

Expression Profile of Nerve Growth Factor after Muscle Incision in the Rat

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Background: Previous studies have demonstrated that nerve growth factor (NGF) is an important mediator of pathologic pain. Many studies have focused on cutaneous mechanisms for NGF-induced hyperalgesia; few have examined its contribution in deeper tissues like muscle. This study examined pain behaviors and the expression of NGF in incised hind paw flexor digitorum brevis muscle.

Methods: Adult Sprague-Dawley rats were pretreated with anti-NGF peptibody and underwent skin or skin plus deep fascia and muscle incision. Guarding pain behaviors were measured. Muscle NGF messenger RNA (mRNA) was measured by reverse-transcriptase polymerase chain reaction. Changes in NGF protein expression were measured using Western blot, enzyme-linked immunosorbent assay, and immunohistochemistry. *In situ* hybridization for NGF mRNA was also performed.

Results: Pretreatment with anti-NGF peptibody (100 mg/kg) decreased the guarding behavior caused by deep fascia and muscle incision. Muscle NGF mRNA increased abruptly 2 h after incision and was the same as control by postoperative day 1. NGF protein increased from 4 h after incision and was sustained for several days. NGF was localized in many calcitonin gene-related peptide-positive axons, few N52-positive axons, but not isolectin B4-positive axons in incised muscle. The sources of NGF mRNA included keratinocytes in epidermis and fibroblasts in deeper tissues.

Conclusion: Fibroblasts adjacent to the injury are sources of NGF in incised muscle. NGF is upregulated by incision of muscle and contributes to guarding pain behavior.

NERVE growth factor (NGF) promotes survival of nociceptors and the sympathetic nervous system during late embryonic development.¹ In the adult, NGF is essential for the sprouting of nerve fibers and induction of inflammatory pain; from this, NGF has garnered considerable interest in pain research.²

Administration of exogenous NGF has been shown to produce hyperalgesia in rats³ and humans,^{4,5} and neutralization of NGF by trkA-IgG fusion molecule prevents

the sensitization of nociceptors in some pain models.⁶ The most direct evidence for sensitization of cutaneous afferents by NGF was demonstrated in electrophysiological recordings of nociceptors using the *in vitro* skin-nerve preparation.⁷ Application of NGF to the receptive fields of cutaneous C-mechanoheat nociceptors resulted in sensitization to noxious heat.

Interest in postoperative pain management has prompted further investigation for novel therapies that will facilitate better pain control and limit side effects. NGF is one mediator that likely contributes to nociceptor sensitization, hyperalgesia, and clinical pain after surgery.⁸ Previous studies on skin demonstrated upregulation of NGF mRNA immediately after skin incision; increased NGF protein is sustained for several days.⁹ The increase in NGF occurs at times that are coincident with its role in pain-related behaviors,¹⁰ indicating a relationship between NGF, cutaneous incision, and pain.

In contrast to skin, deep tissue like muscle also contributes to postoperative pain. For example, after abdominal surgery, patients have pain with deep breathing, during cough and with rising from a supine position. Muscle pain is often dull and aching, and difficult to localize. Indeed, muscle afferents form fewer synapses on spinal cord neurons than cutaneous afferents, and descending inhibition of nociceptive spinal cord neurons is stronger for muscle input than for cutaneous input, suggesting different nociceptive processes from muscle compared to skin.¹¹ Muscle incisional pain is likely unique and different than pain from skin incision. Nothing is known about the expression pattern of NGF in incised muscle.

In this study, we compared the effect of NGF blockade on pain-related behaviors after skin incision and after skin and deep incision of the hind paw fascia and flexor digitorum brevis muscle. NGF expression in the hind paw flexor digitorum brevis muscle after plantar incision was measured using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), *in situ* hybridization, enzyme-linked immunosorbent assay, Western blot, and immunohistochemistry. The source of NGF mRNA in normal and incised muscle, changes in NGF protein and mRNA at different times after muscle incision, and distribution of muscle NGF immunoreactivity after incision were determined.

Materials and Methods

This study was approved by the institutional Animal Care and Use Committee at the University of Iowa (Iowa

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City, Iowa). Adult male Sprague-Dawley rats, 225–275 g, were used for all the experiments. Postoperatively, the rats were housed in groups of two or three in clear plastic cages with food and water available *ad libitum*.

Incision, Anti-NGF Peptibody, and Guarding Pain Scores

Rats were acclimated to the testing environment. After acclimation, 100 mg/kg anti-NGF peptibody¹² or vehicle was administered subcutaneously at the neck. The next day, the baseline pain score was measured and the rat underwent plantar incision.¹³ Under isoflurane anesthesia (1.5–2.0%) and using aseptic conditions, a 1-cm longitudinal incision was made 0.5 cm from the heel on the plantar aspect of the right hind paw. The underlying flexor muscle was dissected and longitudinally incised, and the wound was closed with 5-0 nylon suture. Immediately after surgery, antibiotic ointment was applied to the incision. Sutures were removed on postoperative day (POD) 2. A separate group of rats underwent incision of skin only; no fascia or muscle was incised.

A cumulative pain score was used to assess nonevoked pain behaviors as described previously.¹⁴ Unrestrained rats were placed on a plastic mesh floor (8 × 8 mm). The incised and nonincised paws were viewed. Both paws of each animal were closely observed during a 1-min period repeated every 5 min for 1 h. Depending on the position in which each paw was found during the majority of the 1-min scoring period, a 0, 1, or 2 is given. Full weight-bearing of the paw (score = 0) was present if the wound was blanched or distorted by the mesh. If the paw was completely off the mesh, a score of 2 was recorded. If the area of the wound touched the mesh gently without any blanching or distorting, a 1 was given. The sum of the 12 scores (0–24) obtained during the 1-h session for each paw was obtained. The difference between the scores from the incised paw and nonincised paw is the cumulative pain score for that 1 h period. The person performing the behavioral experiments was blinded to the type of incision and the drug (or vehicle) administered.

Quantitative RT-PCR Analysis

Total ribonucleic acid (RNA) from incised rat flexor digitorum brevis muscle tissue was prepared according to manufacturer's instructions using the RNeasy mini kit (Qiagen, Valencia, CA). Some muscle samples from sham-incised rats were used as control. The RNA was quantified using Ribogreen reagent (Molecular Probes, Eugene, OR). Two-step RT-PCR was performed on the 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) by using the TaqMan Gold RT-PCR kit (Applied Biosystems). The RNA was reverse transcribed using random hexamers, and the complementary deoxyribonucleic acid (cDNA) was amplified using a primer/probe set specific for NGF (RatNGF-fwd: TGACTCCAAGCACTGGAATCAT, complementary to 693–715 nucleo-

tides in the rat NGF mRNA; RatNGF-rev: GTTTGTCGTCTGTTGTCAACGC, complementary to 947–926 nucleotides in the rat NGF mRNA; RatNGF-Tamra: TG-CACCACGACTCACACCTTTGTCA, complementary to 718–742 nucleotides in the rat NGF mRNA). The resulting PCR product was 309 bp in length. Thermal cycling was initiated with an initial incubation at 95°C for 10 min. After that, 40 PCR cycles were performed; each cycle consisted of heating at 95°C for 15 s for melting and at 60°C for 1 min for annealing and extension. The samples were analyzed in triplicate from the reverse-transcription step and normalized to the internal control, a housekeeping gene, β -actin. No template samples were used as controls. PCR data were analyzed by the $\Delta\Delta C_t$ method described in detail by Livak *et al.*¹⁵

Enzyme-linked Immunosorbent Assay

Flexor digitorum brevis muscle samples were taken from control rats and injured rats at 4 h, and 1, 2, 5, and 10 days after plantar incision. Muscle tissue from origin to insertion was harvested from each animal after they were euthanized with carbon dioxide. Immediately after extraction, the tissue samples were homogenized and lysed in ice-cold radioimmunoprecipitation assay buffer containing protease inhibitors. The homogenates were centrifuged, and the supernatant was taken. Total protein concentration in each sample was determined using a modified Lowry protein assay. The NGF concentration was measured using a commercially available assay per manufacturer's instructions (NGF Emax Immunoassay; Promega, Madison, WI). This assay exhibits very low crossreactivity with structurally related growth factors, and a minimum of 7.8 pg/ml NGF can be quantified.

Western Blot Analysis

After assay of total protein, 20 μ g of protein in 20 μ l of volume from each sample was electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Bio-Rad Lab, Hercules, CA). Overnight incubation of the membranes in rabbit anti-NGF polyclonal antibody (1:1000, Cat # sc-548; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C was followed by incubation in peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:10,000; Jackson ImmunoRes, West Grove, PA) for 4 h. Membranes were then developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology Inc., Rockford, IL) and scanned by Kodak Image Station 440 scanner (Eastman Kodak Company, Rochester, NY). After the membranes were scanned, they were incubated in stripping buffer at 50°C for 30 min and probed for actin as a loading control. Immunoreactive NGF band intensity was quantified and normalized with actin bands by Image J software (NIH, Bethesda, MD). In a previous study, we demonstrated that the antibody was immunoreactive to mature rat NGF

(N2513; Sigma, St. Louis, MO); no immunoreactive bands were noted when we added the blocking hapten peptide (cat. No. sc-548 P; Santa Cruz Biotechnology Inc.) containing the antigen (100 μ g/0.5 ml) used to raise the antibody.⁹

Immunohistochemistry

Briefly, rats were anesthetized with sodium pentobarbital (150 mg/kg) and transcardiacally perfused with 0.1 M phosphate-buffered saline followed by 4% paraformaldehyde. The glabrous plantar skin samples together with deep tissue and flexor muscle were removed from sham and incised hindpaws and postfixed in the same fixative for 4 h. After washing in gradually increasing concentrations of sucrose, samples were rapidly frozen in 2-methylbutane chilled at -80°C . Consecutive sections (10 μm) were prepared and frozen at -80°C until usage. NGF expression was visualized by NGF immunohistochemistry using the ABC method. The following antibodies were used: rabbit anti-NGF antibody (1:2000, Cat # sc-548; Santa Cruz Biotechnology Inc.) and biotinylated anti-rabbit IgG (1:500; Vector Laboratories, Burlingame, CA). For controls, some sections were processed after preabsorption of the anti-NGF antibody with the blocking peptide (4 $\mu\text{g}/\mu\text{l}$). Other sections were processed without the primary or the secondary antibody as controls.

Immunofluorescence

Double-labeling confocal microscopy and immunofluorescent staining were performed for NGF and protein gene product 9.5 (PGP 9.5, a panaxonal marker), calcitonin gene-related peptide (CGRP, a peptidergic C-fiber marker), isolectin B4 (IB4, a nonpeptidergic C-fiber marker), or neurofilament 200 (N52, a myelinated A-fiber marker) to examine the colocalization of NGF and sensory fibers. To block nonspecific reactions, sections were incubated in 0.1 M phosphate-buffered saline containing 1.5% bovine serum albumin and 0.1% Tween-20 for 1 h. Sections were processed for NGF and PGP 9.5, CGRP, IB4, or N52 double-labeling. The following primary antibodies were used: rabbit anti-NGF polyclonal antibody (1:1000, Cat # sc-548; Santa Cruz Biotechnology Inc), guinea pig anti-PGP 9.5 polyclonal antibody (1:1000, Cat # AB5898; Chemicon, Temecula, CA), rabbit anti-CGRP polyclonal antibody (1:1000, Cat # T-4032; Peninsula Laboratories Inc, San Carlos, CA), biotinylated griffonia simplicifolia lectin I (IB4, 1:8000, Cat # B-1105; Vector Laboratories), mouse antineurofilament 200 monoclonal antibody (1:1000, Cat # N0142; Sigma). Sections were then incubated in the following secondary antibodies, Cy3-conjugated goat anti-rabbit IgG, Cy2-conjugated donkey anti-guinea pig IgG, Cy2-conjugated donkey anti-rabbit IgG, Cy2-conjugated streptavidin and Cy2-conjugated goat antimouse IgG. All the secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA) and applied to sections with a concentration of

1:200. Sections were mounted and observed under Zeiss 510 confocal microscopy (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Digital images were collected in the LSM 510 software (Carl Zeiss MicroImaging, Inc). For controls, sections were stained without primary or secondary antibodies.

In Situ Hybridization

Rat hind paw flexor digitorum brevis muscle was taken 1 h after incision. Total RNA was isolated using RNeasy mini kit (Qiagen). One microgram of RNA was used for RT-PCR to amplify a 416-bp band of NGF using primers: forward, 5'-CCATGGTACAATCTCCTTCA-3'; reverse, 5'-TGCAGGCAAGTCAGCCTCTT-3'. The product was purified using QIAquick PCR purification kit (Qiagen) and then subcloned into T-easy vector (Promega). After sequencing, the template was linearized with Sal I digestion and gel purified using QIAquick Gel Extraction kit (Qiagen). Digoxigenin-labeled probes were made using DIG RNA Labeling Kit (Roche, Mannheim, Germany), and the labeling efficiency was quantified using DIG Luminescent Detection Kit (Roche). All procedures were performed following the manufacturers' instruction.

Sections were postfixed in 4% paraformaldehyde for 10 min and permeabilized by treatment with 0.002% proteinase K for 5 min. Fixation in 4% paraformaldehyde again for 5 min and underwent acetylation (0.5% acetic anhydride in 0.1 M triethanolamine) for 10 min. Probes were diluted to 10 ng/ml in hybridization buffer (50% formamide, 4 \times standard saline citrate, 0.5 \times Denhardt solution, 0.025% yeast tRNA, 0.025% PolyA, 0.025% salmon sperm DNA, 20% dextran sulfate, and 0.1 M dithiothreitol). The sections were hybridized in a humidified chamber at 55°C overnight with sense, antisense or internal control (actin) probes. Some sections were also incubated in a hybridization solution containing no probe. Adult rat cortex sections were hybridized with NGF sense or antisense probes as positive control because it is well accepted that NGF is produced by neurons in cortex.

After hybridization, sections were subjected to post-hybridization washes to remove unbound probes. Endogenous alkaline phosphatase was blocked with blocking reagent (Roche) dissolved in 1 \times MABT (0.88% NaCl, 1.16% maleic acid, 0.72% NaOH) containing 0.01% Tween-20 and 20% goat serum for 60 min. Signal was detected by incubating sections with sheep antidigoxigenin-AP, Fab fragments antibody (1:2000, Cat# 11093274910; Roche) at 4°C overnight, visualized with alkaline phosphatase color substrates, nitroblue tetrazolium/5-Bromo-4-Chloro-Indolyl-Phosphatase for 2 h. The color reaction was stopped by immersing sections in 0.1 M phosphate-buffered saline. Sections were then counterstained in nuclear fast red for 1 min, dehydrated, and mounted for microscopic examination.

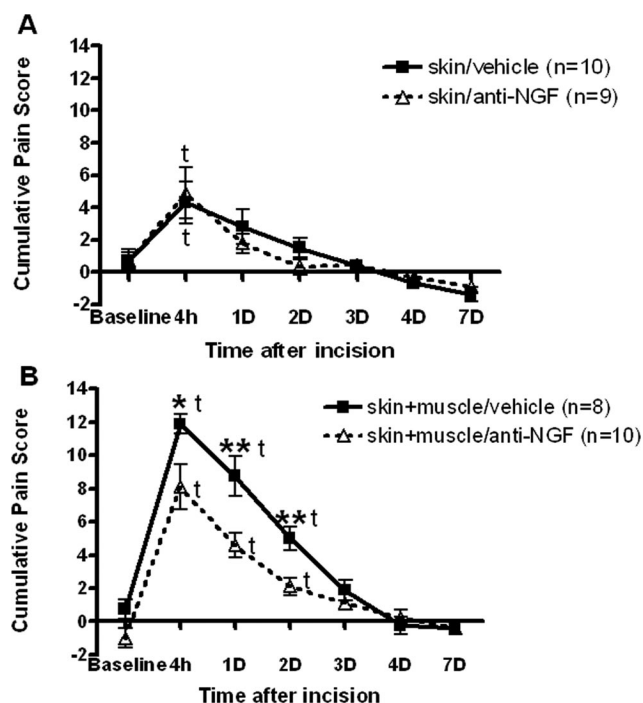


Fig. 1. Effect of anti-nerve growth factor (NGF) peptibody on guarding pain behavior caused by plantar incision. (A) Cumulative pain score after anti-NGF treatment in rats that underwent only skin incision. (B) Cumulative pain score after anti-NGF treatment in rats that underwent skin, fascia, and muscle incisions. $t = P < 0.05$ versus baseline. $* P < 0.05$ and $** P < 0.01$ versus vehicle, respectively.

Statistical Analyses

All statistical analyses were performed using Prism 4.0 (GraphPad Software, Inc., San Diego, CA). Data were expressed as mean \pm SEM. Differences between means were determined by 1-way ANOVA followed by Dunnett *post hoc* test for comparisons of incision versus sham. Guarding pain scores were analyzed by ANOVA. Subse-

quent 1-way ANOVA for repeated measures and *post hoc* t tests compared the effect of drug and Dunnett test versus baseline examined the effect of incision. $P < 0.05$ was considered statistically significant.

Results

Anti-NGF and Guarding Behavior

Incision of skin and treatment with vehicle resulted in a cumulative pain score of 4.3 ± 1.3 (fig. 1A; $P < 0.05$ vs. baseline) 4 h after incision. There was no significant guarding thereafter. Treatment with the anti-NGF peptibody (100 mg/kg) and skin incision were not different than vehicle and skin incision.

In rats that underwent incision of skin, fascia, and muscle and were treated with vehicle, guarding was increased at 4 h, 1 day, and 2 days to 11.9 ± 0.6 , 8.8 ± 1.2 , 5.0 ± 0.7 , respectively (fig. 1B; $P < 0.05$ vs. baseline). Guarding after pretreatment with anti-NGF peptibody (100 mg/kg) and skin, fascia, and muscle incision was 8.1 ± 1.4 , 4.6 ± 0.7 , 2.1 ± 0.5 , at 4 h, 1 day, and 2 days, respectively. The anti-NGF peptibody decreased guarding by skin fascia and muscle incision at 4 h, 1 day, and 2 days ($P < 0.05$ vs. vehicle).

RT-PCR Of mRNA Levels for NGF in Incised Hind Paw Muscle

Compared to the β -actin control, NGF mRNA increased 6.6 ± 1.3 -fold ($P < 0.01$ vs. sham) in incised flexor muscle at 2 h after incision compared with sham control skin (fig. 2A; $n = 6$ per group). The level of NGF mRNA was not significantly increased on POD 1 and thereafter and was similar to the contralateral, nonincised hind paw flexor muscle.

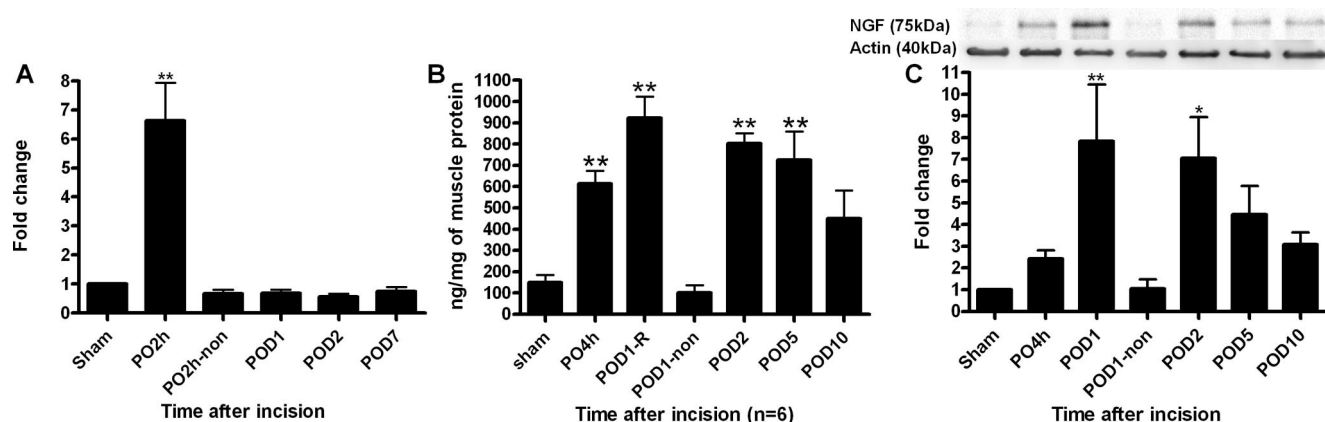


Fig. 2. Nerve growth factor (NGF) expression in incised or sham-operated plantar flexor digitorum brevis muscle. (A) Changes in NGF messenger RNA (mRNA) relative to β -actin mRNA examined by real-time quantitative-polymerase chain reaction. Data are expressed as mean \pm SEM of ratio of incised flexor muscle to sham controls. (B) NGF protein determined by enzyme-linked immunosorbent assay. Data are shown as mean \pm SEM of nanogram of NGF protein per milligram of protein. (C) Western blots for NGF and actin immunoreactivity. Top: Example of Western blot showing immunoreactive NGF and actin control bands in detailed time course after plantar incision. Bottom: Summary of relative changes in NGF protein expression after plantar incision. Data are expressed as mean \pm SEM of ratio of incised flexor muscle to sham controls. Non = contralateral nonincised muscle. PO = post operative. POD = postoperative day. $* P < 0.05$, $** P < 0.01$ versus sham. $N = 6$ per group.

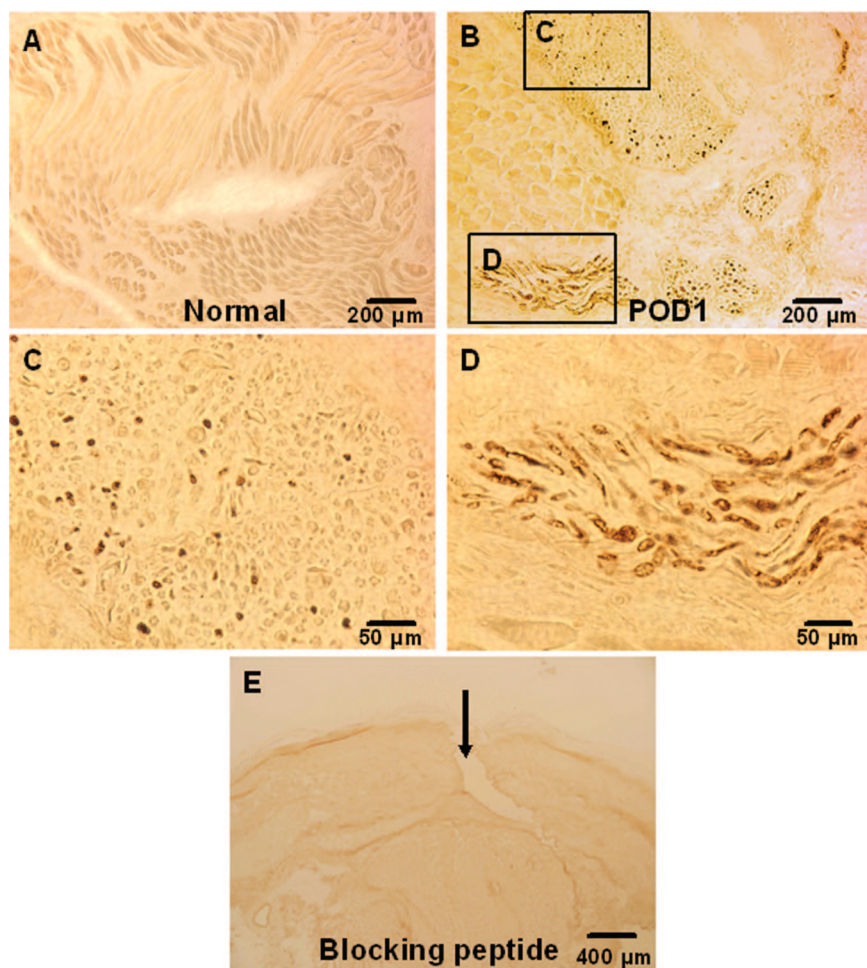


Fig. 3. 3,3'-diaminobenzidine immunohistochemistry for nerve growth factor (NGF) in plantar flexor digitorum brevis muscle. (A) NGF immunohistochemistry in sham-operated flexor muscle. (B) NGF in nerve-like structures from incised muscle on POD (postoperative day) 1. (C) A higher magnification of the rectangular area C in panel B, showing immunoreactivity for NGF within a transverse sectioned nerve-like structure. (D) A higher magnification of the rectangular area D in panel B, showing immunoreactivity for NGF within a longitudinal sectioned nerve-like structure. (E) NGF immunohistochemistry in skin, fascia, and muscle after preabsorption with blocking peptide. The arrow indicates the incision.

NGF Protein Expression in Incised Hind Paw Muscle

As shown in figure 2B, basal NGF expression was 149.3 ± 35.9 ng/mg protein in sham-operated muscle. NGF levels increased to 614.3 ± 61.3 ng/mg ($P < 0.01$ vs. sham) by 4 h and peaked at 923.4 ± 101.3 ng/mg on POD 1 in incised muscle. After POD 1, levels of NGF expression were 803.3 ± 47.3 ng/mg, and 725.1 ± 135.5 ng/mg on POD 2 ($P < 0.01$ vs. sham, $n = 6$ per group), and POD 5 ($P < 0.01$ vs. sham, $n = 6$ per group), respectively. NGF was not different than control on POD 10 (449.8 ± 131.6 ng/mg) and at the contralateral hind paw muscle (100.1 ± 35.1 ng/mg).

Western blot analysis revealed a single high-molecular weight (MW) NGF increased by incision. Representative blots were shown in figure 2C top. This large MW form of NGF protein, approximately 75 kDa, was increased in incised muscle. A summary quantifying the NGF immunoreactive bands for different times after incision is shown in figure 2C bottom. The data were normalized to actin ($n = 6$ per group). In comparison to sham, NGF expression in the incised hind paw increased initially 2.4 ± 0.4 -fold by 4 h to 7.8 ± 2.6 -fold ($P < 0.01$ vs. sham) on POD 1. Intensity of NGF expression was in-

creased 7.0 ± 1.9 -fold ($P < 0.05$ vs. sham) on POD 2, 4.5 ± 1.3 -fold on POD 5, and 3.1 ± 0.5 -fold POD 10. NGF expression in the contralateral, unincised hind paw flexor muscle (1.0 ± 0.4 -fold) was the same as sham.

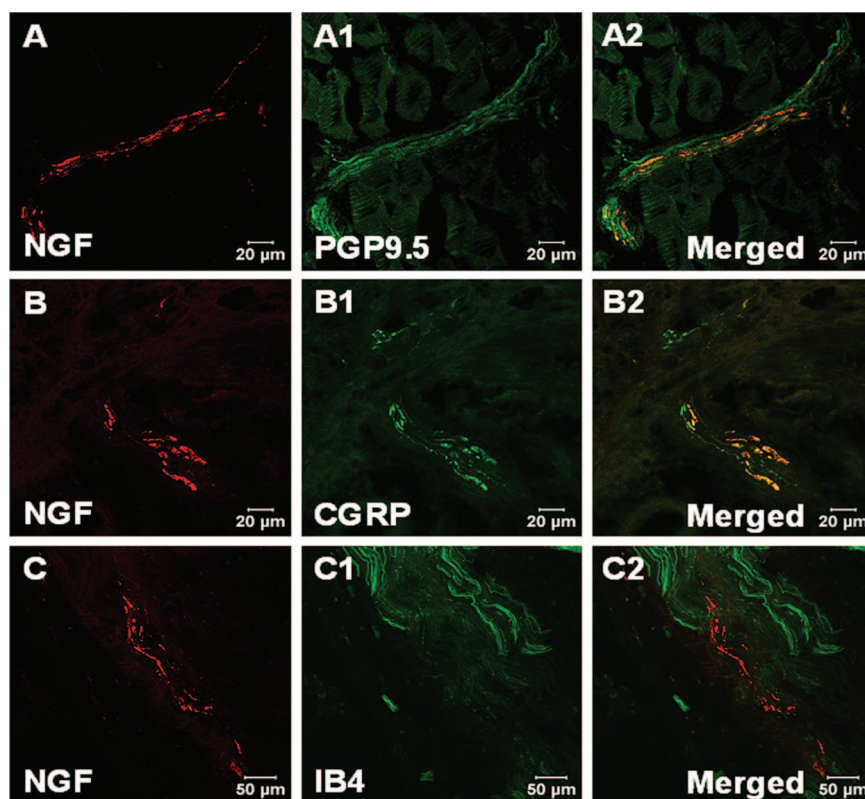
Immunohistochemistry of Incised Hind Paw Muscle for NGF

There was no NGF immunoreactivity detected in sham-operated, control flexor muscle (fig. 3A). NGF immunoreactivity was found in apparent nerve-like structures in incised muscle on POD 1 (fig. 3B). Higher magnifications of the rectangular areas marked in figure 3B are shown in figure 3C and 3D. NGF immunoreactive elements were localized within transverse and longitudinal sections of nerve-like structures (fig. 3, C and D). After preabsorption of the anti-NGF antibody with the hapten-blocking peptide, there was no NGF immunoreactivity detected in skin, fascia, and muscle (fig. 3E).

Immunofluorescent Double-Labeling For NGF In Incised Hind Paw Muscle

NGF and PGP 9.5, CGRP, or IB4 double-labeling was performed to confirm the immunoreactive NGF in nerve structures. NGF-positive staining was found in nerve

Fig. 4. Confocal image of nerve fibers in muscle tissue for nerve growth factor (NGF) and protein gene product 9.5 (PGP 9.5), calcitonin gene-related peptide (CGRP), and isolectin B4 (IB4) fluorescent immunohistochemistry. (A, B, and C) NGF fluorescent immunohistochemistry (red) within incised flexor muscle on postoperative day (POD) 1. (A1) PGP 9.5 immunohistochemistry for nerve axons (green) in incised flexor muscle. (A2) Merged image shows that NGF colocalizes with PGP 9.5 immunoreactive axons (yellow). (B1) CGRP immunohistochemistry for axons (green) within incised flexor muscle. (B2) Merged image shows that NGF colocalizes with CGRP immunoreactive axons (yellow). (C1) IB4 labeling (green) in incised flexor muscle. (C2) Merged image shows that NGF does not colocalize with IB4 axons (yellow).



axons labeled with PGP 9.5 in incised muscle 1 day after incision (fig. 4, A-A2). These NGF-positive axons were colocalized with CGRP-positive fibers (fig. 4, B-B2), but not IB4-positive non-peptidergic C-fibers (fig. 4, C-C2). NGF-positive staining could also be found in large nerve bundles in deep tissue, which innervates the flexor digitorum brevis muscle (fig. 5) and possibly other deep structures. In these bundles, NGF immunoreactivity was colocalized with CGRP-positive fibers (fig. 5, A-A2). There was no colabeling of NGF with IB4-positive C-fibers (fig. 5, B-B2), but colabeling was present on some N52-labeled myelinated axons (fig. 5, C-C2).

In Situ Hybridization For NGF mRNA Expression in Incised Hind Paw

Adult rat brain cortex sections were subjected to *in situ* hybridization with NGF antisense and sense probes to examine the specificity of the NGF probes (fig. 6). NGF mRNA signals (blue) could be observed in cortex (fig. 6A) by antisense probe hybridization, and most of the signals were localized in cytoplasm surrounding the nucleus (red) of neurons (fig. 6B). There was no NGF mRNA staining in cortex when hybridized with the sense probe (fig. 6C); only nuclear staining (red) was observed (fig. 6D).

NGF mRNA expression could be found in sections obtained from sham-incised hind paw (fig. 7A). The sources of NGF mRNA included keratinocytes in the epidermis and fibroblasts in dermis (fig. 7B). In deeper tissue, fibroblasts in dense connective tissue (fig. 7C),

perimysium of muscle (fig. 7D), perivascular connective tissue (fig. 7E), and a large nerve bundle (fig. 7F) innervating the muscle were positive for NGF mRNA.

NGF mRNA labeling was observed in sections obtained at 2 h after plantar incision (fig. 8A) when NGF mRNA by RT-PCR was increased. In addition to structures labeled in the unincised tissue (fig. 8, B-F), fibroblasts in the subcutaneous region adjacent to the dissected tissue and the incision (fig. 8G) and fibroblasts adjacent to the muscle incision (fig. 8H) produced NGF mRNA. *In situ* hybridization with NGF sense probe at 2 h after incision did not show NGF mRNA-positive signals (fig. 8I).

Discussion

In this study, incision that included hind paw fascia and flexor digitorum brevis muscle produced guarding behavior that was reduced by blockade of NGF. NGF expression was examined in normal and in incised hind paw muscle. NGF mRNA was increased at 2 h after incision and returned to normal on POD 1. NGF protein increased 4 h after muscle incision, was increased for 5 days, and was not different than control on POD 10. A large MW form of NGF was produced and increased by incision. NGF immunoreactivity was not identified in normal muscle; however, NGF-immunoreactive structures were observed in nerve bundles from incised muscle. Further delineation of the immunoreactive structures revealed that NGF staining was present in CGRP- and

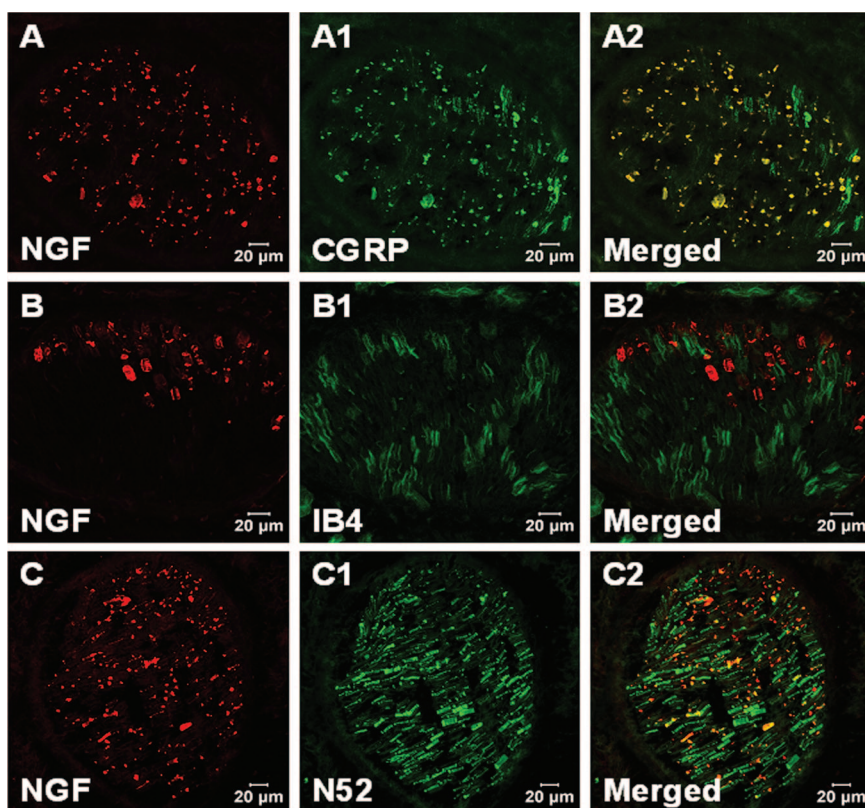


Fig. 5. Confocal image for nerve growth factor (NGF) and calcitonin gene-related peptide (CGRP), isolectin B4 (IB4), and neurofilament 200 (N52) fluorescent double-labeling immunohistochemistry in large nerve in deep tissue. (A, B, and C) NGF fluorescent immunohistochemistry 1 day after plantar incision. (A1) CGRP immunohistochemistry for axons (green) in large nerve. (A2) Merged image shows that NGF colocalizes with CGRP immunoreactive axons (yellow). (B1) IB4 labeling for axons (green) in large nerve. (B2) Merged image shows that NGF does not colocalize with IB4 axons (yellow). (C1) N52 labeling for axons (green) in large nerve. (C2) Merged image shows that NGF colocalizes with some N52 axons (yellow).

N52-positive axons. IB4-positive axons did not stain for NGF. NGF mRNA was present in normal hind paw sections. Fibroblasts were the sources of NGF mRNA in perimysium, perivascular connective tissue, and nerve adjacent to the incised muscle.

NGF is an important pain mediator in skin,² but NGF and muscle have been studied to a lesser extent. NGF is synthesized in normal skeletal muscle tissue, but the cellular elements producing NGF are not known.^{16,17}

Studies by others indicate that NGF contributes to muscle healing after injury.¹⁸ NGF is also upregulated in muscle after intramuscular formalin injection.¹⁹

Previously conducted human studies indicate the potential role of NGF in muscle pain. Local injection of NGF into the masseter muscle produces long-term local signs of mechanical allodynia and hyperalgesia and is associated with pain during jaw movement.²⁰ In trials of parenterally administered NGF for patients with diabetic

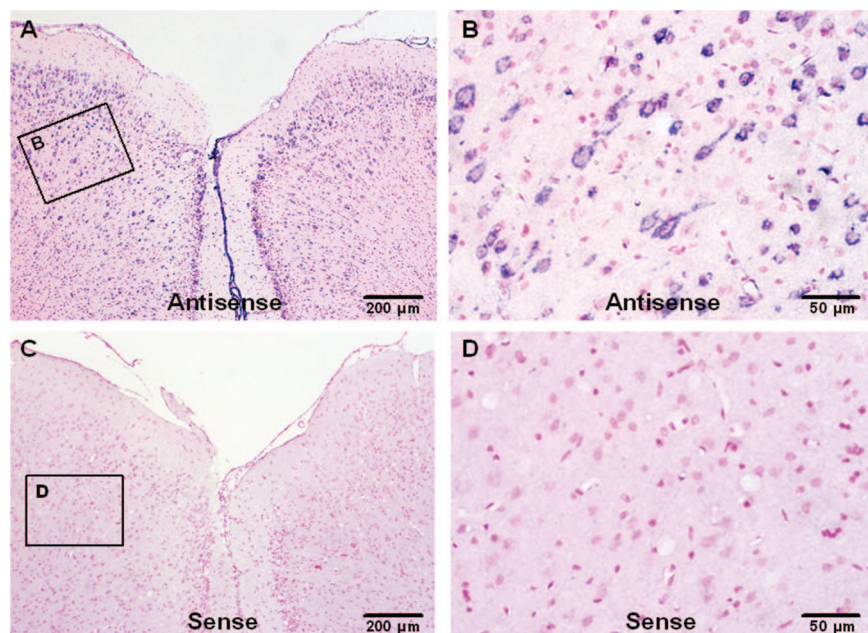
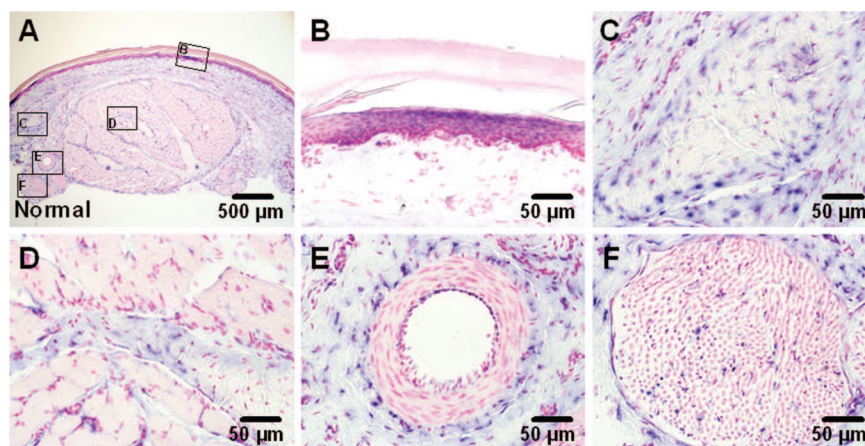


Fig. 6. *In situ* hybridization for nerve growth factor (NGF) mRNA in cortex of adult rat brain. Sections are counterstained with fast red for nuclei. (A) *In situ* hybridization (ISH) for NGF mRNA with antisense probe. (B) A higher magnification of the rectangular area in A, showing NGF expression (blue) in neuron cytoplasm. (C) ISH for NGF mRNA with sense probe. (D) A higher magnification of the rectangular area in C.

Fig. 7. *In situ* hybridization (ISH) for nerve growth factor (NGF) messenger RNA (mRNA) in sham-operated plantar skin and flexor digitorum brevis muscle. Sections are counterstained with fast red for nuclei. (A) ISH for NGF mRNA with antisense probe. (B–F) Higher-magnification photographs show NGF mRNA expression (blue) within epidermis and dermis (B), dense connective tissue in subcutaneous tissue (C), perimysium that separates muscle fibers (D), perivascular connective tissue (E), and large nerve bundle (F) in deep layer that innervates plantar skin and flexor muscle.



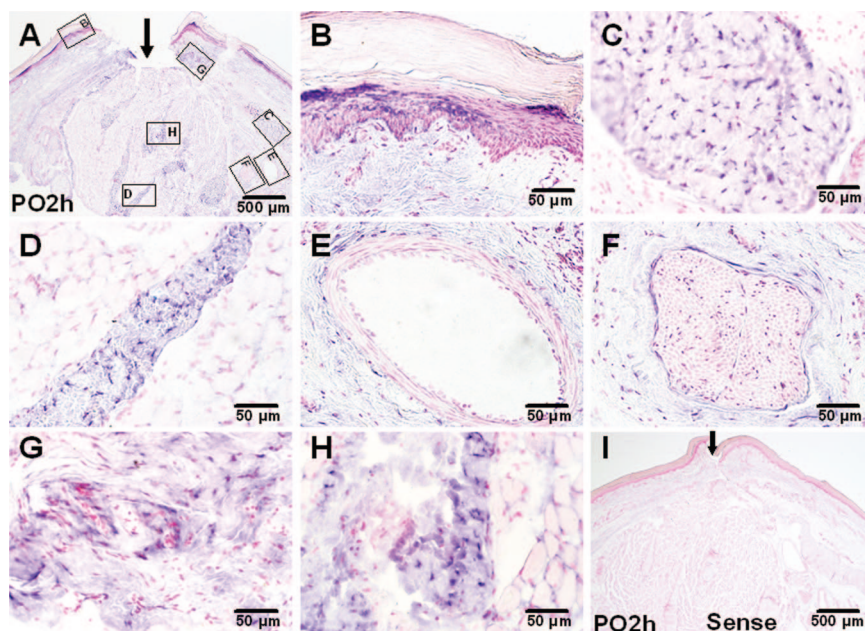
neuropathy, NGF did not improve symptoms. Rather, administration of NGF elicited whole-body myalgias in patients.⁴ The duration and severity of these myalgias were dose-dependent.

Muscle nerves contain motor axons, sensory axons, and sympathetic axons; the sensory fibers are both myelinated and unmyelinated.²¹ Our data have shown immunocytochemical labeling for NGF in CGRP-positive peptidergic axons that may also express the high-affinity NGF receptor trkA.^{22–24} NGF labeling was not found in IB4-positive nonpeptidergic axons. In incisions, NGF was colabeled with the myelinated axon marker N52-positive in a few fibers. There is approximately 10% overlap between CGRP- and N52-positive axons; therefore, these NGF-positive, N52-positive fibers may also have the trkA receptor.²⁵ In animal studies, intramuscular injection of NGF excites primary afferent muscle nociceptors²⁶ and dorsal horn neurons transmitting nociception from muscle.^{27,28} One study was not able to detect sensitization of nociceptors by NGF injected into rat mas-

seter muscle.²⁹ Muscle afferents transmit both mechanosensitivity and chemosensitivity.^{30–33} There is also evidence that muscle afferents have thermosensitivity.³⁴

NGF released by muscle incision likely influences the sensitivity of nociceptors to a variety of mediators, although no systematic studies of NGF and muscle sensory ion channels and receptors have been described. In other tissues, NGF regulates a number of ligand-gated ion channels, including purinergic receptor of ATP (P2X3), acid-sensing ion channel 3, transient receptor potential vanilloid 1, and G protein-coupled receptors, including bradykinin B2 receptors.³⁵ NGF also increases voltage-gated sodium channels, including tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels (Nav 1.3, 1.8, and 1.9). Whether these mechanisms through which NGF contributes to nociception in other tissues also influence nociception from muscle is not known. We speculate that NGF influences chemosensitivity of muscle afferents after incision, potentiating its responses to mediators like prostaglandins, acid, and lactate.

Fig. 8. *In situ* hybridization (ISH) for nerve growth factor (NGF) messenger RNA (mRNA) in incised plantar skin and flexor digitorum brevis muscle. Sections are counterstained with fast red for nuclei. (A) ISH for NGF mRNA with the antisense probe 2 h after incision. (B–H) Higher-magnification photographs show NGF mRNA expression within epidermis and dermis (B), dense connective tissue in subcutaneous region (C), perimysium (D), perivascular connective tissue (E) and large nerve bundle (F) in deep tissue layer. (G) ISH in subcutaneous region adjacent to skin incision. (H) Flexor muscle fibers adjacent to muscle incision. (I) ISH for NGF mRNA with sense probe at 2 h after incision showing no positive staining. The arrows indicate the incision. PO = postoperative.



NGF is produced in several forms by a variety of tissues and cells depending on the conditions. A large MW form of NGF, approximately 75 kDa, was shown after muscle incision in the current study and after skin incision in a previous study.⁹ Mature NGF is approximately 16 kDa; therefore, this large MW form is likely a glycosylated precursor, like preproNGF, or a dimer of a precursor form.^{36,37} Isaacson *et al.*^{37,38} demonstrated that mature forms of the lower MW NGF proteins are uncommon in tissues, and each tissue exhibits a characteristic NGF expression pattern. In adults, muscle and skin have the same large MW form, and both increase in response to incision.

In vitro studies of primary cell cultures indicate that NGF is produced by fibroblasts,³⁹ keratinocytes,⁴⁰ and T cells.⁴¹ In inflammatory pain models, NGF is likely produced by macrophages⁴² and mast cells⁴³; after nerve injury, Schwann cells are a source of NGF.⁴⁴

In the current study, NGF mRNA was identified using *in situ* hybridization in normal plantar skin and flexor muscle as well as in incised tissue. Fibroblasts from fascia and connective tissues surrounding muscle express NGF in the area injured by the incision. NGF mRNA was found in nonneuronal cells, whereas NGF protein was localized in nerve fibers. NGF expression by inflammatory cells was not prominent. The different NGF source in our incisional pain model compared with other inflammatory pain models supports the unique characteristics for incisional pain mechanisms *versus* inflammatory pain. Our data are supported by another study of cutaneous wound healing,⁴⁵ which demonstrates that NGF is produced by keratinocytes and fibroblasts and contributes to cutaneous wound healing.

In previously performed studies in skin,⁹ we demonstrated that NGF protein is present in the area surrounding incised skin. In transverse hind paw skin incisions, immunoreactive NGF was prominent in distal axons in skin but not found in axons proximal to the incision. On the basis of these previous results, the current study, and work by others,^{46,47} we propose the following model. NGF is synthesized in fibroblasts and connective tissue in incisions, is retrogradely transported to the dorsal root ganglia in intact axons, and sensitizes these nociceptors. The distal portions of cut axons take up NGF but do not transport it retrogradely.⁴⁶ These distal portions of the cut axons are immunoreactive for NGF. The colabeling of NGF with CGRP indicates that these are Trk A-positive axons. In incised skin, NGF produces heat sensitization.¹⁰ In deep muscle incision, we propose that NGF produces spontaneous activity resulting in ongoing pain.

In summary, NGF contributes to guarding pain behaviors caused by deep muscle incision. In incised muscle, NGF mRNA increases 2 h after incision, and increased protein expression is evident for several days. Immunohistochemical studies of incisions reveals NGF protein in CGRP-positive but not IB4-positive nerve fibers. Fibro-

blasts adjacent to the injury are sources of NGF in incised muscle. NGF is not only important for cutaneous pain mechanism but also for pain from incised muscle.

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