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Mechanisms of nerve growth factor signaling in bone nociceptors and in an animal model of inflammatory bone pain

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Abstract

Sequestration of nerve growth factor has been used successfully in the management of pain in animal models of bone disease and in human osteoarthritis. However, the mechanisms of nerve growth factor-induced bone pain and its role in modulating inflammatory bone pain remain to be determined. In this study, we show that nerve growth factor receptors (TrkA and p75) and some other nerve growth factor-signaling molecules (TRPV1 and Nav1.8, but not Nav1.9) are expressed in substantial proportions of rat bone nociceptors. We demonstrate that nerve growth factor injected directly into rat tibia rapidly activates and sensitizes bone nociceptors and produces acute behavioral responses with a similar time course. The nerve growth factor-induced changes in the activity and sensitivity of bone nociceptors we report are dependent on signaling through the TrkA receptor, but are not affected by mast cell stabilization. We failed to show evidence for longer term changes in expression of TrkA, TRPV1, Nav1.8 or Nav1.9 in the soma of bone nociceptors in a rat model of inflammatory bone pain. Thus, retrograde transport of NGF/TrkA and increased expression of some of the common nerve growth factor signaling molecules do not appear to be important for the maintenance of inflammatory bone pain. The findings are relevant to understand the basis of nerve growth factor sequestration and other therapies directed at nerve growth factor signaling, in managing pain in bone disease.

Keywords

Bone, bone pain, skeletal, NGF, nerve growth factor, inflammatory pain

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Introduction

Nerve growth factor (NGF) is a neurotrophin known for its role in the development of sympathetic and sensory neurons,[1] but has more recently been implicated as a mediator of inflammatory pain.[2,3] NGF acts through two cell surface receptors: p75 and TrkA. Mutations of TrkA genes have been reported in individuals who have congenital insensitivity to pain.[4–6] There is an increase in NGF levels in painful conditions that are characterized by inflammation, such as arthritis,[7,8] and NGF applied exogenously to human skin or muscle produces hyperalgesia or allodynia.[9–11] TrkA receptor knockout mice are hypoalgesic[12] and transgenic animals over-expressing NGF are hyperalgesic.[13] Furthermore, NGF is elevated in animal models of acute and chronic pain.[14–18] Importantly, most of these studies have used inflammatory pain models or examined conditions that have a significant inflammatory component, reinforcing a role for NGF in inflammatory pain.

NGF can affect the excitability of peripheral sensory neurons in a number of ways. Binding of NGF to TrkA can lead to rapid post-translational modifications that increase the activity of some ion channels, including transient receptor potential vanilloid type 1 (TRPV1) and voltage-gated Na^+ channels, at peripheral sensory nerve terminals.[19–23] These changes at nerve terminals

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are evident within minutes of applying NGF and increase their activation by sensory stimuli. There is also evidence that internalization and retrograde transport of the NGF/TrkA complex to the soma of peripheral sensory neurons induce delayed changes in expression of TRPV1 and voltage-gated Na^+ channels.[24–28] The increase in membrane expression of these ion channels occurs over hours to days and increases the excitability of peripheral sensory neurons by lowering their sensory thresholds.

The dominant feature common to most conditions that produce bone pain is inflammation resulting from the release of inflammatory mediators by cells associated with bone disease.[29–33] Agents known to act by reducing inflammatory processes (e.g. NSAIDs) produce partial analgesia in animal models of bone cancer pain[34] and pro-inflammatory cytokines contribute to nociceptive responses in bone fracture models.[35] The TrkA receptor is localized to peripheral nerve terminals in bone,[36–39] and TrkA expression has been reported in the soma of bone afferent neurons, at least in very young animals.[40] However, only a few studies have investigated the mechanisms of NGF signaling in bone pain. These have shown that sequestering NGF by administration of anti-NGF antibodies can alleviate, in part, pain-like behaviors in animal models of bone cancer and skeletal fracture.[41–43] While these studies clearly indicate a role for NGF signaling in bone pain, an understanding of the mechanisms underlying the effects of NGF in the context of bone pain is still lacking.

The objective of the present study was to clarify the mechanisms by which NGF interacts with bone nociceptors to signal bone pain. We aimed to determine if, and what proportion of, bone nociceptors express NGF receptors (TrkA and p75) and associated signaling molecules (TRPV1 and the sodium channels Nav1.8 and Nav1.9). We also aimed to determine if NGF rapidly activates and/or sensitizes bone nociceptors and if its time course of action is consistent with acute behavioral effects induced by NGF injected directly into bone. Finally, we aimed to determine if increased expression of NGF-signaling molecules occurs in the longer term in a model of inflammatory bone pain.

Methods

Naïve, male Sprague-Dawley rats weighing between 200 and 350 g were used in this study. Animals were housed in pairs or groups of four, in a 12/12 h light/dark cycle and were provided with food and water ad libitum. All experiments conformed to the *Australian National Health and Medical Research Council* code of practice for the use of animals in research and were approved by the University of Melbourne *Animal Experimentation Ethics Committee*.

In the current study, we focused on innervation of the bone marrow, not the periosteum. Almost all bony pathologies, including those with a significant inflammatory component, involve the bone marrow. Sensory neurons that innervate the bone marrow are described as small-diameter myelinated or unmyelinated fibers,[44–48] contain neuropeptide markers for nociceptors, such as substance P (SP) and calcitonin gene-related peptide (CGRP),[45,48,49] and are responsive to noxious (but not innocuous) chemical and mechanical stimuli.[46,47,50] Taken together, these findings indicate that sensory neurons that innervate the bone marrow are nociceptors, and we refer to them as bone nociceptors throughout.

Retrograde tracing

Animals were anesthetized with isofluorane (4% induction; 2.5% maintenance). A skin incision was made over the medial aspect of the tibia and a small hole was made in the cortical bone on the medial aspect of the tibial diaphysis using a sterile 26 gauge needle. A Hamilton syringe was used to inject the retrograde tracer Fast Blue (2 µl FB; 10% in dH_2O Illing Plastics GmbH) through the hole and directly into the medullary cavity. The hole was sealed with bone wax and covered to prevent leakage into surrounding tissues and the entire area was washed extensively with 0.1 M phosphate-buffered saline (pH 7.4; PBS) and inspected for tracer leakage using a hand-held UV illumination device. Animals that showed evidence of tracer leakage to surrounding tissues were excluded from this study. Skin incisions were closed with stainless steel autoclips. For studies of constitutive expression of NGF-signaling molecules in bone nociceptors, animals were left for a seven-day survival period to allow for transport of the tracer to neuronal cell bodies in the dorsal root ganglion (DRG). For studies of changes in expression of NGF-signaling molecules in inflammatory bone pain, an inflammatory agent (see below) or saline was co-injected with the retrograde tracer and the animals were left for a four-day survival period chosen to coincide with the peak pain time-point in the animal model.

Tissue preparation

Each animal was given an overdose of sodium pentobarbitone (Lethobarb; 80 mg/kg; i.p.) and was perfused via the ascending aorta with 500 ml of heparinized PBS followed by 500 ml of 4% paraformaldehyde in PBS. For studies of peripheral nerve terminals in bone, the tibiae of animals were removed and carefully cleaned of surrounding soft tissue. They were decalcified in a solution containing 20% EDTA in PBS for two weeks. The solution was changed every three days over this time. For studies of retrograde-labeled bone nociceptors, lumbar

DRG L3 and L4 were dissected. After dissection and/or decalcification, all tissues were left overnight in a solution containing 20% sucrose in PBS, and were sectioned at 14 μm (DRG) or 20 μm (bone) using a cryostat the next day. Multiple series of sections were collected on gelatinized glass slides (0.1% chrome alum and 0.5% gelatin) and processed for immuno-labeling.

Immunohistochemistry

Sections were immuno-labeled to determine whether peripheral nerve terminals in bone expressed protein gene product 9.5 (PGP9.5), TrkA and/or tyrosine hydroxylase (TH), or if retrograde-labeled neurons expressed TrkA, p75, CGRP, TRPV1, Nav1.8 and/or Nav1.9. Details of the primary and secondary antisera, including references to work that cite specificity of the antibodies, are given in Table 1. All antisera were diluted in PBS containing 0.3% Triton X-100 and 0.1% sodium azide. Sections were washed three times in PBS and incubated overnight in the primary antisera at room temperature. Following three further washes in PBS, they were incubated in secondary antibody for 2 h, and washed again three times in PBS. The slides were cover-slipped using DAKO fluorescence mounting medium.

Image acquisition and data analysis

Sections of bone or DRG were examined and photographed with a 20× objective using a Zeiss Axioskop fluorescence microscope (Zeiss, Oberkocken, Germany) fitted with an AxioCam MRm camera. FITC, Texas Red, Cy5 and UV filter sets were used to discriminate labelling with the AlexaFluor 488, 594 and 647 fluorophores, and Fast Blue, respectively. Immuno-labelling of retrograde-labeled bone nociceptors was examined in a separate series of DRG sections for each marker. Counts, soma size measurements (cross-sectional area of soma) and intensity measurements were made directly from the images using Zen lite software. For constitutive expression analysis, we determined the proportion of retrograde-labeled neurons that expressed each antibody marker for each animal. For analysis of changes associated with inflammatory bone pain, we tested whether there was a difference in the proportion of bone nociceptors expressing each marker and/or the intensity of labelling in neurons. Changes in the proportion of neurons that express each molecule likely reflect de novo expression and/or retrograde transport from the periphery to the soma. If there is no change in the proportion, then changes in the intensity of immuno-labelling likely reflect increased expression in neurons that already contain the protein, not de novo expression. Intensity of immunolabelling was determined for each cell by calculating the mean pixel intensity in the soma and was expressed relative to the average of the mean pixel intensities of five surrounding cells, in the same section, that were clearly not immuno-labeled. To prevent double counting and to avoid cell size bias, only cells with a nucleus visible under the microscope were examined. Figures were prepared using CorelDraw software. Individual images were contrast and brightness adjusted. No other manipulations were made to the images. A Mann-Whitney test was used to test for statistical differences between inflamed and saline-injected animals. $P < 0.05$ was used to define statistical significance. For this comparison, the group sizes required to demonstrate a statistical difference ($n = 4$ per group) were determined using a power of 0.8 and an alpha of 0.05, with one mean and standard deviation for this power calculation taken from the studies of the constitutive protein expression (Table 2), and the other mean being at least double this value but with the same standard deviation.

Electrophysiological recordings using an in vivo bone-nerve preparation

Electrophysiological experiments were performed in rats anesthetized with urethane (50% w/v, 1.5 g/kg i.p). We identified a fine branch of the tibial nerve that innervates the marrow cavity of the rat tibia. The nerve was carefully teased away from its associated blood vessels and surrounding tissues over a distance of approximately 1 cm and placed over a platinum hook electrode for electrophysiological recordings. Whole-nerve electrical activity was amplified (1000×) and filtered (high-pass 100 Hz, low pass 3 kHz) using a differential amplifier (DP-311, Warner Instruments), sampled at 20 kHz (Powerlab, ADInstruments, Australia) and stored to PC using the recording software LabChart (ADInstruments). Mechanical stimulation was delivered by increasing tibial intra-osseous pressure to 400 mmHg and maintaining this pressure for 15 s. This was achieved by injecting heparinized physiological saline (0.9% sodium chloride) into the marrow cavity through a needle, implanted in a small hole in the proximal tibia (size: 0.8 mm), that was connected to a syringe pump via polyethylene tubing. Preliminary experiments revealed that this magnitude of pressure (400 mmHg) provides supra-threshold stimulation for single bone nociceptors. Changes in the intra-osseous pressure were measured using a bridge amplified (Quad Bridge, ADInstruments) signal derived from a pressure transducer (BP transducer, ADInstruments) placed to measure the input pressure to the bone. These data were also stored to PC in parallel with the nerve recordings. NGF (1 or 5 μg in 10 μl; Sigma NGF-7 S) was delivered to the marrow cavity through a second needle and hole (size: 0.5 mm) using a Hamilton syringe attached with polyethylene tubing. The concentrations of NGF used are in the range that produces behavioral

Table 1. Source and concentrations of the primary and secondary antisera used in this study.

Primary antibody antigen	Immunogen	Manufacturer and product code	Dilution used	Specificity/ characterization
Protein gene product 9.5 (PGP9.5)	Human PGP9.5 purified from pathogen-free human brain	Ultraclone; Rabbit polyclonal; #RA95101	1:1000	[26]
Calcitonin gene-related peptide (CGRP)	Synthetic rat Tyr-CGRP (23–37)	Biogenesis; Goat polyclonal; #1720–9007	1:1000	[109] Manufacturer's information
Calcitonin gene-related peptide (CGRP)	Synthetic rat CGRP conjugated to KLH	Sigma, Rabbit polyclonal; #C8198	1:500	[84,111] manufacturer's information
Transient receptor potential Vanilloid 1 (TRPV1)	Intracellular C-terminus of rat TRPV1 (824–838)	Alomone Labs; Rabbit polyclonal; #ACC-030	1:500	[1,30] Manufacturer's information
Voltage-gated sodium channel $Na_V1.8$	Intracellular, C-terminus of rat $Na_V1.8$. (1943–1956)	Alomone Labs; Guinea pig polyclonal; #AGP-029	1:500	Manufacturer's information
Voltage-gated sodium channel $Na_V1.9$	Intracellular, C-terminus of rat $Na_V1.8$. (1748–1765)	AlomoneLabs; Rabbit polyclonal; # ASC-017	1:2000	[98,110]
Tyrosine receptor kinase A (TrkA)	Extracellular domain of embryonic chick TrkAisolated from e8 DRG	Louis Reichardt; rabbit polyclonal	1:2000	[32,63,90]
Tyrosine receptor kinase A (TrkA)	Purified recombinant rat TrkA extracellular domain (Ala33-Pro-418)	R&D Systems; Goat polyclonal; #AF1056	1:500	[59,76] Manufacturer's information
Nerve growth factor receptor (p75)	Cytoplasmic domain of human p75	Promega; rabbit polyclonal #G323A	1:1000	Manufacturer's information
Tyrosine hydroxylase	Denatured tyrosine hydroxylase from rat pheochromocytoma	Millipore; rabbit polyclonal; #AB152	1:250	[49] Manufacturer's information
Secondary antibody		Manufacturer	Dilution used	
Donkey anti-Rabbit 594		Molecular probes, Invitrogen; #A21207	1:200	
Donkey anti-Goat 488		Molecular probes, Invitrogen; #A11055	1:200	
Donkey anti-Guinea pig 647		Jackson Immuno Research; #706-606-148	1:500	

Table 2. The percentage of retrograde-labeled bone nociceptors that express various NGF-signaling molecules, and the proportion of TrkA+ bone nociceptors that express CGRP.

	Number of animals	Number of retrograde-labeled bone nociceptors	Percentage (mean ± SEM)
Percentage of bone nociceptors that are:			
TrkA+	3	106	67 ± 1.3
p75+	3	411	65 ± 5.8
TRPV1+	3	106	30 ± 0.6
Nav1.8+	3	146	40 ± 5
Nav1.9+	3	146	7 ± 3
Proportion of TrkA+ bone nociceptors that express CGRP	3	183	85 ± 2
Proportion of TrkA+ bone nociceptors that express TRPV1	3	154	38 ± 3
Proportion of TrkA+ bone nociceptors that express Nav1.8	3	146	53 ± 3
Proportion of TrkA+ bone nociceptors that express Nav1.9	3	146	6 ± 3

pain-like responses when applied to the hindpaw footpad[51] and the tibial marrow cavity (see Results section) of naïve rats. However, we do not know the actual concentration of NGF at the nerve terminal endings in the bone marrow because there is undoubtedly some dilution in the bone marrow compartment. The tube used for delivery of NGF was clamped before and after its delivery to avoid leakages during pressurization. A function blocking anti-TrkA antibody (15 μg i.v.; MNAC13; Absolute Antibody, Oxford, UK) or a mast cell stabilizer (20 mg/kg i.v.; cromolyn; Sigma-Aldrich, Castle Hill, AUS) was delivered systemically via a cannula inserted into the jugular vein. Only nerve impulses that were at least twice the amplitude of the background noise (~40 μV peak-to-peak) were used for assessing changes in activity. Single units were discriminated in the whole nerve recordings according to their amplitude and duration using Spike Histogram software (LabChart 8, ADInstrument). Threshold for activation and discharge frequency after injection of NGF were expressed relative to pre-injection baseline levels.

In preliminary experiments, single unit conduction velocities were determined using two recording electrodes with a distance of 7 to 10 mm between electrodes. Pressure stimuli were delivered at 400 mmHg as above, and single units were discriminated on each electrode during mechanical stimulation. Impulses recorded at the second electrode, that were time-locked to those recorded at the first electrode, were considered as originating in the same axon. For these units conduction velocities were estimated by dividing conduction time between the electrodes by the distance between electrodes. Units with Aδ conduction velocities (2 to 12.5 m/s) had action potential amplitudes between 47 and 145 μV (peak-to-peak; n = 16) and were readily discriminated in our recordings. Units with C conduction velocities (<2 m/s) had small amplitude action potentials (<40 μV, n = 5). These were excluded from further analysis because variation in their amplitude and duration made them difficult to isolate unambiguously. Thus, the units we have presented in this paper are exclusively Aδ fibres.

The first series of experiments were conducted to determine if NGF can rapidly activate bone nociceptors in whole-nerve recordings (Saline n = 6; NGF 5 μg in 10 μl n = 6; NGF 1 μg in 10 μl n = 6). In these experiments, to assess the effects of the treatments on ongoing (whole-nerve) activity, the frequency (Hz) of impulses was determined over 3-min intervals at the 5, 15, 30, 45 and 60 min time-points.

A second series of experiments was conducted to determine if single mechanically activated bone nociceptors could be sensitized by NGF (Saline n = 10; NGF 5 μg in 10 μl n = 12; NGF 1 μg in 10 μl n = 7). Sensitization to mechanical stimulation was explored because intra-osseous pressure is a mechanical stimulus that directly activates the nerve fibers in the rat tibia and we are able to control changes in intra-osseous pressure in our preparation. In these experiments, responses of single units to pressure (400 mmHg) were assessed before, 15 and 30 min after application of NGF or saline. Decreases in the mechanical threshold assessed on the rising phase of the pressure ramp and/or increases in overall discharge frequency during the pressure stimulus were used as indicators of sensitization. There was some variability in discharge frequency of mechanically activated units before and after saline injection, but none had increases that were >15% above the baseline

frequency of discharge. Thus, single units were classified as being sensitized by NGF if they increased their discharge frequency by $\geqslant 20\%$ relative to the baseline level.

A third series of experiments was conducted to determine if NGF-induced changes in the activity or sensitivity of bone nociceptors were dependent on signaling through the TrkA receptor or mast cells. To do this, we compared NGF-induced activity in whole-nerve recordings, and NGF-induced changes in the sensitivity of single, mechanically activated bone nociceptors, in the presence and absence of a function blocking anti-TrkA antibody or a mast cell stabilizer. The anti-TrkA antibody (15 μg i.v.; MNAC13; Absolute Antibody, Oxford, UK) blocks NGF binding to TrkA in rats[52] and prevents licking behavior when delivered systemically at this dose in a mouse formalin-induced inflammatory pain model.[53] In the present study, it was delivered intravenously, 30 min before application of NGF. The mast cell stabilizer (20 mg/kg i.v.; cromolyn; Sigma-Aldrich, Castle Hill, AUS) inhibits mast cell degranulation, has a short half-life with peak activity at approximately 15 min, and is delivered systemically at this dose to treat asthma.[54,55] In the present study, it was delivered intravenously, 15 min before application of NGF.

Repeated measures ANOVA with Dunn's post hoc test, or a one- or two-way ANOVA with Bonferroni's post hoc test were used for statistical analyses of electrophysiological data as required. Post hoc testing was only performed if the ANOVA indicated a significant difference. $P < 0.05$ was used to define statistical significance.

Surgery to inject NGF for behavioral testing

NGF was injected into the rat tibia to test whether it produces rapid pain-like behaviors when applied directly to bone. Animals were anesthetized and prepared as above for retrograde tracing. A Hamilton syringe was used to inject NGF (5 μg/10 μl) or saline (10 μl volume control) directly into the medullary cavity. Recovery from isofluorane anesthesia occurred within a few minutes of the end of the surgery, allowing behavioral testing to be performed within 15 min of surgery.

Complete Freund's adjuvant-induced inflammatory bone pain

Complete Freund's adjuvant (CFA) was injected into the rat tibia to test whether inflammation in bone, that develops over a longer time course (days to weeks), produces delayed and persistent pain-like behaviors that can be correlated with changes in expression of NGF-signaling molecules. Animals were anesthetized and prepared as above for retrograde tracing. A Hamilton syringe was used to inject CFA (50 μl; Mycobacterium tuberculosis, suspended in a 1:1 oil/saline mixture; Sigma, Australia) or saline (volume control) through the hole directly into the medullary cavity. To identify bone nociceptors, the retrograde tracer Fast blue (2 μl; 10% in dH_2O) was co-injected with CFA (or saline). Animals that showed evidence of Fast blue leakage to surrounding tissues were excluded from this study.

A series of experiments were performed to confirm inflammation was present in the tibiae of CFA-injected animals at the peak pain time-point. CFA ($n = 3$) was injected as above and behavioral testing (see below) used to confirm pain-like behavior. Animals were then perfused, bones collected and prepared as above, and embedded in paraffin blocks. Hematoxylin and eosin staining was performed on sections cut at 20 μm. Sections were examined for cells that indicate the presence of inflammation including myeloid and erythroid cells, neutrophils and megakaryocytes. Saline-injected animals without pain behavior at the same time-points were used as controls ($n = 3$). Observations were made of the bone marrow at a point 6 to 10 mm distal to the proximal tibial metaphysis. Qualitative increases (⇑) or decreases (⇓) relative to saline-injected control animals were assessed by a trained histo-pathologist.

Behavioral testing

Changes in weight bearing, assessed with an incapacitance meter (IITC Life Science Inc., California, US), were used to assay pain-like behavior in response to injection of NGF (at 0, 15 and 30 min, 1, 2 and 5 h, and 1 day after injection) and CFA (0, 4, 7, 10, 14, 18, 22, 26 days after injection). The incapacitance meter independently measures weight bearing on the injected and un-injected limb. Testing was always performed at the same times each day. Animals were first habituated with exposure to the equipment for 15 min per day over three days prior to the surgery. At each testing time-point, five weight bearing readings of 3 s duration were collected. The weight bearing on the injected hindlimb of each animal was then calculated as a percentage of the total weight bearing, using the following equation

$$\frac{\text{average weight on injected hindlimb}}{\left\{\begin{array}{c}\text{average weight on injected hindlimb} \\ +\,\text{average weight on un-injected hindlimb}\end{array}\right\}} \times 100$$

For experiments that explored changes in expression of NGF-signaling molecules associated with CFA-induced inflammatory bone pain, behavioral testing was performed to confirm pain-like behaviors in each animal injected with CFA, and lack of pain-like behaviors in those injected with saline. Testing for this was performed before (baseline) and at day 4 (peak pain

time-point determined from timeline studies above). Weight bearing of less than 45% on the injected hindlimb was used as confirmation of pain behavior in CFA-injected animals because most of these animals bore less weight than this on their injected limb at the four-day time-point, while saline-injected animals had an even distribution of weight between the hindlimbs at this same time-point.

Friedman's tests with Dunn's post hoc analysis were used for statistical comparisons of behavioral data within each experimental group, and Mann-Whitney U-tests were used for comparisons between the groups. Post hoc testing was only performed if the Friedman's test indicated a significant difference. $P < 0.05$ was used to define statistical significance.

Results

TrkA is expressed in peripheral nerve terminals in bone

We used immunohistochemistry to identify TrkA expression in peripheral nerve terminals in bone. Immunolabeling with a pan-neuronal marker (PGP9.5) (Figure 1(a)) showed the full extent of nerve terminal labeling in the marrow cavity. PGP9.5+ nerve fibers were clearly identified in large nerve bundles as they entered the bone through nutrient foramina. Nerve fibers branched from these bundles and extended into the marrow cavity, where they could be seen ending in close association with blood vessels or often as free endings away from blood vessels. TrkA+ nerve fibers were relatively sparse in relation to PGP9.5+ fibers. They were often found in association with blood vessels (Figure 1(c), large arrows) but could also be found away from blood vessels in the marrow cavity (Figure 1(b) and (c); small arrows). Double labeling confirmed that some of the TrkA+ nerve terminals co-expressed TH, the rate limiting enzyme in dopamine synthesis, and are therefore most likely sympathetic nerve terminals in bone (Figure 1(c) to (e)). Those that were not TH+ are presumed sensory in origin. The latter appeared to be located more often away from blood vessels in the marrow cavity (Figure 1(e); small arrows). We attempted but were unable to immuno-label nerve terminals in bone marrow using the antibodies directed against p75, TRPV1, Nav1.8 and Nav1.9 available to us. This may have been because our protocol was not

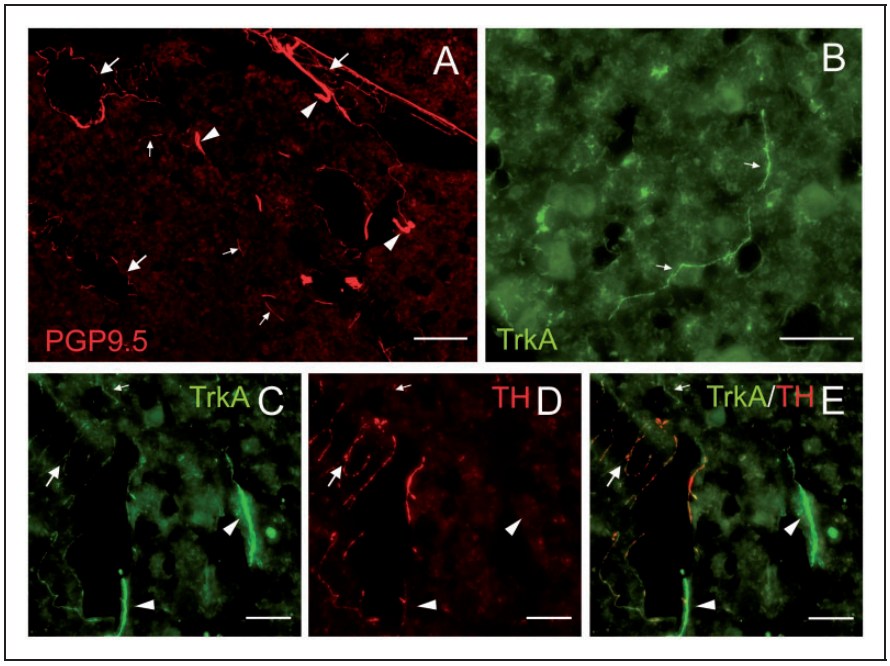

Figure 1. Immuno-labeling of nerve terminals in the rat tibial marrow cavity. (a) PGP9.5+ nerve fiber bundles (arrowheads), nerve fibers in close association with blood vessels (large arrows) and nerve fibers located between blood vessels (small arrows). (b) TrkA+ nerve fibers located between blood vessels (small arrows). (c to e) Images of the same field of view captured with different filter settings to demonstrate the degree of co-expression of TrkA and TH in nerve fibers in the marrow cavity. (c) TrkA+ nerve fiber bundles (arrowheads), TrkA+ nerve fibers around blood vessels (large arrows), and TrkA+ nerve fibers located between blood vessels (small arrow). (d) TH+ nerve terminals around blood vessels (large arrow). (e) merged image showing that most of the TrkA+ nerve fibers around blood vessels expressed TH (large arrow), but that those away from blood vessels did not (small arrow). Scale bar in A = 100 μm and in B–E = 50 μm.

sensitive enough to identify expression in the nerve terminal endings of rat bone nociceptors, or because our decalcification procedure destroyed antigenicity.

TrkA, p75 and other NGF-signaling molecules are constitutively expressed in a substantial proportion of sensory neurons that innervate bone

We used a combination of retrograde tracing and immunohistochemistry in order to confirm the sensory neuron origin of some TrkA+ nerve terminals in the marrow cavity and to determine the proportion of bone nociceptors that express the TrkA and p75 receptors, and/or other NGF-signaling molecules (TRPV1, Nav1.8 and Nav1.9). Retrograde-labeled bone nociceptors were predominantly small or medium-sized neurons (>93% of those counted were less than 1800 μm^2). We found that approximately two-thirds of the bone nociceptors expressed TrkA ($67 \pm 1.3\%$; $n = 3$) and a similar proportion expressed p75 ($65 \pm 5.8\%$; $n = 3$) (Figure 2, Table 2). Almost all of the TrkA+ bone nociceptors ($85 \pm 2\%$; $n = 3$) co-expressed CGRP (Figure 2; Table 2). Thus, not all bone nociceptors express TrkA, but those that do are predominantly peptidergic (express

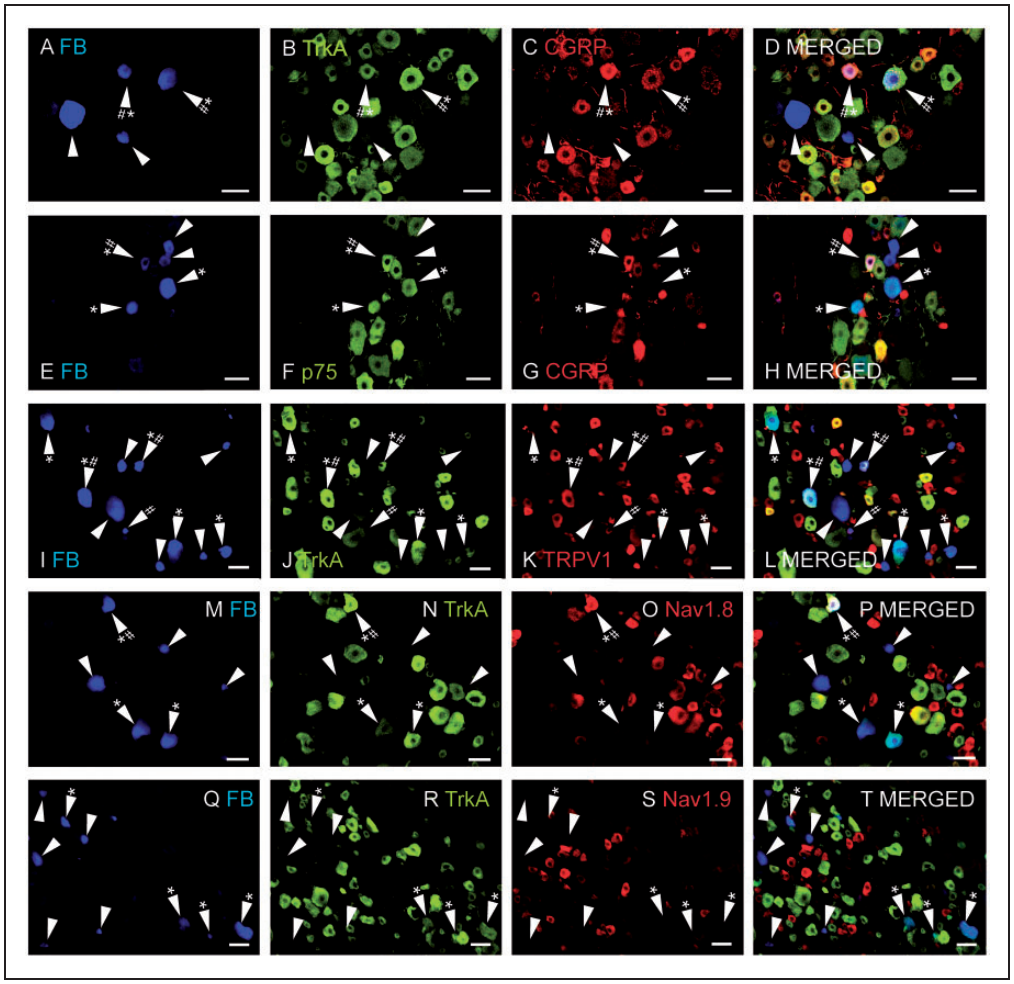

Figure 2. Images of retrograde (Fast blue; FB) and immuno-labeled bone nociceptors in sections through the DRG. Each horizontal panel shows the same field of a single section. Arrowheads identify retrograde-labeled bone nociceptors throughout. A–D shows FB (a), TrkA immuno-labeling (b), CGRP immuno-labeling (c) and a merged image (d). Asterisks (*) indicate bone nociceptors that are TrkA+, and hashes (#) indicate bone nociceptors that are CGRP+. E-H shows FB (e), p75 immuno-labeling (f), CGRP immuno-labeling (g) and a merged image (h). Asterisks (*) indicate bone nociceptors that are p75+, and hash (#) indicates a bone nociceptor that is CGRP+. I–L shows FB (i), TrkA immuno-labeling (j), TRPV1 immuno-labeling (k) and a merged image (l). Asterisks (*) indicates bone nociceptor that are TrkA+, and hash (#) indicates bone nociceptors that are TRPV1+. M–P shows FB (m), TrkA immuno-labeling (N), Nav1.8 immuno-labeling (o) and a merged image (p). Asterisks (*) indicate bone nociceptors that are TrkA+, and hash (#) indicates a bone nociceptor that is Nav1.8+. Q–T shows FB (q), TrkA immuno-labeling (r), Nav1.9 immuno-labeling (s) and a merged image (t). Asterisks (*) indicate bone nociceptors that are TrkA+. Bone nociceptors in this field do not express Nav1.9. Scale bars = 50 μm.

CGRP). A substantial proportion of bone nociceptors expressed TRPV1 (30 ± 0.6%; n = 3) and Nav1.8 (40 ± 5%; n = 3), but relatively few expressed Nav1.9 (7 ± 3%; n = 3) (Figure 2, Table 2). TrkA+ bone nociceptors had enriched expression of TRPV1 (38 ± 3%; n = 3) or Nav1.8 (53 ± 3%; n = 3), but few expressed Nav1.9 (6 ± 3%; n = 3) (Figure 2, Table 2).

NGF rapidly activates bone nociceptors in vivo

We used an *in vivo* electrophysiological bone-nerve preparation to determine if NGF can rapidly activate bone nociceptors. Whole-nerve ongoing activity prior to and after application of NGF was recorded as frequency of action potential discharge (Figure 3(a)). Application of NGF (5 μg in 10 μl) to the marrow cavity resulted in increased ongoing activity in whole-nerve recordings that began between 50 and 165 s of NGF application (Figure 3(b)) (85 ± 20 s; mean ± SEM; n = 6). The peak in the frequency of ongoing discharge occurred within the first 5 min and then slowly decreased to pre-application levels by 30 min (Figure 3(b)). This pattern of NGF-induced activity was observed in all six whole-nerve recordings made. Figure 3(c) shows group data for the 5, 15, 30, 45 and 60 min time-points and reveals a significant increase in ongoing activity at 5 and 15 min but not at later time-points after application of NGF. The magnitude of the discharge at the 5 min time-point was up to five times that of the baseline, and at the 15 min time-point was double that of baseline. Injections of saline (n = 6) or of a lower dose of NGF (1 μg in 10 μl; n = 6) did not change the frequency of ongoing discharge at any time-point.

NGF acutely sensitizes single mechanically activated bone nociceptors in vivo

We also tested whether NGF could acutely sensitize mechanically activated bone nociceptors in our bone-nerve preparation. Whole-nerve recordings showed increased activity during mechanical stimulation after application of NGF (5 μg in 10 μl), relative to before application of NGF (Figure 4(a)). Single units could easily be isolated from the whole-nerve recordings using spike discrimination software and many showed increased discharge frequency during mechanical stimulation after application of NGF (Figure 4(a) and (b)). NGF (5 μg in 10 μl) increased the response of two-thirds of the single units to mechanical stimulation at the 15 min, but not 30 min, time-point (Figure 4(b)). On average the number of impulses evoked at the 15 min time-point was almost double that of pre-injection values. The other third of the single mechanically activated units tested did not have altered activity after

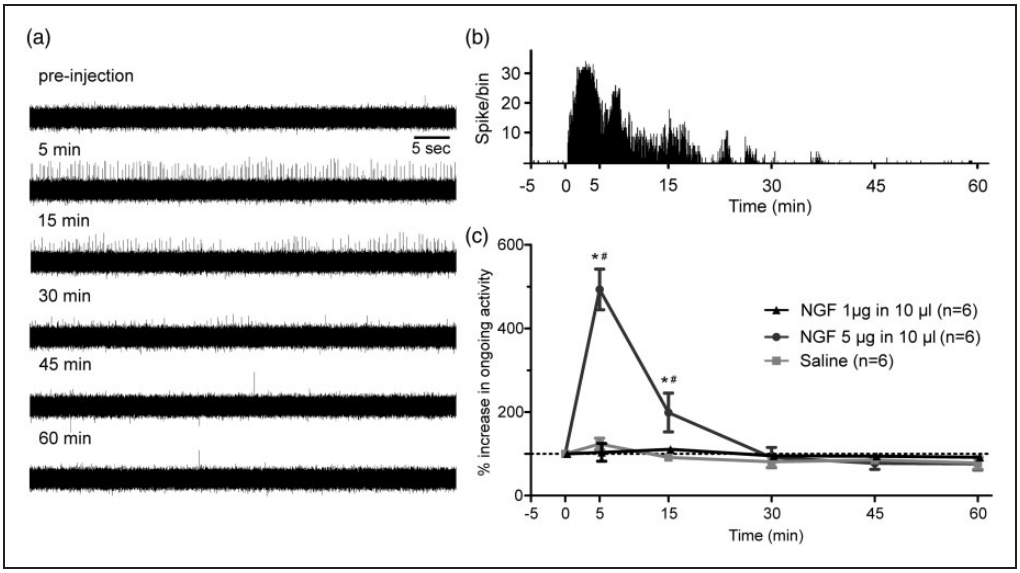

Figure 3. NGF rapidly activates bone nociceptors. (a) Raw data for a whole-nerve recording of the nerve to the rat tibia in response to application of NGF (5 μg in 10 μl). Each trace represents a 1 min segment of the recording before, 5, 15, 30, 45 and 60 min after application of the NGF. (b) Frequency histogram of the entire recording presented in (a). Bin width = 5 s. (c) Group data showing discharge frequency (mean ± SEM) in response to application of NGF 5 μg in 10 μl (n = 6), NGF 1 μg in 10 μl (n = 6) or saline (n = 6). Application of NGF 5 μg in 10 μl resulted in a significant increase in whole-nerve activity at 5 and 15 min, both relative to pre-injection baseline levels (Dunn's post-hoc analysis #$P < 0.05$) and to levels of activity in the saline control or NGF 1 μg in 10 μl (Bonferroni's post hoc test *$P < 0.05$). Saline or NGF 1 μg in 10 μl injections did not change nerve activity at any time-point (Repeated measures ANOVA $P > 0.05$).

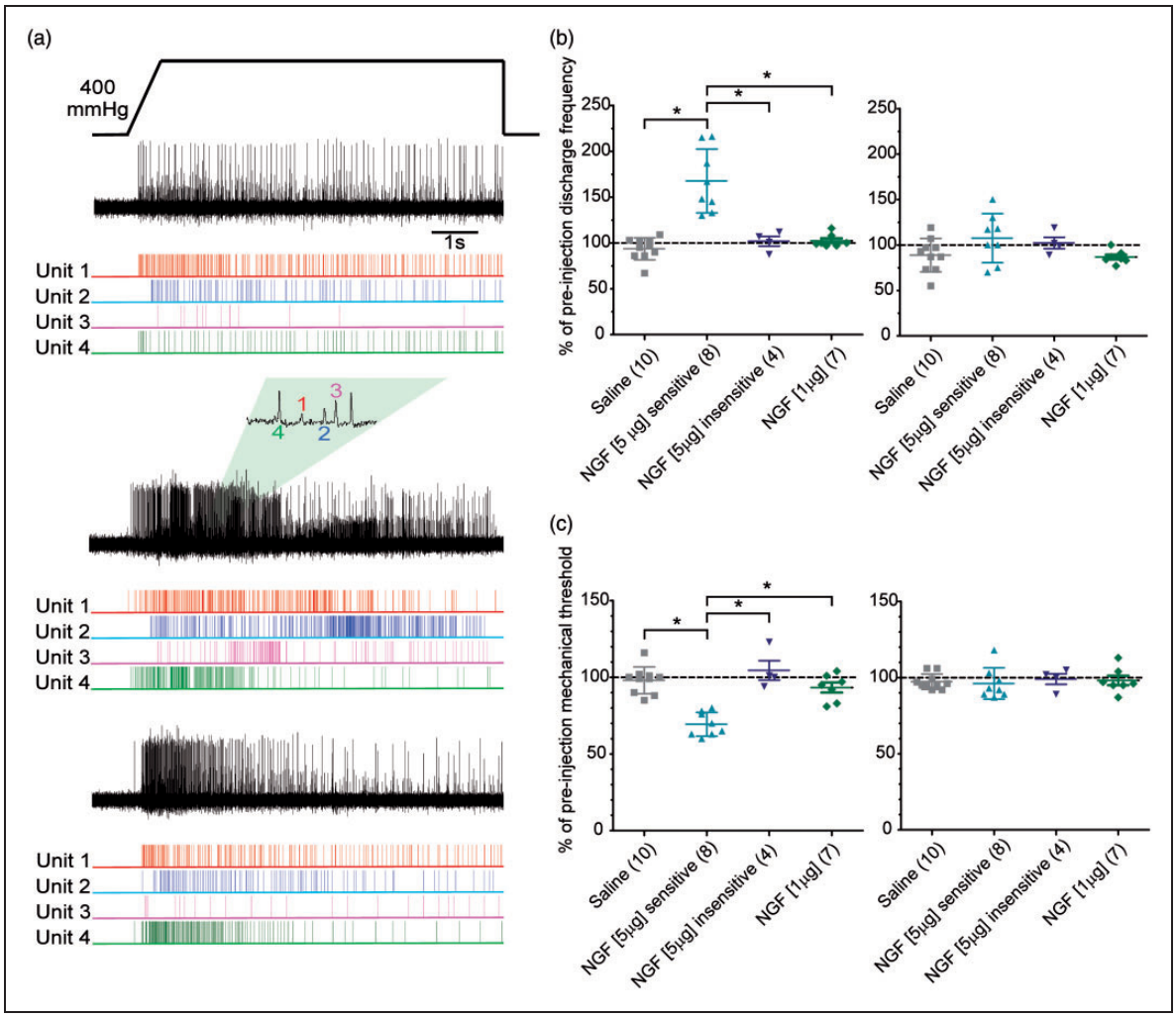

Figure 4. NGF sensitizes mechanically activated bone nociceptors. (a) Example of a whole-nerve recording and rasters of isolated single units in response to a 400 mmHg pressure stimulus before (top), 15 min (middle) and 30 min (bottom) after application of NGF (5 μg in 10 μl) to the marrow cavity. NGF application results in increased frequency of discharge and reduced threshold for activation in response to a 400 mmHg pressure stimulus. (b) Discharge frequency and (c) threshold for activation for single mechanically activated units at 15 min (left panels) and 30 min (right panels) after injection of NGF (5 μg in 10 μl) (n = 12), NGF (1 μg in 10 μl) (n = 7) or saline (n = 10), expressed as a percentage of pre-injection values. In experiments where NGF (5 μg in 10 μl) was applied, the single units were separated into those that were sensitized by NGF (n = 8) and those that were not (n = 4). There was a significant increase in discharge frequency in response to the pressure stimulus at 15 min, but not 30 min, after application of NGF (5 μg in 10 μl), relative to levels of activity in the saline control and NGF (1 μg in 10 μl) groups (Bonferroni's post hoc test *$P < 0.05$). At the 15 min time-point, the units that responded to NGF (5 μg in 10 μl) with increased discharge frequency had significantly lower thresholds for activation than those that did not respond to NGF, or that were recorded subsequent to injections of saline and NGF (1 μg in 10 μl) (Bonferroni's post hoc test *$P < 0.05$). There were no differences in thresholds for activation in any of the units at 30 min (Repeated measures ANOVA $P > 0.05$). Data in (b) and (c) are for single units and error bars represent mean ± SEM.

application of NGF (5 μg in 10 μl) (Figure 4(b)). The threshold for activation during the rising phase of the pressure stimulus was determined for all the mechanically activated units recorded. Units that responded to NGF (5 μg in 10 μl) with increased discharge frequency had significantly lower thresholds than those that did not respond to NGF with increased discharge frequency at the 15 min, but not 30 min, time-point (Figure 4(c)). Thus, the sensitization of bone nociceptors involved both an increase in discharge frequency and a reduction in threshold for activation at the 15 min time-point. Neither saline nor a lower dose of NGF (1 μg in 10 μl) changed the mechanically evoked increase in discharge frequency (Figure 4(b)) or the activation threshold of single, mechanically activated bone nociceptors (Figure 4(c)).

NGF-induced changes in ongoing activity and mechanical sensitivity are dependent on signaling through the TrkA receptor, but are not affected by the activity of mast cells

Systemic delivery of a function blocking anti-TrkA antibody (MNAC13; 15 μg) 30 min before application of NGF (5 μg in 10 μl) entirely prevented NGF-induced changes in whole-nerve ongoing activity (Figure 5(a)). This treatment also prevented the NGF-induced increase in the mechanically evoked discharge frequency (Figure 5(b)) and decrease in the thresholds for activation (Figure 5(c)) of single, mechanically activated units. In contrast, delivery of a mast cell stabilizer (cromolyn, 20 mg/kg) did not alter the NGF-induced increase in ongoing activity in whole-nerve recordings (Figure 5(d)) and did not prevent the NGF-induced increase in mechanically evoked discharge frequency (Figure 5(e)) or decrease threshold for activation (Figure 5(f)) in single, mechanically activated units.

NGF induces acute changes in pain behavior at a time consistent with sensitization of bone nociceptors

To test whether NGF can produce pain-like behavior in conscious animals, we applied NGF to the marrow cavity of anesthetized rats and measured the percentage of the total weight bearing on the injected hindlimb in animals after wound closure and recovery from anesthesia (Figure 6(a)). NGF (5 μg in 10 μl)-injected animals (n = 5) had a significant reduction in the percentage of the total weight bearing on the injected hindlimb at 15 min compared to pre-injection baseline values for the same animals (Figure 6(b)). At this time-point, there was also a significant reduction in the percentage of the total weight bearing on the injected hindlimb in the NGF-injected animals compared to the saline-injected animals (n = 5; Figure 6(b)). This effect disappeared by 30 min (Figure 6(b)). Thus, the time course is similar to that for the sensitization of mechanically activated bone nociceptors produced by the same concentration of NGF in the electrophysiological experiments above (Figure 4). We could not test weight bearing behavior earlier than the 15 min time-point because we could not be sure that the animals were fully recovered from anesthesia.

CFA-induced inflammation produces robust and delayed changes in weight bearing that can be used to model inflammatory bone pain

We developed an animal model to explore delayed changes in protein expression in bone nociceptors that might be associated with inflammatory bone pain. Inflammation was induced by injection of 50 μl of CFA into the marrow cavity of anesthetized rats (n = 14) and the presence of inflammation-induced bone pain was determined by measuring the percentage of the total weight bearing on the injected hindlimb in animals for up to 26 days following surgery. We found that the measured percentage of the total weight bearing on the injected hindlimb steadily decreased for up to four days post-CFA injection and then returned slowly to normal over the next three weeks (Figure 7(a)). At the peak of inflammatory bone pain (day 4), weight bearing on the injected hindlimb was significantly reduced compared to pre-injection values for the same animals. At this time-point, there was also a significant difference between the CFA- and saline-injected groups. Importantly, there was no change in weight distribution in saline-injected control animals, relative to pre-injection values, at any time-point (n = 10). Histology showed that inflammatory cells are increased in the bone marrow of CFA-injected animals at the four-day time-point when the pain-related behavior peaked (Figure 7(b)), with a definite and diffuse increase in neutrophils (thus raising the myeloid/erythroid ratio) and megakaryocytes relative to saline-injected controls. These findings confirm that our animal model is appropriate to study inflammatory bone pain.

Inflammatory bone pain is not associated with changes in expression of NGF-signaling molecules in bone nociceptors

To determine if inflammatory bone pain is associated with changes in expression of TrkA and/or other NGF-signaling molecules (TRPV1, Nav1.8 and Nav1.9), we compared expression of each of these in animals that displayed CFA-induced reductions in weight bearing (inflammatory bone pain) with those that had no reduction in weight bearing following saline injection (control). Expression was quantified in two ways. First, we determined if there was a change in the proportion of retrograde-labeled bone nociceptors that expressed each marker. Second, we determined if there was an increase in the level of expression (assessed by fluorescence intensity).

There were no significant differences in the proportions of retrograde-labeled bone nociceptors that expressed TrkA, TRPV1, Nav1.8 or Nav1.9 in animals that had CFA-induced reductions in weight bearing (n = 4) relative to animals that were injected with saline and did not (n = 4) (Table 3). In both these groups of animals, there were also no significant differences in the intensity of immuno-labeling for TrkA, TRPV1, Nav1.8 or Nav1.9, regardless of whether all retrograde-labeled cells were analyzed or only those that were clearly immuno-labeled were analyzed (Table 4). We were unable to test whether inflammation affected the expression of TRPV1, Nav1.8 or Nav1.9 in TrkA+ bone

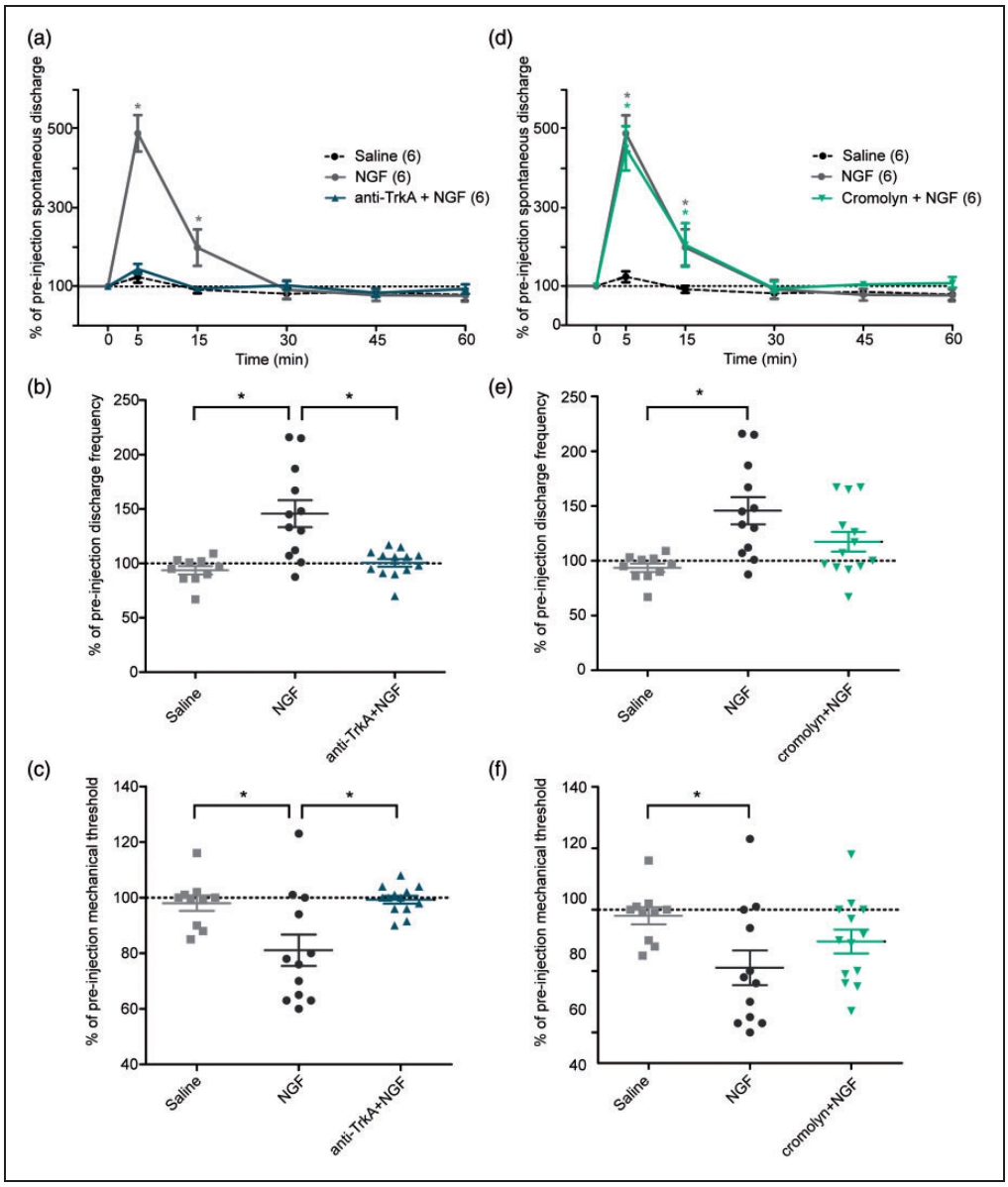

Figure 5. Effects of anti-TrkA and cromolyn on NGF-induced changes in the activity and sensitivity of bone nociceptors. (a) Group data showing whole-nerve ongoing discharge frequency in response to application of NGF (n = 6), saline (n = 6) or NGF+anti-TrkA (n = 6). Animals injected with NGF+anti-TrkA had significantly reduced whole-nerve ongoing activity relative to animals injected with NGF alone (Bonferroni's post hoc test *$P < 0.05$), and were indistinguishable from saline-injected controls. (b) Discharge frequency and (c) threshold for activation of single, mechanically activated units after application of NGF (n = 12), saline (n = 10) or NGF+anti-TrkA (n = 13). Single units in animals injected with NGF+anti-TrkA had significantly reduced discharge frequencies, and significantly greater thresholds for activation, in response to mechanical stimulation relative to those in animals injected with NGF alone (Bonferroni's post hoc test *$P < 0.05$), and were indistinguishable from those in saline-injected control animals. (d) Group data showing whole-nerve ongoing discharge frequency in response to application of NGF (n = 6), saline (n = 6) or NGF+cromolyn (n = 6). Animals injected with NGF, or NGF+cromolyn had significantly increased whole-nerve ongoing activity relative to animals injected with saline (Bonferroni's post hoc test *$P < 0.05$). There were no significant differences in whole-nerve ongoing activity between animals injected with NGF or NGF+cromolyn. (b) Discharge frequency and (c), threshold for activation of single, mechanically activated units after application of NGF (n = 12), saline (n = 10) or NGF+cromolyn (n = 13). While there was a significant increase in discharge frequency and reduction in threshold for activation in single, mechanically activated units in animals injected with NGF alone, relative to saline-injected controls (Bonferroni's post hoc test *$P < 0.05$), units in animals injected with cromolyn+NGF were indistinguishable from those injected with NGF alone. All data are normalized to pre-injection values. Data in (a) and (d) are represented as mean ± SEM. Data in (b), (c), (e) and (f) are for single units and error bars represent mean ± SEM. NGF was applied at 5 μg in 10 μl.

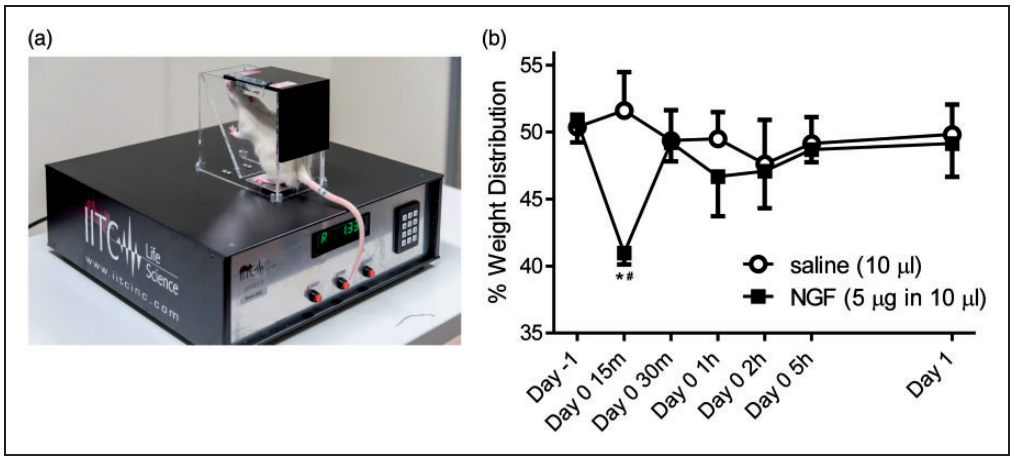

Figure 6. NGF injection into the rat tibia produces rapid pain-like behavior. (a) Weight bearing was assessed using an incapacitance meter that measures the distribution of weight bearing across each hindlimb. (b) There was a significant reduction in weight bearing on the injected hindlimb, relative to the un-injected hindlimb, at 15 min (but not at later time-points) after application of NGF (Dunn's post-hoc analysis #$P < 0.05$) and a significant reduction in weight bearing on the injected hindlimb relative to the un-injected hindlimb, in NGF-injected animals compared to that in saline-injected animals at this same time-point (Mann-Whitney *$P < 0.05$).

nociceptors because in both treatment groups a relatively small number of retrograde-labeled bone nociceptors co-expressed TrkA and either TRPV1, Nav1.8 or Nav1.9 at the 4 day time-point when pain peaked. When the number of cells used to calculate proportions is low, small changes in expression produces too much variability to produce meaningful comparisons between CFA- and saline-injected animals.

Discussion

NGF-signaling molecules in bone nociceptors and their nerve terminal endings

We have shown TrkA expression in peripheral nerve endings in the rat tibia, where it is likely activated by NGF released from inflammatory cells, and we confirmed a sensory origin for some of these endings with retrograde tracing to the DRG. Our finding that approximately two-thirds of the retrograde-labeled bone nociceptors expressed TrkA or p75 further suggests they are predominantly NGF-sensitive. Castaneda-Corral et al. have clearly demonstrated TrkA+ nerve terminals in murine long bones and have suggested that sensory neurons supplying bone are restricted almost entirely to a population that is NGF-sensitive.[36] Our findings suggest there is a greater diversity in the rat tibia, because up to one-third of bone nociceptors do not express detectable levels of TrkA or p75. A similar proportion is reported for sensory neurons supplying

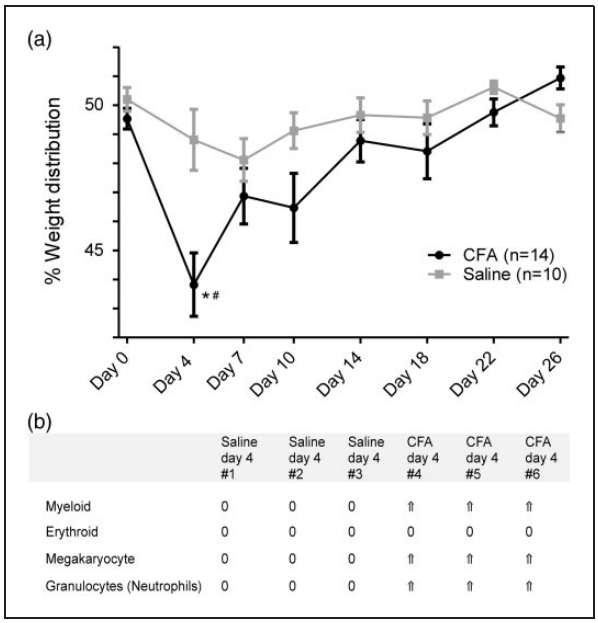

Figure 7. Model of CFA-induced inflammatory bone pain. (a) Time-course of CFA-induced inflammatory bone pain. The peak of inflammatory bone pain at day 4 was significantly different to pre-injection values for the same animals (Dunn's post-hoc analysis #$P < 0.05$) and the values for the saline-injected control group at this time-point (Mann-Whitney *$P < 0.05$). Importantly, there was no significant change in weight distribution in control animals, relative to pre-injection values, at any time-point. (b) CFA-induced inflammation increases the number of inflammatory cells in the bone marrow of CFA-injected animals, relative to saline-injected animals, at the peak pain time-point (day 4).

Table 3. The proportion of retrograde-labeled bone nociceptors that express various NGF-signaling molecules in saline vs. CFA injected animals.

	Saline: number of animals	Saline: number of retrograde-labeled bone nociceptors	Saline: percentage (Mean ± SEM)	CFA: number of animals	CFA: number of retrograde-labeled bone nociceptors	CFA: percentage (mean ± SEM)	Significant difference between saline and CFA injected animals (y/n)
TrkA	4	257	56 ± 6.8	4	122	60 ± 8.9	n
TRPV1	4	272	31 ± 6.4	4	127	37 ± 3.6	n
Nav1.8	4	79	30 ± 4.5	4	76	35 ± 4.4	n
Nav1.9	4	77	2.7 ± 1.6	4	63	1.3 ± 1.3	n

Table 4. The intensity of retrograde-labeled bone nociceptors that express various NGF-signaling molecules in saline vs. CFA injected animals. Means are presented for all retrograde-labeled neurons and for retrograde-labeled neurons that clearly expressed each marker.

	Saline: number of animals	Saline: number of retrograde-labeled bone nociceptors	Saline: intensity (mean ± SEM)	CFA: number of animals	CFA: number of retrograde-labeled bone nociceptors	CFA: intensity (mean ± SEM)	Significant difference between saline and CFA injected animals (y/n)
All retrograde-labeled cells							
TrkA	4	257	2 ± 0.1	4	122	1.9 ± 0.02	n
TRPV1	4	272	2.0 ± 0.3	4	127	1.8 ± 0.3	n
Nav1.8	4	79	1.4 ± 0.03	4	76	1.5 ± 0.01	n
Nav1.9	4	77	1.2 ± 0.04	4	63	1.2 ± 0.02	n
Retrograde-labeled cells that clearly expressed each marker							
TrkA	4	138	2.4 ± 0.1	4	72	2.4 ± 0.1	n
TRPV1	4	75	4.2 ± 1.1	4	47	2.9 ± 0.6	n
Nav1.8	4	21	1.8 ± 0.06	4	25	1.7 ± 0.03	n
Nav1.9	4	3	1.6 ± 0.1	4	3	1.6 ± 0.04	n

subchondral bone of the rat femur.[40] Whether this reflects species differences, or the different methodologies used, remains to be determined. However, what is clear is that the proportions of TrkA+ sensory neurons that innervate bone are significantly greater than published reports of TrkA+ sensory neurons that innervate skin, muscle and joints,[40,56,57] suggesting that NGF-signaling may be more important in bone pain compared to pain from other somatic tissues.

There are a number of studies suggesting that TrkA+ nerve endings in bone are almost exclusively peptidergic,[36,58] and we have confirmed that this is also the case at their soma in the DRG. In addition, approximately one-third of the bone nociceptors we retrogradely labeled constitutively expressed TRPV1 or Nav 1.8, but relatively few expressed Nav1.9. This further suggests that TRPV1 and/or Nav 1.8 may contribute to bone nociception. The possibility that this occurs as a result of rapid, post-translational modifications in peripheral nerve terminals of bone nociceptors, or through changes in the expression of these channels subsequent to inflammation in bone, is discussed below.

NGF rapidly activates and sensitizes bone nociceptors and produces pain-like behavioral responses with a similar time course

We have demonstrated that NGF applied directly to bone rapidly activates bone nociceptors and sensitizes many that respond to mechanical stimulation. Increased ongoing action potential discharge was observed within minutes of NGF application and lasted for up to 15 min. Single mechanically activated bone nociceptors showed reduced thresholds for

activation and increased discharge frequency in response to pressure stimuli within minutes of NGF application, and these changes lasted for up to 30 min. NGF applied to skeletal muscle and urinary bladder of rats is known to rapidly increase ongoing action potential discharge in primary sensory neurons.[59–61] In addition, NGF rapidly sensitizes mechanically sensitive muscle and bladder afferents[59–62] and can quickly induce mechanical hyperalgesia in rodents.[51,63] When we applied NGF directly to bone in behavioral experiments, we observed rapid changes in weight-bearing at 15 min (the first timepoint we could assay) that lasted for up to 30 min. This acute time course of pain behavior is entirely consistent with the rapid changes in the activity of bone nociceptors we noted in our electrophysiological experiments. Together the findings suggest that acute behavioral responses to NGF in bone can be explained at least in part by the rapid activation and/or sensitization of mechanically activated bone nociceptors.

Rapid activation and sensitization of peripheral sensory neurons can be achieved by binding of NGF to TrkA on peripheral nerve terminals and post-translational modifications of ion channels and/or receptors. Rapid increases in mechanical sensitivity induced by NGF have been shown to require the presence of TrkA in peripheral sensory neurons[63] and a number of studies have provided evidence for direct, rapid interactions of TrkA with ion channels.[19–23] Our finding that a function blocking anti-TrkA antibody prevents the rapid changes in mechanical sensitivity of bone nociceptors confirms that the NGF-induced changes we have reported in bone nociceptors are TrkA dependent. TRPV1, which we found is expressed in ~30% of bone nociceptors, is an example of an ion channel known to interact with TrkA. NGF rapidly increases both the membrane current carried by TRPV1 channels[64,65] and rises in intracellular calcium levels evoked by the TRPV1 agonist capsaicin.[66] These changes might reduce the temperature threshold of bone nociceptors, increasing their sensitivity to physiological temperatures in the bone marrow and could, at least in part, account for the increased ongoing activity of bone nociceptors we observed after application of NGF. However, TRPV1 does not contribute to the transduction of mechanical stimuli,[67] so it is unlikely that TRPV1 mediates the rapid sensitization of mechanically activated bone nociceptors we observed. The TTX-resistant Na^+ channel Nav1.8, that was expressed in approximately 40% of bone nociceptors, is another ion channel that might be involved. Inflammatory mediators, including NGF, are known to increase the magnitude of the TTX-resistant Na^+ current and shift the voltage threshold for activation of Nav1.8 channels to more negative values.[68–71] These changes would lower the voltage threshold for action potential initiation and could account for the sensitization of mechanically activated bone nociceptors we observed after application of NGF.

NGF can also exert effects via indirect interactions with a number of different cell types in peripheral tissues, including mast cells, leukocytes and sympathetic neurons.[72–77] In response to NGF, these can release a variety of substances, including inflammatory mediators that directly affect ion channels and receptors on peripheral nerve endings. Importantly, most of these cell types are resident in bone marrow, or can be recruited to bone marrow under pathological conditions such as inflammation. For example, we have shown that sympathetic nerve endings are located in the bone marrow of rats (Figure 1) and that inflammatory cells are increased in bone marrow after CFA-induced inflammation (Figure 7(b)). The bone marrow contains mast cells and there is good evidence that mast cell products are capable of sensitizing nociceptors.[78–81] Mast cell depletion prevents NGF-induced reductions in heat, but not mechanical thresholds in the skin-nerve preparation,[82] and depleting mast cells does not prevent NGF-induced mechanical hyperalgesia at the rat footpad,[78] suggesting that mast cell degranulation may not to be relevant to mechanical sensitization. In the present study, we used mast cell stabilization to show that mast cells do not contribute to NGF-induced changes in ongoing activity or mechanical sensitivity of Aδ-fiber bone nociceptors, but it remains a possibility that they do affect the activity of C-fiber bone nociceptors.

Inflammatory bone pain is not associated with changes in expression of some common NGF-signaling molecules

Internalization and retrograde transport of the NGF/TrkA complex can result in up-regulated expression of a number of NGF-signaling molecules, including TRPV1 and voltage-gated Na^+ channels,[24–28] and increased membrane expression of these ion channels can alter the excitability of nociceptors. In the current study, we used an animal model to test whether these mechanisms are relevant to inflammatory bone pain. CFA is used routinely in studies of experimental inflammatory pain and it drives changes in sensitivity to both thermal and mechanical stimuli that can last for days to weeks.[83–88] Sequestration of NGF blocks CFA-induced inflammatory pain,[89] reinforcing a potential role for NGF in our model.

However, we did not observe significant differences in expression of TRPV1, Nav1.8 or Nav1.9 between animals with, and those without, CFA-induced inflammatory bone pain. This is despite analyzing our data in a number of different ways, including assessing proportions of neurons that expressed these molecules to explore likely *de novo* expression, and immuno-labeling

intensity to explore changes in expression in neurons that already expressed each molecule. Furthermore, the failure to detect an increase in TrkA protein in the soma of bone nociceptors suggests that the NGF/TrkA complex may not be retrogradely transported from the periphery to the soma in this model. Thus, inflammatory bone pain does not appear to involve long-term changes in expression of some of the common NGF-signaling molecules. This is notably different to the changes in expression of these same ion channels that have been observed at peak pain time-points in animal models of CFA-induced cutaneous inflammatory pain[68,83,87,90–93] and likely reflects significant tissue-dependent differences.

NGF sequestration to treat pain of bony origin

NGF is up-regulated in peripheral tissues after inflammation[15,94–97] and sequestering NGF with function blocking antibodies significantly attenuates pain behaviors in animal models of bone disease.[3,38,41–43,98,99] NGF sequestration has been successfully applied in clinical trials to treat osteoarthritic pain.[100–104] While there have been some concerns about safety, the benefits for pain management are clear. Interestingly, sequestering NGF after inflammatory pain has already developed attenuates pain behaviors in animal models of bone cancer and skeletal fracture,[105] suggesting that NGF is present and contributing to sensitization of peripheral nociceptors at the time when the antibodies are delivered. In the present study, we have demonstrated that NGF rapidly activates and alters the excitability of bone nociceptors, and that it produces acute behavioral responses in animals when injected directly into bone. These changes occur within minutes and resolve within half an hour, suggesting that NGF needs to be present for pain to be maintained. In our animal model of inflammatory bone pain, we failed to find evidence for a delayed increase in the expression of TrkA, TRPV1, Nav1.8 or Nav1.9 in the soma of bone nociceptors. Thus, signaling by retrograde transported NGF/TrkA complexes does not appear to be important in this model.

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Declaration of Conflicting Interests

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