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Signaling pathways that mediate NGF-induced increase in expression and release of calcitonin gene-related peptide from sensory neurons

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Abstract

Nerve growth factor (NGF) can augment transmitter release in sensory neurons by acutely sensitizing sensory neurons and by increasing the expression of calcitonin gene-related peptide (CGRP) over time. The current study examined the intracellular signaling pathways that mediate these two temporally distinct effects of NGF to augment CGRP release from sensory neurons. Growing sensory neurons in 30 or 100 ng/ml of NGF for 7 days increases CGRP content and this increase augments the amount of CGRP that is released by high extracellular potassium. Overexpressing a dominant negative Ras, Ras(17N) or treatment with a farnesyltransferase inhibitor attenuates the NGF-induced increase in CGRP content. Conversely, overexpressing a constitutively active Ras augments the NGF-induced increase in content of CGRP. Inhibiting MEK activity also blocks the ability of NGF to increase CGRP expression. In contrast to the ability of chronic NGF to increase peptide content, acute exposure of sensory neurons to 100 ng/ml NGF augments capsaicin-evoked release of CGRP without affecting the content of CGRP. This sensitizing action of NGF is not affected by inhibiting Ras, MEK, or PI3 kinases. In contrast, the NGF-induced increase in capsaicin-evoked release of CGRP is blocked by the PKC inhibitor, BIM and the Src family kinases inhibitor, PP2. These data demonstrate that different signaling pathways mediate the alterations in expression of CGRP by chronic NGF and the acute actions of the neurotrophin to augment capsaicin-evoked release of CGRP in the absence of a change in the content of the peptide.

Keywords

Ras; sensitization; inflammatory pain; ERK; PKC; Src

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In adult animals, the neurotrophin, nerve growth factor (NGF) is an inflammatory mediator that contributes to hypersensitivity by acting directly on small-diameter sensory neurons (Shu and Mendell, 1999). During tissue injury or inflammation, NGF levels are elevated in peripheral tissues (Woolf et al., 1994, Safieh-Garabedian et al., 1995). In animal models of inflammation, treatment with antibodies that block the actions of NGF or with the fusion protein TrkA-IgG, which sequesters NGF, attenuates hypersensitivity to noxious thermal and mechanical stimuli (Lewin et al., 1993, Woolf et al., 1994, Andreev et al., 1995). Acute exposure to NGF produces hyperalgesia (Lewin et al., 1993) and lowers the firing threshold of small-diameter sensory neurons (Shu and Mendell, 1999). On the other hand, chronic exposure to the neurotrophin, which occurs during inflammation, initiates a number of changes in sensory neurons that contribute to hypersensitivity, one of which is an increase in the expression of substance P (SP) and calcitonin gene-related peptide (CGRP) in sensory neurons (Lindsay and Harmar, 1989, Noguchi et al., 1995, Bowles et al., 2006). In an analogous manner, peripheral inflammation increases the expression of neuropeptides in sensory neurons, and these alterations are reversed by an NGF-blocking antibody (Donnerer et al., 1992, Leslie et al., 1995). Both acute exposure to NGF, which enhances excitability of sensory neurons, and long term exposure, which increases the content of neuropeptide transmitters, can result in an increase in transmitter release from sensory nerve endings in response to a given stimulus. An increase in release of SP and CGRP from peripheral endings of sensory neurons contributes to neurogenic inflammation (Richardson and Vasko, 2002), whereas an increase in release from central terminals of sensory neurons augments nociception and contributes to the development of central sensitization (Sun et al., 2004).

Nerve growth factor binds to two receptors, trkA and p75, and both are localized on small-diameter sensory neurons (Verge et al., 1989, Sofroniew et al., 2001). These receptors, in turn are linked to a number of downstream signaling pathways (see reviews by Sofroniew et al., 2001; Nicol and Vasko, 2007). For example, the binding of NGF to the trkA receptor activates phospholipase C γ , PI3 kinases, Src kinases and the small G-proteins Ras and Rap1 which lead to the activation of MAP kinases (Sofroniew et al., 2001). Activation of the p75 receptor increases sphingolipid synthesis, JNK activity, PI3 kinase activity and NF κ B activity (Dobrowsky et al., 1994, Nicol and Vasko, 2007). Controversy remains as to the cellular mechanisms by which NGF augments excitability of sensory neurons since a number of these signaling pathways have been implicated in NGF-induced sensitization (Nicol and Vasko, 2007). For example, a number of studies suggest that NGF-induced sensitization of the TRPV1 response is attenuated by PI3 kinase inhibitors (Bonnington and McNaughton, 2003, Zhuang et al., 2004, Zhu and Oxford, 2007). Other studies have shown that MEK inhibitors block the sensitizing actions of NGF on TRPV1 (Zhuang et al., 2004, Zhu and Oxford, 2007), but some do not see an effect of blocking ERK phosphorylation (Shu and Mendell, 2001, Bonnington and McNaughton, 2003).

Despite the importance of NGF-induced increases in transmitter release in the pathophysiology of inflammation, the intracellular signaling mechanisms regulating release have yet to be determined. Indeed, since release represents an integrated response of the neuron subsequent to stimulation, signaling pathways controlling this function may differ from those controlling other endpoints of excitability. Consequently, we examined in detail which signal transduction cascades mediate the ability of NGF to augment CGRP release by two mechanisms, acute sensitization and long-term upregulation of CGRP expression. We used genetic manipulations of signaling pathways and inhibitors of signaling effectors to manipulate pathways. Our results show that NGF-induced increases in immunoreactive CGRP (iCGRP) release from sensory neurons involves both an increase in peptide expression (after long-term exposure) and sensitization (after acute exposure) and these actions are mediated by different signaling pathways. Activation of the small G-protein, Ras mediates in part the NGF-induced increase in iCGRP expression in sensory neurons, but is

not involved in the ability of an acute exposure to NGF to augment capsaicin-evoked release of iCGRP. This acute sensitizing action of NGF on release is dependent on PKC and activity of Src family kinases.

Experimental Procedures

Isolation and culture of adult sensory neurons from rats

Adult male Sprague-Dawley rats (150–175 g) were purchased from Harlan (Indianapolis, IN). Cell cultures were derived from dorsal root ganglia (DRGs) and were prepared as previously described (Southall and Vasko, 2000). Briefly, the animals were euthanized by CO₂ asphyxiation, the DRGs were collected from the entire length of the spinal column, incubated in F-12 media containing 0.01% collagenase for 2 hours in 3% CO₂ at 37°C, and mechanically dissociated. Cells were plated at a density of approximately 15,000 cells in each well (16 mm diameter) of 24-well culture plates precoated with poly-D-lysine and laminin. The cells were maintained in F-12 media supplemented with 10% horse serum, 2 mM glutamine, 100 µg/ml normocin-O, 50 µg/ml penicillin, 50 µg/ml streptomycin, 50 µM 5-fluoro-2'-deoxyuridine, 150 µM uridine, and the indicated amount of NGF in ng/ml. Cells were placed in an incubator at 3% CO₂ at 37°C. Growth medium was changed every other day. Cells were used 7–12 days after plating depending on the experimental protocol.

Cellular content of immunoreactive calcitonin gene-related peptide (iCGRP)

To determine content of iCGRP, the growth media was removed and the cells were washed with HEPES buffer consisting of (in mM): 25 HEPES, 135 NaCl, 3.5 KCl, 2.5 CaCl₂, 1 Mg₂Cl₂, 3.3 dextrose, and 0.1% (w/v) bovine serum albumin, pH 7.4, and maintained at 37°C. The cells were exposed to 400 µL of 0.1 N HCl for 10 min, scraped, and the solution centrifuged for 5 minutes at 1,500 × g. Aliquots of the supernatant were diluted in HEPES buffer and assayed for iCGRP using radioimmunoassay. The protein content was determined using a Bradford assay. The content of iCGRP in each well of cells is expressed as fmol/µg protein.

Release of iCGRP from sensory neurons in culture

After 7–12 days in culture, studies examining iCGRP release were performed on the cells as previously described (Vasko et al., 1994). Briefly, the neuronal cultures were washed twice with HEPES buffer then incubated for successive 10 min intervals with 0.4 ml of buffer. Basal or resting release was determined by exposing the cells to HEPES buffer alone. Cells were subsequently exposed to successive 10 min incubations in HEPES buffer containing NGF (100 ng/ml) and/or other drugs as indicated for each group of experiments. Drugs not soluble in water were prepared in 1-Methyl-2-pyrrolidone (MPL). The maximal concentration of MPL was 0.1% by volume. This concentration has no effect on iCGRP release from sensory neurons in culture. In all instances, the maximum concentration of drug used in experiments was included in a standard curve to assure that the drug did not influence the results of the RIA. Capsaicin (30 nM) or increasing concentrations of KCl substituted for equimolar NaCl in HEPES buffer with or without NGF or other drugs was used to evoke the release of iCGRP from the neuronal cultures. At the completion of the experiments, cells were exposed to HEPES buffer alone for a 10 min incubation to reestablish resting release. During incubations, the cells were maintained at 37°C.

At the end of each release protocol, the total content of iCGRP was determined after the last incubation by scraping the cells in 400 µl of 0.1 N HCl. The suspension was then centrifuged for 5 minutes at 1,500 × g, and the supernatant was diluted and analyzed for iCGRP content. Release values are presented as fmol of iCGRP released/well/10 min incubation for experiments involving acute drug treatments. In the case of chronic

treatments, release was presented as percent of the total content of iCGRP in the cultures released during the 10 min incubation.

Radioimmunoassay of iCGRP

The amount of iCGRP was assayed from 50–200 μ l of undiluted release buffer or from 50 μ l of a 1/8 dilution of cell lysate to determine the total content of the peptide using RIA as previously described (Vasko et al., 1994). Added to the sample aliquots was 25 μ l of a 1:70,000 dilution of CGRP antibody (a generous gift from Dr. M. Iadarola from the NIH) and a 25 μ l of 125 I-[Tyr⁰] CGRP. After incubation at 4°C for 16–20 hours, 0.5 ml of a 0.1M phosphate buffer (pH 7.4) containing 1% charcoal was added and the mixture was centrifuged at $1,500 \times g$ for 20 minutes. In this manner, the unbound radiolabeled peptide was trapped in the charcoal and the radiolabeled peptide bound to antibody remained in the supernatant. The supernatant was decanted and the radioactivity was measured using gamma scintillation spectrometry. The amount of iCGRP was determined by comparing the amount of radioactivity in unknown samples to values of a standard curve generated for each experiment.

Measurement of RasGTP

Ras activity assays were conducted with a Ras Activation Assay Kit from Upstate Biotechnology (Lake Placid, NY). Cells were maintained in the absence or presence of various concentrations of NGF for 7 days. After 7 days, the media was removed, the cells were washed with PBS, and then lysed with 0.5 ml/well of the following buffer: 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% Igepal CA-630, 50 mM MgCl₂, 5 mM EDTA, 10% glycerol, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM Na₃VO₄, and 25 mM NaF. For positive and negative controls, either 2.5 μ l of 10 mM GTP γ S or 2.5 μ l of 100 mM GDP, respectively, was added to 250 μ g of protein from control samples. The RasGTP was measured relative to the total Ras protein in each sample following kit instructions. The values for each sample were normalized to the value for the sample incubated with GTP γ S (positive control).

Cell viability assay

To determine if viral infection or treatment for 7 days with FTI-276 affects cell viability, we measured the relative numbers of metabolically active cells under various conditions using the Cell Titer 96[®] Aqueous One Solution Assay from Promega (Madison, WI). In this assay, cells were incubated for 4 hours with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). MTS is taken up by cells and bio-reduced to a colored formazan product by dehydrogenase enzymes, NADPH or NADH in metabolically active cells. The formazan product is soluble in tissue culture medium, and its quantity as measured by the absorbance at 492 nm is directly proportional to the number of living cells in culture. Absorbance was measured using a SpectraFluor Plus spectrophotometer with Magellan 5.03 software from Tecan (Crailsheim, Germany). Coated wells containing no cells were used as blanks for the spectrophotometer. As a positive control for cell death, cells were killed by 10 min exposure to 4% paraformaldehyde in PBS. Other controls included wells treated with media alone, vehicle (0.1% MPL), or viral vector.

Development of viral constructs

The dominant negative Ras, (S17N isoform) and the constitutively active Ras (CA Ras, Q61L isoform) isoforms were generous gifts of Dr. L. Quilliam (Indiana Univ. School of Medicine). We chose to over express Ras(17N), which substitutes an asparagine for a serine at position 17, because this mutant protein forms a stable complex to its guanine nucleotide exchange factor (GEF) and results in lower amounts of activated Ras in the cell (Feig and

Cooper, 1988, Quilliam et al., 1994). In the case of CA Ras, a mutation of a glutamine to leucine disrupts the GTPase activity of the protein, preventing hydrolysis of GTP and maintaining Ras in the active state (Moodie et al., 1995). Fragments containing the Ras(17N) and CA Ras sequences were amplified by PCR from the original plasmids with restriction sites, *EcoRI* and *SacII* added that were compatible with the multiple cloning site of the pIRES2-EGFP vector (BD Biosciences Clontech). The PCR primers used to add the restriction sites were: 5'-CCGGA-ATTCACCATGACGGAATATAAGCTG-3' and 5'-ATTAGGCCGCGGTCAGGAGAGCACACTT-3'. The PCR protocol was 95°C for 3 minutes, followed by 30 cycles with the following conditions: 94°C for 1 minute; 57°C for 1 minute, and then 65°C for 2.5 minutes. These cycles were followed by an elongation phase of 65°C for 10 minutes. Products were resolved on a 1% agarose gel. After digestion with *EcoRI* and *SacII* enzymes, the PCR fragment was inserted into the pIRES2-EGFP vector via ligation using T4 DNA ligase (Invitrogen Life Technologies) according to the manufacturer's instructions. The plasmids were then transformed into OneShot® Top10 *E. coli* following the manufacturer's instructions. Colonies were selected based on kanamycin resistance. Plasmid DNA was isolated from bacterial cultures grown from original kanamycin-resistant colonies using a Hurricane® Mini-prep kit (Gerard Biotech, Oxford, OH). The appropriate Ras fragment was sequenced to ensure there were no mutations generated during PCR or during insertion of constructs into plasmids. The sequencing primers were 5'-TAACAACTCCGCCCCATTGACG-3' and 5'-GACGGCAATATGGTGGAAAATA-3'. Sequencing was performed at the Biochemistry Biotechnology Facility of the IU School of Medicine, and the results were analyzed using Chromas 2.31 chromatogram viewer software (Technelysium, Australia).

Ras(17N)-IRES-EGFP or CA Ras-IRES-EGFP constructs were subcloned into an entry vector, pENTRA 1A (Invitrogen Life Technologies). The individual Ras constructs were inserted into separate pENTRA 1A vectors via ligation using T4 DNA ligase according to the manufacturer's instructions. The plasmids were then transformed into OneShot® Top10 *E. coli* per manufacturer's instructions. Colonies were selected by kanamycin resistance, and plasmid DNA was isolated from cultures using a Hurricane® Mini-prep kit (Gerard Biotech, Oxford, OH). The GatewayR LR Clonase II (Invitrogen Life Technologies) reaction was used to move the isolated Ras(17N)-IRES-EGFP or the isolated CA Ras-IRES-EGFP into the lentiviral transfer vector, pCSCGW obtained from the Vector Production Facility (Indiana University School of Medicine). The construct was transformed into STBL3 *E. coli* (Invitrogen Life Technologies) and then selection was performed using zeocin resistance and ccdB^s sensitivity selection. Plasmid was isolated using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA). An aliquot of plasmid was used for PCR to ensure the Ras insert could be amplified from the destination vector. The same primers and PCR protocol from the above amplification of Ras were used. Products were resolved on a 1% agarose gel. With confirmation that the destination vector contained the Ras insert, the remaining plasmid was used for production of lentivirus containing the CMV promoter, Ras(17N)-IRES-EGFP or CA Ras-IRES-EGFP, IRES and EGFP (Vector Production Facility, IU School of Medicine). To determine the quantity of lentivirus particles contained in solution produced by the Vector Production Facility, IU School of Medicine, the QuikTiter Lentivirus Quantitation Kit (HIV p24 ELISA) was used per manufacturer's instructions (CellBioLabs, San Diego, CA).

Lentivirus Infection

Isolated sensory neurons were incubated in F-12 media with or without added NGF for 2 days after plating. The cultures then were exposed to various lentivirus constructs diluted in media at a final concentration of 150 viral particle/cell for 48 hours. After 48 hrs, media containing virus was removed and fresh F-12 media with or without added NGF was added

to wells for an additional 7 days with the media changed every other day. In all instances, we confirmed that 90–95% of neurons expressed EGFP, as indicated by green fluorescence prior to conducting experiments.

Western Blotting

Cells were collected and lysed in the following buffer: 50 mM Trizma base, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM Na₃VO₄, and 25 mM NaF. Samples were loaded onto 4–12% gradient gels (for ERK) or 10% precast polyacrylamide gels (for Ras) and electrophoresis was performed at 75V for 1hr. Proteins were transferred onto a PVDF membrane at 100V for 1hr. Immunoblotting was performed using mouse anti-Ras antibody (1:1000 dilution; Upstate, Lake Placid, NY), rabbit pERK antibody (1:2000 dilution; Cell Signaling Technologies, Danvers, MA); rabbit total ERK antibodies (1:2000 dilution; Cell Signaling Technologies, Danvers, MA), or mouse anti-actin antibody (1:2000; Upstate). Secondary antibody was a 1:5000 dilution of HRP-conjugated goat anti-mouse antibody (Bio-Rad) or 1:2000 dilution of Anti-rabbit IgG, HRP linked antibody (Cell Signaling Technologies, Danvers, MA). To detect proteins on PVDF membranes, Western Lightning® chemiluminescent HRP substrate solution was used. Membranes were incubated with the solution for 1 minute and signal was detected on radiographic film. Densitometric analysis was performed using QuantityOne® software from Bio-Rad (Hercules, CA). The Ras signal was normalized to the signal from the loading control actin, and pERK normalized to total ERK. To measure the amount of farnesylated Ras and unfarnesylated Ras, a 15% gel was used to resolve the unfarnesylated Ras from farnesylated Ras proteins and electrophoresis was performed at 75V over 2 hours.

Statistics

All data was analyzed using Prism 3.0 by GraphPad. All graphs are presented as the mean ± the standard error of the mean (S.E.M.). Statistical significance was established using one-way ANOVA with Tukey's analysis, or two-way ANOVA with Bonferroni's analysis, as indicated. The n represents the number of wells of neurons per treatment condition from at least three separate harvests of cells. The significance level for all tests was $p < 0.05$.

Materials

Mouse nerve growth factor (7S) was purchased from Harlan (Indianapolis, IN). LY294004, wortmannin, PP2, and PP3 were from EMD Biosciences (San Diego, CA). F-12 medium, Mg²⁺- and Ca²⁺-free Dulbecco's phosphate buffered saline (PBS), Hank's Balanced Salts solution (HBSS), horse serum, L-glutamine, penicillin/streptomycin, plasmids, antibiotics, and chemically competent *E.coli* were purchased from Invitrogen (Carlsbad, CA). Normocin O™ was purchased from InvivoGen (San Diego, CA). Bovine IgG protein, Bradford reagent, pre-cast polyacrylamide gels, PVDF membranes, Laemmli buffer, HRP-conjugated goat anti-mouse and anti-rabbit antibodies were from Bio-Rad (Hercules, CA). CGRP was from Tocris (Ellisville, MO). Radiolabeled NaI (NaI¹²⁵) for iodination of CGRP and Western Lightning® chemiluminescent HRP substrate solution were from Perkin-Elmer (Shelton, CT). NeuroPorter® was from Gene Therapy Systems (San Diego, CA). Restriction enzymes were from New England Biolabs (Ipswich, MA). All other chemicals were from Sigma (St. Louis, MO). Farnesyltransferase inhibitor (FTI-276) was dissolved in 1-methyl-2-pyrrolidinone (MPL), and then diluted to the appropriate concentrations in media. LI-COR Odyssey blocking solution was from LI-COR Biosciences (Lincoln, NE). All procedures were approved by the Animal Care and Use Committee at Indiana University School of Medicine, Indianapolis, IN.

Results

Chronic exposure to NGF increases the expression of iCGRP and augments potassium-stimulated release of the neuropeptide

Previous work has shown that long-term exposure to NGF increases the content of neuropeptides in sensory neurons (Lindsay and Harmar, 1989, Donnerer et al., 1992, Noguchi et al., 1995). We confirmed these findings using adult sensory neurons grown in culture. As shown in Figure 1A, growing sensory neurons with various amounts of NGF in the culture media resulted in an increase in the content of iCGRP. The content of iCGRP in neuronal cultures grown for 7 days without the addition of NGF was 3.4 ± 0.3 fmol/ μ g protein. The content of the iCGRP in the cultures increased significantly to 6.6 ± 0.5 and 8.1 ± 0.6 fmol/ μ g protein when maintained for 7 days in the presence of 30 and 100 ng/ml NGF respectively (figure 1A). The content of iCGRP, however, was not significantly different between these two concentrations of NGF. Growing cultures in various concentrations of NGF did not affect the total amount of protein in the cultures. Protein content was 2.7 ± 0.7 , 3.0 ± 0.8 , and 3.2 ± 0.7 mg/ml for cells grown in 0, 30, or 100 ng/ml NGF, respectively.

We next determined whether increasing the content of iCGRP would augment iCGRP release evoked by high extracellular potassium. Exposure to high extracellular potassium produced a concentration-dependent increase in the fmol of iCGRP released in neurons grown in the absence of NGF or in 30 ng/ml NGF (figures 1B). The total amount of iCGRP released by 30 mM, 40 mM and 50 mM potassium was significantly higher, however, in cells grown in 30 ng/ml NGF compared to those grown in the absence of NGF. For example, exposing cultures to 50 mM KCl released 96 ± 12 fmol of iCGRP/well/10 min in cells grown without NGF, whereas in cells grown in 30 ng/ml of the neurotrophin, 50 mM KCl increased release to 256 ± 20 fmol/well/10 min (figure 1B). The basal release of iCGRP (as indicated by the use of 2.5 mM KCl) was not affected by growing cells in the absence or presence of NGF (data not shown).

To determine whether the NGF-induced increase in potassium-stimulated release of iCGRP was secondary to an increase in expression of the neuropeptide, we normalized the release data to the total content of the peptide within each well (figure 1C). Potassium-evoked release measured as percent of the total peptide content was not significantly different in cells grown without added NGF compared to those grown in 30 ng/ml NGF (figure 1C). Release evoked by exposure to 50 mM KCl was 12.7 ± 0.9 % of total content in the 10 min incubation period in cells grown without added NGF and 13.7 ± 1.2 % of total content per 10 min incubation in cells grown in 30 ng/ml NGF. These data demonstrate that the increase in the release of iCGRP by high extracellular potassium in cells grown in NGF is secondary solely to changes in the content of iCGRP.

Inhibiting Ras activation attenuates the NGF-induced increase in iCGRP content

As an initial step in determining whether activation of Ras is involved in NGF-induced increases in the expression of CGRP, levels of total Ras and RasGTP were measured by Western blotting in cultures grown in the absence or presence of NGF. Although increasing amounts of NGF in the culture media did not alter the expression of Ras in sensory neuronal cultures (figure 2A), there was a significant increase in the ratio of RasGTP to total Ras in cells grown in 30, 100 or 250 ng/ml NGF compared to those grown in the absence of added NGF or to those grown in 10 ng/ml NGF (figure 2B). These data indicate that growing cells in sufficient concentrations of NGF will activate Ras in cells in culture.

To determine whether activation of Ras mediates the NGF-induced increases in iCGRP content, a dominant negative isoform of Ras, Ras(17N), was expressed in isolated sensory neurons to inhibit Ras activity (Quilliam et al., 1994). As previously observed, when

untreated cells are grown in the presence of 30 or 100 ng/ml NGF there is a significant increase in the content of iCGRP compared to cells grown in the absence of NGF (figure 3A; open columns). Exposing cells to control virus did not alter the content of iCGRP in cells grown in the presence or absence of NGF (figure 3A; hatched columns). In contrast, infecting cells with lentivirus containing the Ras(17N) construct significantly attenuated the NGF-induced increase in iCGRP content (figure 3A; shaded columns). In cells exposed to control virus and grown in the presence of 30 ng/ml NGF the content of iCGRP was 9.1 ± 0.9 fmol/ μ g protein, whereas in cells expressing Ras(17N) the content was 5.3 ± 0.6 fmol/ μ g protein. In a similar manner, exposing neuronal cultures to virus containing the Ras(17N) construct reduced the level of iCGRP in cells grown in 100 ng/ml NGF from 7.5 ± 0.8 fmol/ μ g protein (viral control) to 4.0 ± 0.4 fmol/ μ g protein. Over expression of Ras(17N) did not alter the content of iCGRP in cells grown in the absence of added NGF. Viral infection increased Ras expression from 1 to 3 ± 0.5 when compared to control (Figure 3B). Infection of neurons with control virus or virus expressing Ras (17N) does not alter overall protein expression in neuronal cultures (data not shown). We also confirmed that the Ras(17N) was capable of inhibiting Ras activity. For these studies, cultures were grown in the absence of NGF for 7–9 days and infected with virus on days 2–4 in culture. On day 7 or day 9 they were exposed to 30 ng/ml NGF and the amount of pERK determined on day 12. Previous studies have shown that NGF increases ERK phosphorylation through activation of Ras, thus the ability of Ras(17N) to attenuate pERK in the sensory neuronal cultures grown in 30 ng/ml NGF demonstrates that the infection with dominant negative Ras suppresses Ras activity (Figure 3B).

As another way to reduce Ras activity, we inhibited farnesyltransferase, the enzyme that catalyzes the addition of a farnesyl group to Ras. Inhibiting this enzyme decreases the ability of Ras to localize to the membrane, thereby decreasing its activity (Sebti and Hamilton, 1997). Neuronal cultures were treated with the farnesyltransferase inhibitor (FTI-276; 5 μ M) or vehicle (0.01% MPL) for four days in the absence of NGF, then treated with FTI-276 or vehicle in the presence or absence of NGF for an additional 6–7 days. Cells were collected and iCGRP content was measured. When sensory neuron preparations were treated with 5 μ M FTI-276 in the presence of NGF, there was a significant reduction in the neurotrophin-induced up regulation of iCGRP (Figure 3C). In cells grown in 30 ng/ml NGF and treated with vehicle, the content of iCGRP was 6.3 ± 0.2 fmol/ μ g protein. Treating cultures with FTI-276 reduced this content to 3.8 ± 0.5 fmol/ μ g protein. In a similar manner, FTI-276 reduced the iCGRP content in cells grown in 100 ng/ml NGF from 9.9 ± 1.7 fmol/ μ g protein to 6.7 ± 1.3 fmol/ μ g protein. This treatment did not completely block the ability of NGF to increase iCGRP content, since growing cells in 100 ng/ml NGF still significantly increased peptide content over levels seen in cells grown in the absence of added NGF.

To confirm that exposing neuronal cultures to 5 μ M FTI-276 inhibited farnesyltransferase, we measured the levels of farnesylated Ras and unfarnesylated Ras in untreated cultures and those treated with the inhibitor. In cultures grown in 30 or 100 ng/ml NGF, there was a significant (~2-fold) increase in the ratio of unfarnesylated to farnesylated Ras (from 0.7 ± 0.2 to 2.7 ± 0.2 and from 1 ± 0.1 to 2.5 ± 0.7 , respectively). We also determined whether treating sensory neuronal cultures with various concentrations of FTI-276 for 9 days could affect cell viability. Using the MTS assay, the absorbance at 490 nm for cells grown in media alone was not significantly different from cells grown in 1 μ M or 10 μ M FTI-276 (Table 1). These results confirm that up to 10 μ M FTI-276 does not alter cell viability and that cell death cannot account for the reduction in iCGRP content after FTI-276.

Blocking MEK activity inhibits NGF-induced increases in iCGRP content

One important downstream pathway activated by Ras is the MEK/ERK cascade. To ascertain whether the NGF-induced increase in iCGRP content is mediated by activation of

the MEK/ERK cascade, we determined whether growing neurons in the presence of the MEK inhibitor, U0126, would alter the NGF-induced increase in iCGRP. As can be seen in figure 3D, growing sensory neurons in 30 ng/ml NGF significantly increased iCGRP content from 3.1 ± 0.5 fmol/ μ g protein to 5.6 ± 0.9 fmol/ μ g protein. Treating cells grown in 30 ng/ml NGF for 7 days with 1 μ M U0126 blocked the neurotrophin-induced increase in iCGRP content (3.2 ± 0.4 fmol/ μ g protein). In cultures grown in 100 ng/ml NGF in the absence of U0126, iCGRP content increased from a control level of 3.1 ± 0.5 to 7.0 ± 1.0 fmol/ μ g protein (figure 3D). When neurons were grown in 100 ng/ml NGF in the presence of U0126, the content of iCGRP was reduced significantly to 4.3 ± 0.6 fmol/ μ g protein (figure 3D). Treatment with U0126 did not completely block the ability of 100 ng/ml NGF to increase iCGRP content since the content was still significantly greater than that observed in cells grown in 1 μ M U0126 with no added NGF (2.1 ± 0.7 fmol/ μ g protein).

Enhanced Ras activity augments the NGF-induced increase in the content of CGRP

We next performed studies to determine if Ras activation could augment the ability of NGF to increase the content of iCGRP in sensory neuronal cultures. For these studies we used a constitutively active form of Ras (CA Ras) (Moodie et al., 1995). Cultures were grown for 2 days in media without added NGF and then exposed for 48 hours to 150 pfu of lentivirus containing the CMV promoter, CA Ras, IRES, and EGFP in the absence of NGF. Cells not infected or those exposed to a viral construct containing the CMV promoter and EGFP served as controls.

Independent of viral infection, when cells were grown in the presence of 10, 30 or 100 ng/ml NGF, there was a significant increase in the content of iCGRP compared to cells grown in the absence of NGF (figure 4; open columns). There was, however, no significant difference in the content of iCGRP in untreated cells compared to those treated with control virus (figure 4; grey columns) in cultures grown in the absence or presence of NGF. In contrast, when cells were exposed to the lentivirus containing the CA Ras construct, there was a significant augmentation in the effect of NGF compared to controls (figure 4; black columns). In cells grown in 10 ng/ml NGF, treatment with lentivirus containing the CA Ras construct significantly increased iCGRP content from 3.1 ± 0.7 fmol/ μ g protein in vector controls to 8.3 ± 2.4 fmol/ μ g protein. In cells grown in 30 ng/ml NGF, introduction of CA Ras increased iCGRP content from 5.5 ± 1.0 fmol/ μ g protein (control virus) to 10.5 ± 2.5 fmol/ μ g protein. In an analogous manner, iCGRP content in cells grown in 100 ng/ml NGF and treated with control virus was 6.0 ± 1.0 fmol/ μ g protein, whereas the content was 12.8 ± 1.4 fmol/ μ g protein in cells treated with lentivirus containing the CA Ras construct.

Viral infection significantly increased Ras expression by approximately 30% when compared to control (Figure 4B). In an additional series of experiments, we confirmed that infection with the CA Ras viral construct is functionally active. Increasing the expression of CA Ras in sensory neuronal cultures grown in the absence of NGF, results in a significant increase in pERK levels (Figure 4B). Infection with control virus or the CA Ras-containing virus does not alter the total protein levels measured in cell cultures (data not shown). Furthermore, cell viability is not affected by the expression of CA Ras (Table 1), therefore changes in levels of iCGRP are not likely secondary to changes in cell numbers.

The RAS/MEK/ERK and PI3-Kinase pathways do not mediate NGF-induced increases in iCGRP release

To determine if short term exposure of sensory neurons to NGF alters the release of iCGRP, rat sensory neurons grown in culture in the presence of 30 ng/ml NGF were washed in buffer then exposed to 100 ng/ml NGF for 10 min prior to and throughout treatment with capsaicin. As seen in figure 5A, although there was no effect of acute NGF treatment on

basal (resting) release of iCGRP (figure 5A, open columns), the release of iCGRP elicited by 30 nM capsaicin increased two-fold from 6.0 ± 1.5 % of the total content of iCGRP in the cultures to 12.3 ± 2.2 % (figure 5A, shaded columns). There was no effect of this exposure to 100 ng/ml NGF on peptide content. In the cultures shown in figure 5A, the CGRP content in cells exposed to only capsaicin was 2305 ± 193 fmol/well, whereas in cells exposed to NGF and capsaicin the content was 2030 ± 68 fmol/well. In addition, short term exposure to NGF did not augment the release of iCGRP when the cells were stimulated with 30 mM KCl (data not shown)

To determine whether activation of Ras mediates the NGF-induced increase in iCGRP release, a dominant negative isoform of Ras, Ras(17N), was expressed in isolated sensory neurons. As can be seen in figures 5B and 5C, infecting the cells in culture with control virus or virus containing the Ras(17N) construct did not attenuate the NGF-induced increase in capsaicin-evoked release of iCGRP. In cells infected with control virus, acute exposure to 100 ng/ml NGF increased release from 7.5 ± 1.4 percent of total content to 15.1 ± 1.0 percent (figure 5B). In cultures expressing Ras(17N), NGF significantly increased capsaicin-evoked release from 4.3 ± 0.6 to 7.7 ± 1.1 percent of total content (figure 5C). Although, the total amount of iCGRP released by capsaicin is reduced in cells expressing Ras(17N) compared to untreated cells and cells treated with control virus, acute exposure to 100 ng/ml NGF still increased evoked release approximately two-fold. The effect of reduced Ras activity on the capsaicin-stimulated release could be secondary to a decrease in the expression of TRPV1 in the cultures grown in NGF since previous work suggests that Ras is involved in the NGF-induced increase in TRPV1 receptor expression (Bron et al., 2003).

We also examined whether pretreating sensory neurons with the MEK inhibitor, U0126 would block the ability of acute NGF to augment iCGRP release. In this series of experiments, exposing sensory neurons to 100 nM NGF did not alter basal release but increased capsaicin-stimulated release approximately 2.5-fold. When cells were incubated with 1 μ M U0126 for 10 min prior to and throughout the NGF and capsaicin treatments, U0126 did not alter the NGF-induced augmentation of capsaicin-stimulated iCGRP release (Table 2).

The PI-3 kinase signaling cascade also can be activated by NGF and there is growing evidence that PI3-K can alter the activity of the capsaicin receptor, TRPV1 (Bonnington and McNaughton, 2003). To determine if the PI-3K pathway is involved in the acute sensitization of sensory neurons, the inhibitors wortmannin and LY294002 (Powis et al., 1994, Vlahos et al., 1994) were used in an attempt to block NGF-induced enhancement of iCGRP release. As in previous experiments, acute treatment with 100 ng/ml NGF significantly augmented capsaicin-evoked iCGRP release from isolated sensory neurons (Table 2). Pretreating the neuronal cultures with 3 μ M LY294002 or 3 nM wortmannin did not alter the basal or the capsaicin-stimulated release of iCGRP. These treatments also did not attenuate the NGF-induced augmentation of capsaicin-stimulated iCGRP release (Table 2).

The involvement of protein kinase C in NGF-induced increase in the release of iCGRP

Activation of the TrkA receptor by NGF can signal through phospholipase C (PLC) to produce IP₃ and DAG, which in turn can increase the activity of protein kinase C (PKC). To determine if activation of PKC is involved in acute NGF-induced sensitization of sensory neurons, the capsaicin-stimulated release of iCGRP was measured in the presence of NGF and the PKC inhibitor, bisindolylmaleimide I (BIM). Similar to experiments described above, sensory neurons in culture were exposed to 100 ng/ml NGF in the presence or absence of 30 nM BIM and capsaicin-evoked iCGRP release was measured. By itself, BIM had no significant effect on basal or capsaicin-stimulated release of iCGRP (figure 6).

Exposing sensory neurons to 100 ng/ml NGF increased capsaicin-evoked iCGRP release from 209 ± 25 to 320 ± 41 fmol/well/10 minutes (figure 6). Pretreating the neuronal cultures with 30 nM BIM, however, blocked the ability of NGF to augment evoked peptide release (figure 6). The capsaicin-evoked release after BIM and NGF treatment was 247 ± 27 fmol/well/10min. These results suggest that PKC is involved in the acute action of NGF to increase capsaicin-stimulated release of iCGRP from sensory neurons.

Inhibiting Src family kinases abolishes NGF-induced increases in the release of iCGRP

Recent work has shown that Src family kinases (SFKs) can alter the sensitivity of sensory neurons (Jin et al., 2004, Zhang et al., 2005) and NGF can signal through activation of Src family kinases. Thus, we examined whether inhibition of SFKs with PP2 (Hanke et al., 1996) would inhibit the NGF-induced increase in capsaicin-evoked iCGRP release from sensory neurons. Again, acute treatment of sensory neurons with 100 ng/ml NGF caused a significant increase in the capsaicin-evoked release of iCGRP from 107 ± 8 to 169 ± 6 fmol/well/10min (figure 7). Exposing neurons to 10 μ M PP2 or to 10 μ M of the inactive PP2 analog, PP3, did not significantly alter the basal or capsaicin-stimulated release of iCGRP in neurons grown in the absence of added NGF (figure 7). Pretreating the sensory neurons with PP2 abolished the NGF-mediated increase in capsaicin-stimulated release of iCGRP (109 ± 7 fmol/well/10min in the presence of PP2 and NGF compared to 107 ± 8 fmol/well/10min in the presence of PP2 alone), whereas PP3 did not significantly alter the NGF-induced sensitization of sensory neurons. When cells were treated with NGF in the presence of either PP2 or PP3, there was a small but significant increase in the resting release of iCGRP (figure 7, open columns). These observations suggest an important role for SFKs in the NGF-induced sensitization of sensory neurons.

Discussion

The main findings in this manuscript are three-fold. First, although it has been demonstrated previously that the activity of the promoter for CGRP and CGRP mRNA are increased with NGF treatment in a MEK-dependent manner, our experiments are the first to establish that activation of Ras is a necessary upstream component of the MEK activation involved in NGF-mediated increases in CGRP expression. Second, we demonstrate that Src family kinases and PKC are signaling cascades which mediate the acute NGF-initiated sensitization of sensory neurons, as measured by stimulus-evoked release of CGRP. Third, and most importantly, we are the first to demonstrate that the signaling cascades by which NGF acutely enhances stimulus-evoked release of CGRP are different and distinct from those by which NGF increases CGRP expression. By demonstrating the Ras-dependence of the increases in CGRP expression and the contrasting independence of enhanced stimulus-evoked release of CGRP from changes in Ras, MEK or PI3K pathways, we are able to show a shift in NGF signaling from acute to chronic exposures.

Our results show that the ability of chronic NGF to increase the content of CGRP, thereby augmenting the total outflow of the peptide in response to stimulation with high extracellular potassium, was mediated largely through activation of Ras. This conclusion is based on the use of novel genetic methods and more established pharmacological methods to manipulate the Ras signaling cascade in sensory neurons. Growing sensory neurons with concentrations of NGF that increased iCGRP content also increased the levels of RasGTP without altering the expression of Ras. Inhibiting Ras activity by over expressing a dominant negative Ras or by inhibiting farnesyltransferase with FTI-276 significantly attenuated the ability of long term exposure to NGF to increase iCGRP content. Over expression of a constitutively active Ras isoform, RasQ61L, caused a significant augmentation in the ability of NGF to increase peptide content compared to untreated cells or those treated with vector alone. Constitutively active Ras, in the absence of NGF, did not increase the content of CGRP suggesting that the

full consequences of this mutation on iCGRP content may require initial upstream activation of the Ras pathway by NGF. In effect, CA Ras may serve to prime the neuron to respond to NGF more strongly.

Although treatments that inhibit Ras activity significantly attenuated the NGF-induced increases in iCGRP content in sensory neuronal cultures, they did not completely abolish the effect of the neurotrophin. There are two possible explanations to account for the lack of complete inhibition by over expressing Ras(17N) or treatment with FTI-276. First, the activity of Ras may not be completely blocked with these treatments. After FTI-276 treatment, there remained a significant amount of farnesylated Ras suggesting that our treatment only partially inhibited farnesyltransferase. Higher concentrations (30 μ M) of FTI-276 reduced cell viability and thus could not be used. It also is possible that there are other signaling pathways in sensory neurons involved in regulating CGRP content. In PC12 cells, phospholipase C γ is essential for NGF activation of the Raf/MEK/MAPK pathway upstream of Ras/Rap1 activation (Rong et al., 2003). Therefore, Rap1 may also be important in NGF's effects on sensory neurons and further studies are warranted to examine the role of this small G-protein.

Our results are the first to provide conclusive evidence that activation of Ras is a critical component for the NGF-induced increase in expression of CGRP and this is likely secondary to an increase by the neurotrophin in the promoter activity of the CGRP gene, an effect that is blocked by MEK inhibitors (Freeland et al., 2000, Durham and Russo, 2003). Using the MEK inhibitor, U0126 at a concentration that is selective (Favata et al., 1998), we demonstrated that this drug treatment completely blocks the increase in peptide content in cells grown in 30 ng/ml NGF, while significantly attenuating the increase in content in cells grown in 100 ng/ml NGF. Previous work also has shown that chronic NGF increases CGRP mRNA in sensory neurons and this effect also is blocked by MEK inhibition (Xu and Hall, 2007). Because changes in mRNA expression do not always reflect actual changes in protein expression (Gygi et al., 1999, Tian et al., 2004), our current data, measuring CGRP content clearly establish that the RAS/MEK/ERK pathway plays a critical role in the ability of NGF to increase expression of the peptide itself.

Another important finding of the current work is that acute exposure of sensory neurons in culture to NGF augments the capsaicin-evoked release of iCGRP. To our knowledge, this is the first demonstration of an acute effect of NGF augmenting the release of neuropeptides from peripheral sensory neurons. Previous work has shown that chronic administration of NGF causes an increase in evoked peptide release from dorsal roots of the dorsal spinal cord and from isolated biopsies of hindpaw skin, but whether the increase was secondary to an increase in content (as we observe with potassium stimulated release) was not determined (Malcangio et al., 1997, Bowles et al., 2006). Acute administration of NGF had little effect on peptide release, even though the same acute treatment with NGF elicits a large behavioral response (Lewin et al., 1993, Andreev et al., 1995, Malcangio et al., 1997).

Although chronic NGF increases peptide content in sensory neurons through activation of Ras, this signaling pathway does not mediate the ability of acute NGF exposure to augment peptide release. Inhibition of Ras activity by over expression of a dominant negative Ras or inhibition of MEK activity does not block the ability of acute NGF to augment capsaicin-induced iCGRP release. The negative results in these studies confirm some studies in the literature which suggest that the Ras/MEK/ERK pathway does not mediate sensitization of sensory neurons by acute exposure to NGF and conflict with those which suggest that this pathway is necessary. For example, inhibition of MEK attenuates the ability of acute NGF to prevent the desensitization of the capsaicin-induced inward current (Zhu and Oxford, 2007) and the NGF-induced increase in TRPV1 localization in the cell membrane, increased ERK

phosphorylation and the capsaicin-induced increase in cobalt uptake in sensory neurons (Bron et al., 2003). These latter effects were also blocked by a dominant negative Ras. In another study, inhibition of MEK did not alter the ability of NGF to prevent capsaicin-induced desensitization (Shu and Mendell, 2001) or prevent NGF-induced augmentation of the capsaicin-induced entry of calcium into sensory neurons (Bonnington and McNaughton, 2003). There are two possible explanations for the differences in various experiments. First, it seems likely that different signaling cascades mediate different endpoints of sensitization. Since release represents an integrated response in neurons, the NGF-induced signaling pathways mediating release are likely different from signaling that controls the opening of the TRPV1 channel. It also is likely that differences can occur with different culture conditions. Sensory neurons used in the studies by Bron et. al. (2003) were grown for 4–5 days in serum free media or were serum starved for at least 24 hours prior to their experiments. In contrast, neurons in the current studies were grown for 7 days in 30 ng/ml NGF in media with serum. The question remains which conditions better mimic what likely occurs in neurons *in situ*.

Inhibitors of the PI3 kinase pathway also did not affect the NGF-induced increase in capsaicin-evoked iCGRP release (table 2). For these studies we used concentrations of inhibitors that were near the IC₅₀ of the drugs (Powis et al., 1994, Vlahos et al., 1994) and did not alter the resting release of iCGRP when given alone. Our results contrast with the findings of others that brief exposure to NGF increases the phosphorylation of Akt, an indicator of activation of PI3 kinases and that PI3 kinases inhibitors block the ability of acute NGF to prevent desensitization or to increase TRPV1-mediated calcium entry (Bonnington and McNaughton, 2003, Zhu and Oxford, 2007). Again, it is possible that the differences between our results and these studies are the endpoints measured or the cell preparation used. For example, in the study by Bonnington and McNaughton (2003) cells were derived from neonatal mice rather than adult rats. Signaling in neurons from neonatal mice may not be identical to neurons from adult rats. It is known that signaling in response to NGF undergoes a developmental switch between postnatal days 4–10 from the neurotrophin being required for survival to it being an inflammatory mediator (Zhu et al., 2004).

We found that the Src family kinases inhibitor, PP2 (10 μ M), completely blocked the NGF-induced increase in capsaicin-evoked iCGRP release, whereas the inhibitor had no effect on capsaicin-evoked release in the absence of NGF. In contrast, PP3, the inactive analogue to PP2, did not affect the NGF-induced increase in capsaicin-evoked release and did not alter basal release. These results suggest that Src family kinases mediate the NGF-induced augmentation of transmitter release from sensory neurons and this conclusion is supported by the observation that PP2 decreased capsaicin-evoked currents in dorsal root ganglion neurons, and that TRPV1 and Src kinase co-precipitated in lysates from a heterologous expression system (Jin et al., 2004). NGF-induced sensitization of iCGRP release also was inhibited by the PKC inhibitor, BIM. The mechanism by which PKC controls NGF-induced sensitivity in sensory neurons is not known, but PKC can phosphorylate and sensitize the TRPV1 receptor. In light of the data showing Src family kinases as regulators of sensitivity, it may be that PKC is downstream from Src family kinases. It has been demonstrated in cell lines that Src can phosphorylate and activate PKC δ (Joseloff et al., 2002), a novel PKC isoform which is inhibited by BIM.

Our findings demonstrate that NGF enhances the capsaicin evoked outflow of CGRP from sensory neurons acutely through augmentation of release and increases potassium evoked release after chronic exposure by increasing the expression of the neuropeptide. The question remains whether the concentrations of NGF used in the present study are similar to those obtained under physiological or pathological conditions. Previous studies by others

have shown that intraplantar injection of complete Freund's adjuvant or of capsaicin induced increases of NGF in the paw from levels of ~7 ng/hindpaw in uninjured tissues to 12 ng/hindpaw or 25 ng/hindpaw for CFA or capsaicin treated, respectively (Safieh-Garabedian et al., 1995, Woolf et al., 1997, Saade et al., 2002). We have measured the volume of an inflamed hindpaw and found it to be ~0.5 ml. Thus, the approximate tissue levels of NGF after inflammation range from ~24–50 ng/ml.

It is interesting to speculate that the mechanism mediating the sensitizing action of acute NGF is not through regulating voltage gated calcium channels since acute NGF does not augment potassium-stimulated peptide release. Rather the effect of NGF appears to be via activation of TRPV1. This observation is consistent with a role of NGF as an inflammatory mediator in the periphery regulating TRPV1 rather than a mediator in the spinal cord where release is secondary to invasion of action potentials. The dichotomy of NGF effects presented in this paper also may contribute to clarifying mechanisms by which this neurotrophin induces hyperalgesia. Lewin et al. (1993) demonstrated that administration of NGF produces thermal and mechanical hyperalgesia. The thermal hyperalgesia has its onset within 1 h after NGF injection, while mechanical hyperalgesia typically has a latency of several hours, suggesting a more complex mechanism, probably involving central processes (Shu and Mendell, 2001). The changes in sensitization of sensory neurons, *i.e.*, increased release of CGRP, may account for the rapid onset NGF-induced hyperalgesia through direct stimulation of the nociceptive terminals. The increase in CGRP content could result in an increase in CGRP outflow in the spinal cord, contributing to the establishment of NGF-induced mechanical hyperalgesia. In this context, it is important to note that long-term exposure to NGF also has been shown to increase the content of SP in sensory neurons (Woolf et al., 1994, Leslie et al., 1995), but chronic NGF does not appear to alter glutamate release in sensory neurons (Rydh-Rinder et al., 2001). Since both SP and CGRP augment NMDA-induced excitability of dorsal horn neurons (Dougherty and Willis, 1991, Rusin et al., 1992, Ebersberger et al., 2000), the ability of NGF to increase peptide release from sensory neurons can increase excitability at central synapse of the sensory neurons. Thus, knowing the signaling pathways that mediate the different actions of NGF is critical to developing new ways to regulate the acute and chronic components of inflammatory pain.

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Abbreviations

NGF	nerve growth factor
CGRP	calcitonin gene-related peptide
iCGRP	calcitonin gene-release peptide like immunoreactivity
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
BIM	bisindolylmaleimide
PP2	4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine

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Figure 1.

Nerve growth factor (NGF) increases expression and release of iCGRP from sensory neurons in culture. A. Cell lysates were obtained after growing neurons for 7 days in culture in the absence of added NGF (open column) or presence of 30 or 100 ng/ml NGF as indicated (shaded columns). Each column is the mean $\pm$ S.E.M. of iCGRP in fmol/ μ g protein. An asterisk indicates a significant difference in iCGRP levels compared to cells grown in the absence of added NGF ($p < 0.05$, $n = 12$, using one-way ANOVA with Tukey's post-hoc test). B. Each column represents the mean $\pm$ S.E.M. of iCGRP in fmol/well/10 min in cells grown in the absence of added NGF (open columns) or in the presence of 30 ng/ml NGF (shaded columns). An asterisk indicates a significant increase in potassium stimulated release compared to release in the presence of 2.5 mM KCl, whereas a cross indicates a significant increase in release compared to cells grown in the absence of added NGF ($p < 0.05$, $n = 9$, using two-way ANOVA with Bonferroni's post-hoc test). C. Each column represents the mean $\pm$ S.E.M. of iCGRP as % of total content for 10 min incubation in cells grown in the absence of added NGF (open columns) or in the presence of 30 ng/ml NGF (shaded columns). An asterisk indicates a significant increase in potassium stimulated release compared to release in the presence of 2.5 mM KCl ($p < 0.05$, $n = 11$, using two-way ANOVA with Bonferroni's post-hoc test).

Figure 2.

Chronic exposure to NGF enhances Ras activity in sensory neuronal cultures without increasing total Ras protein levels. A. Sensory neurons were grown in the indicated amount of NGF for 7 days, then collected, lysed and blotted for total Ras protein and actin. A representative western blot is shown in the top panel, whereas the bottom panel shows the mean $\pm$ S.E.M. density of the Ras band normalized to actin from 3 separate harvests. B. Each column represents the mean $\pm$ S.E.M. of the ratio of RasGTP to total Ras normalized to the amount of RasGTP generated by incubating lysates with GTP γ S (hatched column, see Methods). Cells are grown in the absence of added NGF (open column) or the presence of various concentrations of NGF as indicated (shaded columns). Lysate from cells was also exposed to GDP, which served as a negative control (lightly shaded column). An asterisk indicates a significant difference in the ratio of RasGTP to total Ras compared to cells grown in the absence of NGF ($p < 0.05$, $n = 3$ from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

Figure 3.

Inhibition of Ras or MEK attenuates NGF-induced increases in iCGRP content in sensory neurons in culture. A. Each column represents the mean $\pm$ S.E.M. of iCGRP content in fmol/ μ g protein for untreated cells (media: open columns), cells treated with lentivirus containing the CMV promoter and the EGFP construct (control virus: hatched columns), or cells treated with lentivirus containing the CMV promoter, the dominant negative Ras (17N) construct, IRES, and the EGFP construct (shaded columns). Cells are harvested, grown for 48 hrs, then exposed to virus for 48 hours. After viral exposure, cells were grown in the absence or presence of NGF for 7 days as indicated. An asterisk indicates a significant increase in iCGRP content in neurons grown in the presence of added NGF compared to those grown without added NGF, whereas a cross indicates a significant difference in iCGRP expression between cells receiving control virus and those receiving virus containing the Ras(17N) construct ($p < 0.05$, $n = 9$, from 3 separate harvests using two-way ANOVA with Bonferroni's post-hoc test). B. The top panels show representative Western blots of Ras and actin expression (left) or pERK and total ERK (right) in untreated cells, in cells infected with the lentivirus construct containing CMV-EGFP and a construct containing CMV-Ras (17N)-IRES-EGFP as indicated. The lower panels show the mean $\pm$ S.E.M. expression of Ras measured as the ratio of the density of the Ras bands to the actin bands and normalized to the control (left) or the ratio of pERK to total ERK (right). An asterisk indicates a significant change in Ras expression or pERK compared to control using one-way ANOVA with Tukey's post-hoc test. C. Each column represents the mean $\pm$ S.E.M. of iCGRP content in fmol/ μ g protein for cells treated with vehicle (open columns) or cells treated with 5 μ M FTI-276 (shaded columns) in the presence or absence of increasing concentrations of NGF as indicated. An asterisk indicates a significant increase in iCGRP content in neurons grown in the presence of added NGF compared to those grown without added NGF, whereas a cross indicates a significant difference in iCGRP expression between cells receiving vehicle and those receiving FTI-276 ($p < 0.05$, $n = 12$, from 3 separate harvests using two-way ANOVA with Bonferroni's post-hoc test). D. Each column represents the mean $\pm$ S.E.M. of iCGRP content in fmol/ μ g protein for cells treated with vehicle (open columns) or cells

treated with 1 μ M U0126 (shaded columns) and grown in the presence or absence of NGF for 7 days as indicated. An asterisk indicates a significant increase in iCGRP content in neurons grown in the presence of added NGF compared to those grown without added NGF, whereas a cross indicates a significant difference in iCGRP expression between cells receiving vehicle and those receiving U0126 ($p < 0.05$, $n = 18$, from 3 separate harvests using two-way ANOVA with Bonferroni's post-hoc test).

Figure 4.

Treatment with constitutively active Ras augments NGF-induced increases in the expression of iCGRP in sensory neurons in culture. A. Each column represents the mean $\pm$ S.E.M. of iCGRP content in fmol/ μ g of protein for cells not exposed to virus (no infection; open columns), cells treated with lentivirus containing the CMV promoter and the EGFP construct (control virus; grey columns), or cells treated with lentivirus containing the CMV promoter, a constitutively active Ras construct, IRES, and the EGFP construct (CA Ras; black columns). Cells are harvested, grown for 48 hrs, then exposed to virus for 48 hours. After viral exposure, cells were grown in the absence or presence of NGF for 7 days as indicated. An asterisk indicates a significant increase in iCGRP content in neurons grown in the presence of added NGF compared to those grown without added NGF. A cross indicates a significant difference in iCGRP expression between cells receiving control virus and those receiving virus containing the CA Ras construct ($p < 0.05$, $n = 4$, from 4 separate harvests using two-way ANOVA with Bonferroni's post-hoc test). B. The top panels show representative Western blots of Ras and actin expression (left) or pERK and total ERK (right) in untreated cells, in cells infected with the lentivirus construct containing CMV-EGFP and a construct containing CMV-CA-Ras-IRES-EGFP as indicated. The lower panels show the mean $\pm$ S.E.M. expression of Ras measured as the ratio of the density of the Ras bands to the actin bands and normalized to the control (left) or the ratio of pERK to total ERK (right). An asterisk indicates a significant change in Ras expression or pERK compared to control using one-way ANOVA with Tukey's post-hoc test.

Figure 5.

Treatment with a dominant negative Ras, Ras(17N), does not alter NGF-induced sensitization of sensory neurons. Each column represents the mean $\pm$ S.E.M. of iCGRP release as percent of total content of iCGRP in cultures for cells not exposed to virus (A), cells treated with lentivirus containing the CMV promoter and the EGFP construct (B), or cells treated with lentivirus containing the CMV promoter, the Ras(17N) construct, IRES, and the EGFP construct (C). Cultures were exposed for 10 min to HEPES alone (basal), or HEPES in the presence of 30 nM capsaicin (Cap) in the absence or presence of 100 ng/ml NGF as indicated. An asterisk indicates a significant increase in iCGRP release in neurons exposed to NGF compared to those without added NGF, whereas a cross indicates a significant difference in iCGRP release between cells receiving control virus and those receiving virus containing the Ras(17N) construct ($p < 0.05$, $n = 9$, from 3 separate harvests using two-way ANOVA with Bonferroni's post-hoc test).

Figure 6.

Inhibition of protein kinase C blocks NGF-induced sensitization of sensory neurons. Isolated sensory neurons in culture were incubated for 10 min with either HEPES alone; HEPES containing NGF (100 ng/ml) or BIM (30 nM; or a combination of NGF and BIM, as indicated; open columns). Cells were then exposed for 10 min to 30 nM capsaicin in the presence or absence of NGF and BIM, as indicated (shaded columns). Each column represents the mean $\pm$ S.E.M. of iCGRP release in fmol/well of cells/10min. An asterisk indicates a significant difference in stimulated release of iCGRP compared to neurons treated with capsaicin alone ($p < 0.05$, $n = 9$ wells from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

Figure 7.

Inhibition of Src family kinases abolishes NGF-induced sensitization of sensory neurons. Isolated sensory neurons in culture were incubated for 10 min with either HEPES alone; HEPES containing NGF (100 ng/ml), PP2 (10 μM), or PP3 (10 μM); or a combination of NGF with the inhibitors as indicated (open columns). Cells were then exposed for 10 min to 30 nM capsaicin in the presence or absence of NGF, PP2, or PP3, as indicated (shaded columns). Each column represents the mean $\pm$ S.E.M. of iCGRP release in fmol/well of cells/10min. An asterisk indicates a significant difference in stimulated release of iCGRP compared to neurons treated with capsaicin alone and an (#) indicates a significant increase in resting release of iCGRP compared to resting release in the presence of HEPES alone ($p < 0.05$, $n = 9$ wells from 3 separate harvests, using one-way ANOVA with Tukey's post-hoc test).

Table 1

Viability of cells in sensory neuronal cultures after chronic changes in RAS-activity

Treatment	n	Absorbance at 492 nm (mean $\pm$ S. E. M. normalized to the control)
No virus	12	1.0
Vector control virus	12	0.84 $\pm$ 0.11
CA RAS virus	12	1.33 $\pm$ 0.15
Vehicle	16	1.0
1 μ M FTI	16	1.08 $\pm$ 0.19
10 μ M FTI	16	1.09 $\pm$ 0.18
30 μ M FTI	16	0.8 $\pm$ 0.11

Statistical significance was established using one-way ANOVA with Tukey's analysis.

Table 2

Inhibition of MEK or PI3 kinase does not alter the NGF-induced increase in capsaicin-evoked release of iCGRP from sensory neurons.

Treatment	n	iCGRP release (fmol/well/10 min)	
		Basal	Capsaicin-stimulated
Control	9	20 ± 7	144 ± 26
NGF (100 nM)	9	21 ± 7	374 ± 61*
NGF (100 nM) + U0126 (1mM)	9	14 ± 2	348 ± 57*
Control	9	11 ± 2	185 ± 39
LY 294002 (3 mM)	9	11 ± 2	202 ± 39
Wortmannin (3 nM)	9	13 ± 3	155 ± 23
NGF (100 nM)	9	21 ± 4	391 ± 50*
NGF (100 nM) + LY294002 (3 mM)	9	23 ± 4	428 ± 79*
NGF (100 nm) + Wortmannin (3 nM)	9	23 ± 5	370 ± 40*

Statistical significance was established using one-way ANOVA with Tukey's analysis. An asterisk indicates a significant increase in capsaicin-stimulated release compared to control.