

## Research article

## NGF and BDNF expression in mouse DRG after spared nerve injury

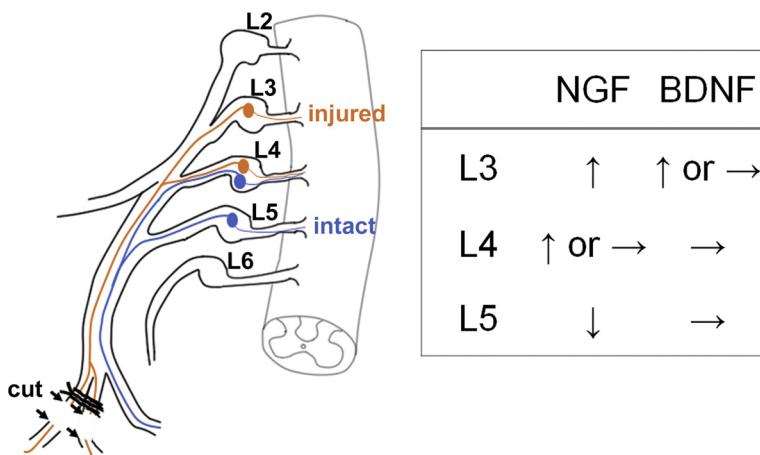
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## GRAPHICAL ABSTRACT



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## ABSTRACT

Neuropathic pain is initiated by a primary lesion in the peripheral nervous system and spoils quality of life. Neurotrophins play important roles in the development and transmission of neuropathic pain. There are conflicting reports that the dorsal root ganglion (DRG) in an injured nerve contribute to neuropathic pain, whereas several studies have highlighted the important contribution of the DRG in a non-injured nerve. Clarifying the role of neurotrophins in neuropathic pain is problematic because we cannot distinguish injured and intact neurons in most peripheral nerve injury models. In the present study, to elicit neuropathic pain, we used the spared nerve injury (SNI) model, in which injured DRG neurons are distinguishable from intact ones, and mechanical allodynia develops in the intact sural nerve skin territory. We examined nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) expression in the DRGs of SNI model mice. NGF and BDNF levels increased in the injured L3 DRG, while NGF decreased in the intact L5 DRG. These data offer a new point of view on the role of these neurotrophins in neuropathic pain induced by peripheral nerve injury.

**Abbreviations:** BDNF, brain-derived neurotrophic factor; DRG, dorsal root ganglion; NGF, nerve growth factor; SNI, spared nerve injury

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## 1. Introduction

Neuropathic pain is a complex chronic condition resulting from nerve injury. Mechanical allodynia, a hypersensitivity to sensory stimuli, is a typical symptom of neuropathic pain. Many reports have shown that neuropathic pain is caused by the injured nerve itself [for a review, see 1]. The dorsal root ganglion (DRG) in an injured nerve exhibits abnormal gene expression and protein products that may contribute to neuropathic pain [2,3]. On the other hand, several studies have highlighted the important contribution of non-injured neurons to neuropathic pain. For example, after partial nerve injury, the expression of the sensory neuron-specific cation channel vanilloid receptor 1 was greater in the intact DRG somata than in those from naïve animals [4]. In addition, the expression of tumor necrosis factor-alpha receptor 1 and 2 was upregulated in uninjured L4 DRGs after L5 and L6 spinal nerve ligation [5]. It has also been reported that intact nociceptors adjoining denervated areas are sensitized and have spontaneous activity [6,7]. These changes in intact neurons can induce pain and certain aspects of hyperalgesia.

Neurotrophins, which are essential for neuronal development, survival, and differentiation, have been reported to be involved in pain. For example, nerve growth factor (NGF) expression is reportedly upregulated in ipsilateral DRG neurons after chronic constriction injury of the sciatic nerve [8]. NGF regulates brain-derived neurotrophic factor (BDNF) expression in the peripheral nervous system [24]. In the spinal nerve ligation model, both NGF and BDNF expression were increased in DRGs [9]. BDNF synthesis in the ipsilateral DRG is increased in the sciatic nerve crush model [10]. After a peripheral nerve injury, injured neurons seek to regenerate and neurotrophins exert beneficial effects in this process. Conversely, NGF activates nociceptive neurons that express high-affinity NGF receptors, and antagonists of NGF relieve established neuropathic pain [11]. Controversies over the effects of neurotrophins on neuropathic pain should be resolved to establish effective pain therapy. In peripheral nerve injury models such as chronic constriction injury, sciatic nerve crush, and partial sciatic nerve ligation, injured and intact neurons are mixed in a single DRG and this situation makes it difficult to elucidate detailed expression patterns of neurotrophins.

In the present study, we used spared nerve injury (SNI) model mice [12]. The SNI model involves the lesion of two terminal branches of the sciatic nerve, the common peroneal and tibial nerves, sparing the sural nerve, and inducing mechanical hypersensitivity in the spared sural nerve territory [13]. It has been reported that the injured common peroneal and tibial nerves project into the L3 and L4 DRGs, and the non-injured sural nerve projects into the L4 and L5 DRGs in C57BL/6 J mice as illustrated in the graphical abstract [14]. Using the SNI model, we investigated NGF and BDNF expression in the L3, L4, and L5 DRGs individually.

## 2. Materials and methods

### 2.1. Animals

Eight-week-old male mice (C57BL/6 J) were purchased from CLEA Japan (Tokyo, Japan). The animals were housed in a colony room with a 12-h light/12-h dark cycle, given access to commercial chow and tap water ad libitum, and maintained under pathogen-free conditions. Animal care and the experiments were conducted in accordance with the guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals and the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. The Animal Care Committee of Nara Medical University approved the experimental protocol.

### 2.2. Surgery of SNI model

The surgery of the SNI model was conducted as described previously [12]. Surgical procedures were performed under 1.3% isoflurane anesthesia. SNI was made by a 6-0 polypropylene thread with tight ligation of the two branches of the right sciatic nerve, the common peroneal and the tibial nerves, followed by transection and removal of a 2-mm nerve portion. The sural nerve remained intact and any contact with or stretching of this nerve was carefully avoided. Muscle and skin were closed in two distinct layers. The animals in the sham operation group received the same operation but without ligation and transection of the nerves. Instead, a 2-mm-long polypropylene thread was placed longitudinally at the level of the trifurcation. Streptomycin was used for infection prophylaxis before surgery.

### 2.3. Behavioral testing

Mechanical sensitivity of both hind paws was tested at days 1 and 3 before SNI in all mice, which were divided into an SNI group and a sham operation group. Behavioral evaluation was done blindly with respect to the condition of the mice (SNI vs. sham operation). Mechanical sensitivity was again examined at days 1, 3, and 7 after SNI. Mice were placed individually in transparent plastic boxes (100 mm × 100 mm × 100 mm) on a metal mesh floor with a hole size of 5 × 5 mm. After a 20-min habituation, the threshold for paw withdrawal (both ipsilateral and contralateral sides) was measured by grade-strength von Frey monofilaments (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, 2.0, and 4.0 g) [15]. Monofilaments were perpendicularly applied to the lateral plantar surface of the hind paw with sufficient force to cause filament bending. Testing started with filament 0.008 g and a positive response was determined by a paw withdrawal response to any two of 10 repetitive stimuli. In the case of a negative response, the next stiffer monofilament was applied. The monofilament that first evoked a positive response was designated as the threshold in grams [13]. The sample size for behavioral testing was five animals for each group.

### 2.4. Western blotting

For Western blot analysis, mice were transcardially perfused with PBS (pH 7.4) under deep pentobarbital anesthesia. L3-5 DRGs were then rapidly dissected. Lumbar segmentation differs among different strains of mice [16], and even among individuals [17]. We therefore exposed the sciatic nerve completely, and confirmed that the trifurcation of common peroneal, tibial, and sural nerves gathered. When the sciatic nerve is traced back to the central side, the first branch on the caudal side leads to the L5 DRG. Following the identification of L5 DRGs, the L4 and L3 DRGs were sequentially confirmed to the head side, and the same was done on both sides. The DRGs were rapidly removed and frozen in liquid nitrogen and stored at –70 °C. Proteins were extracted by ultrasonication in a lysis buffer containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, and protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The homogenates were then centrifuged at 20,000g for 30 min at 4 °C. Equal amounts of each protein sample were denatured in Laemmli sample buffer containing 6% β-mercaptoethanol for 3 min at 100 °C, separated on 5–20% SDS-polyacrylamide gradient gels, and then transferred onto polyvinylidene fluoride membranes (Immobilon-PSQ; Millipore, Hessen, Germany). After transfer, the membranes were blocked for 30 min with Blocking One-P (Nacalai Tesque), and incubated overnight at 4 °C with rabbit antibodies raised against BDNF at 1:400 (ANT-010, Alomone, Jerusalem, Israel) and NGF at 1:1000 (clone H-20, sc-548, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in phosphate-buffered saline containing 0.05% Tween-20 (PBS-T). Mouse IgG against GAPDH (MAB374, Millipore) was used as a loading control. After overnight incubation with primary antibodies, membranes were washed three

times with PBS-T (10 min each) and then incubated with their respective secondary horseradish peroxidase-conjugated antibodies at 1:2000 (Cell Signaling Technology, Beverly, MA, USA) for 60 min. Membranes were then washed three times with PBS-T for 15 min each, and the immunoreactivity of bands was visualized with ImmunoStar Z (Wako Pure Chemical Industries, Osaka, Japan). Bands were quantified using ImageJ software (National Institutes of Health). The densitometry values were normalized against the GAPDH values. Control conditions were considered to be 1 and experimental variables were normalized with respect to this value. We used one DRG for one sample (did not use a pool of DRGs). The sample size for western blotting was from seven to ten DRGs for each group.

### 2.5. Light microscopic immunohistochemistry and confocal observation

Mice were transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep pentobarbital anesthesia. The dissected DRGs were postfixed for 6 h, cryo-protected with 30% sucrose in PBS, and frozen at  $-80^{\circ}\text{C}$  in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan). Sections were cut at a thickness of 30  $\mu\text{m}$  with a cryostat (Leica, Heidelberg, Germany) at  $-15^{\circ}\text{C}$ . For immunofluorescence detection, we processed free-floating sections as described previously [18]. In brief, sections were washed with PBS and incubated with 5% normal donkey serum in PBS containing 0.3% Triton X-100 for 1 h and then with rabbit IgG against NGF (Santa Cruz Biotechnology; 1:1000) and BDNF (Santa Cruz Biotechnology; 1:200). The primary antibody was visualized by incubating the sections in Alexa 488-conjugated donkey IgG against rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:1000) in PBS containing 0.3% Triton X-100 for 1 h. Nissl staining of the total neuronal population was carried out using NeuroTrace 530/615 Red Fluorescent Nissl Stain (Invitrogen, Carlsbad, CA, USA; 1:200).

Specimens were placed on slides and coverslips sealed with Vectashield (Vector Labs), and then observed using a laser-scanning confocal microscope (FV1000, Olympus, Tokyo, Japan). To minimize observation bias among different sections, the parameters of the confocal microscope (such as pinhole size, brightness, and contrast setting) were maintained. Images were obtained ( $512 \times 512$  pixels) and saved as TIF files using Olympus FV10-ASW Ver 1.7 Viewer. The sample size for immunohistochemical analysis was from six DRGs for each group.

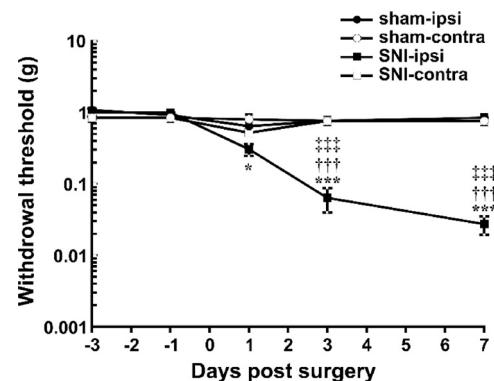
### 2.6. Statistical analysis

All statistical analyses were carried out in SPSS (Version 23.0, IBM SPSS Statistics, Ehningen, Germany). Statistical comparisons were performed using one-way ANOVA with Scheffé's post hoc test for behavioral testing and with Dunnett t post hoc test for Western blot and immunohistochemical analysis. Difference was assessed at a significance level of  $P < 0.05$ . Data are shown as mean  $\pm$  SEM.

## 3. Results

### 3.1. Behavioral assessment of SNI mice

After SNI surgery, mice developed mechanical hypersensitivity to von Frey monofilament stimulation as shown by a decrease in the paw withdrawal threshold ipsilateral to the nerve injury (Fig. 1). The mechanical hypersensitivity manifested only in the lateral surface of the hind paw (sural nerve skin area) and was present ipsilaterally at day 1 after SNI surgery as compared to the sham group. It became more marked at days 3 and 7 after SNI surgery. Seven days after surgery, the withdrawal threshold value for the ipsilateral paw was  $0.027 \pm 0.018$  g, which was significantly lower than that of the sham-operated animals ( $0.84 \pm 0.22$  g,  $P < 0.001$ ). Similarly, a significant difference was observed between ipsilateral and contralateral paws in



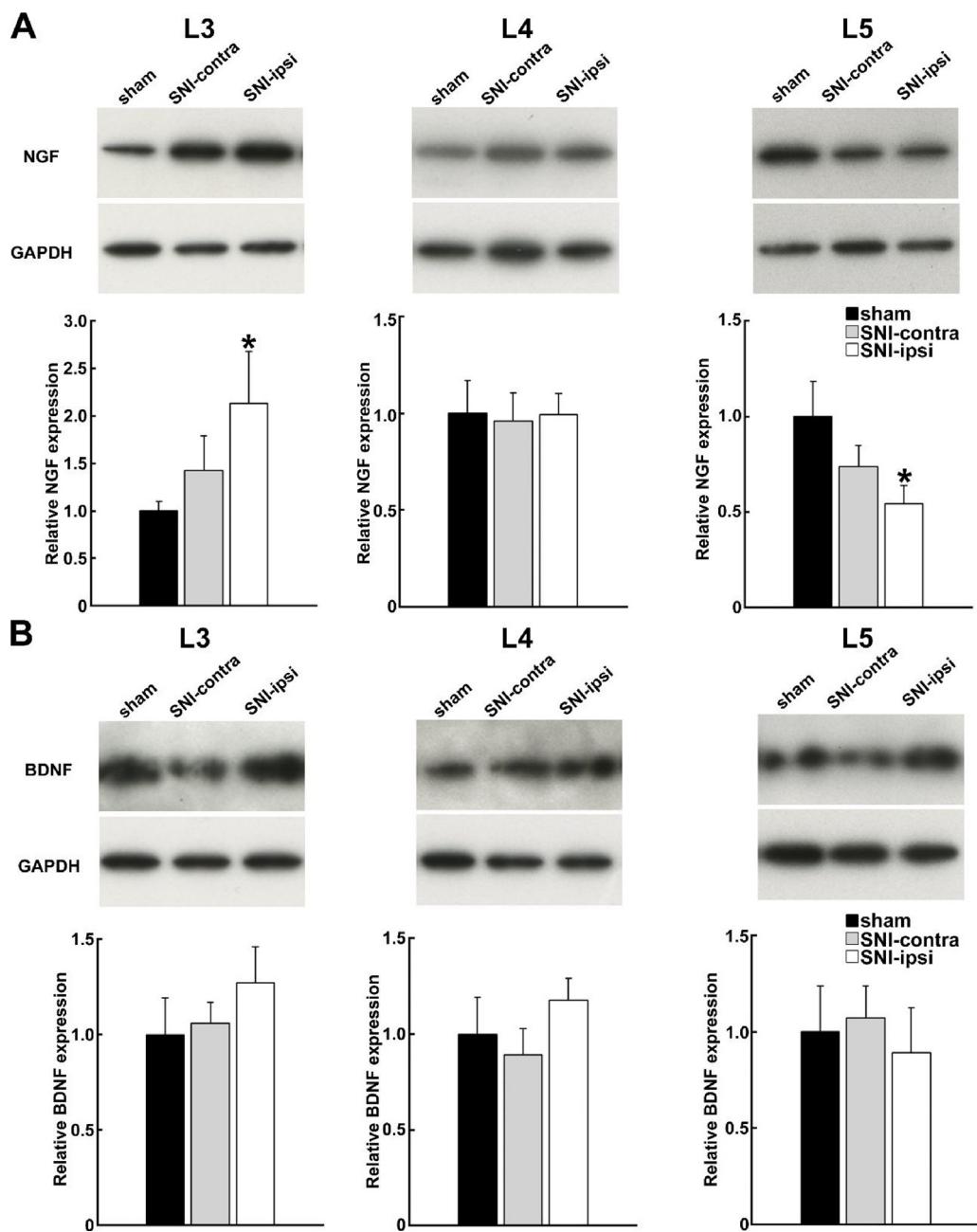
**Fig. 1.** Development of mechanical allodynia after SNI. Mechanical allodynia was tested with von Frey monofilaments within 7 days after SNI surgery. The withdrawal threshold of the SNI-ipsilateral paw significantly decreased from day 1 after SNI surgery and continued to decrease at days 3 and 7 after SNI surgery, compared to that of the sham-contralateral paw. No significant change occurred in the contralateral paw after SNI or in the sham group. Values are means  $\pm$  SEM,  $N = 5$  animals in each group. We measured both hind paws (ipsi and contra). \* $P < 0.05$ , \*\* $P < 0.001$ , compared to sham-contralateral paw. \*\*\* $P < 0.001$ , compared to sham-ipsilateral paw. \*\*\*\* $P < 0.001$ , compared to SNI-contralateral paw, one way ANOVA with Scheffé's post hoc test.

the SNI group ( $P < 0.001$ ). On the other hand, there was no significant change in the withdrawal thresholds of either ipsilateral or contralateral paws of the sham group from the pre-surgery baseline.

### 3.2. Effect of SNI on the expression of NGF and BDNF in the L3-5 DRG

In this SNI procedure, it is known that the L3 DRG is enriched in injured fibers, the L4 DRG has a mixture of injured and non-injured fibers, and the L5 DRG is enriched in non-injured fibers [14]. NGF and BDNF protein expression levels in each of the L3, L4, and L5 DRG were quantified in sham-ipsilateral (sham), SNI-contralateral (SNI-contra), and SNI-ipsilateral (SNI-ipsi) at day 7 after SNI surgery by Western blotting (Fig. 2). In the L3 DRG, densitometric analysis of the protein bands showed that NGF expression significantly increased ( $F_{2,18} = 3.1$ ,  $P = 0.043$ ) in SNI-ipsi than in sham. In the L4 DRG, there was no significant change in NGF expression in SNI-ipsi compared to sham. On the other hand, in the L5 DRG, NGF expression in SNI-ipsi showed a significant decrease ( $F_{2,27} = 2.9$ ,  $P = 0.042$ ) (Fig. 4). BDNF expression did not significantly change in the L3-L5 DRGs. In all DRG levels, there was no significant difference in NGF or BDNF expression between sham and SNI-contra.

Then, we performed immunohistochemical analysis using an antibody to NGF or BDNF and fluorescent Nissl to detect neurons in the L3-5 DRGs at day 7 after SNI surgery. Some Nissl-positive neurons expressed NGF or BDNF in the sham DRG as demonstrated in previous studies [19,20], but we did not find NGF and BDNF immunoreactivity in other cells such as satellite glial cells (Figs. 3–5). In the L3 DRG, NGF-positive neurons and BDNF-positive neurons significantly increased in SNI-ipsi than in sham (NGF:  $F_{2,15} = 6.7$ ,  $P = 0.006$ , BDNF:  $F_{2,15} = 5.8$ ,  $P = 0.008$ ). In the L4 DRG, NGF-positive neurons significantly increased in SNI-ipsi than in sham ( $F_{2,15} = 4.8$ ,  $P = 0.023$ ) and in SNI-contra ( $P = 0.047$ ). In the L5 DRG, NGF-positive neurons significantly decreased in SNI-ipsi than in sham (NGF:  $F_{2,15} = 9.8$ ,  $P = 0.003$ ). BDNF-positive neurons did not significantly change in the L4 and L5 DRG. NGF and BDNF immunoreactivity were observed in both small- and medium-sized neurons, as shown in the histogram of soma diameter distribution, and increased or decreased regardless of the cell size.



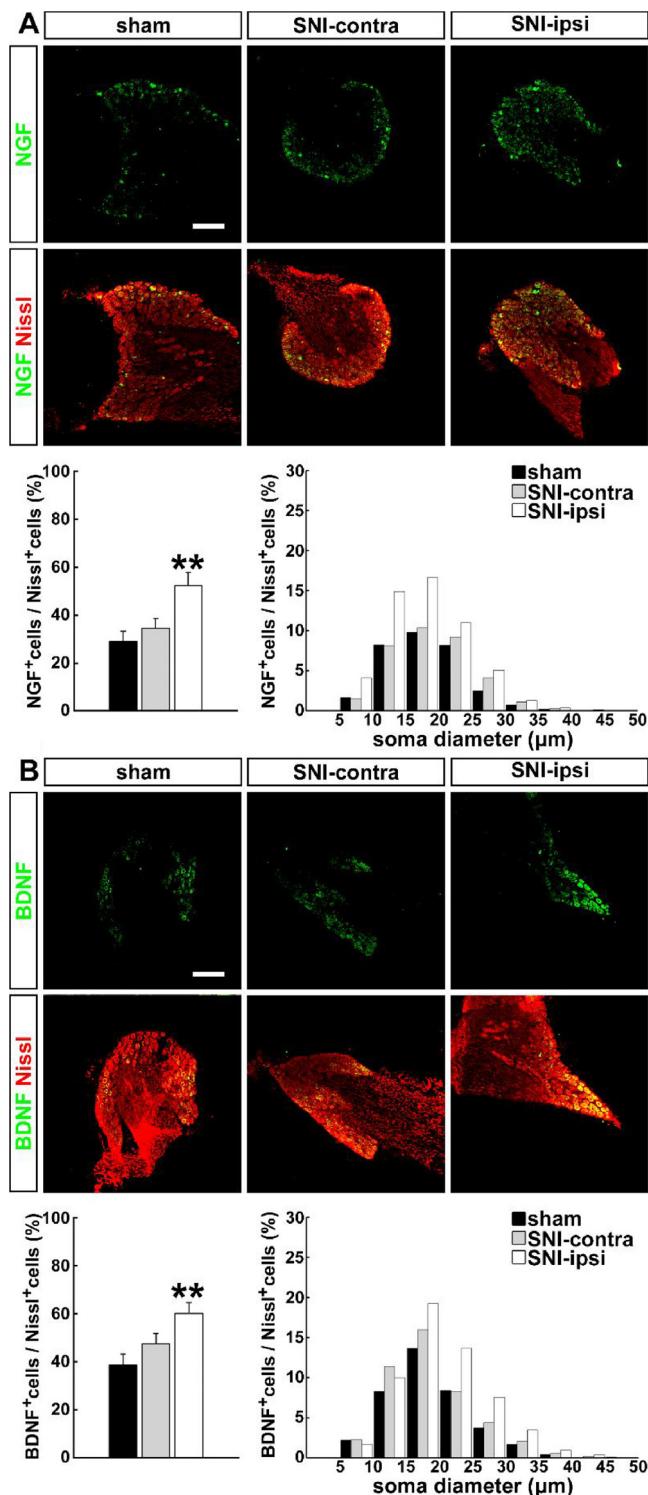
**Fig. 2.** Western blot analysis of NGF and BDNF expression in the L3-5 DRG. Relative NGF and BDNF expression were quantified by densitometry. (A) Representative immunoblots of NGF and GAPDH bands and semi-quantitative densitometric data for NGF expression. N = 7 (L3), 10 (L4), 10 (L5) animals in each group. (B) Representative immunoblots of BDNF and GAPDH bands and semi-quantitative densitometric data for BDNF expression. N = 10 (L3), 8 (L4), 9 (L5) animals in each group. Values are shown as the mean  $\pm$  SEM. \*P < 0.05, one way ANOVA with Dunnett t post hoc test.

#### 4. Discussion

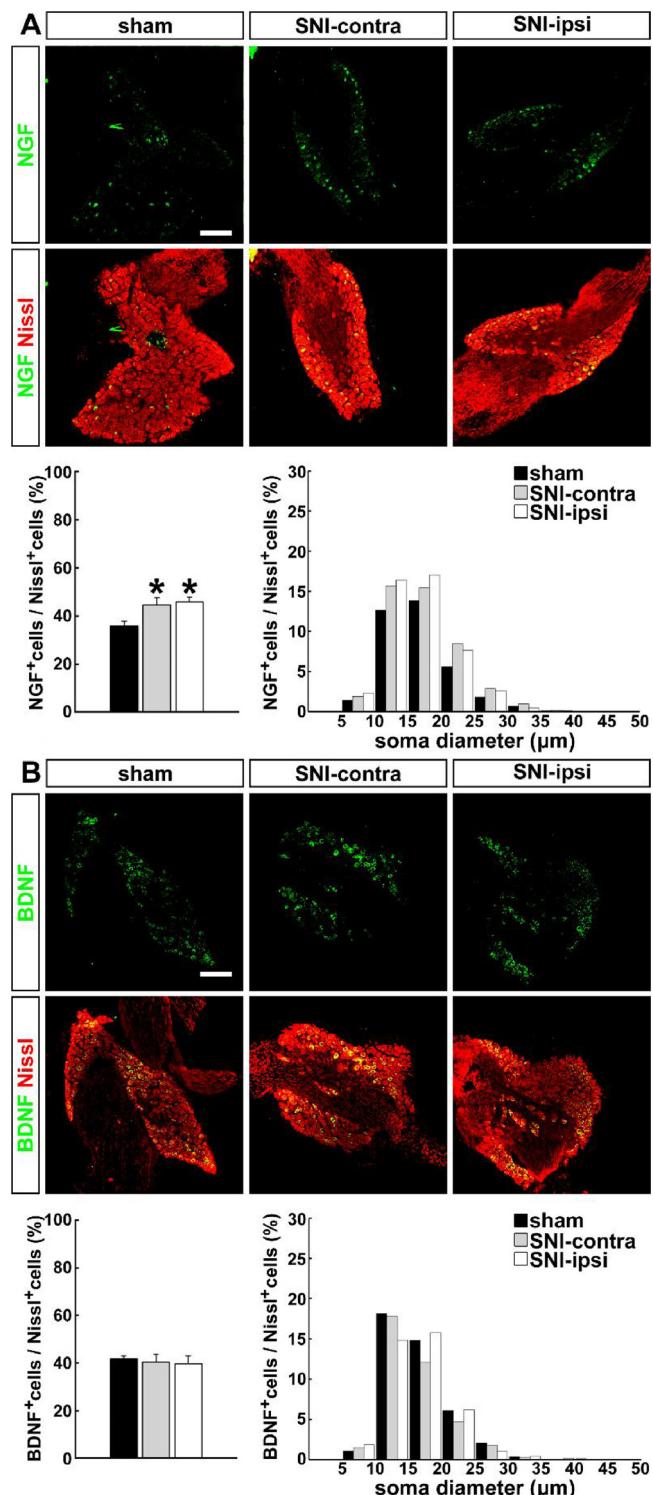
In the present study, we used SNI as a peripheral nerve injury model that induces mechanical allodynia in the non-injured sural nerve territory [19]. We investigated NGF and BDNF expression in the L3, L4, and L5 DRG individually, since it is known that the injured common peroneal and tibial nerves project into the L3 and L4 DRG, and the non-injured sural nerve projects into the L4 and L5 DRG in this model [14]. In agreement with previous studies, NGF expression in the SNI model was upregulated in the L3 DRG, which consisted primarily of injured neurons. On the other hand, NGF expression was significantly decreased in the L5 DRG, composed mostly of intact neurons. In the L4 DRG, there was no visible change in the expression of neurotrophins by Western blotting analysis, but NGF expression was significantly

increased by immunohistochemistry. This may result from the mixture of injured and non-injured fibers in the L4 DRG. In immunohistochemical analysis, BDNF<sup>+</sup> neurons in L3 DRG increased, but in L5 DRG did not increase by SNI. Although both NGF and BDNF expression were increased after SNI in injured L3 DRG as in the spinal nerve ligation model [9], decrease of NGF in L5 DRG may independent of BDNF expression in L5 DRG. These results indicate that the expression pattern of neurotrophins differs between injured neurons and intact neurons.

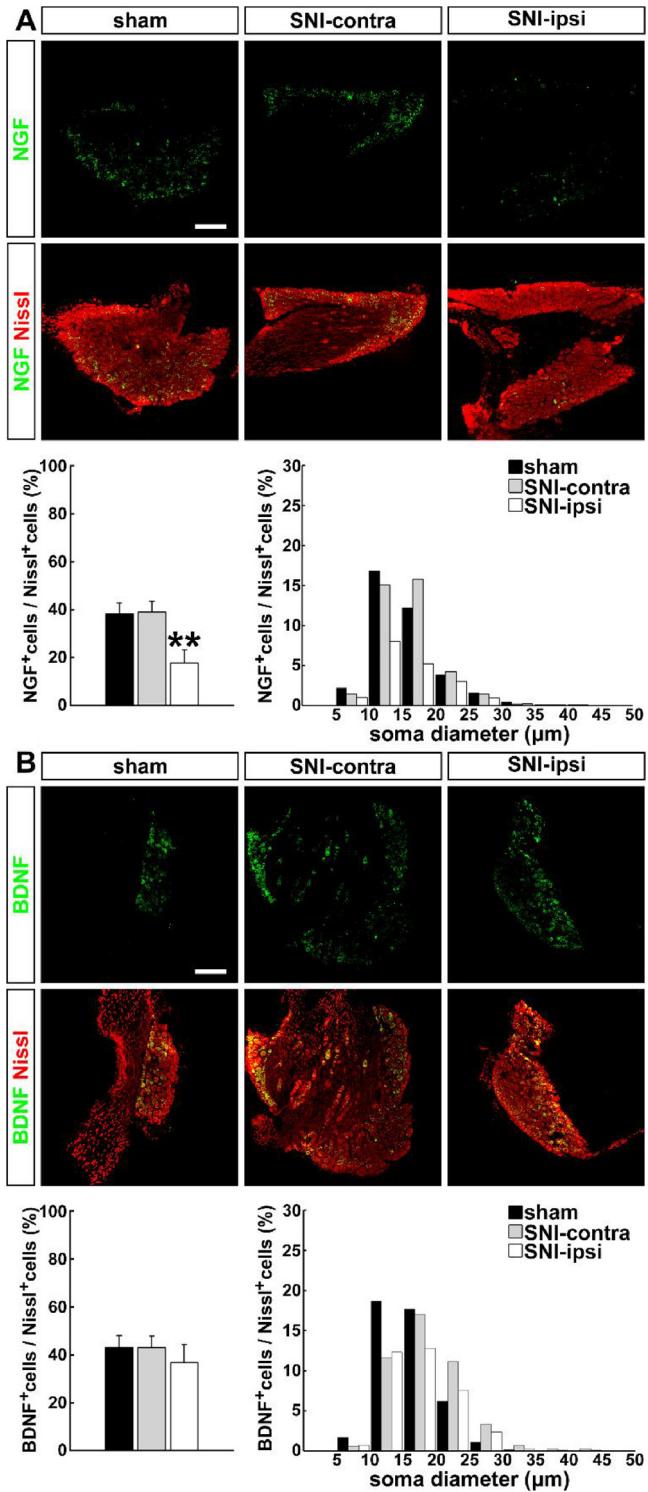
As a cause of hyperalgesia, intact neurons have been proposed to be involved in the main mechanism of neuropathic pain. In the SNI model, mechanical hypersensitivity is expressed predominantly in the skin territory of the non-injured sural nerve [12,13,21]. The expression of NGF was low in the L5 DRG of SNI model, into which neurons are



**Fig. 3.** Immunohistochemical analysis of NGF and BDNF expression in the L3 DRG. (A) Typical confocal images showing NGF distribution with Nissl staining in the sham, SNI-contra, and SNI-ipsi L3 DRG, quantitative data for percentage of NGF<sup>+</sup> cells to the total neurons, and its histogram of soma diameter distribution. (B) Typical confocal images showing BDNF distribution with Nissl staining in the sham, SNI-contra, and SNI-ipsi L3 DRG, quantitative data for percentage of BDNF<sup>+</sup> cells to the total neurons, and its histogram of soma diameter distribution. N = 6 animals in each group. Values are shown as the mean  $\pm$  SEM. \*P < 0.05, one way ANOVA with Dunnett t post hoc test. Scale bars = 200  $\mu\text{m}$ .



**Fig. 4.** Immunohistochemical analysis of NGF and BDNF expression in the L4 DRG. (A) Typical confocal images showing NGF distribution with Nissl staining in the sham, SNI-contra, and SNI-ipsi L4 DRG, quantitative data for percentage of NGF<sup>+</sup> cells to the total neurons, and its histogram of soma diameter distribution. (B) Typical confocal images showing BDNF distribution with Nissl staining in the sham, SNI-contra, and SNI-ipsi L4 DRG, quantitative data for percentage of BDNF<sup>+</sup> cells to the total neurons, and its histogram of soma diameter distribution. N = 6 animals in each group. Values are shown as the mean  $\pm$  SEM. \*P < 0.05, one way ANOVA with Dunnett t post hoc test. Scale bars = 200  $\mu\text{m}$ .



**Fig. 5.** Immunohistochemical analysis of NGF and BDNF expression in the L5 DRG. (A) Typical confocal images showing NGF distribution with Nissl staining in the sham, SNI-contra, and SNI-ipsi L5 DRG, quantitative data for percentage of NGF<sup>+</sup> cells to the total neurons, and its histogram of soma diameter distribution. (B) Typical confocal images showing BDNF distribution with Nissl staining in the sham, SNI-contra, and SNI-ipsi L5 DRG, quantitative data for percentage of BDNF<sup>+</sup> cells to the total neurons, and its histogram of soma diameter distribution. N = 6 animals in each group. Values are shown as the mean  $\pm$  SEM. \*P < 0.05, one way ANOVA with Dunnett t post hoc test. Scale bars = 200  $\mu\text{m}$ .

projected from the region showing mechanical hypersensitivity. In the diabetic neuropathic pain model, the expression of neurotrophins is downregulated. For example, NGF expression is downregulated in the DRG, and this was positively correlated with the mechanical pain threshold of diabetic neuropathic pain model rats [22]. NGF expression is also decreased in the vagus nerve [23], the calf muscle, and the sciatic nerve [24] of the diabetic animals. In addition, the administration of NGF has been reported to attenuate functional neuronal deficits characteristic of diabetic neuropathy and could mitigate neuropathic pain [25]. Both peripheral nerve injury and diabetic neuropathy induce mechanical allodynia, however, and the reason for this discrepancy is not yet known. Despite their opposite effects on neurotrophin expression, both the mechanical nerve injury model and the diabetic neuropathic pain model induce allodynia. Since NGF downregulation precedes damage to DRG neurons in the diabetic neuropathic pain model [22,26], we propose that the downregulation of neurotrophins in non-injured DRG neurons contributes to neuropathic pain in peripheral nerve injury. Considering NGF as therapeutic target, the present study highlights the need for region-specific therapy.

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