Rescue of α-SNS Sodium Channel Expression in Small Dorsal Root Ganglion Neurons After Axotomy by Nerve Growth Factor In Vivo

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Dib-Hajj, S. D., J. A. Black, T. R. Cummins, A. M. Kenney, J. D. Kocsis, and S. G. Waxman. Rescue of α-SNS sodium channel expression in small dorsal root ganglion neurons after axotomy by nerve growth factor in vivo. J. Neurophysiol. 79: 2668–2676, 1998. Small (18–25 μm diam) dorsal root ganglion (DRG) neurons are known to express high levels of tetrodotoxin-resistant (TTX-R) sodium current and the mRNA for the α-SNS sodium channel, which encodes a TTX-R channel when expressed in oocytes. These neurons also preferentially express the high affinity receptor for nerve growth factor (NGF), TrkA. Levels of TTX-R sodium current and of α-SNS mRNA are reduced in these cells after axotomy. To determine whether NGF participates in the regulation of TTX-R current and α-SNS mRNA in small DRG neurons in vivo, we axotomized small lumbar DRG neurons by sciatic nerve transection and administered NGF or Ringer solution to the proximal nerve stump using osmotic pumps. Ten to 12 days after pump implant, whole cell patch-clamp recording demonstrated that TTX-R current density was decreased in Ringer-treated axotomized neurons (154 ± 45 pA/pF; mean ± SE) compared with nonaxotomized control neurons (865 ± 123 pA/pF) and was restored partially toward control levels in NGF-treated axotomized neurons (465 ± 78 pA/pF). The \( V_{1/2} \) for steady-state activation and inactivation of TTX-R currents were similar in control, Ringer- and NGF-treated axotomized neurons. Reverse transcription polymerase chain reaction revealed an upregulation of α-SNS mRNA levels in NGF-treated compared with Ringer-treated axotomized DRG. In situ hybridization showed that α-SNS mRNA levels were decreased significantly in small Ringer-treated axotomized DRG neurons in vivo and also in small DRG neurons that were dissociated and maintained in vitro, so as to correspond to the patch-clamp conditions. NGF-treated axotomized neurons had a significant increase in α-SNS mRNA expression, compared with Ringer-treated axotomized cells. These results show that the administration of exogenous NGF in vivo, to the proximal nerve stump of the transected sciatic nerve, results in an upregulation of TTX-R sodium current and of α-SNS mRNA levels in small DRG neurons. Retogradely transported NGF thus appears to participate in the control of excitability in these cells via actions that include the regulation of sodium channel gene expression in vivo.

INTRODUCTION

Dorsal root ganglion (DRG) neurons, with axons that project from the periphery to the spinal cord, exhibit a variety of electrophysiological phenotypes related to their function. Two broad classes of sodium currents, tetrodotoxin-sensitive (TTX-S) and TTX-resistant (TTX-R) (Caffrey et al. 1993; Elliott and Elliott 1993; Kostyuk et al. 1981; Roy and Narahashi 1992), have been recorded in DRG neurons, and these cells express the mRNAs for at least six sodium channel α-subunits (Black et al. 1996). Unmyelinated C-type and myelinated cutaneous afferent DRG neurons produce relatively high levels of TTX-R sodium current (Cummings and Waxman 1997; Honmou et al. 1994; Rizzo et al. 1994). The expression of TTX-R sodium currents in C-type DRG neurons, in particular, is associated with high levels of mRNA for the α-SNS sodium channel (Black et al. 1996; Dib-Hajj et al. 1996), which is expressed preferentially in these cells and encodes a TTX-R sodium current when expressed in oocytes (Akopian et al. 1996; Sangameswaran et al. 1996). After axotomy, the density of TTX-R sodium current decreases in myelinated cutaneous afferent (Rizzo et al. 1995) and C-type DRG neurons (Cummings and Waxman 1997) and, concomitantly, levels of α-SNS mRNA are reduced in these cells (Dib-Hajj et al. 1996).

The regulatory mechanisms that trigger and maintain the expression of the multiple sodium channel isoforms that are present in DRG neurons (Black et al. 1996) are not fully understood. Nevertheless, there is evidence indicating that nerve growth factor (NGF) may be involved in this process, at least for several of the isoforms expressed in these cells. The electrophysiological phenotype of nociceptive and cutaneous afferent DRG neurons is regulated by NGF during development (Ritter and Mendell 1992; Ritter et al. 1991). NGF induces increases in sodium channel expression in several types of cells (D’Arcangelo et al. 1993; Fanger et al. 1995; Kalman et al. 1990; Mandel et al. 1988; Toledo-Aral et al. 1995), including increases in the expression of TTX-R sodium current in PC-12 cells (Rudy et al. 1987) and in DRG neurons (Aguayo and White 1992). NGF also has been shown to upregulate the expression of mRNA for sodium channel α-subunits II (Mandel et al. 1988) and PN1 (D’Arcangelo et al. 1993; Toledo-Aral et al. 1995) in PC-12 cells and α-subunit II in embryonic DRG neurons (Zur et al. 1995).

The decrease in TTX-R sodium current expression that occurs after axotomy in myelinated cutaneous afferent DRG neurons (Rizzo et al. 1995) is reversed partially by administration of NGF to the injured nerve stump in vivo (Oyelese et al. 1997). However, the effect of NGF on sodium channel gene expression in these cells has not been studied, and the contribution of changes in α-SNS mRNA expression to the effects of NGF on sodium currents in DRG neurons is not known. A similar decrease in TTX-R sodium current density occurs after axotomy in C-type DRG neurons (Cummings and Waxman 1997) and is associated with a decrease in α-
SNS mRNA levels in these cells (Dib-Hajj et al. 1996). Thus C-type DRG neurons present a cell type in which it is possible to study the expression of both a particular class of sodium current (TTX-R) and a mRNA that encodes a TTX-R sodium channel. Small DRG neurons are known to preferentially express the high affinity receptor for NGF, TrkA (Kashiba et al. 1995; McMahon et al. 1994). It has been shown that NGF, delivered directly to cell bodies, acts to maintain high levels of α-SNS mRNA expression in C-type DRG neurons in an in vitro model that mimics axotomy (Black et al. 1997). However, the effects of NGF on axotomized C-type DRG neurons have not been studied previously in vivo, and the effects of peripheral delivery of NGF to the transected nerve stump have not been examined. In the present study, small DRG neurons were axotomized within the sciatic nerve of adult rats, and exogenous NGF was administered to the proximal peripheral nerve stump using osmotic pumps. Patch-clamp recording examined the expression of TTX-R sodium current, and reverse transcription polymerase chain reaction (RT-PCR) and in situ hybridization were used to measure the expression of α-SNS mRNA in these cells. The results demonstrate that exogenously administered NGF, delivered to the transected nerve, mitigates the effects of axotomy on C-type DRG neurons, maintaining high levels of TTX-R sodium current and α-SNS mRNA in these cells.

METHODS

Surgery

Mouse 2.5 S NGF (Upstate Biotechnology) was diluted in filtered Ringer solution immediately before use in Alzet osmotic pumps (Alza Scientific Products). Pumps designed for 14-day use were filled with NGF for a delivery rate of 125 ng/h; this rate falls within the range of exogenous NGF administration that has been shown to be effective when applied to the axotomized adult rat sciatic nerve in vivo (Fitzgerald et al. 1985; Munson et al. 1997; Verge et al. 1995; Wong and Oblinger 1991). Experiments (Oyalese et al. 1997) in which pumps filled with 4% Fluorogold were implanted and the L4/L5 DRG then were removed, sectioned, and examined by fluorescent microscopy resulted in high levels of staining of DRG neurons but not of satellite cells, making it unlikely that delivery of NGF from the pump site to the DRG was limited to passive diffusion. For comparison with NGF, additional pumps were filled with sterile Ringer solution. A polyethylene catheter connected to a silicone cuff was attached to each pump, and the units were allowed to equilibrate 5 h to overnight at 37° C in sterile Ringer solution. Adult Sprague-Dawley female rats were anesthetized with ketamine/ xylazine (40/2.5 mg/kg ip), and the NGF pump with catheter and cuff was implanted in a subcutaneous pocket in the abdominal region on the right side. The Ringer pump was implanted similarly in the left side. For axotomy and connection with the pump, the sciatic nerve was exposed, ligated with 4.0 silk suture, and transected at the level of the pyriform tendon. The ligated nerve stump was drawn into the silicon cuff and sutured in place. Ten to 12 days after pump implantation, animals were anesthetized with ketamine/xylazine and the animals were exsanguinated and L4/L5 DRG excised and processed for cell culture (Black et al. 1996) for patch-clamp recording or in situ hybridization, exsanguinated and L4/L5 DRG rapidly dissected and placed on dry ice for extraction of RNA for RT-PCR (Dib-Hajj et al. 1996), or intracardially perfused, first with phosphate-buffered saline and then with 4% paraformaldehyde in 0.14 M Sorenson’s phosphate buffer, and the L4/L5 DRGs removed and processed for in situ hybridization cytochemistry (Black et al. 1996; Waxman et al. 1994). In all cases, the pumps were examined to verify that they remained intact and connected to the sciatic nerve stump for the duration of implantation.

The experimental procedures described here were approved by the institutional animal use and care committee and meet all guidelines for the humane treatment of animals as approved by the Association for Accreditation of Laboratory Animal Care.

Cell culture

 Cultures of DRG neurons from adult rats were established as described previously (Rizzo et al. 1994). Briefly, lumbar ganglia (L4, L5) from control (no pump implant or axotomy) and NGF- and Ringer-pump–implanted adult Sprague Dawley female rats were freed from their connective sheaths and incubated sequentially in enzyme solutions containing collagenase and then papain. The tissue was triturated in culture medium containing 1:1 Dulbecco’s modified Eagle’s medium and Hank’s F12 medium and 10% fetal calf serum, 1.5 mg/ml trypsin inhibitor, 1.5 mg/ml bovine serum albumin, 100 U/ml penicillin, and 0.1 mg/ml streptomycin and plated at a density of 500–1,000 cells/mm² on polyornithine/ laminin coated coverslips. The cells were maintained at 37°C in a humidified 95% air-5% CO2 incubator overnight and used for electrophysiological recording or in situ hybridization within 24 h of plating.

Electrophysiology

Whole cell patch-clamp recordings were conducted at room temperature (21°C) using an EPC-9 amplifier. Data were acquired on a Macintosh Quadra 950 using the Pulse program (v 7.52, HEKA Electronic, Germany). Fire-polished electrodes (0.8–1.5 MΩ) were fabricated from 1.65-mm capillary glass (WPI) using a Sutter Science pipette puller. Fire-polished electrodes (0.8 ± 1.5 MΩ) were used for recording or in situ hybridization within 24 h to measure the expression of α-SNS mRNA in these cells. The liquid junction potential for these solutions was adjusted to 310 mosM (Wescor 5550 osmometer). Membrane currents were also corrected to account for this offset. The osmolarity of all patch clamp electrophysiology solutions was measured by EPC-9 and data were not used if resistance changes of >20% occurred. The average access resistance was 2.1 ± 0.6 MΩ (mean ± standard deviation, n = 137). Voltage errors were minimized using 80% series resistance compensation, and the capacitance artifact was canceled using the computer-controlled circuitry of the patch clamp amplifier. Linear leak subtraction, based on resistance estimates from four to five hyperpolarizing pulses applied before the depolarizing test potential, was used for all voltage-clamp recordings. Membrane currents were filtered at 5 kHz and sampled at 20 kHz. The pipette solution contained (in mM) 140 CsF, 2 MgCl2, 1 ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, and 10 Na-A2- hydroxyethylpi- perazine-N’-2-ethanesulfonic acid (HEPES) (pH 7.3). The standard bathing solution contained (in mM) 140 NaCl, 3 KCl, 2 MgCl2, 1 CaCl2, 0.1 CaCl2, 10 HEPES, and 10 glucose (pH 7.3). The liquid junction potential for these solutions was <5 mV; data were not corrected to account for this offset. The osmolarity of all solutions was adjusted to 310 mosM (Wescor 5550 osmometer). The offset potential was zeroed before patching the cells and checked after each recording for drift: if the drift was >5 mV/h, the recording was not used.

RT-PCR

For RT-PCR, four animals were killed at 10–12 days after implantation of NGF and Ringer pumps, and the L4/L5 DRGs from the respective sides were pooled. Total cellular RNA was isolated by the single step guanidinium isothiocyanate–acid phenol procedure (Chomczynski and Sacchi 1987). Extraction buffer was used at 25 μl per 1 mg of tissue. The quality of the RNA was assessed by electrophoresis in a 1.2% agarose gel. First strand cDNA was reverse transcribed in a 25 μl final volume using 1 μM random primer and the SuperScript II reverse transcriptase (Invitrogen, San Diego, CA) with 10 μM dNTPs. PCR was performed with 2× PCR Master Mix (Qiagen) containing 0.2 μM primers for β-actin and 1 μM α-SNS primers. The conditions were initial denaturation at 95°C for 8 min, followed by 35 cycles of 1 min at 95°C, 1 min at 58°C, and 2 min at 72°C, and a final extension at 72°C for 5 min. The primer sequences for α-SNS and β-actin were 5′-GCCGACACCTTCTCTAGAC-3′ (forward) and 5′-AAGCTGATGGCAACATAA-3′ (reverse) and 5′-AGGGTGTGAAAGAGGATGG-3′ (forward) and 5′-TCCGAGAGTGCTCGTTTCT-3′ (reverse), respectively. For the determination of α-SNS expression, 20–30 cycles of PCR were run, and the reactions were resolved on agarose gels and stained with ethidium bromide. For β-actin, primer sequences were used for the standard 25-cycle PCR reaction. A 3-kb PCR product for α-SNS was predicted from the 1.6-kb RT-PCR products. Experiments were repeated three times, and each experiment was run in triplicate. All bands were densitometrically analyzed using ImageJ software (NIH). The mean and standard error of the mean (SEM) were calculated using the Student t test for each experiment. Statistical significance was determined using an unpaired t test.
hexamer (Boehringer Mannheim), 500 units SuperScript II reverse transcriptase (Life Technologies) and 100 units of RNase inhibitor (Boehringer Mannheim). The buffer consisted of (in mM) 50 tris (hydroxymethyl) aminomethane (Tris)–HCl (pH 8.3), 75 KCl, 3 MgCl₂, 10 DTT, and 5 dNTP. The reaction was allowed to proceed at 37°C for 90 min and 42°C for 30 min and then was terminated by heating to 65°C for 10 min.

For α-SNS RT-PCR, primers were used that were complementary to sequences in domain I (D1) and which amplify a 479-bp product that corresponds to nucleotides 762-1241 (accession number: SNS X92184). The amplified product spans multiple exon/intron boundaries (Souslova et al. 1997). Forward primer F1 (5’ GAC-CCRTGGAGATGTTTGGA 3’) matches multiple subunits with a T-to-C mismatch at position 3 compared with α-SNS. Reverse primer R1 (5’ GAGGAATGCCAACCAGGAATC 3’) specifically matches subunit α-SNS. The F1/R1 primer pair amplified only one product from a DRG cDNA template; this product matched the predicted fragment properties of length and restriction enzyme polymorphism (data not shown). 

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to minimize the effects of variations in quantity of input RNA, efficiency of reverse transcription and/or amplification among the samples. Rat GAPDH sequences were amplified using primers that amplify a 666-bp product that corresponds to nucleotides 328-994 (accession number: M17701). The amplified product spans multiple exon/intron splice sites, based on the structure of the human gene (Ercolani et al. 1988). 

PCR treatment was routinely performed before reverse transcription to prevent amplification of GAPDH sequences from genomic processed pseudogenes that might have contaminated the total RNA preparation (Benham et al. 1984).

PCR conditions for simultaneous linear amplification of α-SNS and GAPDH were determined empirically. To prevent inhibition of the amplification of α-SNS template by GAPDH, we delayed addition of the GAPDH primers until later stages of amplification (Kinoshita et al. 1992). GAPDH and α-SNS primers were used at final concentrations of 0.5 and 3.3 μM, respectively. Amplification typically was performed in 60 μl volume using 1.75 units of Expand Long Template DNA polymerase enzyme mixture (Boehringer Mannheim). Control PCR with water or RNA template produced no amplification products (data not shown). The PCR reaction buffer consisted of 50 mM Tris–HCl (pH 9.2), 16 mM (NH₄)₂SO₄, 2.25 mM MgCl₂, 2% (vol/vol) dimethyl sulfoxide, and 0.1% Tween 20. Amplification was carried out in three stages: first, denaturation at 94°C for 4 min, annealing at 60°C for 2 min, and elongation at 72°C for 90 s, pause at 20°C to allow addition of the GAPDH primers; second, denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 90 s, repeated 26 times; and third, elongation at 72°C for 10 min. Multiple amplifications were performed for each RNA sample.

Amplification products were separated on a 1.6% agarose gel supplemented with 0.25 mg/ml ethidium bromide. Gel images were digitized using a GelBase 7500 system (UVP) with gray scale converted to false colors. Gel tracks were scanned and peaks corresponding to the GAPDH and α-SNS quantified in auto-analysis mode. The level of the α-SNS in the cDNA pool was expressed as the ratio ‘a/b’, where a and b are the areas under the peaks of the α-SNS and GAPDH, respectively. Four independent amplifications were performed on each template and the results analyzed for statistical significance by two-tail t-test (2 samples assuming unequal variance) using Microsoft Excel software.

**In situ hybridization**

For the study of cells in DRG sections, 14-μm cryosections were mounted on poly-L-lysine-coated glass slides and processed for in situ hybridization as previously described (Black et al. 1994a). Briefly, the sections were sequentially incubated in 1) 4% paraformaldehyde in 0.14 M phosphate buffer, 5 min; 2) phosphate-buffered saline (PBS), three times, 2 min each; 3) proteinase K (10 μg/ml; Boehringer-Mannheim) in 100 mM Tris/50 mM ethylenedinitrilotetraacetic acid, disodium salt (EDTA), pH 8.0, 25 min at 37°C; 4) PBS, three times, 2 min each; 5) 0.1 M methanol (TEA), pH 8.0, 2 min; 6) 0.25% acetic anhydride in 0.1 M TEA, 10 min; 7) 2× saline-sodium citrate buffer (SSC), three times, 2 min each; 8) prehybridization solution, containing 50% formamide, 5× SSC, 5× Denhardt’s, and 100 μg/ml salmon sperm DNA (Sigma), for 1–2 h; 9) hybridization solution, containing 50% formamide, 1 mg/ml dextran sulfate, 5× SSC, 1× Denhardt’s, 100 μg/ml salmon sperm DNA, and 0.25 ng/μl α-SNS sense or antisense riboprobe overnight at 56°C in a humidified chamber; 10) 4× SSC, 5 min; 11) 2× SSC, twice, 10 min each; 12) RNase A (20 μg/ml in 10 mM Tris/500 mM NaCl, pH 8) at 37°C for 30–45 min; 13) 2× SSC, 10 min, twice; 14) 0.2× SSC, 20 min at 56°C, three times; 15) 100 mM Tris/150 mM NaCl, pH 7.5, 2 min; 16) 100 mM Tris/150 mM NaCl, pH 7.5, containing 2% normal sheep serum and 1% bovine serum albumin, 15 min; 17) anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer-Mannheim; 1:500 in blocking solution) overnight at 4°C; 18) 100 mM Tris/150 mM NaCl, three times, 5 min each; 19) 100 mM Tris/100 mM NaCl/50 mM MgCl₂, pH 9.5, three times, 5 min each; and 20) alkaline phosphatase color substrate (NBT/X-phos) for 1 h. The reaction was stopped with 10 mM Tris/1 mM EDTA, pH 8, and the glass slides were coverslipped with poly-aqua-mount (Polysciences).

In situ hybridization cytochemistry also was performed on cultures derived from control and NGF- and Ringer-pump L₄/L₅ DRG as previously described (Black et al. 1994b). Briefly, coverslips were washed twice with Hank’s balanced salt solution (HBSS) and then fixed with 4% paraformaldehyde in HBSS for 10 min at 4°C. The coverslips were then rinsed several times in HBSS, incubated for 15 min in HBSS containing 0.1% Triton, rinsed several times in HBSS and then 2× SSC, and incubated for 1 h in prehybridization solution (50% formamide, 5× SSC, 5× Denhardt’s solution, and 100 μg/ml salmon testis DNA) at room temperature. The coverslips were hybridized overnight at 56°C in hybridization solution containing 50% formamide, 5× SSC, 1× Denhardt’s solution, and 100 μg/ml salmon testis DNA, and 0.25 ng/μl α-SNS antisense or sense riboprobe. The coverslips then were processed as described above for tissue sections.

For each cell culture or tissue section in situ hybridization experiment, the control and NGF- and Ringer-derived DRG cells or sections were incubated in the chromogen solution for the same length of time. Only background levels of hybridization signal were detected in cultured cells or tissue sections when hybridized with sense riboprobes.

**Data analysis**

To obtain a quantitative measurement of hybridization signal, images of cultured or tissue section DRG neurons were captured with a ComputerEyes/1024, Version 1.07, image capture board. In tissue sections, only those small neurons the nuclei of which were apparent were included in the analysis. Optical density (OD) measurements of each neuron were obtained using the NIH Image program; the program was calibrated with images of neutral density filters of 0.1, 0.3, and 0.6 OD and fitted to a straight line (R² ≥ 0.999). All signals measured were within the linear calibration range. The means ± SD for optical densities for small (<25 μm diam) neurons of control (no pump implant), NGF-treated axotomized and Ringer-treated axotomized DRG were determined. Student’s t-test analyses were performed with Microsoft Excel software. The data in vivo and in vitro experiments were analyzed for the expression of α-SNS mRNA in small DRG neurons. Analysis of the pooled data from the in vivo or in vitro experiments, and of individual experiments, yielded similar results.
RESULTS

**TTX-R sodium currents are increased by NGF treatment after axotomy**

Using patch-clamp recording, control (no sciatic nerve transection), NGF-treated axotomized and Ringer-treated axotomized [10–12 days post axotomy (dpa)] neurons were examined. Sodium currents were recorded from small (18–25 μm) DRG neurons after 12–24 h in culture using whole cell patch-clamp techniques. For each of the three experimental groups, 5–12 cells per culture were studied from at least four different cultures. Figure 1 shows recordings from a typical neuron for each group. The sodium currents in control neurons were similar to those previously described in small DRG neurons (Caffrey et al. 1992; Elliott and Elliott 1993; Rizzo et al. 1994; Roy and Narahashi 1992), with most control neurons expressing both fast-inactivating TTX-S and slow-inactivating TTX-R sodium currents (Fig. 1). Because TTX-S currents inactivate at potentials ~35 mV more negative than TTX-R currents, prepulse inactivation can be used to separate the TTX-R and TTX-S current components (McLean et al. 1988; Roy and Narahashi 1992). TTX-subtraction and prepulse-inactivation methods give essentially the same results (Cummins and Waxman 1997), but prepulse-inactivation is simpler than TTX subtraction. Therefore, the relative contributions of the fast and slow currents were estimated using the inflection point in the steady-state inactivation curves (Fig. 1) (Cummins and Waxman 1997). The inflection point was usually near ~50 mV, and the TTX-R current amplitude typically was measured using a 20-ms test pulse to ~10 mV after a 500-ms prepulse to ~50 mV. The TTX-S current amplitude was estimated by subtracting the TTX-R current (typically obtained with the ~50 mV prepulse) from the current elicited with a 20 msec test pulse to ~10 mV after a 500-ms prepulse to ~120 mV.

Although control small neurons express both TTX-S and TTX-R currents, the amount of TTX-R current in small neurons cultured from Ringer-treated axotomized DRG is decreased, and these cells expressed predominantly TTX-S currents (Fig. 1B). The TTX-R current density was 865 ± 123 pA/pF (mean ± SE; n = 27) in control neurons and 154 ± 45 pA/pF (n = 50) in Ringer-treated axotomized neurons; the ratio of TTX-R to TTX-S current was 0.79 in control and 0.13 in Ringer-treated axotomized neurons. By contrast, neurons cultured from NGF-treated axotomized DRG typically expressed both TTX-S and TTX-R sodium currents (Fig. 1C). The TTX-R current density was significantly (P < 0.01) increased in NGF-treated axotomized neurons (465 ± 78 pA/pF; n = 60) compared with Ringer-treated neurons. Although the majority of control neurons expressed a high TTX-R current density and the majority of Ringer-treated axotomized neurons expressed a low TTX-R current density, the majority of NGF-treated axotomized neurons expressed an intermediate TTX-R current density (Fig. 2A). The ratio of TTX-R to TTX-S current in NGF-treated axotomized neurons (0.43; n = 60) was also significantly greater compared with Ringer-treated axotomized neurons (0.13) but was less than that of control neurons (0.79; Fig. 2B). Thus the in vivo administration of exogenous NGF appears to partially, but not fully, restore the level of TTX-R current in axotomized neurons.

The properties of the TTX-R currents in both Ringer- and NGF-treated axotomized neurons were similar to those of the TTX-R currents in control neurons (Fig. 3). The V_{1/2} for activation obtained by prepulse subtraction (Cummins and Waxman 1997) was ~23 ± 2 mV (n = 15) for control TTX-R currents, ~21 ± 2 mV (n = 11) for Ringer-treated axotomized TTX-R currents, and ~21 ± 1 mV (n = 15) for NGF-treated axotomized TTX-R currents (Fig. 3D). The V_{1/2} for steady-state inactivation was ~37 ± 1 mV (n = 25) for control TTX-R currents, ~36 ± 1 mV (n = 18) for Ringer’s-treated axotomized TTX-R currents, and ~37 ± 1 mV (n = 41) for NGF-treated axotomized TTX-R currents (Fig. 3E). Thus the TTX-R currents in NGF-treated axotomized neurons appear to have similar properties to those of control neurons.
SNS mRNA expression is increased by NGF treatment

Previous work has shown that the expression of sodium channel α-SNS mRNA in Xenopus oocytes is accompanied by the appearance of TTX-resistant, slow sodium currents (Akopian et al. 1996; Sangameswaran et al. 1996). Moreover, axotomy of DRG neurons results in a downregulation of α-SNS mRNA that parallels the reduction in TTX-R sodium currents (Dib-Hajj et al. 1996). To determine whether the changes in sodium currents observed in axotomized in vivo DRG neurons provided with exogenous NGF were accompanied by alterations in α-SNS mRNA levels, these cells were examined by RT-PCR and in situ hybridization cytochemistry.

RT-PCR

α-SNS and GAPDH, which was used as an endogenous internal control, amplification products migrate slightly faster than the 500- and 700-bp-size markers, respectively, consistent with their predicted sizes of 479 and 666 bp, respectively (Fig. 4A). An increase in α-SNS transcript levels in NGF-treated DRG is reflected by an increased competition with GAPDH manifested as a reduction in the amplifi-
A total of eight independent amplifications for four separate experiments were analyzed. Analysis of the relative RT-PCR results indicates that NGF-treated axotomized DRG results in higher levels (1.9-fold increase) of α-SNS transcripts compared with those in Ringer-treated axotomized DRG. The mean ± SD optical density (OD) signal for α-SNS was significantly lower (P < 0.01) in Ringer-treated axotomized neurons, compared with control neurons. The α-SNS signal was enhanced significantly (P < 0.01) in NGF-treated, compared with Ringer-treated, axotomized neurons (Fig. 7). Similar trends were present and reached statistical significance (P < 0.01) when each of the three individual experiments was analyzed independently. These results are consistent with our RT-PCR observations in demonstrating that exogenous NGF administered to transected sciatic nerve maintains a high level of α-SNS mRNA in small DRG neurons.

To examine α-SNS mRNA expression in a population of cells more closely corresponding to those that we studied by patch-clamping, in situ hybridization also was performed on cultured DRG neurons derived from control, NGF-treated, and Ringer-treated axotomized DRG. As with the electrophysiological recordings, in situ hybridization studies were performed on cultured neurons within 24 h of plating.

In cultures derived from control DRGs, many small (<25 μm diam) neurons exhibit high levels of α-SNS hybridization signal (Fig. 6A) similar to those observed within normal DRG tissue. Small neurons cultured from Ringer-treated axotomized DRG show a reduction in the level of α-SNS mRNA expression (Fig. 6B). In contrast, high levels of α-SNS hybridization signal are observed in most small neurons derived from NGF-treated axotomized DRG (Fig. 6C).

The α-SNS hybridization signal of cultured small DRG neurons derived from control, Ringer-treated, and NGF-treated axotomized DRG also was quantitated by microdensitometry. A total of 120 control, 219 Ringer-treated, and 108 NGF-treated axotomized small DRG neurons from four separate cultures were studied. The mean ± SD OD signal for α-SNS was significantly lower (P < 0.01) in Ringer-treated axotomized neurons compared with control neurons. The α-SNS signal was enhanced significantly (P < 0.01) in NGF-treated compared with Ringer-treated axotomized neurons (Fig. 7). A decrease in α-SNS signal in Ringer-treated axotomized compared with control neurons and an increase in α-SNS signal in NGF-treated axotomized compared with Ringer-treated axotomized neurons also were present and reached statistical significance (P < 0.05) when each of the four individual experiments was analyzed independently, with one exception in one experiment where the Ringer-treated OD was not significantly lower than the OD for control neurons.
FIG. 5. α-SNS mRNA expression in small neurons in vivo from control and Ringer-treated axotomized and NGF-treated axotomized DRG. A: many small neurons (→) in control DRG express high levels of hybridization signal for α-SNS mRNA. B: signal for α-SNS mRNA is attenuated in small neurons (→) in Ringer-treated axotomized DRG. C: NGF-treated axotomized small DRG neurons (→) express high levels of α-SNS mRNA. Magnification, ×300. Bar, 25 μm.

DISCUSSION

This study, which focused on C-type (small) DRG neurons, which include nociceptive and temperature-sensitive cells and which preferentially express the α-SNS sodium channel, demonstrates that the in vivo administration of exogenous NGF to the proximal peripheral nerve stump of the transected sciatic nerve results in an elevation of the level of TTX-R sodium current after axotomy of these cells. Patch-clamp studies have demonstrated that, in the absence of exogenous NGF, TTX-R current levels are reduced substantially in C-type DRG neurons after axotomy (Cummins and Waxman 1997), and the present results thus demonstrate a partial rescue of TTX-R current expression in small DRG neurons after axotomy by NGF. The results also demonstrate that administration of exogenous NGF to transected sciatic nerve results in an elevation in levels of mRNA encoding the α-SNS sodium channel in small DRG neurons after axotomy. Previous studies have shown that, in the absence of exogenous NGF, α-SNS mRNA levels are decreased substantially in small DRG neurons after axotomy (Dib-Hajj et al. 1996). The present results extend, to the in vivo situation, earlier studies that showed that exogenously added NGF attenuates the drop in α-SNS mRNA expression that occurs in C-type DRG neurons in an in vitro model of axotomy after dissociation and cell culturing (Black et al. 1997). The present results also provide a parallel, in C-type DRG neurons, for previous reports that exogenous NGF attenuates the loss of TTX-R sodium current in myelinated cutaneous afferent DRG neurons (Oyelese et al. 1997).

In contrast to earlier in vitro experiments in which dissociated DRG neurons were bathed in exogenously added NGF-containing medium (Black et al. 1997), in the present experiments NGF was not applied directly to the DRG neuron cell body but rather administered to its peripheral axon, ~4 cm distal to the soma. These results are consistent with the model that NGF acts as a target-derived trophic factor (Mendell 1995) provided by cells in the distal nerve stump after injury (Heumann et al. 1987; Meyer et al. 1992). Because, in the present study, NGF was applied to the nerve at the site of injury, the effects of NGF on sodium channel gene expression presumably reflect retrograde transport, which has been demonstrated previously (Bhattacharya et al. 1997; DiStefano et al. 1993; Matheson et al. 1997).

Previous studies have shown that Schwann cells produce a factor that upregulates α-SNS expression in DRG neurons (Hinson et al. 1997), and it is well established that Schwann cells can act as sources of neurotrophic factors including NGF (Bandtlow et al. 1987; Heumann et al. 1987; Meyer et al. 1993). The effects of exogenously delivered NGF on axotomized DRG neurons in this study were demonstrated both by patch-clamp recording of TTX-R sodium currents and by examination of the expression of α-SNS mRNA with RT-PCR and in situ hybridization. The patch-clamp recordings on these cells reported here confirmed the marked reduction in TTX-R current that has been observed previously in these neurons after axotomy (Cummins and Waxman 1997) and demonstrated a threefold increase in TTX-R current density in NGF-treated compared with Ringer-treated neurons. However, TTX-R current densities were not fully restored to control values and reached only 54% of the level that was observed in control neurons. Consistent with the electrophysiological observations, our RT-PCR and in situ hybridization experiments both showed a significant upregulation of α-SNS mRNA in NGF-treated compared with Ringer-treated DRG after axotomy. The in situ hybridization experiments might be interpreted as suggesting a restoration of α-SNS mRNA expression, after NGF treatment, to levels close to those in controls. The quantitative difference between the degree of recovery of SNS mRNA levels and TTX-R sodium current levels may reflect technical limitations inherent in the quantitation of in situ hybridization. It is unlikely that our patch-clamp recordings failed to detect electrotonically distant sodium current in cell processes because, with the short culture times that we used,
accompanied by a substantial increase in

significant in indicating that the NGF-induced increases in

and NGF-treated axotomized (NGF) small DRG neurons in vivo and in vitro is shown graphically. For both in vivo and in vitro conditions, the mean OD for NGF-treated neurons is significantly (P < 0.01; *) greater than that of Ringer-treated neurons.

Changes in the expression of TTX-R sodium currents are likely to be significant with respect to the electrogenic properties of DRG neurons. Action potential configuration appears to be correlated with the level of TTX-R current in these cells (Ogata and Tatebayashi 1992; Oyalese et al. 1997). TTX-R channels display steady-state inactivation that is shifted in a depolarized direction compared with TTX-S channels in DRG neurons (Caffrey et al. 1992; Cummins and Waxman 1997; Elliott and Elliott 1993). Moreover, TTX-R channels underlie a persistent sodium current that may have a depolarizing influence on resting potential, thereby regulating the availability of TTX-S channels in DRG neurons (Cummins and Waxman 1997). The ratio of TTX-S to TTX-R currents in DRG neurons may be an important determinant of firing properties in these cells.

Although NGF also has been shown to upregulate the expression of mRNA for sodium channel α-subunits II (Mandel et al. 1987; Zur et al. 1995) and PN1 (D’Arcangelo et al. 1993; Toledo-Aral et al. 1995), its effect does not appear to be nonspecific. The mRNA for sodium channel α-subunit III is upregulated in DRG neurons after axotomy (Dib-Hajj et al. 1996; Waxman et al. 1994). This upregulation of α-III mRNA expression is opposed by NGF, which reduces α-III mRNA expression in DRG neurons that have been

there was very little neuritic growth. It is possible that the difference between patch-clamp and in situ hybridization results may reflect a contribution of posttranscriptional or posttranslational mechanisms to the regulation of functional channel incorporation into the membrane. Precedent for this is provided by observations that suggest that some neurons express γ-aminobutyric acid-A (Hales and Tyndale 1994) or NMDR1 (Sucher et al. 1993) mRNAs but do not express functional receptors on their surfaces. Deployment of TTX-R sodium channels in DRG neurons, although dependent on NGF, also may require additional neurotrophic factors or other factors. Irrespective of this, the present results are significant in indicating that the NGF-induced increases in TTX-R sodium currents in axotomized DRG neurons are accompanied by a substantial increase in α-SNS mRNA levels. These results suggest that the action of NGF on sodium channel expression in vivo is mediated, at least in part, by transcriptional mechanisms that regulate sodium channel gene expression.


