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Glial Cell Line-Derived Neurotrophic Factor Family Members Sensitize Nociceptors *In Vitro* and Produce Thermal Hyperalgesia *In Vivo*

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Nerve growth factor (NGF) has been implicated as an effector of inflammatory pain because it sensitizes primary afferents to noxious thermal, mechanical, and chemical [e.g., capsaicin, a transient receptor potential vanilloid receptor 1 (TRPV1) agonist] stimuli and because NGF levels increase during inflammation. Here, we report the ability of glial cell line-derived neurotrophic factor (GDNF) family members artemin, neurturin and GDNF to potentiate TRPV1 signaling and to induce behavioral hyperalgesia. Analysis of capsaicin-evoked Ca^{2+} transients in dissociated mouse dorsal root ganglion (DRG) neurons revealed that a 7 min exposure to GDNF, neurturin, or artemin potentiated TRPV1 function at doses 10–100 times lower than NGF. Moreover, GDNF family members induced capsaicin responses in a subset of neurons that were previously insensitive to capsaicin. Using reverse transcriptase-PCR, we found that artemin mRNA was profoundly upregulated in response to inflammation induced by hindpaw injection of complete Freund's adjuvant (CFA): artemin expression increased 10-fold 1 d after CFA injection, whereas NGF expression doubled by day 7. No increase was seen in neurturin or GDNF. A corresponding increase in mRNA for the artemin coreceptor GFRα3 (for GDNF family receptor α) was seen in DRG, and GFRα3 immunoreactivity was widely colocalized with TRPV1 in epidermal afferents. Finally, hindpaw injection of artemin, neurturin, GDNF, or NGF produced acute thermal hyperalgesia that lasted up to 4 h; combined injection of artemin and NGF produced hyperalgesia that lasted for 6 d. These results indicate that GDNF family members regulate the sensitivity of thermal nociceptors and implicate artemin in particular as an important effector in inflammatory hyperalgesia.

Key words: **artemin; NGF; GDNF; neurturin; pain; TRPV1**

Introduction

Nociceptive sensory neurons, those responsive to noxious stimuli, are dependent on nerve growth factor (NGF) for survival during development (Lindsay, 1996; Fundin et al., 1999; Mendell et al., 1999; Baudet et al., 2000). Nociceptors are absent in mice lacking NGF (Crowley et al., 1994) or its receptor tyrosine kinase TrkA (Silos-Santiago et al., 1995), and, in humans, loss-of-function mutations in the *trkA* gene cause congenital insensitivity to pain syndrome (Shatzky et al., 2000). During the perinatal period, approximately half of these neurons downregulate TrkA and begin to express Ret, the receptor tyrosine kinase for the GDNF family of ligands (GFL): GDNF, artemin, and neurturin (Molliver and Snider, 1997). Signaling by each of the GDNF family members also requires a preferred coreceptor glial cell line-derived neurotrophic factor (GDNF) family receptor α 1 (GFRα1) to GFRα3], which binds the GFL and activates Ret.

Although initial studies of sensory neuron growth factors focused on their importance for developmental survival, we now know that they also regulate functional properties of sensory neurons (Stucky et al., 1999; Ritter et al., 2001; Albers et al., 2006). In both rodents and humans, *in vivo* injection of NGF produces thermal hypersensitivity within 30 min and mechanical sensitivity within hours (Lewin et al., 1994; Dyck et al., 1997). *In vitro*, NGF sensitizes the transient receptor potential vanilloid type 1 (TRPV1) receptor (Shu and Mendell, 1999a, 2001; Zhu et al., 2004). TRPV1 is a nonselective cation channel gated by noxious heat, protons, and vanilloid compounds such as capsaicin (Caterina et al., 1997; Tominaga et al., 1998) and is required for inflammation-induced heat hyperalgesia (Caterina et al., 2000; Davis et al., 2000). Under normal conditions, repeated activation results in progressive desensitization of TRPV1 (tachyphylaxis). NGF potentiates TRPV1 function, antagonizing or blocking tachyphylaxis, and is upregulated in the periphery after inflammation; these results lead to the hypothesis that NGF plays a major role in acute inflammatory hyperalgesia (Shu and Mendell, 1999b).

Several recent reports suggest that GDNF family members may also modulate thermal sensation. Mice overexpressing arte-

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min in the skin have increased thermal sensitivity and decreased thermal thresholds in C-fiber nociceptors compared with wild-type mice (Elitt et al., 2006); increased thermal responsiveness has also been reported in mice overexpressing NGF (Stucky et al., 1999). Extensive overlap between TRPV1 and the artemin-selective coreceptor GFRα3 has been reported in sensory neurons (Orozco et al., 2001).

This study was designed to test the hypothesis that, like NGF, GDNF family members acutely sensitize TRPV1 in sensory neurons. We were surprised to find that acute application of artemin, neurturin, or GDNF induces significant potentiation of TRPV1 function at doses 10–100 times lower than that effective for NGF. In addition, artemin mRNA increased rapidly and dramatically in inflamed skin. Finally, hindpaw injections of artemin or neurturin produced acute hyperalgesia lasting up to 4 h, whereas combined injections of NGF and artemin produced heat hyperalgesia that lasted 6 d. These results indicate that nociceptor responsiveness can be regulated by GDNF family members.

Materials and Methods

Cell culture. Six to 8-week-old adult male C57BL/6 mice obtained from The Jackson Laboratory (Bar Harbor, ME) were used for all experiments. Mice were given an overdose of Avertin anesthetic and perfused transcardially with 4° Ca^{2+}/Mg^{2+}-free HBSS. All dorsal root ganglia (DRGs) were rapidly dissected and cleaned in HBSS. Ganglia were dissociated as described previously (Molliver et al., 2002). Cells were plated in HBSS containing 5% fetal calf serum and antibiotics (penicillin/streptomycin, 50 U/ml). No additional growth factors were added to the culture medium. Ca^{2+} imaging was performed 18–24 h after plating. In several experiments, TRPV1 knock-out mice were used. These mice were generously provided by Dr. Michael Caterina (Johns Hopkins University, Baltimore, MD). These studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Ca^{2+} imaging. Isolated sensory neurons were loaded with 2 μM fura-2 in HBSS containing 5 mg/ml BSA for 30 min at 37°C and then mounted on an Olympus Optical (Thornwood, NY) upright microscope stage with constantly flowing buffer at 5 ml/min. Perfusion rate was controlled with a gravity flow system (VC66; Warner Instruments, Hamden, CT), and perfusate temperature was maintained at 30°C using a heated stage and an in-line heating system (PH1, SHM-6, TC344B; Warner Instruments). Drugs were delivered with a rapid-switching local perfusion system. Firmly attached, refractile cells were identified as regions of interest in the software (Simple PCI, C-Imaging; Compix Imaging Systems, Sewickley, PA). All fields were first tested with brief application of 50 mM K^+ (high K^+), and Ca^{2+} transients were imaged to standardize pipette placement and to ensure that cells were healthy and responsive. Responses were measured as the ratio of absorbance at 340 nm to that obtained at 380 nm ($\Delta F_{340/380}$) [DG4 (Sutter Instruments, Novato, CA); Retiga 1300 (Burnaby, British Columbia, Canada)]; peak responses were >0.1 $\Delta F_{340/380}$ and were easily distinguished from optical noise (<0.02 $\Delta F_{340/380}$). Cells not responsive to high K^+ application were not analyzed further. In the standard protocol, cells were first tested with two to three presentations of capsaicin (1 μM) at 10 min intervals. Concentrations of 10 nM to 2 μM capsaicin were tested. Capsaicin at 1 μM elicited responses >0.1 $\Delta F_{340/380}$ from the maximal number of cells and could be applied repeatedly without significant loss in the number of responding cells over three applications. However, response magnitudes decreased with each subsequent agonist presentation. This does not reflect fatigue of Ca^{2+}-handling machinery because the magnitude of high K^+-induced Ca^{2+} transients did not decrease when elicited over the same time course. In this study, capsaicin response data were obtained before and after a 7 min perfusion of growth factor (NGF, GDNF, artemin, or neurturin). Ca^{2+} response peak and area data are presented as the ratio of post-growth factor capsaicin responses to naive capsaicin responses in individual cells (ΔF_{peak}, ΔF_{area}). Response areas were calculated using Microsoft (Seattle, WA) Excel for suprathreshold responses (peak response >0.1 $\Delta F_{340/380}$) as a measure of total Ca^{2+} influx. The portion of the calcium response that was used for this measurement included the entire curve from the initiation of the response until the point at which the calcium signal returned to the prestimulus baseline. Typically, this occurred in <90 s because the dosage and duration of the capsaicin application was chosen to produce a brief response. Any response lasting longer than 90 s was removed for the analysis.

Mean ± SEM data are ratios from all capsaicin-responsive neurons exposed to a given growth factor. Responses were allowed to recover to determine the time course of growth factor action on TRPV1. Capsaicin responses were evaluated after several growth factor concentrations to generate a dose–response curve. Additional experiments used the lowest effective dose to minimize concerns regarding nonspecific effects. Growth factors were also tested sequentially to determine the extent to which capsaicin responses are modulated by multiple trophic factors. Capsaicin at 10 mM (Sigma, St. Louis, MO) in 1-methyl-2-pyrrolidinone was used as a stock solution; 1.0 μM capsaicin was made fresh daily in HBSS. NGF (Harlan, Indianapolis, IN), artemin (R & D Systems, Minneapolis, MN), neurturin (R & D Systems), and GDNF (Calbiochem, La Jolla, CA) were aliquoted at 10 μg/ml in HBSS, stored at −20°C, and diluted in HBSS immediately before use. Growth factor concentrations in the text are given in nanograms per milliliter; these factors have similar molecular weights (in kDa: 26 NGF, 24 artemin, 23 neurturin, and 30 GDNF). However, it is worth noting that, because GDNF weighs slightly more than NGF, neurturin, or artemin, the molar concentration of GDNF is slightly less than the other growth factors at the same nanograms per milliliter dose.

Labeling cutaneous afferents. Detailed methods have been reported previously (Christianson et al., 2006). In brief, mice were deeply anesthetized using 5% halothane, and saphenous nerves were exposed on both sides at the midthigh level. To prevent leakage of the tracer to surrounding tissues, Parafilm (Fisher Scientific, Pittsburgh, PA) was inserted under the nerve before injection of ~10 μl Alexa Fluor 488-conjugated wheat germ agglutinin (WGA) (2% in sterile saline; Invitrogen, Carlsbad, CA) into the saphenous nerve. A picospritzer was used to inject tracer into the nerve; injection was visually confirmed and ~50 μl gelatin (10% in 0.1 M phosphate buffer) was applied after the injections to seal the nerves. The wounds were sutured, the animals were allowed to recover for 3 d, and neuronal cultures were prepared from L2, L3, and L4 DRGs. Labeled cells were identified in culture before imaging. Very few (<1%) back-labeled neurons were detected in culture when dye was spritzed onto the outside of the nerve, suggesting that leakage effects were minimal.

Complete Freund's adjuvant inflammation. Detailed methods for this procedure have been reported previously (Zwick et al., 2003). Briefly, 20 mice were anesthetized with isoflurane, and 20 μl of complete Freund's adjuvant (CFA) emulsion was injected into the plantar surface of both hindpaws. Paws were measured for edema with a caliper micrometer; all mice showed substantial edema after CFA injection. At each survival time point (naive baseline and 1, 3, 7, and 14 d after CFA injection), five adult male mice were given an overdose of Avertin anesthetic and perfused transcardially with 25 ml of ice-cold 4° Ca^{2+}/Mg^{2+}-free HBSS. Paw skin and L3–L5 DRGs were collected on dry ice and immediately processed for RNA isolation.

RNA isolation. Tissue from each animal was processed separately. RNA was isolated by homogenizing frozen tissue in 2 ml of Trizol reagent (Invitrogen), followed by isopropanol precipitation. Pellets were washed with 70% ethanol and suspended in RNase-free water, and the concentration was determined using a GeneQuant RNA/DNA calculator (Amersham Biosciences, Piscataway, NJ). RNA (5 μg) was treated with DNase (Invitrogen) to remove genomic DNA, and then 1 μg was reverse-transcribed using Superscript II reverse transcriptase (RT) (Invitrogen).

Radioactive RT-PCR. RT-PCR reactions were done in the presence of ^{32}P-dCTP, and aliquots of the reaction run on 8% polyacrylamide gels in Tris borate EDTA buffer. Gels were dried and placed against a PhosphorImager screen, and the relative level of incorporated label was determined using a Bio-Rad (Hercules, CA) PhosphorImager. The cycle number was optimized for each set of primers by first running PCR reactions

at different cycle numbers to establish the midphase of the reaction. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sets were generated using Macvector software (Accelrys, San Diego, CA). Routine control reactions included PCR reactions on DNased RNA (without RT) and reactions run without templates to test for contamination. Primer sequences included the following (5′ and 3′, respectively): NGF, tccaatcctgttgagagtgg and caggctgtgtctatgcggat; artemin, ctcagtctcctcagcccg and tccacggtcctccaggtg; GDNF, aaggtcaccagataaacaagcgg and tcacaggagccgctgcaatatc; neurturin, ggatgtgccaggagggtctg and caggtcgtagatgcggatgg; and GAPDH, atgtgtccgtcgtggatctga and gctgttgaagtcgcaggagaca.

Real-time PCR. Real-time PCR was used as described previously (Molliver et al., 2005) to determine the extent of expression of growth factor receptors in sensory neurons after CFA-induced inflammation. Primers optimized for real-time PCR were designed using Oligo software (Molecular Biology Insights, Cascade, CO). SYBR Green PCR amplification was performed using an Applied Biosystems (Foster City, CA) 5700 real-time thermal cycler. After amplification, a dissociation curve was plotted against melting temperature to ensure amplification of a single product. All samples were run in triplicate, and control reactions were run without template and with the reverse-transcriptase control reaction products as negative controls with every amplification run. The relative fluorescence of SYBR Green bound to double-stranded DNA was compared with a passive reference for each cycle. Threshold cycle (Ct) values, the cycle number in which SYBR Green fluorescence rises above background, were recorded as a measure of initial template concentration. Relative fold changes in RNA levels were calculated by the ΔΔCt method using GAPDH as a reference standard: Ct values from triplicate samples were averaged and then subtracted from the reference standard, yielding ΔCt. The difference between the ΔCt of the different genes or of the experimental and control groups were then calculated (ΔΔCt). The relative fold change was determined as $2^{-\Delta\Delta Ct}$. Statistical significance was determined by ANOVA. Primer sequences were as follows (5′ and 3′, respectively): TrkA, agagtggcctccgctttgt and cgcattggaggacagattca; GFRα1, gtgtgcacatggtgtggactag and ttcagtggttcagacccacttg; GFRα2, gagaggcgggaggtcacag and tgacggagggtgaggagttct; GFRα3, agattcattttccagggtttgc and cttggtgactacgagttggatgtc; Ret, gcgaagcctgggtctgtct and tgagtgcaccaagcttcagta; TRPV1, cccattgtgcagattgagcat and ttcctgcagaagagcaagaagc; and GAPDH, atgcctgcttcaccaccttctt and atgtgtccgtcgtggatctga.

Immunohistochemistry. Mice were given an overdose of Avertin anesthetic and killed by transcardial perfusion with 4°C saline. DRGs were dissected and embedded in OCT mounting medium on dry ice. Sections were cut at 12 μm (DRG) or 30 μm (skin) on a cryostat, collected on Superfrost microscope slides, and then kept at −20°C until used. Slides were thawed, fixed in 4% paraformaldehyde for 10 min, and then placed in blocking solution (2% normal horse serum and 0.2% Triton X-100 in PBS, pH 7.4) for 60 min. Sections were then incubated in primary antibodies diluted in blocking solution overnight at room temperature. The following antibodies were used: rabbit anti-TRPV1 at 1:500 (Calbiochem), goat anti-GFRα1 and goat anti-GFRα2 at 1:500, and goat anti-GFRα3 at 1:100 (R & D Systems). Antibodies were visualized with donkey anti-rabbit or donkey anti-goat secondary antibodies conjugated to cyanine 3 or 2 (Jackson ImmunoResearch, West Grove, PA), diluted 1:500 and applied for 60 min. Slides were coverslipped in Slow-Fade (Invitrogen) and photographed under epifluorescence with a Retiga digital camera. Images were captured using Capture Q software on an Apple Computers (Cupertino, CA) Macintosh G5 and processed for brightness, contrast, and color using Adobe Photoshop (Adobe Systems, San Jose, CA).

The extent of colocalization between TRPV1 and either GFRα2 or GFRα3 was calculated in L4 DRG from three mice using systematic random sampling (Pakkenberg and Gundersen, 1988). Six evenly spaced sections (every *n*th section, where n = total number of sections/six) were chosen, starting with a randomly chosen section between 1 and *n*. Cellular profiles containing robust immunoreactivity with a clearly defined nucleus were considered positive. Images taken with one wavelength of fluorescence were scored and then overlaid with images of the other wavelength, and then single- and double-labeled cells were counted.

Behavior: thermal sensitivity (Hargreaves test). Adult male mice (10 per condition) were placed in individual Plexiglas chambers on a glass plate maintained at 30°C and allowed to acclimate for 90 min before testing. This was repeated for 3 d before actual testing began. Response latencies to noxious thermal stimulation were measured by applying a radiant heat stimulus (15% intensity on the Hargreaves apparatus; IITC, Woodland Hills, CA) to each hindpaw. The heat source was activated with an electric trigger coupled to a timer, and the latency to stimulus response (flinching or lifting the paw) was recorded to the nearest 0.1 s. Mice were tested three times, and the responses for each paw were averaged. Mice were then given an injection of saline or growth factor in one paw under light isofluorane anesthesia (mice recovered within 20 s), and thermal responses were tested at 30 min, 60 min, 4 h, and 24 h after injection. The experimenter conducting the behavioral testing was blind to which paw was injected. Previous studies demonstrated that intraplantar injection of 250 ng of NGF produces robust thermal hyperalgesia (Woolf et al., 1994; Andreev et al., 1995). Because our *in vitro* dose–response curve suggested that GFLs may be more potent than NGF in sensitizing primary afferents *in vivo*, we initially tested 200 ng of growth factor, slightly below published concentrations for NGF-induced sensitization. This concentration produced robust hyperalgesia for all growth factors and therefore was used for our studies.

Results

Neurturin and artemin receptors colocalize with TRPV1 in some DRG neurons

Initial experiments in this study were based on previous work demonstrating that acute NGF application potentiates TRPV1 responses in sensory neurons (Shu and Mendell, 1999a, 2001; Zhu et al., 2004). Widespread expression of TrkA in TRPV1-expressing neurons (Michael and Priestley, 1999; Orozco et al., 2001) provides a mechanism for the modulation of TRPV1 by NGF. Extensive colocalization of immunoreactivity for TRPV1 and GFRα3 in both rat and mouse DRG neurons has also been described previously (Orozco et al., 2001). To probe coexpression of TRPV1 with GFRα1–GFRα3, double-labeling immunohistochemistry was performed in sections of lumbar DRG. Staining for the GDNF receptor GFRα1 was not adequate for quantification and therefore was not pursued further (data not shown). Examples of GFRα2 and GFRα3 staining are shown in Figure 1; quantification is provided in Table 1. Whereas staining for GFRα2 and TRPV1 were primarily nonoverlapping, a subset of neurons (~22% of TRPV1-positive cells) were clearly positive for both receptors; note the double-labeled cells in Figure 1 (arrows). In contrast, the majority (>65%) of TRPV1-positive cells coexpressed GFRα3 (Fig. 1; Table 1). These immunohistochemical data support the hypothesis that subsets of the TRPV1-expressing population of nociceptors are capable of responding to artemin or neurturin *in vivo*. Because both the GFRα2 and GFRα3 antibodies were generated in goat, we did not attempt to determine the overlap between the expression of GFRα2 and GFRα3. However, the functional studies described below suggest that, among cells that express TRPV1, there is little coexpression for GFRα1–GFRα3; a majority of cells respond to both NGF and artemin, but only 10 and 5% respond to NGF and GDNF or neurturin, respectively (see below), suggesting that coexpression of GFRα1–GFRα3 occurs in a minority of TRPV1-expressing cells.

Growth factor potentiation of capsaicin responses in DRG neurons

The next experiments were designed to test the hypothesis that GDNF family members modulate TRPV1 signaling. Ca^{2+} responses to 1 μM capsaicin were recorded in isolated sensory neurons using fura-2 imaging before and after a 7 min exposure to NGF, artemin, neurturin, or GDNF (each at 100 ng/ml). Capsa-

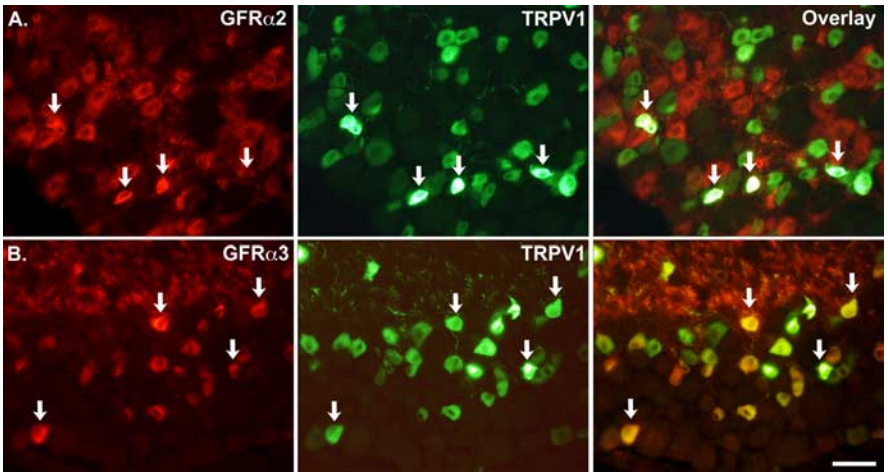

Figure 1. GFRα2 and GFRα3 colocalize with TRPV1. Images show immunohistochemical staining for GFRα2 or GFRα3 (red) with TRPV1 (green) in sections of mouse L4 DRG. ***A***, TRPV1 labeled small- and medium-sized neurons with a range of staining intensity, as widely described in the literature. Staining for GFRα2 was intense in axons and neuronal cell bodies of small and medium size. Only a minority of TRPV1-positive neurons was also GFRα2 positive. Arrows in the merged panel indicate double-labeled neurons. ***B***, GFRα3 stained cell bodies and axons of a subset of small neurons. In contrast to GFRα2 staining, most GFRα3-positive neurons also expressed TRPV1 (see arrows in merged panels). Scale bar, 50 μm. Quantification of staining is provided in Table 1.

Table 1. Extensive overlap occurs between GFRα2, GFRα3, and TRPV1

% TRPV1 that are GFRα2$^+$	22.3 ± 1.1%
% TRPV1 that are GFRα3$^+$	67.1 ± 0.6%[a]
% GFRα2 that are TRPV1$^+$	18.2 ± 1%
% GFRα3 that are TRPV1$^+$	94.3 ± 0.9%[a]

Data are mean ± SD of the percentage of neurons immunoreactive for TRPV1 and GFRα2 or GFRα3 in sections of L4 DRG ($n = 3$ mice, 6 sections per ganglion)

[a]C. Elitt, unpublished observations.

icin at 1 μM administered at 10 min intervals elicited responses that showed significant tachyphylaxis resulting from desensitization of the TRPV1 channel (Table 2) (Shu and Mendell, 1999a). NGF given during this 10 min interval maintained and, in some cases, potentiated TRPV1 response magnitude (Table 2) (Shu and Mendell, 1999a). Responses from representative cells are shown in Figure 2. The percentage of capsaicin-sensitive neurons potentiated by the indicated growth factors is given above the records. Potentiation was defined as a response to subsequent capsaicin applications greater in magnitude (peak or area) than that elicited by the first capsaicin application. All four growth factors significantly potentiated capsaicin-evoked Ca^{2+} transients in some neurons, although the magnitude and frequency of potentiation varied. NGF and artemin each potentiated a majority (>65%) of capsaicin responses, whereas neurturin- and GDNF-responsive cells were less numerous (29 and 39%, respectively).

The magnitude of capsaicin response potentiation also varied with growth factor. Potentiation was calculated as the change in peak (ΔF_{peak}) and area under the curve (ΔF_{area}) of the capsaicin-evoked Ca^{2+} transient elicited before and after growth factor application. Fold change in area (ΔF_{area}) values for all capsaicin responses after treatment with NGF, artemin, neurturin, GDNF, or buffer (untreated) are shown in Figure 2*B*, and average data are shown in Table 2. Capsaicin was applied up to five times at 10 min intervals to examine the time course of growth factor effects on TRPV1. Tachyphylaxis was prominent in cells that received no growth factor treatment (Fig. 2*B*, Untreated), whereas potentiation was often evident for multiple capsaicin applications after growth factor treatment (Fig. 2*A*). The largest response for each neuron was used to calculate potentiation (Fig. 2*B*). NGF potentiated 68% of all capsaicin responders; the mean maximal potentiation was a 2.03 ± 0.23-fold increase in area (ΔF_{area}) over the initial capsaicin response. An almost identical percentage of neurons exhibited potentiation in response to artemin (66 ± 7%), but the magnitude of potentiation elicited by artemin (2.93 ± 0.41) was significantly ($p < 0.05$) greater than that elicited by NGF (Table 2). It is not surprising that both NGF and artemin potentiate a majority of capsaicin-responsive neurons because the NGF receptor TrkA and the artemin receptor GFRα3 are extensively coexpressed with each other (80% of GFRα3-immunoreactive neurons are positive for TrkA) and with TRPV1 (Michael and Priestley, 1999; Orozco et al., 2001). Neurturin and GDNF potentiated capsaicin responses in fewer neurons (29 and 39%, respectively) than either NGF or artemin. However, increases in capsaicin response magnitude induced by neurturin or GDNF (1.89 ± 0.52-fold and 1.65 ± 0.7-fold, respectively) were significant ($p < 0.001$ vs untreated), although not different in magnitude from that induced by NGF.

The mean fold increases in TRPV1 response include all capsaicin responders, both potentiated and unaffected, in the population. As a result, neurturin and GDNF appear to have less effect than NGF or artemin because of population sampling. When the analysis is restricted to potentiated cells, artemin, neurturin, and GDNF sensitize capsaicin responses significantly ($p < 0.05$) more than NGF (fold increases: NGF, 2.49 ± 0.27; artemin, 4.21 ± 0.55; neurturin, 4.19 ± 1.3; and GDNF, 4.13 ± 0.93). These data suggest a significant modulatory role for GDNF family members in sensory neurons and demonstrate that artemin is the most efficacious of the growth factors tested to sensitize TRPV1.

In addition to potentiating capsaicin responses, all of the growth factors tested also caused some neurons that were previously unresponsive to capsaicin ($\Delta F_{340/380} < 0.05$) to display robust capsaicin-evoked Ca^{2+} transients. NGF increased the number of capsaicin-sensitive cells by 46 ± 15%, artemin by 32 ± 8%, neurturin by 29 ± 19%, and GDNF by 21 ± 9%. Note that initial (nonpotentiated) capsaicin responses in some of these cells may be below the detection threshold for Ca^{2+} imaging. However, we report that ~50% of small cells are capsaicin sensitive (47 ± 2%; $n = 63$ mice); similar percentages have been reported previously using electrophysiology (Stucky and Lewin, 1999), suggesting that most capsaicin responses are reliably detected using Ca^{2+} imaging. However, data from these possibly recruited cells were not averaged into the analysis of potentiation because no baseline response was detectable.

Several controls ensure that the effects of acute growth factor treatment on capsaicin responses reported here are specific to TRPV1 and not a result of changes in intrinsic electrical or Ca^{2+}-handling properties. Depolarization-induced Ca^{2+} transients are highly reproducible in our system when elicited with the same time course as capsaicin in this study. Therefore, we attribute the amplitude reduction in later capsaicin responses to desensitization of TRPV1 channels rather than fatigue of Ca^{2+}-handling

processes. Importantly, responses to 50 mM K^+ and 100 μM ATP (a purinergic P2X/P2Y receptor agonist) before and after growth factor treatment were compared. We did not find any significant changes in K^+ or ATP responses after growth factor treatment ($n = 30$ cells), suggesting that acute application of NGF, artemin, neurturin, or GDNF does not significantly affect intracellular Ca^{2+}-handling properties in sensory neurons. Also, depolarization-induced Ca^{2+} transients were routinely elicited during and after capsaicin potentiation trials and were not altered in shape or magnitude in the course of the experiment. In addition, no capsaicin responses were observed in DRG neurons isolated from TRPV1 knockout mice before or after treatment with any of the growth factors tested in this study (NGF, artemin, neurturin, and GDNF; $n = 62$ cells). Therefore, the growth factor responses reported in this study are presumed to be specific to the TRPV1 channel.

Table 2. Frequency and magnitude of capsaicin responses are potentiated by growth factors in sensory neurons

Growth factor (*n* cells)	% Cap responders potentiated	ΔF_{peak} (all)	ΔF_{area} (all)	ΔF_{peak} (pot)	ΔF_{area} (pot)
— (96 cells)	0%	0.62 ± 0.03	0.40 ± 0.03		
NGF (107 cells)	68 ± 8%	1.45 ± 0.24*	2.03 ± 0.23*	2.08 ± 0.25*	2.49 ± 0.27*
Artemin (99 cells)	66 ± 7%	2.24 ± 0.37*	2.93 ± 0.41*,**	3.01 ± 0.53*,**	4.21 ± 0.55*,**
Neurturin (50 cells)	29 ± 19%	0.91 ± 0.11*	1.89 ± 0.52*	2.67 ± 0.39*,**	4.19 ± 1.3*,**
GDNF (55 cells)	39 ± 13%	1.98 ± 0.42*	1.65 ± 0.7*	2.75 ± 0.7*	4.13 ± 0.93*,**

Magnitudes (peak and area) of Ca^{2+} transients elicited by brief (2 s) application of 1 μM capsaicin to isolated DRG neurons were measured. Data presented are the mean ± SEM ratio of values obtained before and after growth factor treatment in all capsaicin responders tested [ΔF(all)] and in the subset of responsive neurons potentiated by the given growth factor [ΔF(pot)]; $n > 6$ mice for each growth factor. Representative records are shown in Figure 2. Potentiation was never seen without growth factor addition. All growth factors potentiated capsaicin responses in subsets of neurons; however, the frequency and magnitude of potentiation varied. Artemin and NGF potentiated capsaicin responses in the majority of cells (>60%), whereas GDNF- and neurturin-induced potentiation was more rare. When potentiation was observed, the magnitudes of artemin-, neurturin- and GDNF-induced potentiation were significantly ($p < 0.05$) greater than NGF-induced potentiation. *$p < 0.001$ versus untreated; **$p < 0.05$ versus NGF treated

The time course of potentiation varies with growth factor

As shown in Figure 3, the time course of potentiation of capsaicin responses was growth factor dependent. Capsaicin responses elicited at 10 min intervals after growth factor treatment were compared with those obtained before growth factor exposure in the same cells. The subsets of cells responsive to each growth factor were analyzed independently. Percentage of cells maximally potentiated at each time point after growth factor treatment are given in Figure 3*A*. Most cells exposed to NGF, artemin, and GDNF showed maximal potentiation 10 min after addition of growth factor (Fig. 3*A*). In contrast, most (>70%) neurturin-responsive cells were not maximally potentiated until the second capsaicin application, 20 min after growth factor treatment was initiated. Neurturin- and GDNF-induced potentiation subsided 30 min after growth factor treatment, but some cells responsive to artemin or NGF were maximally potentiated at this late time point.

The magnitude of capsaicin response potentiation for each growth factor as a function of time was also investigated (Fig. 3*B*). The effects of artemin and GDNF are more transient than those of NGF: NGF-induced potentiation was relatively constant over 30 min in affected cells, whereas artemin- and GDNF-induced potentiations peaked at 10 min and then declined over the next 20 min. In contrast, the magnitude of the neurturin-induced potentiation peaked at 20 min, when the highest percentage of neurturin-responsive cells were at maximal potentiation (Fig. 3*A*). Thus, each of the four growth factors examined (NGF, artemin, neurturin, and GDNF) potentiates TRPV1 with slightly different amplitude and time course profiles.

GDNF family members are more effective modulators of TRPV1 than NGF

Dose–response experiments were performed to compare the relative potency of NGF, artemin, neurturin, and GDNF in the potentiation of TRPV1. The maximal concentration for each growth factor in this series of experiments was 100 ng/ml; higher

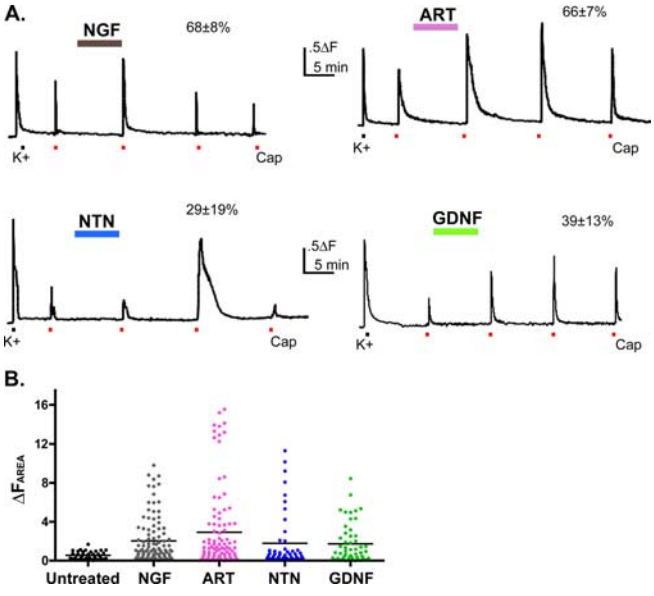

Figure 2. Acute potentiation of TRPV1 by growth factors. ***A***, Ca^{2+} influx after 1 μM capsaicin (Cap) treatment before and after 7 min perfusion of NGF, GDNF, artemin (ART), or neurturin (NTN) (all at 100 ng/ml) was examined in isolated DRG sensory neurons. Each coverslip received a single growth factor perfusion. Growth factor treatment increased capsaicin responses (both peak and area measurements) in many cells (see Table 1); percentage of capsaicin responders potentiated is given above each record. The red bar indicates capsaicin application. ***B***, Fold change in capsaicin response area (ΔF_{area}) measurements for each cell after treatment with NGF, artemin, neurturin, GDNF, or buffer (Untreated).

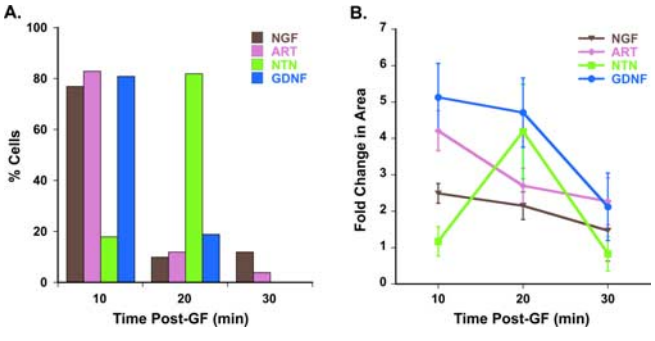

Figure 3. Time course of TRPV1 potentiation varies with growth factor. Capsaicin-induced Ca^{2+} influx was recorded before and 10, 20, and 30 min after 7 min perfusion of growth factors (GF) on isolated DRG neurons. Potentiation of capsaicin-evoked Ca^{2+} transients by growth factors are presented as the percentage of cells at each time point at peak potentiation (***A***) and the percentage magnitude of potentiation at each time point (***B***). The greatest effects of NGF, artemin (ART), and GDNF on TRPV1 occur at 10 min, whereas the action of neurturin (NTN) on TRPV1 is more delayed. Interestingly, although the magnitude of the potentiation by NGF (in potentiated cells) remains relatively constant over 20 min, the percentage of neurons affected decreases substantially over this time period.

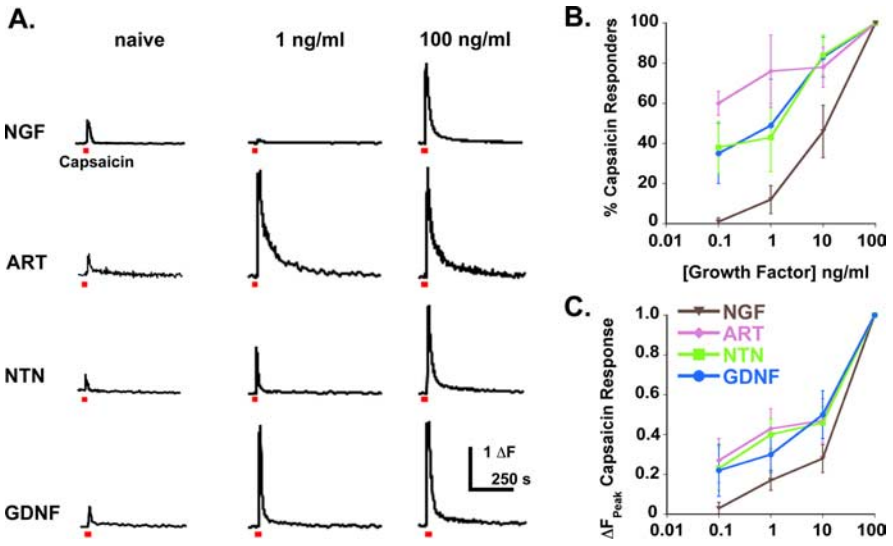

Figure 4. GDNF family members are more potent than NGF in TRPV1 potentiation. Capsaicin-evoked Ca^{2+} influx was recorded before and 10 min after perfusion of growth factors on isolated DRG neurons. Each growth factor concentration (0.1, 1, 10, and 100 ng/ml) was tested on individual coverslips, cells were allowed to recover for 30 min, and maximal doses of each growth factor were given to identify all potential responders in the field and to determine maximal potentiation magnitude for each responsive cell. Representative traces are shown in ***A***. Note that the magnitude of potentiation increases with increasing growth factor concentration (***A***, ***C***), as does the number of cells potentiated (***B***). Although all four factors potentiated capsaicin-evoked responses, all GDNF family members were substantially more potent than NGF. ART, Artemin; NTN, neurturin.

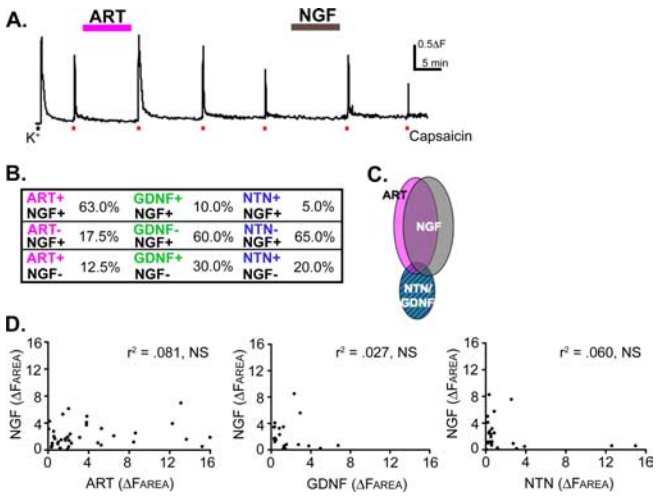

Figure 5. Growth factors affect overlapping subsets of capsaicin-responsive neurons. Growth factors were given serially to investigate the action(s) of multiple growth factors in a single cell. Capsaicin-induced Ca^{2+} influx was recorded before and 10 min after perfusion of artemin (ART), neurturin (NTN), or GDNF (each at 100 ng/ml) on isolated DRG neurons. Cells were allowed to recover for 30 min and tested for NGF-induced potentiation of capsaicin responses. Capsaicin responses obtained from a dually potentiated cell are shown in ***A***. Percentage of overlap in NGF/artemin, NGF/GDNF, and NGF/neurturin responses are shown in ***B***. There is substantial overlap in the actions of NGF and artemin, as predicted by the overlap in their receptor expression. However, the overlap between NGF and neurturin or GDNF is low, suggesting that these growth factors act on distinct subsets of neurons (***B***). These data are graphed as a Venn diagram in ***C***. Neurturin and GDNF populations are graphed together because they showed similar overlap with NGF. ***D***, ΔF_{area} of capsaicin responses after NGF application is graphed versus ΔF_{area} after artemin, neurturin, or GDNF application in the same cell. Potentiation magnitudes were not correlated for these growth factor pairs.

concentrations (200–400 ng/ml) did not elicit greater potentiation or identify additional responsive cells (NGF, artemin, neurturin, and GDNF each tested singly, $n = 15$ each; data not shown). Growth factors were tested singly on individual capsaicin-responsive cells, first at low (0.1, 1.0, or 10 ng/ml) and then high (100 ng/ml) concentrations (Fig. 4*A*). Note that NGF robustly potentiated capsaicin responses at 100 ng/ml but not at 1 ng/ml, whereas artemin, neurturin, and GDNF all caused significant potentiation of capsaicin at 1 ng/ml. Also, potentiation was dose dependent: magnitude increased with increasing growth factor concentration. The proportion of cells responding to 100 ng/ml of a given growth factor that also responded to each of the low concentrations is shown in Figure 4*B*. At 0.1 ng/ml, NGF did not potentiate capsaicin responses in any cells, but artemin, neurturin, and GDNF all potentiated many cells at this concentration (percentage responders potentiated: artemin, 60 ± 6; neurturin, 38 ± 13; and GDNF, 35 ± 15). Even at 10 ng/ml, GDNF family members were more broadly effective than NGF and potentiated most responsive cells (~80% in each case), whereas NGF affected only 46% of responsive neurons. In addition, the magnitude of potentiation seen at the "low" growth factor concentration was compared with that obtained with the maximal concentration in the same cell (Fig. 4*C*). Whereas artemin, neurturin, and GDNF all elicited significant potentiation at the lowest dose tested (0.1 ng/ml), NGF was ineffective at this dose (percentage max ΔF_{area}: NGF, 0; artemin, 47 ± 14; neurturin, 27 ± 9; and GDNF, 22 ± 15). These data demonstrate that artemin, neurturin, and GDNF are ~10–100 times more potent than NGF in the potentiation of capsaicin responses in sensory neurons.

Artemin and NGF modulate TRPV1 in the same subset of sensory neurons

To examine the distribution of growth factor responsiveness, cells were tested with pairs of growth factors presented sequentially. Additional capsaicin presentations were given in these studies to ensure return of the capsaicin response to baseline before application of the second growth factor. Results of such an experiment are shown in Figure 5*A*; tabulated data from all cells are given in Figure 5*B*. In initial experiments, NGF was paired with each of the GDNF family members. All growth factors were given at maximal effective concentrations (100 ng/ml). Most cells (63%) potentiated by NGF were also potentiated by artemin. NGF/GDNF or NGF/neurturin overlap was rarely detected (10 and 5%, respectively), but there was great overlap in GDNF- and neurturin-responsive subsets of cells (80% of neurturin-responsive cells were also GDNF sensitive) (Fig. 5*C*, Venn diagram). Surprisingly, the magnitudes of TRPV1 potentiation induced by artemin, NGF, neurturin, and GDNF were not correlated (Fig. 5*D*). These data define at least two subsets of TRPV1-positive sensory neurons: a major population responsive to NGF and artemin, presumably coexpressing TrkA and GFRα3, and a minor population responsive to neurturin and GDNF, presumably expressing GFRα1 and/or GFRα2.

Strikingly, cells responsive to both artemin and NGF showed significantly ($p < 0.05$) greater potentiation by artemin than by NGF (ΔF_{area}: artemin, 3.78 ± 0.71; NGF, 2.09 ± 0.27) (Table 2). This effect was independent of the order of growth factor appli-

cation, suggesting that, in the same sensory neurons, artemin is more effective than NGF to potentiate TRPV1.

GDNF family members potentiate in the presence of maximal NGF

Most sensory neurons examined responded to more than one growth factor. To examine the simultaneous effects of multiple growth factors on TRPV1, capsaicin responses were recorded before and after NGF exposure. Cells were allowed to return to baseline and then perfused with all four growth factors (each at 100 ng/ml; Mix), and capsaicin responses were recorded again in the same cells. Representative records are shown in Figure 6, *A* and *B*; capsaicin response potentiations (fold change) in all cells are shown in Figure 6*C*. Ninety-eight percent of capsaicin-responsive cells were sensitized by the growth factor mix, suggesting that virtually all TRPV1-expressing neurons respond to at least one growth factor. Twenty-four percent of capsaicin responders were potentiated by GDNF family members but not by NGF alone (Fig. 6*B*); these likely correspond to GDNF/neurturin-sensitive neurons. As seen above, >70% of capsaicin responders were potentiated by NGF (Fig. 6*A*). Interestingly, perfusion with a combination of NGF, artemin, neurturin, and GDNF (Mix) induced significantly ($p < 0.005$) greater potentiation in these cells than NGF alone (Fig. 6*A*, inset table, *C*). In NGF-responsive cells, the ratio of Mix- to NGF-induced potentiation (ΔF_{area} in the same cells) is 7.74 ± 2.75-fold.

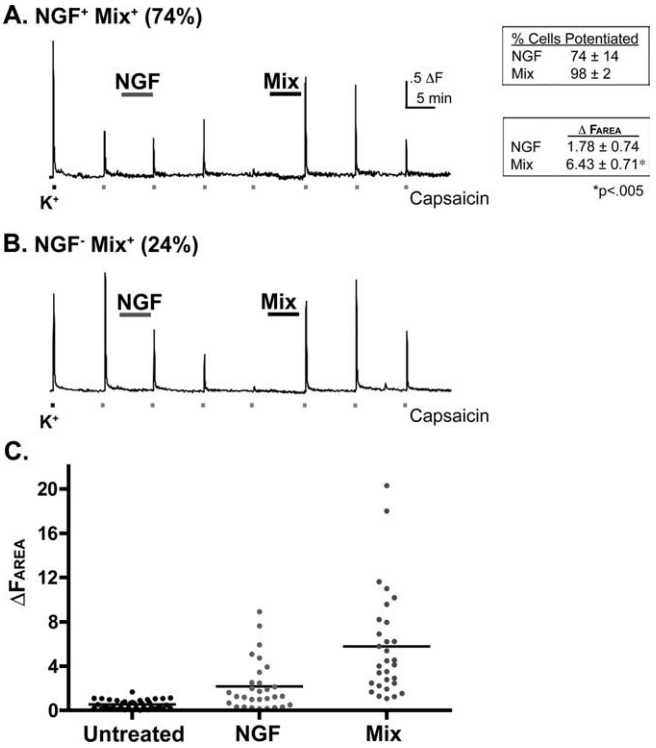

Figure 6. GDNF family members potentiate in the presence of maximal NGF. Potentiation of capsaicin responses by NGF alone and by combined NGF, artemin, neurturin, and GDNF (each at 100 ng/ml; Mix) were examined in single cells to determine whether GDNF family members provide additional sensitization of TRPV1 in the presence of maximal doses of NGF. NGF was given for 7 min, capsaicin responses were examined for 30 min (at 10 min intervals), the growth factor mix was given for 7 min, and capsaicin responses were examined for another 30 min. Representative traces are shown in ***A*** and ***B***. ***A***, Most capsaicin responses (74%) are potentiated by both NGF and GDNF family members. ***B***, Some cells (24%) were not responsive to NGF but were potentiated by GDNF family members. ***C***, ΔF_{area} values for all capsaicin responses in this experiment after initial tachyphylaxis (Untreated), NGF treatment, and treatment with all four growth factors (Mix). In all cells examined, combined NGF and GDNF family members resulted in greater potentiation of TRPV1 than was achieved with NGF alone. Notably, the effect of 400 ng/ml NGF was not greater than that of 100 ng/ml NGF (data not shown).

Table 3. Expression of growth factors and receptors is regulated during inflammation

	Naive	CFA day 1	CFA day 4	CFA day 7	CFA day 14
NGF	0.39 ± 0.05	0.38 ± 0.06	0.52 ± 0.03	0.79 ± 0.05**	0.68 ± 0.07**
Artemin	0.10 ± 0.03	1.08 ± 0.05**	0.58 ± 0.1**	0.20 ± 0.02**	0.12 ± 0.03
GDNF	0.41 ± 0.07	0.15 ± 0.02**	0.55 ± 0.06	0.35 ± 0.05	0.34 ± 0.01
Neurturin	0.53 ± 0.07	0.33 ± 0.08*	0.46 ± 0.06	1.00 ± 0.20	0.39 ± 0.04
TrkA	3.61 ± 0.14	3.61 ± 0.08	3.24 ± 0.18	3.65 ± 0.11	2.77 ± 0.59
RET	3.16 ± 0.15	3.47 ± 0.07*	3.26 ± 0.06	3.12 ± 0.12	3.23 ± 0.1
GFRα1	16.53 ± 0.16	16.46 ± 0.17	17.19 ± 0.37	16.65 ± 0.33	15.03 ± 0.52*
GFRα2	4.35 ± 0.17	3.78 ± 0.03**	3.87 ± 0.1*	4.07 ± 0.11	4.09 ± 0.09
GFRα3	4.32 ± 0.1	4.55 ± 0.11	4.58 ± 0.11	3.87 ± 0.15*	4.31 ± 0.08
TRPV1	5.84 ± 0.21	5.07 ± 0.2*	5.24 ± 0.13*	4.5 ± 0.26**	4.67 ± 0.13*

Mean ± SEM intensity (relative to GAPDH for each sample) is given for products detected using ^{32}P-labeled PCR on mRNA obtained from paw skin of naive and CFA-inflamed mice (NGF, artemin, neurturin, and GDNF). All four growth factors were detected in naive skin, and expression of each was regulated during the course of CFA-induced inflammation in skin, artemin most dramatically (Fig. 8*A*). Mean ± SEM ΔCt values (relative to GAPDH) are given for products detected (TRPV1, GFRα1, GFRα2, GFRα3, Ret, and TrkA) using real-time PCR on mRNA obtained from L3–L5 DRG of naive and CFA-inflamed mice. All growth factor receptors were detected in naive DRG. Expression of all receptors examined, except TrkA, was dynamically regulated during CFA-induced inflammation (Fig. 8*B*). *$p < 0.05$ and **$p < 0.005$ versus naive values each product.

Similar results were obtained in experiments comparing NGF with NGF and artemin (NGF/artemin) treatment in the same cells. Simultaneous presentation of NGF and artemin (each at 100 ng/ml) induced significantly ($p < 0.02$) greater potentiation of TRPV1 than NGF alone (ΔF_{area}: NGF, 1.83 ± 0.49; NGF/artemin, 5.16 ± 0.4; $n = 15$ cells). The average ratio of NGF/artemin-induced to NGF-induced potentiation (in the same cells) was 5.70 ± 2.38, indicating that the combined action of NGF and artemin is greater than that of NGF alone. Importantly, the frequency and the magnitude of potentiation of capsaicin responses when NGF, artemin, neurturin, or GDNF were presented alone at 400 ng/ml was not greater than when these growth factors were presented at 100 ng/ml as part of the growth factor mix. Therefore, increasing the amount of a single growth factor does not produce the level of potentiation seen with presentation of a combination of growth factors.

Artemin potentiation in identified cutaneous afferents

Immunohistochemical staining for TRPV1 labels many peripheral sensory axons in mouse epidermis, a tissue that expresses both NGF and GDNF family members (Snider, 1994; Fundin et al., 1999; Golden et al., 1999). To determine whether artemin acts on cutaneous sensory afferents, double-labeling experiments were performed in mouse hindpaw skin. Representative sections are shown in Figure 7*A*. Coexpression of TRPV1 and GFRα3 was detected in most, but not all, TRPV1-positive cutaneous afferent fibers (Fig. 7*A*, arrows), suggesting that artemin as well as NGF may potentiate cutaneous TRPV1-positive nociceptors. To test this hypothesis, the saphenous nerve (a purely cutaneous nerve of the thigh) was injected with fluorescently labeled WGA 488. The label was readily visible in isolated neurons under epifluorescence, and capsaicin responses were imaged in these retrogradely labeled cutaneous afferents before and after artemin treatment (see example in Fig. 7*B*, arrow; capsaicin responses obtained from this cell are shown in *C*). Artemin robustly potentiated capsaicin responses in 86% of capsaicin-responsive cutaneous afferents ($n = 15$ capsaicin-responsive cells, 4 mice). These

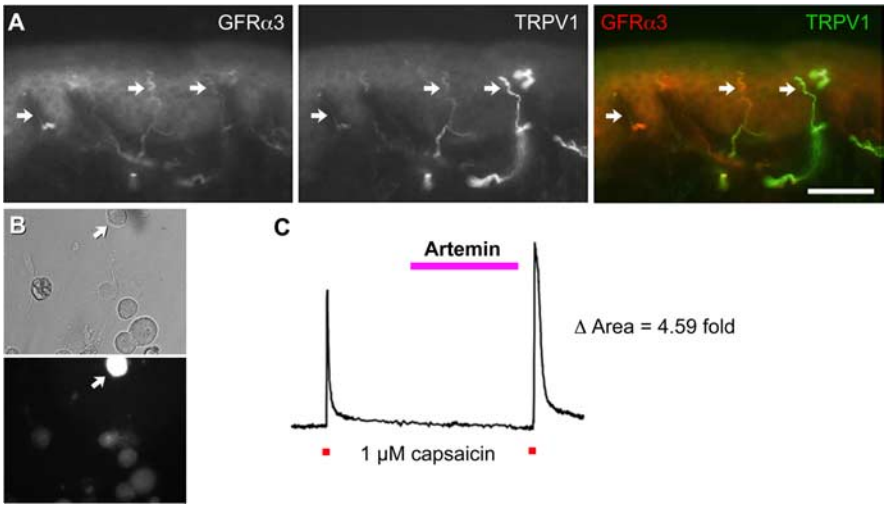

Figure 7. TRPV1 potentiation occurs in cutaneous afferents. ***A***, Immunohistochemical staining for GFRα3 and TRPV1 in hindpaw skin. GFRα3 staining identified numerous fibers entering the epidermis and was extensively colocalized with staining for TRPV1. Scale bar, 50 μm. ***B***, Cutaneous afferents were identified *in vitro* by injecting the retrograde tracer WGA 488 into the saphenous nerve before dissociation of sensory neurons. The top panel is a phase-contrast image of dissociated sensory neurons; the bottom panel is an epifluorescence image of the same field. Arrows indicate the WGA 488-labeled cutaneous afferent. ***C***, Capsaicin responses recorded from the retrogradely labeled cutaneous afferent in ***B***; this response is representative of cutaneous afferents examined in this study. Fold potentiation of the capsaicin response by artemin (100 ng/ml) is given above the trace. Application of artemin substantially increased the magnitude of the capsaicin response in most capsaicin-responsive cutaneous afferents (see Results).

data suggest a prominent role for artemin in the modulation of TRPV1 in cutaneous nociceptors.

Dynamic regulation of growth factor (and receptor) expression during inflammation

One of the most compelling factors supporting a role for NGF in inflammation is its upregulation after injury. Therefore, we examined the expression levels of NGF, artemin, neurturin, and GDNF mRNA in footpad skin during CFA-induced inflammation. CFA injection causes thermal hyperalgesia with a consistent time course that begins within 1 d and lasts for 7–10 d (Fairbanks et al., 2000; Zwick et al., 2003). Expression data are shown in Table 3 and Figure 8*A*: mRNA for all four growth factors is dynamically regulated during inflammation. NGF mRNA increased linearly over the first 7 d to twofold over naive levels. NGF expression then remained constant for the next week. In contrast, artemin increased 10-fold in the first 24 h and remained elevated for 7 d. GDNF and neurturin expression decreased after CFA: GDNF between days 1 and 4, and neurturin at day 1. However, by day 7, neurturin mRNA had doubled and then returned to baseline by day 14. Interestingly, the time course of artemin expression parallels the development of thermal hyperalgesia in inflammation, suggesting that artemin may play a key modulatory role *in vivo*.

We also examined the expression levels of the growth factor receptors in L3–L5 DRG after CFA injection into the hindpaw. Expression data are shown in Table 3 and Figure 8*B*. As reported previously (Molliver et al., 2005), TRPV1 expression increased 2.5-fold during inflammation. Interestingly, GFRα3 and GFRα2 expression was also significantly increased in DRG neurons during inflammation: GFRα3 at day 7 and GFRα2 at days 1 and 4. However, Ret expression was unchanged during this time period. These data further support a role for artemin- and neurturin-induced thermal sensitization of cutaneous afferents during inflammation. TrkA and GFRα1 expression are only affected at day 14, indicating that changes in the expression of these receptors do not contribute to the CFA-evoked hyperalgesia normally seen in the first 2 weeks after injection.

Growth factors directly induce thermal hypersensitivity *in vivo*

To investigate directly the role(s) of NGF, artemin, neurturin, and GDNF in modulating thermal thresholds *in vivo*, growth factors were injected (0.2 μg/20 μl) into the hindpaw, and latency to withdrawal from a noxious thermal stimulus was measured. This concentration was chosen based on previous studies of NGF-induced behavioral thermal hyperalgesia (Amann et al., 1995; Andreev et al., 1995; Bergmann et al., 1998). Injection of growth factors into the skin resulted in thermal hyperalgesia (Fig. 9). The time course of thermal hypersensitivity induced by different growth factors was distinct, and all effects resolved in 24 h. Animals were observed for the first 10 min after growth factor injection of saline or growth factor; no nocifensive behaviors (guarding, lifting, and biting of paw) were observed, suggesting that growth factor injection is not directly painful. At 30 min, 1 h, and 4 h after growth factor injection, NGF, neurturin, and artemin all induced significant ($p < 0.01$) thermal hyperalgesia compared with naive and contralateral thermal thresholds. GDNF effects took longer to develop: GDNF-induced thermal hyperalgesia was not statistically significant until 1 h after injection. Surprisingly, combined injection of NGF and artemin, both of which are upregulated in inflamed skin (Fig. 8), resulted in significant ($p < 0.005$) thermal hyperalgesia lasting 6 d (6 d time point not shown). Also, NGF/artemin-induced hyperalgesia was significantly ($p < 0.05$) greater than that induced by artemin or NGF alone at 4 h after injection. These data suggest that potentiation of TRPV1 by multiple growth factor may play a critical role in modulating thermal sensitivity *in vivo* during inflammation.

Discussion

Previous studies have shown that NGF acutely potentiates TRPV1 function and suggest a role for this trophic factor in the regulation of nociceptor sensitivity. Here, we provide several complementary lines of evidence that members of the GDNF family of trophic factors are also potent regulators of TRPV1 function and likely contribute to the sensitization of primary afferents *in vivo*. Immunoreactivity for GFRα3 was highly colocalized with TRPV1 *in situ*; GFRα2 and TRPV1 overlap is less dramatic but still substantial. All four trophic factors tested (NGF, artemin, neurturin, and GDNF) potentiated capsaicin-evoked TRPV1 signaling in isolated neurons and caused thermal hyperalgesia when injected into the hindpaw. In addition, these factors all potentiated capsaicin responses in a subset of previously unresponsive (at least at the resolution of our calcium imaging protocol) small-diameter neurons. Finally, artemin mRNA (but not GDNF) was substantially upregulated during cutaneous inflammation evoked by hindpaw injection of CFA, suggesting that artemin in particular enhances TRPV1 signaling in response to inflammatory injury. Strikingly, a single combined injection of

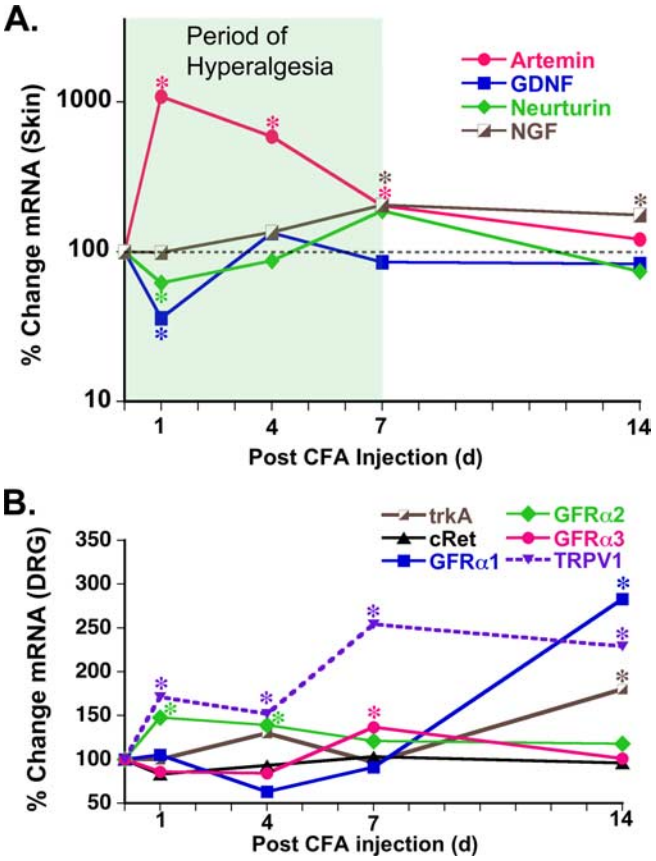

Figure 8. Growth factors are upregulated in the skin during inflammation. Time course and magnitude of changes in growth factor mRNA levels in hindpaw skin during inflammation evoked by injection of CFA and measured by ^{32}P-labeled RT-PCR (***A***). The shaded box indicates the period of thermal hyperalgesia recorded in a previous experiment (Zwick et al., 2003). Artemin is most dramatically upregulated (10-fold) at day 1 after CFA injection; this immediately precedes the time period of greatest thermal hyperalgesia in CFA-induced inflammation (1–4 d). NGF was also upregulated, but at later time points (4, 7, and 15 d) and to a lesser degree (2-fold) than artemin. Neurturin mRNA also doubled by day 7 after showing an initial decrease at day 1. ***B***, Expression levels of growth factor receptors and TRPV1 were measured using real-time PCR in L3–L5 DRG during inflammation evoked by CFA injection. GFRα2, the neurturin receptor, is upregulated during the first week of inflammation; GFRα3, the artemin receptor, is upregulated at day 7, during the period of intense thermal hyperalgesia. Expression of TrkA, the NGF receptor, and GFRα1 are not changed until day 14. *$p < 0.05$ versus naive.

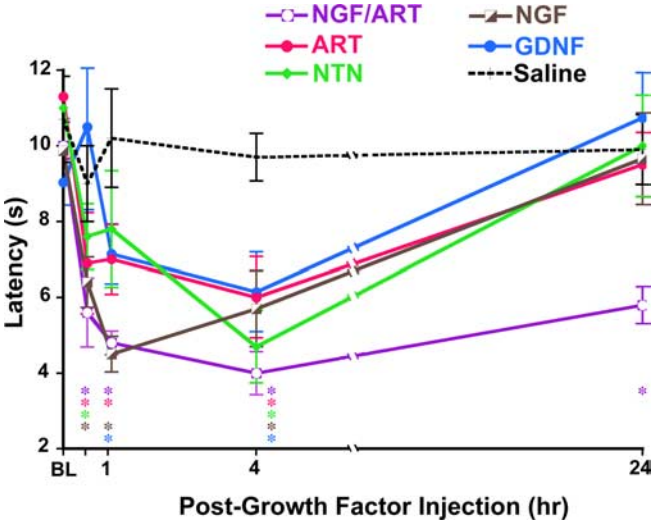

Figure 9. Growth factors acutely potentiate heat responses *in vivo*. Latency to thermal nociceptive response was measured in mice using the Hargreaves apparatus before and after injection of 0.2 μg/20 μl growth factor (or saline control) into the glabrous skin of the hindpaw. All growth factors tested [NGF, artemin (ART), neurturin (NTN), and GDNF] caused significant thermal hyperalgesia during the first 4 h; these effects of single growth factor injection resolved by 24 h after injection. Injection of NGF plus artemin (0.2 μg of NGF/0.2 μg of artemin/20 μl of PBS) resulted in persistent thermal hyperalgesia lasting 6 d (last time point not shown). These data suggest that GDNF family members, as well as NGF, directly potentiate nociceptive thermal signaling *in vivo*. Additionally, the effects of growth factors on thermal signaling are additive *in vivo*. *$p < 0.05$ versus saline control injection.

artemin and NGF produced hyperalgesia that lasted for 6 d, suggesting that the combined elevation of artemin and NGF expression during inflammation evokes prolonged sensitization of pain circuits.

In isolated neurons *in vitro*, the combined application of NGF and artemin potentiated capsaicin responses fivefold more than NGF alone. In the intact animal, robust activation of C-fiber input to the spinal cord (which includes most TRPV1-expressing afferents) produces lasting changes in the efficacy of nociceptive signaling known as central sensitization (Ji and Woolf, 2001). Thus, the long-lasting hypersensitivity seen *in vivo* after coinjection of NGF and artemin may be attributable to a combination of peripheral and central changes. Indeed, inflammatory thermal hyperalgesia does not occur in mice lacking TRPV1. However, additional mechanisms are certainly involved. NGF can also lead to the indirect sensitization of primary afferents through the activation of immune cells (Lewin et al., 1994; Bennett et al., 1998; Lambiase et al., 2004; Vargas-Leal et al., 2005). Furthermore, both neurotrophins and GDNF family members activate transcription in sensory neurons that may lead to lasting changes in nociceptor sensitivity. The *in vitro* and *in vivo* data reported here do support the conclusion that any traumatic insult resulting in a combined increase in expression of NGF and artemin will have profound effects on noxious thermal sensitivity. It will be intriguing to determine whether increased expression of both NGF and artemin is required for inflammatory hyperalgesia.

We were surprised to find that a significant percentage of capsaicin-sensitive neurons were modulated by GDNF and neurturin, because TRPV1 immunostaining is rare in mouse neurons that bind isolectin B4 (IB4), often used as a marker of Ret-expressing small neurons (Molliver and Snider, 1997; Zwick et al., 2002; Woodbury et al., 2004). Several factors may account for this. First, we found immunoreactivity for GFRα2 in 22% of TRPV1-positive neurons. Second, functional assays such as Ca^{2+} imaging are likely to be more sensitive than immunocytochemical detection of protein and therefore more cells may contain functional TRPV1 or Ret than are clearly labeled by antibody visualization (Breese et al., 2005). Finally, there has been substantial variability in the reported population of neurons labeled by IB4, leading to confusion as to the identity of these neurons when comparing reports from different laboratories (Kashiba et al., 2001).

Much attention has been given to possible functional differences between populations of nociceptors delineated by expression of either Ret or TrkA (Snider and McMahon, 1998). However, multiple laboratories have documented that 15–25% of Ret-expressing neurons also express TrkA, and our results suggest that the population of neurons expressing both receptors may be particularly important in responding to injury-evoked changes in their target tissues (Bennett et al., 1998; Kashiba et al., 1998). These data support a functional model in which most TRPV1-positive cells express both TrkA and GFRα3, whereas a less numerous group of cells express TRPV1, GFRα1, and/or GFRα2. In mice, GFRα3 is expressed primarily in TrkA-positive afferents

(80%), and virtually all GFRα3 neurons express TRPV1 (Orozco et al., 2001). These histological results are consistent with our functional data that most neurons potentiated by artemin were also potentiated by NGF. In contrast, GFRα1 and GFRα2 are predominantly expressed in TrkA-negative neurons (Bennett et al., 1998; Leitner et al., 1999; Zwick et al., 2002; Woodbury et al., 2004), consistent with the low coincidence of NGF responsiveness with neurturin or GDNF responsiveness (5 and 10%, respectively) reported here. These two types of TRPV1-expressing neurons would presumably be differentially sensitive to responses of the skin to injury.

NGF, via TrkA, has been shown to potentiate TRPV1 function through phosphorylation of TRPV1 channels by serine/threonine kinases PKC, PKA, and calcium/calmodulin-dependent kinase II (Shu and Mendell, 2001; Bhave et al., 2002, 2003; Bonnington and McNaughton, 2003; Mohapatra and Nau, 2003; Mandadi et al., 2004) and tyrosine kinase c-Src (Zhang et al., 2005). In addition, TRPV1 is tonically inhibited by phosphatidylinositol-4,5-bisphosphate (PIP_2), and depletion of PIP_2 from the plasma membrane by phospholipase Cγ (PLCγ) after NGF treatment leads to increased TRPV1 activation (Prescott and Julius, 2003; Liu et al., 2005). Although TrkA and Ret activate overlapping signaling cascades, including Src and PLCγ (Prescott and Julius, 2003; Paveliev et al., 2004; Liu et al., 2005; Zhang et al., 2005), we show here that artemin is more effective than NGF at potentiating TRPV1 function. One potential difference is that GFRα1–GFRα3/Ret complexes are located in lipid rafts within the plasma membrane and that this location is integral to Ret signaling, whereas TrkA signaling complexes do not appear to be localized to these rafts (Tansey et al., 2000; Encinas et al., 2001). However, the mechanism by which GDNF family members potentiate TRPV1 remains to be established.

Although GDNF, neurturin, and artemin bind very similar receptor complexes, the results of this study demonstrate that these growth factors differentially potentiate TRPV1. The robust potentiating effect of artemin on TRPV1 channels compared with neurturin and GDNF may be partially explained by receptor expression: GFRα3 is a specific receptor for artemin (Baloh et al., 1997; Jing et al., 1997; Klein et al., 1997; Sanicola et al., 1997; Carmillo et al., 2005) and is more highly colocalized with TRPV1 than GFRα2 (this study; Orozco et al., 2001). It is surprising that the frequency and time course of neurturin- and GDNF-induced potentiations of TRPV1 are different, because GDNF and neurturin have been shown to signal via both GFRα1 and GFRα2 *in vitro* (Baloh et al., 1997; Jing et al., 1997; Klein et al., 1997; Sanicola et al., 1997; Carmillo et al., 2005). However, a recent study revealed significant functional differences in signaling of neurturin and GDNF through GFRα1/Ret receptor complexes *in vitro*, suggesting that, although these growth factors both bind GFRα1, they may activate distinct signaling pathways through the same receptor (Lee et al., 2006). Additionally, Leitner et al. (1999) reported evidence that crosstalk is actually limited or nonexistent *in vivo*. Our results support this more selective model of action for GDNF and neurturin; pharmacologic experiments designed to probe these pathways are planned.

Two other studies have examined the effect(s) of artemin injections on nociceptive behaviors. Neither study reported behavioral hyperalgesia after artemin injection, but there were significant protocol differences between those studies and that reported here. Species difference is a confounding factor, because both published studies were conducted in rats. Gardell et al. (2003) reported no change in thermal or mechanical paw sensitivity after subcutaneous artemin injection in the rat (the site of injection was not specified), but they did not test thermal hyperalgesia at the site of artemin injection, as in the experiments reported here. Perhaps the acute affects of artemin we observed represent local action of artemin on nociceptors in the glabrous skin that was directly tested in the Hargreaves apparatus. Also, Gardell et al. (2003) used artemin concentrations that were 300 times greater than that used in this study; this difference raises the possibility that high artemin concentrations have other effects that mask the hyperalgesic action reported here. However, in contrast to Gardell et al. (2003), a similar study found that artemin injection [either intraperitoneally (5 μm/kg) or intrathecally (10 μg/d)] in rats did not block hyperalgesia produced by spinal nerve ligation (Bolon et al., 2004). This suggests that, even in the same species, the method of artemin application appears to dramatically alter efficacy.

Our findings indicate that GDNF family members, and particularly artemin, are capable of modulating TRPV1 activity and cause thermal hyperalgesia when injected into the skin. The prolonged hyperalgesia resulting from combined injection of artemin and NGF and our finding that artemin and NGF are upregulated in the skin in response to inflammation suggest that these two factors, acting in concert, may be particularly important in the regulation of nociceptor responsiveness.

References

Albers KM, Woodbury CJ, Ritter AM, Davis BM, Koerber HR (2006) Glial cell line-derived neurotrophic factor expression in skin alters the mechanical sensitivity of cutaneous nociceptors. J Neurosci 26:2981–2990.

Amann R, Schuligoi R, Herzeg G, Donnerer J (1995) Intraplantar injection of nerve growth factor into the rat hindpaw: local edema and effects on thermal nociceptive threshold. Pain 64:323–329.

Andreev N, Dimitrieva N, Koltzenburg M, McMahon SB (1995) Peripheral administration of nerve growth factor in the adult rat produces a thermal hyperalgesia that requires the presence of sympathetic post-ganglionic neurones. Pain 63:109–115.

Baloh RH, Tansey MG, Golden JP, Creedon DJ, Heuckeroth RO, Keck CL, Zimonjic DB, Popescu NC, Johnson Jr EM, Milbrandt J (1997) TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret. Neuron 18:793–802.

Baudet C, Mikaels A, Westphal H, Johansen J, Johansen TE, Ernfors P (2000) Positive and negative interactions of GDNF, NTN and ART in developing sensory neuron subpopulations, and their collaboration with neurotrophins. Development 127:4335–4344.

Bennett DL, Michael GJ, Ramachandran N, Munson JB, Averill S, Yan Q, McMahon SB, Priestley JV (1998) A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. J Neurosci 18:3059–3072.

Bergmann I, Reiter R, Toyka KV, Koltzenburg M (1998) Nerve growth factor evokes hyperalgesia in mice lacking the low-affinity neurotrophin receptor p75. Neurosci Lett 255:87–90.

Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RWt (2002) cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. Neuron 35:721–731.

Bhave G, Hu HJ, Glauner KS, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RWt (2003) Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). Proc Natl Acad Sci USA 100:12480–12485.

Bolon B, Jing S, Asuncion F, Scully S, Pisegna M, Van GY, Hu Z, Yu YB, Min H, Wild K, Rosenfeld RD, Tarpley J, Carnahan J, Duryea D, Hill D, Kaufman S, Yan XQ, Juan T, Christensen K, McCabe J, Simonet WS (2004) The candidate neuroprotective agent artemin induces autonomic neural dysplasia without preventing peripheral nerve dysfunction. Toxicol Pathol 32:275–294.

Bonnington JK, McNaughton PA (2003) Signalling pathways involved in the sensitisation of mouse nociceptive neurones by nerve growth factor. J Physiol (Lond) 551:433–446.

Breese NM, George AC, Pauers LE, Stucky CL (2005) Peripheral inflammation selectively increases TRPV1 function in IB4-positive sensory neurons from adult mouse. Pain 115:37–49.

Carmillo P, Dago L, Day ES, Worley DS, Rossomando A, Walus L, Orozco O, Buckley C, Miller S, Tse A, Cate RL, Rosenblad C, Sah DW, Gronborg M, Whitty A (2005) Glial cell line-derived neurotrophic factor (GDNF) receptor alpha-1 (GFR alpha 1) is highly selective for GDNF versus artemin. Biochemistry 44:2545–2554.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816–824.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288:306–313.

Christianson JA, McIlwrath SL, Koerber HR, Davis BM (2006) Transient receptor potential vanilloid 1-immunopositive neurons in the mouse are more prevalent within colon afferents compared to skin and muscle afferents. Neuroscience 140:247–257.

Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts-Meek S, Armanini MP, Ling LH, McMahon SB, Shelton DL, Levinson AD, Phillips HS (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76:1001–1011.

Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A, Sheardown SA (2000) Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature 405:183–187.

Dyck PJ, Peroutka S, Rask C, Burton E, Baker MK, Lehman KA, Gillen DA, Hokanson JL, O'Brien PC (1997) Intradermal recombinant human nerve growth factor induces pressure allodynia and lowered heat-pain threshold in humans. Neurology 48:501–505.

Elitt CM, McIlwrath SL, Lawson JJ, Malin SA, Molliver DC, Cornuet PK, Koerber HR, Davis BM, Albers KM (2006) Artemin overexpression in skin enhances expression of TRPV1 and TRPA1 in cutaneous sensory neurons and leads to behavorial sensitivity to heat and cold. J Neurosci 26:8578–8587.

Encinas M, Tansey MG, Tsui-Pierchala BA, Comella JX, Milbrandt J, Johnson Jr EM (2001) c-Src is required for glial cell line-derived neurotrophic factor (GDNF) family ligand-mediated neuronal survival via a phosphatidylinositol-3 kinase (PI-3K)-dependent pathway. J Neurosci 21:1464–1472.

Fairbanks CA, Schreiber KL, Brewer KL, Yu CG, Stone LS, Kitto KF, Nguyen HO, Grocholski BM, Shoeman DW, Kehl LJ, Regunathan S, Reis DJ, Yezierski RP, Wilcox GL (2000) Agmatine reverses pain induced by inflammation, neuropathy, and spinal cord injury. Proc Natl Acad Sci USA 97:10584–10589.

Fundin BT, Mikaels A, Westphal H, Ernfors P (1999) A rapid and dynamic regulation of GDNF-family ligands and receptors correlate with the developmental dependency of cutaneous sensory innervation. Development 126:2597–2610.

Gardell LR, Wang R, Ehrenfels C, Ossipov MH, Rossomando AJ, Miller S, Buckley C, Cai AK, Tse A, Foley SF, Gong B, Walus L, Carmillo P, Worley D, Huang C, Engber T, Pepinsky B, Cate RL, Vanderah TW, Lai J, Sah DW, Porreca F (2003) Multiple actions of systemic artemin in experimental neuropathy. Nat Med 9:1383–1389.

Golden JP, DeMaro JA, Osborne PA, Milbrandt J, Johnson Jr EM (1999) Expression of neurturin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse. Exp Neurol 158:504–528.

Ji RR, Woolf CJ (2001) Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain. Neurobiol Dis 8:1–10.

Jing S, Yu Y, Fang M, Hu Z, Holst PL, Boone T, Delaney J, Schultz H, Zhou R, Fox GM (1997) GFRalpha-2 and GFRalpha-3 are two new receptors for ligands of the GDNF family. J Biol Chem 272:33111–33117.

Kashiba H, Hyon B, Senba E (1998) Glial cell line-derived neurotrophic factor and nerve growth factor receptor mRNAs are expressed in distinct subgroups of dorsal root ganglion neurons and are differentially regulated by peripheral axotomy in the rat. Neurosci Lett 252:107–110.

Kashiba H, Uchida Y, Senba E (2001) Difference in binding by isolectin B4 to trkA and c-ret mRNA-expressing neurons in rat sensory ganglia. Brain Res Mol Brain Res 95:18–26.

Klein RD, Sherman D, Ho WH, Stone D, Bennett GL, Moffat B, Vandlen R, Simmons L, Gu Q, Hongo JA, Devaux B, Poulsen K, Armanini M, Nozaki C, Asai N, Goddard A, Phillips H, Henderson CE, Takahashi M, Rosenthal A (1997) A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature 387:717–721.

Lambiase A, Micera A, Sgrulletta R, Bonini S, Bonini S (2004) Nerve growth factor and the immune system: old and new concepts in the cross-talk between immune and resident cells during pathophysiological conditions. Curr Opin Allergy Clin Immunol 4:425–430.

Lee RH, Wong WL, Chan CH, Chan SY (2006) Differential effects of glial cell line-derived neurotrophic factor and neurturin in RET/GFRalpha1-expressing cells. J Neurosci Res 83:80–90.

Leitner ML, Molliver DC, Osborne PA, Vejsada R, Golden JP, Lampe PA, Kato AC, Milbrandt J, Johnson Jr EM (1999) Analysis of the retrograde transport of glial cell line-derived neurotrophic factor (GDNF), neurturin, and persephin suggests that *in vivo* signaling for the GDNF family is GFRα coreceptor-specific. J Neurosci 19:9322–9331.

Lewin GR, Rueff A, Mendell LM (1994) Peripheral and central mechanisms of NGF-induced hyperalgesia. Eur J Neurosci 6:1903–1912.

Lindsay RM (1996) Role of neurotrophins and trk receptors in the development and maintenance of sensory neurons: an overview. Philos Trans R Soc Lond B Biol Sci 351:365–373.

Liu B, Zhang C, Qin F (2005) Functional recovery from desensitization of vanilloid receptor TRPV1 requires resynthesis of phosphatidylinositol 4,5-bisphosphate. J Neurosci 25:4835–4843.

Mandadi S, Numazaki M, Tominaga M, Bhat MB, Armati PJ, Roufogalis BD (2004) Activation of protein kinase C reverses capsaicin-induced calcium-dependent desensitization of TRPV1 ion channels. Cell Calcium 35:471–478.

Mendell LM, Albers KM, Davis BM (1999) Neurotrophins, nociceptors, and pain. Microsc Res Tech 45:252–261.

Michael GJ, Priestley JV (1999) Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. J Neurosci 19:1844–1854.

Mohapatra DP, Nau C (2003) Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway. J Biol Chem 278:50080–50090.

Molliver DC, Snider WD (1997) Nerve growth factor receptor TrkA is down-regulated during postnatal development by a subset of dorsal root ganglion neurons. J Comp Neurol 381:428–438.

Molliver DC, Cook SP, Carlsten JA, Wright DE, McCleskey EW (2002) ATP and UTP excite sensory neurons and induce CREB phosphorylation through the metabotropic receptor, P2Y2. Eur J Neurosci 16:1850–1860.

Molliver DC, Lindsay J, Albers K, Davis BM (2005) Overexpression of NGF or GDNF alters transcriptional plasticity evoked by inflammation. Pain 113:277–284.

Orozco OE, Walus L, Sah DW, Pepinsky RB, Sanicola M (2001) GFRalpha3 is expressed predominantly in nociceptive sensory neurons. Eur J Neurosci 13:2177–2182.

Pakkenberg B, Gundersen HJ (1988) Total number of neurons and glial cells in human brain nuclei estimated by the disector and the fractionator. J Microsc 150:1–20.

Paveliev M, Airaksinen MS, Saarma M (2004) GDNF family ligands activate multiple events during axonal growth in mature sensory neurons. Mol Cell Neurosci 25:453–459.

Prescott ED, Julius D (2003) A modular PIP2 binding site as a determinant of capsaicin receptor sensitivity. Science 300:1284–1288.

Ritter AM, Woodbury CJ, Davis BM, Albers K, Koerber HR (2001) Excess target-derived neurotrophin-3 alters the segmental innervation of the skin. Eur J Neurosci 14:411–418.

Sanicola M, Hession C, Worley D, Carmillo P, Ehrenfels C, Walus L, Robinson S, Jaworski G, Wei H, Tizard R, Whitty A, Pepinsky RB, Cate RL (1997) Glial cell line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins. Proc Natl Acad Sci USA 94:6238–6243.

Shatzky S, Moses S, Levy J, Pinsk V, Hershkovitz E, Herzog L, Shorer Z, Luder A, Parvari R (2000) Congenital insensitivity to pain with anhidrosis (CIPA) in Israeli-Bedouins: genetic heterogeneity, novel mutations in the TRKA/NGF receptor gene, clinical findings, and results of nerve conduction studies. Am J Med Genet 92:353–360.

Shu X, Mendell LM (1999a) Nerve growth factor acutely sensitizes the response of adult rat sensory neurons to capsaicin. Neurosci Lett 274:159–162.

Shu XQ, Mendell LM (1999b) Neurotrophins and hyperalgesia. Proc Natl Acad Sci USA 96:7693–7696.

Shu X, Mendell LM (2001) Acute sensitization by NGF of the response of small-diameter sensory neurons to capsaicin. J Neurophysiol 86: 2931–2938.

Silos-Santiago I, Molliver DC, Ozaki S, Smeyne RJ, Fagan AM, Barbacid M, Snider WD (1995) Non-TrkA-expressing small DRG neurons are lost in TrkA deficient mice. J Neurosci 15:5929–5942.

Snider WD (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell 77:627–638.

Snider WD, McMahon SB (1998) Tackling pain at the source: new ideas about nociceptors. Neuron 20:629–632.

Stucky CL, Lewin GR (1999) Isolectin B_4-positive and -negative nociceptors are functionally distinct. J Neurosci 19:6497–6505.

Stucky CL, Koltzenburg M, Schneider M, Engle MG, Albers KM, Davis BM (1999) Overexpression of nerve growth factor in skin selectively affects the survival and functional properties of nociceptors. J Neurosci 19:8509–8516.

Tansey MG, Baloh RH, Milbrandt J, Johnson Jr EM (2000) GFRalpha-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. Neuron 25:611–623.

Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 21:531–543.

Vargas-Leal V, Bruno R, Derfuss T, Krumbholz M, Hohlfeld R, Meinl E (2005) Expression and function of glial cell line-derived neurotrophic factor family ligands and their receptors on human immune cells. J Immunol 175:2301–2308.

Woodbury CJ, Zwick M, Wang S, Lawson JJ, Caterina MJ, Koltzenburg M, Albers KM, Koerber HR, Davis BM (2004) Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. J Neurosci 24:6410–6415.

Woolf CJ, Safieh-Garabedian B, Ma QP, Crilly P, Winter J (1994) Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience 62:327–331.

Zhang X, Huang J, McNaughton PA (2005) NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. EMBO J 24:4211–4223.

Zhu W, Galoyan SM, Petruska JC, Oxford GS, Mendell LM (2004) A developmental switch in acute sensitization of small dorsal root ganglion (DRG) neurons to capsaicin or noxious heating by NGF. J Neurophysiol 92:3148–3152.

Zwick M, Davis BM, Woodbury CJ, Burkett JN, Koerber HR, Simpson JF, Albers KM (2002) Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse. J Neurosci 22:4057–4065.

Zwick M, Molliver DC, Lindsay J, Fairbanks CA, Sengoku T, Albers KM, Davis BM (2003) Transgenic mice possessing increased numbers of nociceptors do not exhibit increased behavioral sensitivity in models of inflammatory and neuropathic pain. Pain 106:491–500.