal response to the initially selected hair with these five stimuli, a stronger stimulus was applied. If the animal withdrew its hindpaw in response to any of the five stimuli, the next weaker stimulus was chosen. The mechanical threshold was defined as the minimal force (g) initiating a withdrawal response.

Immunohistochemistry of footpads

Animals were sacrificed by an intracardiac perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. Footpads were fixed for another 6 h and then changed to 0.1 M PB for storage. After a thorough rinsing in PB, samples were cryoprotected with 30% sucrose in 0.1 M PB overnight. Sections of 30 μm perpendicular to the dermis were cut on a sliding microtome, labeled sequentially, and stored at −20 °C. To ensure adequate and systematic sampling, every fourth section of the skin was immunostained (Lin et al., 2001). Sections were treated with 0.5% Triton X-100 in 0.5 M Tris buffer (Tris), pH 7.6, for 30 min and processed for immunostaining. Briefly, sections were quenched with 1% H2O2 in methanol and blocked with 5% normal goat serum in 0.5% nonfat dry milk/Tris. Sections were incubated with primary antiserum overnight. These included protein gene product 9.5 (PGP 9.5, 1:1000, UltraClone, Isle of
Wight, UK), calcitonin gene-related peptide (CGRP, 1:1000, Chemicon, Temecula, CA), and substance P (SP, 1:1000, DiaSorin, Stillwater, MN). After rinsing in Tris, sections were incubated with biotinylated goat antirabbit IgG for 1 h and the avidin–biotin complex (Vector, Burlingame, CA) for another hour. Reaction products were demonstrated with 3, 3’-diaminobenzidine (Sigma, St. Louis, MO).

Quantitation of cutaneous innervation

Epidermal innervation was quantified following the protocols in a coded fashion (Ko et al., 2002). Every fourth section of each tissue was quantified. Nerve fibers immunoreactive for PGP 9.5, SP, and the CGRP in the epidermis of each footpad were counted at a magnification of 400× with an Olympus BX40 microscope (Olympus, Tokyo, Japan). Each individual nerve with branching points after crossing the lower margin of the epidermis was counted as a single nerve. Epidermal nerves splitting below the lower margin of the epidermis were counted as two nerves. The total length of the epidermis along the upper margin of the stratum corneum in each footpad was measured using SigmaScan (SPSS, Chicago, IL). The length of each footpad on the section was 1.5–2 mm, and there were 6–8 footpad sections for each animal with the total length of 1.0–1.5 cm. Epidermal nerves in all these footpads were counted and summed. Epidermal nerve density (END) was therefore derived and expressed as the number of fibers per centimeter of epidermal length. Epidermal innervation was also expressed by the ratio of END (operated side/contralateral side) as the skin innervation index.

Experimental design and statistical analysis

Thermal hyperalgesia and mechanical allodynia were evaluated at the following time points: pre-test of baseline data before CCI (designated POW 0), POW 1, POW 2, POW 4, POW 6, and POW 8. At POW 4, animals were randomly assigned to a decompression group and a CCI group. The examiners were blinded to the grouping information when performing the evaluation and experiments. The grouping information was only decoded when the analyses were completed. Three animals in each group were sacrificed at POW 0, POW 1, POW 2, and POW 4, and five animals in each group were sacrificed at POW 6 and POW 8; behavioral tests were performed in all these animals. All procedures of measurement and quantification were performed in a blinded fashion. Behavioral and morphometric data are presented as the mean±S.E.M. using SPSS for Windows (SPSS) and GraphPad Prism (GraphPad, San Diego, CA). For statistical analysis of the values obtained from the behavioral testing over the experimental period, repeated-measures ANOVA followed by Bonferroni’s post hoc test was used. *p<0.05 was considered significant.

Results

Thermal hyperalgesia

Animals developed typical neuropathic pain behaviors within 1 week after CCI, such as evoking and clenching the hindpaw on the operated hind limb, and sudden licking of the operated hindpaw.

These neuropathic pain behaviors accompanied similar degrees of thermal hyperalgesia (Fig. 1) and mechanical allodynia (Fig. 2) in both the CCI and decompression groups until POW 4. Before CCI, both groups had similar withdrawal latencies to the stimulation of noxious heat on the operated side (8.08±0.23 s in the decompression group, 8.26±0.30 s in the CCI group, p=0.62, Fig. 1). Between POW 1 and POW 4, both groups showed significant reductions of withdrawal latencies, for example, 6.78±0.19 s vs. 6.56±0.26 s, p=0.90 at POW 4.

At POW 4, the ligatures were removed in the decompression group after a behavioral test was conducted. At POW 6, the withdrawal latencies were longer in the decompression group compared to the CCI group (8.01±0.17 s vs. 6.63±0.22 s, p=0.001); this phenomenon still persisted at POW 8 (8.26±0.21 s vs. 6.51±0.22 s, p<0.001).

Mechanical allodynia

Mechanical allodynia also disappeared after decompression with similar temporal changes as those for thermal hyperalgesia (Fig. 2). Before CCI, mechanical thresholds were similar in both groups (17.17±1.71 g in the decompression group and 17.58±2.40 g in the CCI group, p=0.89, Fig. 2). Through POW 1 and POW 4, both groups had reduced mechanical thresholds of similar degrees, e.g., 4.74±0.66 g in the decompression group, and 3.93±0.56 g in the CCI group, p=0.38 at POW 4. After decompression at POW 4, there was a return toward normal mechanical thresholds in the decompression group compared to the CCI group at POW 6 (16.42±1.89 g vs. 5.82±0.72 g, p<0.001). This trend still persisted at POW 8 (17.47±2.56 g vs. 4.87±0.91 g, p=0.002).

![Fig. 1](image-url) Effect of decompression on thermal hyperalgesia. The graph shows temporal changes in thermal hyperalgesia after chronic constriction injury (CCI) and the effect of decompression. Data are presented as the withdrawal latency to the stimulation of noxious heat on the operated side. CCI, the group with ligatures remaining in situ throughout the entire experimental period; Decompression, the group with ligatures removed at postoperative week (POW) 4; *p<0.05.
To understand the effects of CCI and decompression on the cutaneous innervation, we evaluated small-diameter sensory nerve terminals in the skin by immunostaining the skin of the hindpaw (Fig. 3). On the contralateral side, abundant PGP 9.5 (+) fibers originated from the subepidermal nerve plexus, penetrated through the epidermal–dermal junction, and ascended perpendicularly with typical varicosities in the epidermis (Figs. 3A, C, E). Epidermal nerves were reduced after CCI in both the decompression and CCI groups starting from POW 1 to POW 4 (Fig. 3B). At POW 8, there appeared a tendency of increasing PGP 9.5(+) epidermal nerves compared to the skin innervation patterns on the operated side of POW 4 (Figs. 3D, F). These observations, however, required quantitative comparison as described below.

Immunoreactive epidermal nerves on the footpad were quantified as END of PGP 9.5 (Fig. 4A), and also expressed as the skin innervation index of each marker, i.e., the ratio of END (operated side/contralateral side, Fig. 4B). Because the patterns of changes in both END and skin innervation index were the same, only the data of the skin innervation indexes are described here.

Before CCI, the skin innervation indexes for PGP 9.5 were similar in both groups at POW 0 (1.05±0.06 in the CCI group vs. 1.00±0.04 in the decompression group, p=0.52). At POW 1, the skin innervation indexes for PGP 9.5 were significantly reduced after CCI (0.30±0.10 in the CCI group vs. 0.32±0.10 in the decompression group, p=0.87). Skin innervation indexes for PGP 9.5 continued to decline in both groups at POW 2 (0.18±0.03 in the CCI group vs. 0.14±0.02 in the...
decompression group, \( p=0.36 \) and POW 4 (0.16±0.10 in the CCI group vs. 0.13±0.05 in the decompression group, \( p=0.80 \)). Decompression was performed at POW 4. There were trends of an increasing skin innervation index for PGP 9.5 in the decompression group compared to the CCI group at POW 6 and POW 8, but the extent did not reach statistical significance (0.11±0.04 vs. 0.03±0.01, \( p=0.11 \) for POW 6; 0.32±0.09 vs. 0.14±0.04, \( p=0.11 \) for POW 8).

Changes in SP (+) nerves followed a different temporal pattern from PGP 9.5 (+) nerves after decompression (Fig. 5). SP (+) epidermal nerves were reduced after CCI (Figs. 5A, B), but the denervation of SP (+) fibers was reversed after decompression.

Fig. 4. Quantitative analysis of the effects of chronic constriction injury (CCI) and decompression on skin innervation. At various time points, the degree of skin innervation was quantified based on the immunostained sections as described in Figs. 3, 5, and 6. The graphs show comparisons of immunoreactive epidermal nerve densities (ENDs) for protein gene product 9.5 (PGP 9.5, in A and B), substance P (SP, in C and D), and calcitonin gene-related peptide (CGRP, in E and F). (A, C, E) Data are expressed as the END of the CCI group and the decompression group respectively. (B, D, F) The graphs show data as the skin innervation index, i.e., the ratio of END (O/C, operated side/contralateral side). CCI, the group with ligatures remaining in situ throughout the entire experimental period; Decompression, the group with ligatures removed at postoperative week (POW) 4; *\( p<0.05 \). Solid lines indicate the mean skin innervation index of the contralateral side, while dashed lines designate the range of skin innervation indexes of the contralateral side.
sion at POW 8 (Fig. 5F). These changes were confirmed by quantitative analysis of the ENDS of SP fibers (Fig. 4C) and skin innervation indexes of SP fibers (Fig. 4D). At POW 0, the END ratios for SP were similar in both groups (1.01±0.04 in the CCI group vs. 0.94±0.08 in the decompression group, p=0.45). Skin innervation indexes for SP were significantly reduced at POW 1 after CCI (0.24±0.02 in the CCI group vs. 0.29±0.11 in the decompression group, p=0.67). There were continuous reductions in skin innervation indexes for SP at POW 2 (0.16±0.10 in the CCI group vs. 0.11±0.03 in the decompression group, p=0.66) and POW 4 (0.10±0.10 in the CCI group vs. 0.07±0.07 in the decompression group, p=0.80). After decompression, the skin innervation index for SP was higher in the decompression group compared to the CCI group starting from POW 6 (0.21±0.05 vs. 0.02±0.02, p=0.005); the tendency continued at POW 8 (0.45±0.05 vs. 0.16±0.03, p<0.001).

CGRP (+) epidermal nerves showed similar qualitative (Fig. 6) and quantitative (Figs. 4E, F) patterns to PGP 9.5 (+) epidermal nerves, i.e., reduced skin innervation after CCI, but with similar reinnervation between both groups. At POW 0, the skin innervation indexes for CGRP were similar (1.02±0.08 in the CCI group vs. 1.02±0.11 in the decompression group, p=0.99). After CCI, there were similar and significant degrees of decreases in skin innervation indexes for the CGRP at POW 1 (0.24±0.08 in the CCI group vs. 0.29±0.11 in the decompression group, p=0.87). Skin innervation indexes for the CGRP continued to decrease at POW 2 (0.13±0.03 in the CCI group vs. 0.10±0.03 in the decompression group, p=0.53) and POW 4 (0.10±0.06 in the CCI group vs. 0.12±0.03 in the decompression group, p=0.81). After decompression at POW 4, the skin innervation index for the CGRP was similar between the two groups at POW 6 (0.08±0.05 vs. 0.04±0.02, p=0.45) and POW 8 (0.38±0.06 vs. 0.21±0.07, p=0.09).

Discussion

The current report established an animal model of decompression to investigate its effects on neuropathic pain and skin reinnervation. Although decompression is an important surgical procedure to relieve neuropathic pain clinically (Steinberg, 2002; Thoma et al., 2004), the temporal course of its pain-relieving effect remains elusive. Presumably a full reconnection between sensory nerves and their targets is the major mechanism responsible for the disappearance of symptoms. Our current study suggests that the disappearance of neuropathic pain precedes the full reinnervation of the skin.

Skin innervation in painful neuropathy

Partial denervation is the major principle explaining the generation of neuropathic pain in animals (Bennett and Xie, 1988; Kim and Chung, 1992; Seltzer et al., 1990; Woolf, 2000);
this phenomenon is best demonstrated in the epidermis in the territory of a partially injured nerve. Epidermal nerves are reduced in animal models of neuropathic pain during the acute stage (Chiang et al., 2005; Lindenlaub and Sommer, 2002; Ma and Bisby, 2000). There is circumstantial evidence implying that the previously denervated epidermis is not fully reinnervated in the late stage of CCI (Lindenlaub and Sommer, 2002). The present report fully explored this issue by examining the epidermis 2 months after CCI, after ligatures had been removed and animals were completely free of neuropathic pain behaviors. Reinnervation of the epidermis was incomplete even in the decompression group at POW 8. Several mechanisms might account for these findings. First, partial reinnervation of the epidermis of 32–45% of the compression level might be sufficient for proper reestablishment of the correct contacts between sensory nerves and their targets, and therefore this would reverse the changes in neuropathic pain behaviors. Second, changes in antinociceptive molecules and receptors, such as dynorphin and cannabinoid receptors, may alter the transmission of nociceptive impulses in denervated and reinnervated epidermis, thus affecting neuropathic pain behaviors (Ibrahim et al., 2005; Wotherspoon et al., 2005). Alternatively, the central processes of injured dorsal root ganglion neurons may change their synaptic organization in the dorsal horn, which would further modulate nociceptive transmissions.

Two intriguing issues are related to the application of skin biopsies to investigate human painful neuropathy (Kennedy and Said, 1999). First, in neuropathic pain epidermal of different phenotypes are reduced to a similar degree at the peak of CCI at POW 4. Second, the degree of skin reinnervation is different among these markers upon decompression compared to the CCI groups, and only SP-epidermal nerves achieved significant level of difference. SP is a sensory neuropeptide responsible for nociception; the significance of its paradoxical reversal in the dorsal horn after decompression is obscure. A possible explanation involves parallel changes in the delta opioid receptor. The delta opioid receptor is colocalized with SP in dorsal root ganglion neurons, and its trafficking is linked to activation of SP neurons (Guan et al., 2005; Julius and Basbaum, 2005).

Several studies have indicated that skin innervation was reduced in painful neuropathy (Holland et al., 1997; Kennedy and Said, 1999; Periquet et al., 1999). However, the degree of skin denervation apparently did not parallel the neuropathic pain states (Herrmann et al., 2006; Lee et al., 2005; Pan et al., 2003; Shun et al., 2004; Sorensen et al., 2006). The current study explores the influence of decompression on epidermal nerves of different phenotypes. Certainly, further studies are required to investigate whether epidermal nerves of SP or other phenotypes are responsible for neuropathic pain behaviors.

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Fig. 6. Effect of decompression on skin innervation of calcitonin gene-related peptide-immunoreactive nerves. Footpads of the hindpaw were immunostained with markers of sensory nerve terminals, calcitonin gene-related peptide. CCI, the group with ligatures remaining in situ throughout the entire experimental period; Decompression, the group with ligatures removed at postoperative week (POW) 4 (Scale bar=70 μm).
Potential mechanisms for skin reinnervation after decompression

The present report suggests that skin innervation was increased within 2 weeks of decompression. Several possibilities may explain the potential mechanisms of skin reinnervation after decompression including altered SP expression, regeneration of SP (+) epidermal fibers from the previously constricted nerves, and collateral sprouting from the neighboring nerves. Previous studies have indicated that the expression of SP in small-diameter dorsal root ganglia neurons, whose peripheral processes terminates in the skin, was downregulated after nerve injury (Malcangio et al., 2000; Noguchi et al., 1995; Weissner et al., 2006). Thus the reversal of SP expression by decompression is most likely responsible for the increased skin innervation index of SP 2 weeks after decompression. Because significant degeneration of nerve fibers after CCI was documented in previous studies (Lin et al., 2001; Tamura et al., 1998), the likelihood of additional nerve regeneration, however, could not be completely excluded at a lateral phase of decompression. In contrast, the possibility of collateral sprouting is low for two reasons. First, skin innervation index continued to decline within 4 weeks of CCI, when collateral sprouting should have already started. Second, several studies have suggested that collateral sprouting, which depends on nerve growth factor, was insufficient in restoration of skin innervation after nerve transection (Diamond et al., 1992; Hsieh et al., 2000). Certainly, these possibilities require further studies. In conclusion, this study establishes an animal model of decompression for neuropathic pain and provides a new system to investigate skin reinnervation and neuropathic pain.

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References


