Effect of Nerve Growth Factor Treatment on p75^{NTR} Gene Expression in Lumbar Dorsal Root Ganglia of Streptozocin-Induced Diabetic Rats

Jean-Dominique Delcroix, Gregory J. Michael, John V. Priestley, David R. Tomlinson, and Paul Fernyhough

Previous work shows that gene expression for p75^{NTR} in lumbar dorsal root ganglia (DRG) is deficient in streptozocin-induced diabetic rats, while expression of trkA protein is unaffected. This is the first study on the effect of diabetes on immunohistochemical staining and axonal transport of p75^{NTR} in sensory neurons. We also investigated the novel effect of nerve growth factor (NGF) treatment on the levels of mRNA and protein of the NGF receptors, trkA and p75^{NTR}, in normal and diabetic rats. Immunohistochemical staining for p75^{NTR} was significantly reduced in DRG of 12-week streptozocin-induced diabetic rats, confirming the previous work. Anterograde and retrograde axonal transport of p75^{NTR} within the sciatic nerve was similarly decreased in diabetic rats, while levels of transport of trkA were unaffected. Treatment with systemic NGF reversed the diabetes-induced deficit in p75^{NTR} transcripts and protein within the DRG and left expression levels of trkA unchanged. We propose that in sensory neurons of diabetic rats, the ability to capture and retrogradely transport NGF may be impaired because of suboptimal production of p75^{NTR} receptors and that NGF therapy may overcome this deficiency. *Diabetes* 47:1779–1785, 1998

Dysfunction of primary sensory neurons is a consistent feature of diabetic symmetrical polyneuropathy (1). Studies, predominantly in animals with experimental diabetes, indicate a wide range of peripheral neuron deficits, including reduced motor nerve conduction velocity (2), impaired sciatic nerve regeneration (3), axonal shrinkage in association with reduced neurofilament delivery (4), and deficient anterograde axonal transport (5). In addition, there is now mounting evidence that deficient neurotrophic support may play a role in diabetic symmetrical polyneuropathy. Diabetic rats exhibit reduced retrograde transport of nerve growth factor (NGF) (6–9), and peripheral sensory neuron target tissues express lowered levels of NGF mRNA and protein (8,10–12). Sensory neuron gene expression for the neuropeptides substance P and calcitonin gene-related peptide (CGRP) is known to be regulated by NGF in vivo and in vitro (13,14). Recent studies show that neuropeptide gene expression is reduced in diabetic rats and that treatment with exogenous NGF reverses this down-regulation (8,15,16).

NGF mediates effects on sensory neurons via the high- and low-affinity receptors trkA and p75^{NTR} (17–19). The expression of trkA mRNA is restricted to small unmyelinated neurons, including those expressing substance P and CGRP, with additional expression in a cohort of myelinated larger-caliber neurons (20,21). The kinase domain of the trkA receptor undergoes transphosphorylation upon binding of NGF, and this event is believed to trigger subsequent second messenger cascades, resulting in a range of functional alterations in neuron activity (22). The p75^{NTR} receptor binds NGF with low affinity and is expressed in the majority of sensory neurons within the L_4 and L_5 dorsal root ganglia (DRG) (19). The function of p75^{NTR} in the adult nervous system remains controversial; however, recent evidence suggests a role in modulating the affinity of trkA for NGF (23,24).

Previous studies have shown deficient levels of mRNA and protein for trkA and p75^{NTR} in sensory neurons of diabetic rats (25,26). This study aimed to confirm those observations using immunohistochemistry for the receptors in DRG, to determine whether axonal transport of the receptors was affected, and, by treating sensory neurons of normal and diabetic rats with exogenous NGF, to determine the dependence on NGF for maintenance of both transcripts and proteins for the receptors.

RESEARCH DESIGN AND METHODS

Animals, diabetes, and NGF and insulin treatment. In a dose-response study for the effect of NGF, male Wistar rats (300–350 g) were treated with a low (0.2 mg/kg) or high (1.0 mg/kg) dose of human recombinant NGF three times weekly for 4 weeks. Subcutaneous injections were administered at the base of the neck; the control group received saline. In the diabetes studies, male Wistar rats (250–300 g), after an overnight fast, were made diabetic with 65 mg/kg i.p. streptozotocin (Sigma, St. Louis, MO) and maintained without treatment for up to 12 weeks. In the NGF and insulin treatment studies, diabetic and age-matched normal rats received a low-dose (0.2 mg/kg) regimen of NGF treatment for the final 4 weeks of the study (duration of diabetes was 8 weeks by the end of the experiment). Another group of 4-week diabetic rats received an insulin implant (4 U · day^{-1} · rat^{-1}; Linplants; Møllegaard Breeding Centre A/S, Skensved, Denmark) for the final 4 weeks (27). In the sciatic nerve axotomy study, rats were maintained diabetic for 8 weeks, and then the sciatic nerve was crushed for 10 days. In all studies with diabetic rats, age-matched normal rats were used as controls. Blood samples were taken and assayed for blood glucose using the GOD-PERID test kit (Boehringer Mannheim, Mannheim, Germany). All diabetic rats included in the study had plasma glucose levels >20 mmol/l (control range 10–11

From the Departments of Pharmacology (J.-D.D., D.R.T., P.F.) and Anatomy (G.J.M., J.V.P.), St. Bartholomews' and Royal London Hospital School of Medicine and Dentistry, Queen Mary and Westfield College, University of London, London, U.K.

Address correspondence and reprint requests to Dr. Paul Fernyhough, Division of Neuroscience, School of Biological Sciences, University of Manchester, 1.124 Stopford Building, Manchester M13 9PT, U.K.

ANOVA, analysis of variance; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; ELISA, enzyme-linked immunosorbent assay; NFL, neurofilament L; NFM, neurofilament M; NGF, nerve growth factor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; γ-PPT, γ-preprotachykinin; SCG, superior cervical ganglia.

mmol/l) and a body weight range of 300–350 g (control range 400–550 g). Because four independent diabetic studies are included in this investigation, the plasma glucose levels and body weight data have not been shown. All animal procedures followed the guidelines recommended by the U.K. home office.

Sciatic nerve axotomy and measurement of axonal transport. Male Wistar rats (age-matched normal or 8-week diabetic) were subjected to unilateral sciatic nerve crush for 10 days. All operations were performed under deep halothane anesthesia. The right sciatic nerve of the rats was crushed twice at the same point, midthigh level, with watchmaker's forceps, and each crush was sustained for a period of 30 s. The second crush was applied to destroy any axons that might have been missed by the first. The crush site was marked with a fine Prolene suture through the epineurium. Axonal transport in the ipsilateral and contralateral nerves was then measured using a double-ligature paradigm (two ligatures were placed 1 cm apart proximal to the crush on the ipsilateral side for 12 h) (26,28). The accumulation attributable to axonal transport of trkA and $p75^{NTR}$ receptor proteins in the anterograde and retrograde directions was measured and found to be linear up to 12 h after ligation (data not shown). A similar double-ligature paradigm was used to measure axonal transport in sciatic nerve in normal rats treated with NGF for 4 weeks (separate from the axotomy study).

Isolation of total RNA and Northern blotting. Total RNA was isolated essentially as described elsewhere (29). Briefly, the L_4 and L_5 dorsal root ganglia (DRG) were homogenized (Polytron; Kinematica, Lucerne, Switzerland) in 0.8 ml homogenization solution. Total RNA was extracted with phenol:chloroform and quantified by optical density reading at 260 nm, and its purity was estimated by the 260:280 nm ratio. The purified total RNA was subjected to Northern blotting. Equivalent amounts of total RNA (8 µg) were size-fractionated in a 1.0% agarose/formaldehyde gel (30). Ethidium bromide staining was used to determine the position of the 18S and 28S rRNA subunits and to confirm that equivalent amounts of undegraded RNA had been loaded. The fractionated RNA was transferred to Nytran-N nitrocellulose (Schleicher and Schuell, Dassel, Germany) using a Posiblot transfer system (Stratagene, La Jolla, CA) as described (31) and fixed to the membrane by cross-linking under ultraviolet light (Stratalinker, Stratagene).

Detection of specific mRNA species. The trkA probe was a 1.5-kb full-length rat cDNA (32), and the $p75^{NTR}$ probe was a 1.5-kb *Nco*I fragment derived from the rat 3.2-kb cDNA (33). The rat γ-preprotachykinin (γ-PPT), human CGRP, and human neurofilament L (NFL) and neurofilament M (NFM) probes have been described (16,34). The probes were random primer ^{32}P-labeled using a kit (Promega, Madison, WI). The CGRP probe was labeled using asymmetric polymerase chain reaction (PCR) as described (16). Hybridization to nitrocellulose filters proceeded for 16–18 h at 65°C, the solution consisting of 0.5 mol/l Na_2HPO_4, pH 7.0, 7% SDS, 1% bovine serum albumin (fraction V), 1 mmol/l EDTA, and 0.01 mg/ml salmon sperm DNA (35). The blots were washed to a final concentration of 1× or 0.5× SSC (75 mmol/l NaCl, 7.5 mmol/l sodium citrate, 5 mmol/l Na_2HPO_4, 0.015% pyrophosphate, and 0.025% SDS, pH 7.0).

Autoradiograms were prepared on Fuji X-ray film using a single enhancing screen at –70°C (laid down for up to 2 weeks). The level of hybridization detected by the X-ray film was measured using an image analyzer (AI, Cambridge, U.K.). The data are presented per total RNA and expressed relative to control. In the diabetes study, three Northern gels were prepared; three samples common to all gels were included to allow comparison of data between gels.

NGF protein measurement in sciatic nerve. At death, 1 cm of sciatic nerve was frozen in liquid nitrogen and analyzed for NGF protein using an enzyme-linked immunosorbent assay (ELISA) purchased from Boehringer Mannheim (8).

Measurement of trkA and $p75^{NTR}$ protein levels using Western blotting. The L_4 and L_5 DRG and 0.5-cm nerve segments proximal and distal to the sciatic nerve ligatures were removed, frozen in liquid nitrogen, and homogenized according to Filliatreau et al. (36). Briefly, samples were homogenized using a polytron (Kinematica, Lucerne, Switzerland) in 0.1 mmol/l PIPES (pH 6.9), 5.0 mmol/l $MgCl_2$, 5.0 mmol/l EGTA, 0.5% Triton X-100, 20% glycerol, 1.0 mmol/l phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors (1.0 µg/ml pepstatin A, 1.0 µg/ml leupeptin, 10 µg/ml benzoyl-L-arginine methyl ester, 10 µg/ml p-tosyl-L-arginine methyl ester, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml L-1-tosylamide-2-phenylethylchloromethyl ketone, and 7 µg/ml aprotinin). Protein levels were quantified using a Pierce Coomassie Plus assay kit (Pierce & Warriner, Chester, U.K.). SDS-polyacrylamide gel electrophoresis (8% acrylamide) was performed on 10 µg of protein, and the separated proteins were transferred to nitrocellulose (Amersham ECL [enhanced chemiluminescence] membrane; Amersham, U.K.) using a graphite blotter. For trkA and $p75^{NTR}$ protein detection, blots were incubated overnight with a 1:5,000–10,000 dilution of polyclonal antisera against trkA (37) or $p75^{NTR}$ (38), respectively. The trkA antibody was a gift of Dr. Louis F. Reichardt and Dr. Douglas Clary, and the $p75^{NTR}$ was given by Dr. Moses Chao. Detection was achieved using the New England Biolabs phototope-HRP system. The levels of trkA and $p75^{NTR}$ protein were determined using an image analyzer (AI). Nitrocellulose blots were routinely stained with India ink, levels of total protein were measured using image analysis, and Western blot data was then adjusted for any uneven loading of protein.

Immunohistochemistry for $p75^{NTR}$ in lumbar DRG. Three control and three 12-week streptozocin-induced diabetic rats were used for immunocytochemical analysis using standard techniques (39). After being anesthetized with sodium pentobarbital (60 mg/kg), the animals were perfused through the ascending aorta with saline followed by 300 ml of 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4. The L_4 and L_5 DRG were removed, postfixed for 2 h in the same fixative, and cryoprotected in 15% sucrose overnight. Tissues were frozen and sectioned at 8 µm. Sections were stained using indirect immunofluorescence histochemistry with rabbit antiserum directed against $p75^{NTR}$ (anti-REX) diluted to 1:5,000 and a tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antiserum (1:200; Jackson Immunoresearch, Philadelphia) (40). After final washes in phosphate-buffered saline (PBS), sections were mounted on slides in a PBS/glycerol solution (1:3) containing 2.5% 1,4-diazobicyclo(2,2,2)octane (antifading agent; Sigma). Immunoreactivity was visualized on a Leica epifluorescence microscope using the N2 filter block.

Analysis of fluorescence signal intensity and cell size was conducted using Visilog image analysis software. Images at magnification ×25 were captured from the microscope using a Grundig FA87 digital camera with integrating framestore. Positively stained cells were outlined using a computer mouse, and each cell's area and average gray level were calculated. At least 650 cells were analyzed from the control group ($n = 3$), and 780 cells were analyzed from the diabetic group ($n = 3$).

Statistical analysis. Where appropriate, data were subjected to one-way analysis of variance (ANOVA) using the Statistical Package for Social Scientists (SPSS/PC+; SPSS, Chicago). Where the F ratio gave $P < 0.05$, comparisons between individual group means were made by Duncan's multiple range test at significance levels of $P = 0.05$ and 0.01. Levene's test was used to examine the data for homogeneity of variance, accepting $P > 0.05$. When a one-way ANOVA gave an F ratio of $P > 0.05$ or nonhomogeneity of variances prevented a meaningful analysis, single comparisons between groups were made using Student's t test.

RESULTS

Effect of diabetes on immunohistochemical localization of $p75^{NTR}$ in DRG. Immunohistochemical analysis of L_4 and L_5 DRG from control and 12-week diabetic rats is shown in Figs. 1 and 2. Staining for $p75^{NTR}$ was predominantly neuronal in control and diabetic rats, although there was some evidence of satellite cell staining (Fig. 1). The percentage of lumbar DRG sensory neurons positive for $p75^{NTR}$ staining was 65% (~1,000 cells counted; $n = 3$) for control and 53% (~1,200 cells counted; $n = 3$) for diabetic rats. Analysis of the intensity of staining for $p75^{NTR}$ (Fig. 2*A*) showed that the levels of $p75^{NTR}$ were reduced across the whole range of cell sizes in diabetic rats, with large cells having particularly lowered levels of expression (~32% decrease in the 1,500–2,100 µm^2 cell size range). The number of cells larger than 900 µm^2 that stained positive for $p75^{NTR}$ was generally reduced in diabetic rats (control 50.4%, diabetic 35.6%), whereas the number with a cell size in the range of 300–700 µm^2—i.e., small neurons—was elevated (control 40.3%, diabetic 53.2%). There was no indication of any significant cell loss in the DRG of diabetic rats.

Effect of sciatic nerve crush and diabetes on axonal transport of $p75^{NTR}$ and trkA. Fast axonal transport within the sciatic nerve of $p75^{NTR}$ and trkA (145-kDa species; the 110-kDa species was not transported) was measured in normal and diabetic rats, and the effect of sciatic nerve crush was investigated as an analogous model of neurotrophic factor depletion. A double-ligature paradigm was used to adjust for locally synthesized $p75^{NTR}$ produced in response to the 12-h ligature (there was no local synthesis of trkA). Levels of anterograde versus retrograde axonal transport were the same for both proteins (Fig. 3). Neither diabetes nor sciatic nerve crush had any effect on the levels of axonal transport of trkA. Axonal transport of $p75^{NTR}$ was reduced by 45–50% in diabetic rats in both the anterograde and retrograde directions. Sciatic nerve crush reduced axonal transport of $p75^{NTR}$ by 39% in normal rats; in diabetic

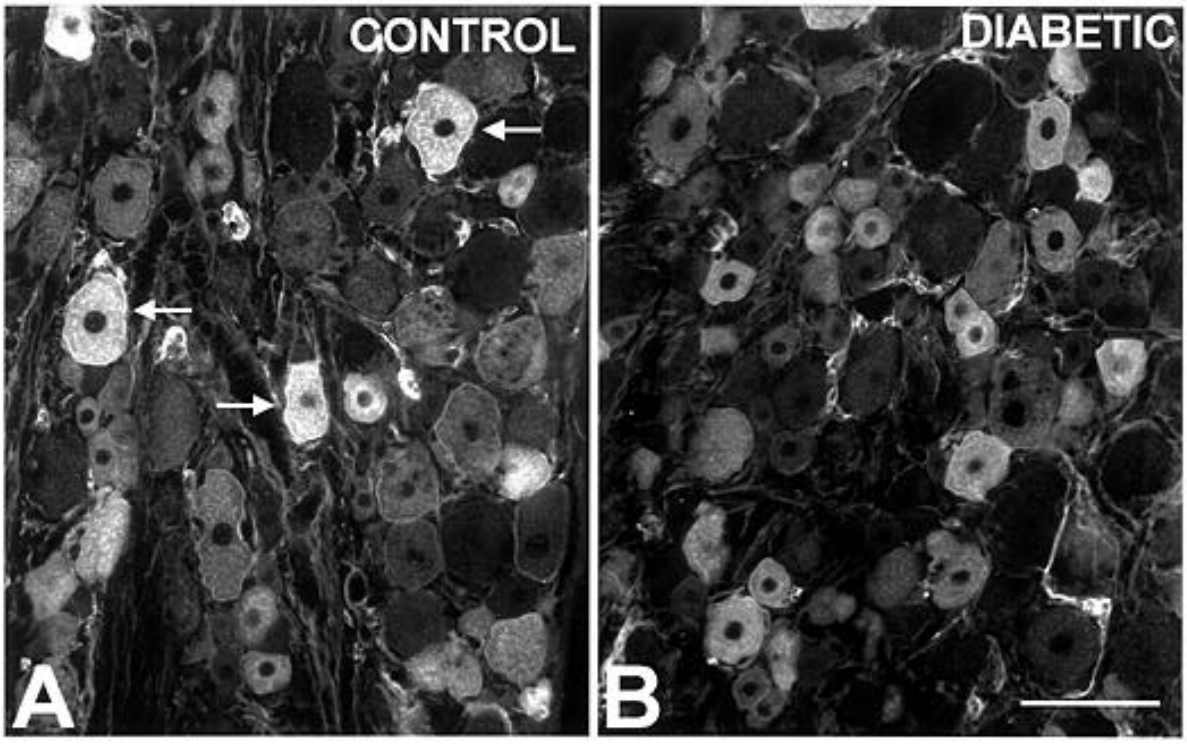

FIG. 1. Effect of diabetes on p75^{NTR} protein immunoreactivity in lumbar DRG. DRG from age-matched control and 12-week diabetic rats were stained for p75^{NTR} protein. *A*: In control rats, cells of all sizes showed immunoreactivity, and some cells (arrows) showed particularly strong labeling. *B*: In diabetic rats, immunoreactivity was still present, but the level of staining in individual cells was much lower. Bar indicates 100 µm.

rats, there was no significant effect beyond that already attributable to diabetes (Fig. 3).

Effect of NGF on mRNA levels in lumbar DRG in normal rats. To discover a suitable NGF dose regimen to reverse diabetes-induced deficits in p75^{NTR} expression, normal rats were injected subcutaneously with 0.2 or 1.0 mg/kg NGF for 4 weeks. The resulting effects on the levels of a range of transcripts in the L_4 and L_5 DRG are shown in Fig. 4. mRNAs for CGRP, γ-PPT, and p75^{NTR} were increased approximately twofold over untreated control rats at both doses of NGF (Fig. 4*A*–*C*; Table 1). Transcripts for trkA, neurofilament M (NFM), and neurofilament L (NFL) were increased by 20–30% upon treatment with 0.2 mg/kg NGF; the 1.0 mg/kg NGF dose had a similar effect. The elevation in trkA mRNA induced by 0.2 mg/kg NGF was significant ($P < 0.05$); at 1.0 mg/kg NGF, however, there was no significant effect (Fig. 4*D*; Table 1). Sciatic nerve levels of NGF protein were elevated in a dose-related manner, with 0.2 mg/kg NGF raising NGF levels approximately threefold and 1.0 mg/kg NGF inducing a sixfold elevation over control levels (Table 1).

Effect of NGF treatment on p75^{NTR} and trkA protein levels in DRG and axonal transport in normal rats. Table 2 shows that systemic treatment with 0.2 mg/kg NGF elevated expression of p75^{NTR} protein in DRG of normal rats. The levels of anterograde and retrograde axonal transport of p75^{NTR} within the sciatic nerve were also increased. TrkA protein expression (145- and 110-kDa species) in the lumbar DRG and the levels of trkA axonally transported (145-kDa species only) were unaffected by NGF treatment (Fig. 5).

Effect of NGF on mRNA levels in diabetic rats. Knowing that 0.2 mg/kg NGF upregulated gene expression and

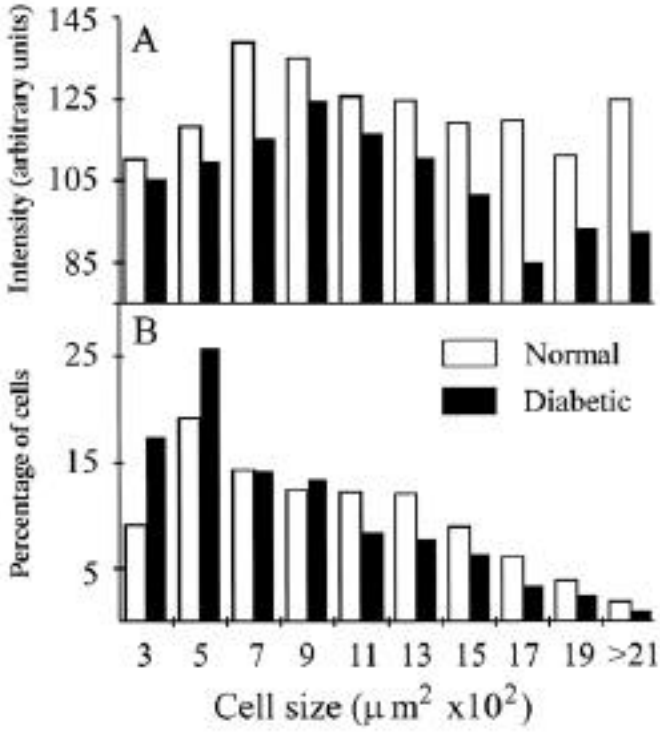

FIG. 2. Immunohistochemical analysis of the effect of diabetes on expression of p75^{NTR} in the neuronal population of lumbar DRG. The DRG sections in Fig. 1 were analyzed for the intensity of p75^{NTR} staining. *A*: Intensity of p75^{NTR} staining plotted against cell size. *B*: Percentage of p75^{NTR}-positive cells plotted against cell size.

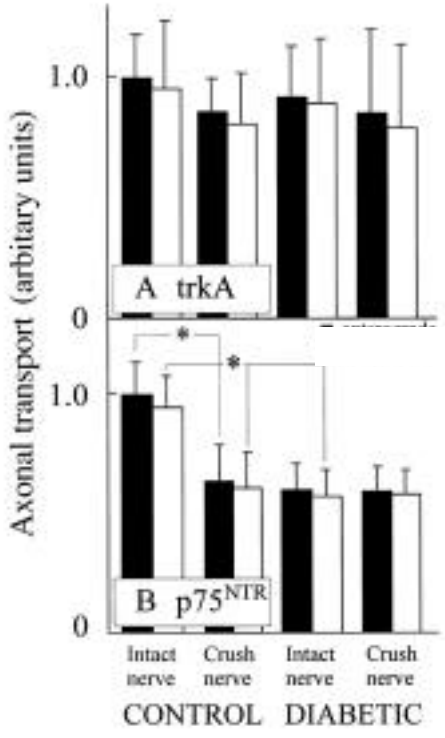

FIG. 3. Effect of diabetes and axotomy on fast axonal transport of p75^{NTR} and trkA proteins in sciatic nerve. Groups of normal and 8-week diabetic rats underwent unilateral sciatic nerve crush for 10 days. Animals then had contralateral and ipsilateral sciatic nerves double-ligated for 12 h to measure axonal transport of proteins. *A*: trkA, 145-kDa form; *B*: p75^{NTR}. ■, anterograde transport; □, retrograde transport. Data are means ± SD (n = 7), and the p75^{NTR} values have been adjusted for local synthesis (there was no local synthesis of trkA). All values have been normalized to the anterograde transport levels for the contralateral nerve of control rats. *P < 0.01 (t test).

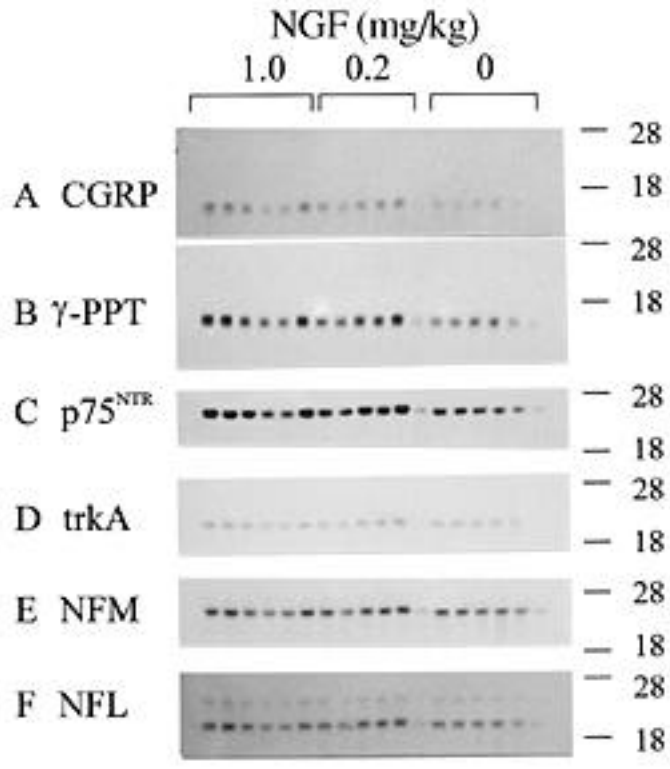

FIG. 4. Effect of NGF treatment on L_4 and L_5 DRG mRNAs in normal rats. Normal rats were injected subcutaneously with 0 (n = 5), 0.2 (n = 5), and 1.0 (n = 6) mg/kg NGF three times weekly for 4 weeks, and total RNA from L_4 and L_5 DRG was isolated and subjected to Northern blotting. The blot was probed sequentially for CGRP (*A*), γ-PPT (*B*), p75^{NTR} (*C*), trkA (*D*), NFM (*E*), and NFL (*F*) mRNAs. The 18S and 28S ribosomal rRNA bands are indicated.

axonal transport of p75^{NTR} in normal rats, we used that dose for further studies of NGF in diabetic rats. The effect of diabetes and NGF treatment on L_4 and L_5 DRG mRNA levels is shown in Table 3. All transcripts in diabetic rats were lowered compared with control rats, the level of reduction ranging from 30% for trkA (P < 0.05) to 50% for p75^{NTR} and NFM (P < 0.05). Insulin treatment of diabetic rats succeeded in completely reversing the deficits for CGRP, p75^{NTR}, and trkA mRNAs but failed to entirely reverse the reduced levels of transcripts for NFM and NFL. Treatment of diabetic rats with 0.2 mg/kg NGF for 4 weeks reversed the reduced levels of mRNA for CGRP (P < 0.05). The effect of NGF treatment on p75^{NTR} mRNA levels was insignificant, the levels being raised by only 21%. The reduced levels of transcripts for trkA, NFM, and NFL in diabetic rats were insensitive to treatment with NGF.

Effect of NGF treatment on NGF protein levels in sciatic nerve of diabetic rats. Table 3 shows that treatment of diabetic rats with 0.2 mg/kg NGF for 4 weeks significantly raised NGF levels in the sciatic nerve above those of untreated diabetic rats.

Effect of NGF treatment on p75^{NTR} and trkA protein levels in lumbar DRG of diabetic rats. Treatment of 8-week diabetic rats with 0.2 mg/kg NGF reversed the deficit in p75^{NTR} protein levels in DRG (Table 4). The trkA protein levels (145- and 110-kDa species) were unaffected by NGF treatment.

DISCUSSION

Diabetes-induced decrease in p75^{NTR} gene expression in the lumbar DRG has been shown previously (25,26). This work is novel in that it shows that this deficiency in gene expression for p75^{NTR} can be reversed by administration of systemic NGF. It also shows that fast anterograde and retrograde axonal transport of p75^{NTR} is decreased in diabetes. The effects of diabetes on the expression of trkA, the specific tyrosine kinase receptor protein for NGF, were more complex. In lumbar DRG, diabetes reduced mRNA expression for trkA, as reported (25), and decreased expression of the 110-kDa

TABLE 1
Effect of NGF treatment on transcript levels in DRG and NGF protein levels in sciatic nerve of normal rats

		NGF (mg/kg)	
	Untreated	0.2	1.0
mRNA			
γ-PPT	1.0 ± 0.32	2.09 ± 0.52*	2.08 ± 0.78*
CGRP	1.0 ± 0.22	1.97 ± 0.43*	2.23 ± 0.83*
p75^{NTR}	1.0 ± 0.39	1.91 ± 0.76*	2.09 ± 1.06*
trkA	1.0 ± 0.14	1.33 ± 0.28*	1.24 ± 0.26
NFM	1.0 ± 0.31	1.34 ± 0.35	1.19 ± 0.38
NFL	1.0 ± 0.28	1.22 ± 0.16	1.12 ± 0.31
NGF (pg/cm of nerve)	28.5 ± 11.2	100.2 ± 9.0*	182.9 ± 76.4

Data are means ± SD (n = 5–6). *P < 0.05 vs. untreated.

TABLE 2
Effect of NGF on p75^{NTR} and trkA protein levels in DRG and axonal transport in normal rats

	L_4 and L_5 DRG		Retrograde transport	
	p75^{NTR}	trkA (145 kDa)	p75^{NTR}	trkA (145 kDa)
Control	1.0 ± 0.36*	1.0 ± 0.06	1.0 ± 0.2*	1.0 ± 0.07
NGF-treated	1.54 ± 0.39	1.08 ± 0.19	1.7 ± 0.66	1.04 ± 0.1

Data are means ± SD (n = 6). NGF-treated rats were given 0.2 mg/kg NGF for 4 weeks. The 110-kDa species of trkA detected in DRG was similarly unaffected by NGF treatment. The levels of p75^{NTR} and trkA in anterograde axonal transport were the same as those in retrograde axonal transport. *P < 0.05 vs. NGF-treated (t test).

receptor protein while leaving expression of the 145-kDa form unaffected, also as reported (26). In this study, NGF treatment failed to reverse those deficits in trkA expression (Table 4).

Immunohistochemical analysis for p75^{NTR} in lumbar DRG confirmed that expression was mainly neuronal in origin in age-matched control and 12-week diabetic rats (Fig. 1). Diabetes caused a clear reduction in the intensity of staining in all cell sizes, with larger cells (1,500–2,100 μm^2) being particularly targeted (Figs. 1 and 2*A*). The percentage of p75^{NTR}-positive neurons was reduced in cells larger than 900 μm^2 but increased in cells from 300 to 700 μm^2—i.e., small cells—in diabetes (Fig. 2*B*). This diabetes-induced shift in p75^{NTR} staining toward cells of a smaller size was probably the result of cellular shrinkage (41) or preferential loss of staining in the larger cells.

The reduction in expression of p75^{NTR} in lumbar DRG of diabetic rats was matched by decreased anterograde and retrograde axonal transport of the protein (Fig. 3). Furthermore, nerve crush failed to further reduce the level of axonal transport of p75^{NTR} in diabetic rats, implying that the level of neurotrophic support responsible for maintenance of p75^{NTR} expression and its axonal transport was at a basal level. The levels of NGF transported in the sciatic nerve of diabetic rats show a remarkably similar pattern (26).

In normal rats, NGF treatment modulated expression and axonal transport of p75^{NTR} without modifying trkA expression or axonal transport in any significant way. (NGF at 0.2 mg/kg did increase trkA mRNA expression by 34% in DRG of normal rats, but at 1.0 mg/kg NGF there was no significant effect; these effects of NGF on trkA mRNA levels were minimal compared with the twofold elevation of p75^{NTR} mRNA observed [Table 1].) These observations suggest a role for NGF in regulating expression of p75^{NTR}, but not trkA, in DRG, thereby complementing results from other studies. For example, during development of mouse trigeminal sensory neurons, the mRNA levels of p75^{NTR} were sensitive to NGF, whereas trkA transcripts appeared to be independent (42). Treatment of PC12 cells or cultured adult rat sensory neurons with NGF resulted in an elevation in p75^{NTR} gene expression (43,44), leaving trkA mRNA levels unchanged (45). NGF treatment of the terminal fields of sympathetic neurons innervating the eye of the rat increased p75^{NTR} mRNA in the superior cervical ganglia (SCG) in parallel with enhanced immunostaining for p75^{NTR} in the terminal neurites (46). Interestingly, in the SCG, treatment of the terminal fields with NGF caused no change in trkA transcript levels while inducing local sprouting, suggesting that changes in p75^{NTR} expression could have functional consequences (46). Studies in vivo using in situ hybridization have shown that intact sensory neurons treated with NGF upregulated the levels of p75^{NTR} transcripts, but only in neurons exhibiting high-affinity binding of ^{125}I-NGF, presumably trkA-positive (47). There was, however, no effect on trkA transcripts.

As observed for normal rats, treatment of diabetic rats with NGF upregulated p75^{NTR} protein levels within the lumbar DRG, leaving expression of trkA unaffected. NGF treatment raised p75^{NTR} mRNA levels by only 21%, suggesting that the effect of NGF may have been targeted mainly at the translational level. The binding of NGF to and activation of trkA may maintain normal expression of p75^{NTR} and, furthermore, mediate the elevation in p75^{NTR} gene expression in DRG of normal and diabetic rats treated with NGF. The failure of NGF to completely reverse the deficit in p75^{NTR} mRNA was possibly a result of cells that express p75^{NTR}, but not trkA, being insensitive to NGF, for example, trkC-positive, NT-3–sensitive neurons that express p75^{NTR}. The downregulation of p75^{NTR} gene expression in the DRG of diabetic rats is likely to be a result of the dramatic reduction in target-derived NGF in the lower limb of diabetic rats (8,12). However, a direct effect of diabetes leading to reduced levels of neuronal expression of p75^{NTR}, independent of diabetes-induced changes in peripherally derived neurotrophic support, may also occur. After all, the reduction in trkA mRNA is unlikely to be consequent on NGF changes, since it was unaffected by administration of NGF. A

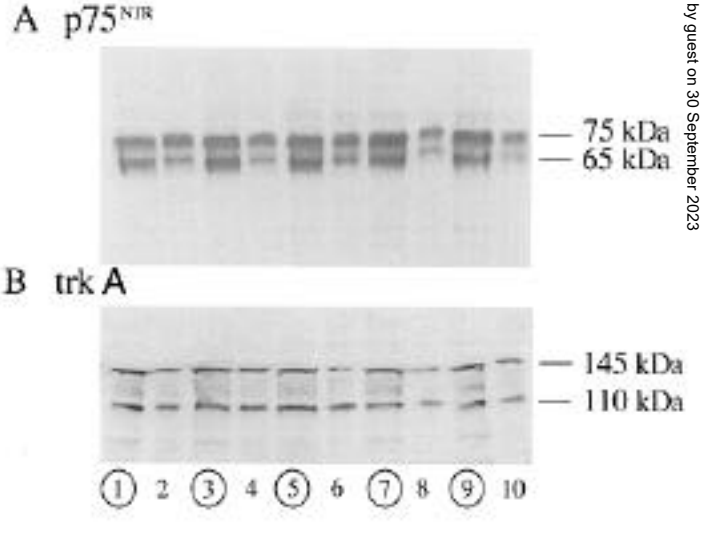

FIG. 5. Effect of NGF treatment on p75^{NTR} and trkA protein levels in DRG of diabetic rats. Diabetic rats (8-week) were treated systemically with 0.2 mg/kg NGF for the final 4 weeks of the study. Lumbar DRG homogenates were then subjected to Western blotting and probed for p75^{NTR} (*A*) and trkA (*B*). Odd numbers (circled), NGF-treated; even numbers, saline-treated.

TABLE 3
Levels of NGF receptor mRNAs and other transcripts in DRG and NGF protein levels in sciatic nerve of normal rats and diabetic rats with or without NGF or insulin treatment

	mRNA					Sciatic nerve NGF
	CGRP	p75	trkA	NFL	NFM	(pg/cm nerve)
Control	1.0 ± 0.67	1.0 ± 0.15†	1.0 ± 0.2†	1.0 ± 0.12†	1.0 ± 0.13†	15.9 ± 8.2‡
Untreated diabetic	0.62 ± 0.22	0.52 ± 0.29	0.7 ± 0.3	0.65 ± 0.27	0.57 ± 0.37	8.2 ± 6.7
NGF-treated diabetic	1.18 ± 0.57*	0.73 ± 0.25	0.74 ± 0.27	0.66 ± 0.18	0.5 ± 0.28	13.2 ± 5.3‡
Insulin-treated diabetic	1.18 ± 0.39	1.06 ± 0.36	0.95 ± 0.41	0.81 ± 0.34	0.79 ± 0.23	25.0 ± 15.2‡

Data are means ± SD. For transcript data, n = 6–7; insulin group, n = 5; for NGF protein data, n = 7–12. *P < 0.05 vs. untreated diabetic (t test); †P < 0.05 vs. diabetic (t test); ‡P < 0.05 vs. untreated diabetic (one-way ANOVA).

neuronal problem with receptor expression would result in reduced sensitivity to target-derived NGF levels and subsequent reductions in retrograde transport of NGF.

The inability of NGF treatment to radically enhance trkA gene expression levels in DRG of normal rats (Fig. 3*D*; Table 1) and diabetic rats (Table 3) suggests that maintenance of trkA expression in the intact peripheral neuron, in both the normal and diabetic state, is independent of NGF. There is little data to indicate the identity of the factors involved in the regulation of trkA expression, but one line of evidence implicates IGFs, based on the colocalization throughout development and in the adult of the insulin receptor-related receptor with trkA in sensory neurons of the DRG (48) and the ability of insulin to potentiate responses to NGF in cultured neurons (49). That evidence could explain the deficit in trkA transcript levels in DRG of diabetic rats because expression of this family of proteins is clearly abnormal in diabetes (50). In this study, the ability of insulin therapy to completely reverse diabetes-induced deficits in $p75^{NTR}$ and trkA mRNA levels supports the suggestion above (Table 3). The effect of insulin therapy was superior to NGF treatment in this regard, probably because it corrected $p75^{NTR}$ deficits in cells that do not respond to NGF—trkC-expressing cells, for example. Therefore, cotreatment with NGF and other neurotrophins remains a useful therapeutic goal.

The reduced expression and axonal transport of $p75^{NTR}$ would presumably reduce the capacity of the sensory neuron terminals to capture and transport NGF. In fact, $p75^{NTR}$ has been shown to modulate transphosphorylation of trkA and, in a related manner, to regulate sensitivity of neurons to NGF (24,51). The reduction in levels of $p75^{NTR}$ receptors observed here could be implicated in the reduced axonal transport of NGF in the sciatic nerve of streptozocin-induced diabetic rats and the correlated deficiency in neuropeptide gene expression within the DRG (8,9,12).

The reduction in $p75^{NTR}$ gene expression in DRG of diabetic rats may well reproduce the type of sensory neuropathy observed in $p75^{NTR}$ knockout mice (52). It is of great interest that the pathology of the neuropathy observed in the knockout mice was very closely related to the presentation of sensory neuropathy in humans with type 1 diabetes, i.e., decreased sensitivity to heat and pain followed by the appearance of skin lesions associated with sensory loss within the skin.

In conclusion, the results of the present study show that the level of $p75^{NTR}$ gene expression in DRG of diabetic rats was reduced compared with age-matched control animals. Systemic administration of NGF upregulated the levels of $p75^{NTR}$ expression in DRG of normal and diabetic rats without similar effects on the transcript levels for trkA, NFL, or NFM. It can be proposed that the deficits in gene expression for $p75^{NTR}$ and trkA in diabetes may cause reduced uptake of NGF; that, in turn, may contribute to the sensory neuropathy observed in the human disease. This study shows that NGF therapy in human diabetic neuropathy would be expected to increase availability of NGF coupled with an increase in capacity of sensory neurons to bind this NGF, the latter being a feature of NGF's ability to upregulate $p75^{NTR}$ gene expression.

ACKNOWLEDGMENTS

This work was supported by grants from the Wellcome Trust (P.F., D.R.T.) and the Medical Research Council (MRC) (J.V.P.).

We thank Genentech, and especially Dr. Heidi Phillips, for the human recombinant NGF. The anti-REX $p75^{NTR}$ and RTA trkA antibodies were a kind gift of Dr. Louis F. Reichardt, Howard Hughes Medical Center, University of California, and Dr. Douglas Clary, Sugen Pharmaceuticals. The $p75^{NTR}$ antibody used for Western blotting was generously given by Dr. Moses Chao, Cornell University. For gifts of probes, thanks go to Dr. Anthony Harmar, MRC Brain Metabolism Unit, Royal Edinburgh Hospital (γ-PPT); Dr. David Craig, Zeneca Pharma, Macclesfield Cheshire (with permission of Professor David Latchman) (CGRP); Professor Eric Shooter, Stanford University School of Medicine ($p75^{NTR}$); Dr. George Yancopoulos, Regeneron Pharmaceuticals (trkA); and Professor Douglas Ishii, Colorado State University (with permission from Dr. Kim Angelides) (NFL and NFM). Thanks also go to Nicola Havenhand and Samina Riaz for providing the NGF ELISA data presented in Table 1.

TABLE 4
Effect of administration of systemic NGF on the diabetes-induced deficit in $p75^{NTR}$ protein expression in DRG

		trkA	
	$p75^{NTR}$	145 kDa	110 kDa
Untreated control	1.0 ± 0.22*‡	1.0 ± 0.06	1.0 ± 0.19
NGF-treated control	1.42 ± 0.23*	1.08 ± 0.19	1.07 ± 0.2
Untreated diabetic	0.54 ± 0.17	0.95 ± 0.26	0.71 ± 0.09†
NGF-treated diabetic	0.91 ± 0.15*	0.98 ± 0.1	0.73 ± 0.06†

Data are means ± SD (n = 4–5) and have been normalized to control (saline-treated). Normal and 8-week diabetic rats were subcutaneously treated with 0.2 mg/kg NGF for 4 weeks. Homogenates of lumbar DRG were subjected to Western blotting. *P < 0.05 vs. untreated diabetic; †P < 0.05 vs. untreated and NGF-treated control; ‡P < 0.05 vs. NGF-treated control (all one-way ANOVA).

REFERENCES

1. Thomas PK, Tomlinson DR: Diabetic and hypoglycaemic neuropathy. In *Peripheral Neuropathy*. 3rd ed. Thomas PK, Tomlinson DR, Dyck PJ, Griffin JW, Low PA, Poduslo JF, Eds. Philadelphia, London, Toronto, Montreal, Sydney, Tokyo, W.B. Saunders Co., 1992, chapter 64, p. 1219–1250
2. Eliasson SG: Nerve conduction changes in experimental diabetes. *J Clin Invest* 43:2353–2358, 1964
3. Ekström PAR, Tomlinson DR: Impaired nerve regeneration in streptozotocin-diabetic rats: effects of treatment with an aldose reductase inhibitor. *J Neurol Sci* 93:231–237, 1989
4. Medori R, Jenich H, Autilio-Gambetti L, Gambetti P: Experimental diabetic neuropathy: similar changes of slow axonal transport and axonal size in different animal models. *J Neurosci* 8:1814–1821, 1988
5. Tomlinson DR, Mayer JH: Defects of axonal transport in diabetes mellitus: a possible contribution to the aetiology of diabetic neuropathy. *J Auton Pharmacol* 4:59–72, 1984
6. Jakobsen J, Brimijoin S, Skau K, Sidenius P, Wells D: Retrograde axonal transport of transmitter enzymes, fucose-labeled protein, and nerve growth factor in streptozotocin-diabetic rats. *Diabetes* 30:797–803, 1981
7. Schmidt RE, Grabau GG, Yip HK: Retrograde axonal transport of [^{125}I]nerve growth factor in ileal mesenteric nerves in vitro: effect of streptozotocin diabetes. *Brain Res* 378:325–336, 1986
8. Fernyhough P, Diemel LT, Hardy J, Brewster WJ, Mohiuddin L, Tomlinson DR: Human recombinant nerve growth factor replaces deficient neurotrophic support in the diabetic rat. *Eur J Neurosci* 7:1107–1110, 1995
9. Hellweg R, Raivich G, Hartung H-D, Hock C, Kreutzberg GW: Axonal transport of endogenous nerve growth factor (NGF) and NGF receptor in experimental diabetic neuropathy. *Exp Neurol* 130:24–30, 1994
10. Hellweg R, Hartung H-D: Endogenous levels of nerve growth factor (NGF) are altered in experimental diabetes mellitus: a possible role for NGF in the pathogenesis of diabetic neuropathy. *J Neurosci Res* 26:258–267, 1990
11. Hellweg R, Wöhrle M, Hartung H-D, Stracke H, Hock C, Federlin K: Diabetes mellitus-associated decrease in nerve growth factor levels is reversed by allogeneic pancreatic islet transplantation. *Neurosci Lett* 125:1–4, 1991
12. Fernyhough P, Diemel LT, Brewster WJ, Tomlinson DR: Deficits in sciatic nerve neuropeptide content coincide with a reduction in target tissue nerve growth factor mRNA in streptozotocin-diabetic rats: effects of insulin treatment. *Neuroscience* 62:337–344, 1994
13. Goedert M, Stoeckel K, Otten U: Biological importance of the retrograde axonal transport of nerve growth factor in sensory neurons. *Proc Natl Acad Sci U S A* 78:5895–5898, 1981
14. Lindsay RM, Harmar AJ: Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. *Nature* 337:362–364, 1989
15. Apfel SC, Arezzo JC, Brownlee M, Federoff H, Kessler JA: Nerve growth factor administration protects against experimental diabetic sensory neuropathy. *Brain Res* 634:7–12, 1994
16. Diemel LT, Brewster WJ, Fernyhough P, Tomlinson DR: Expression of neuropeptides in experimental diabetes; effects of treatment with nerve growth factor or brain-derived neurotrophic factor. *Mol Brain Res* 21:171–175, 1994
17. Meakin SO, Shooter EM: The nerve growth factor family of receptors. *Trends Neurosci* 15:323–331, 1992
18. Barbacid M: The Trk family of neurotrophin receptors. *J Neurobiol* 25:1386–1403, 1994
19. Chao MV, Hempstead BL: p75 and Trk: a two-receptor system. *Trends Neurosci* 18:321–326, 1995
20. McMahon SB, Armanini MP, Ling LH, Phillips HS: Expression and coexpression of Trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. *Neuron* 12:1161–1171, 1994
21. Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV: Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur J Neurosci* 7:1484–1494, 1995
22. Kaplan DR, Stephens RM: Neurotrophin signal transduction by the Trk receptor. *J Neurobiol* 25:1404–1417, 1994
23. Benedetti M, Levi A, Chao MV: Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. *Proc Natl Acad Sci U S A* 90:7859–7863, 1993
24. Barker PA, Shooter EM: Disruption of NGF binding to the low affinity neurotrophin receptor p75^{LNTR} reduces NGF binding to TrkA on PC12 cells. *Neuron* 13:203–215, 1994
25. Maeda K, Fernyhough P, Tomlinson DR: Regenerating sensory neurones of diabetic rats express reduced levels of mRNA for GAP-43, gamma-preprotachykinin and the nerve growth factor receptors, trkA and p75^{NGFR}. *Mol Brain Res* 37:166–174, 1996
26. Delcroix J, Tomlinson DR, Fernyhough P: Diabetes and axotomy-induced deficits in retrograde axonal transport of nerve growth factor correlate with decreased levels of p^{75LNTR} protein in lumbar dorsal root ganglia. *Mol Brain Res* 51:82–90, 1997
27. Stevens EJ, Carrington AL, Tomlinson DR: Nerve ischaemia in diabetic rats: time-course of development, effect of insulin treatment plus comparison of streptozotocin and BB models. *Diabetologia* 37:43–48, 1994
28. Raivich G, Hellweg R, Kreutzberg GW: NGF receptor-mediated reduction in axonal NGF uptake and retrograde transport following sciatic nerve injury and during regeneration. *Neuron* 7:151–164, 1991
29. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
30. Lehrach H, Diamond D, Wozney JM, Boedtker H: Ribonucleic acid molecular weight determination by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* 16:4743–4751, 1977
31. Thomas PS: Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci U S A* 77:5201–5205, 1980
32. Meakin SO, Suter U, Drinkwater CC, Welcher AA, Shooter EM: The rat *trk* protooncogene product exhibits properties characteristic of the slow nerve growth factor receptor. *Proc Natl Acad Sci U S A* 89:2374–2378, 1992
33. Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM: Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature* 325:593–597, 1987
34. Mohiuddin L, Fernyhough P, Tomlinson DR: Reduced levels of mRNA encoding endoskeletal and growth-associated proteins in sensory ganglia in experimental diabetes mellitus. *Diabetes* 44:25–30, 1995
35. Mahmoudi M, Lin VK: Comparison of two different hybridization systems in northern transfer analysis. *Biotechniques* 7:331–332, 1989
36. Filliatreau G, Denoulet P, De Nechaud B, Di Giamberardino L: Stable and metastable cytoskeletal polymers carried by slow axonal transport. *J Neurosci* 8:2227–2233, 1988
37. Clary DO, Weskamp G, Austin LAR, Reichardt LF: TrkA cross-linking mimics neuronal responses to nerve growth factor. *Mol Biol Cell* 5:549–563, 1994
38. Huber LJ, Chao MV: Mesenchymal and neuronal cell expression of the p75 neurotrophin receptor gene occur by different mechanisms. *Dev Biol* 167:227–238, 1995
39. Priestley JV: Immunocytochemical techniques for the study of the nervous system. In *Neurochemistry: A Practical Approach*. 2nd ed. Bachelard HS, Turner AJ, Eds. Oxford, Oxford University Press, 1997, p. 71–120
40. Weskamp G, Reichardt LF: Evidence that biological activity of NGF is mediated through a novel subclass of high affinity receptors. *Neuron* 6:649–663, 1991
41. Sidenius P, Jakobsen J: Reduced perikaryal volume of lower motor and primary sensory neurons in early experimental diabetes. *Diabetes* 29:182–186, 1980
42. Wyatt S, Davies AM: Regulation of expression of mRNAs encoding the nerve growth factor receptors p75 and trkA in developing sensory neurons. *Development* 119:635–647, 1993
43. Doherty P, Seaton P, Flanigan TP, Walsh FS: Factors controlling the expression of the NGF receptor in PC12 cells. *Neurosci Lett* 92:222–227, 1988
44. Lindsay RM, Shooter EM, Radeke MJ, Misko TP, Dechant G, Thoenen H, Lindholm D: Nerve growth factor regulates expression of the nerve growth factor receptor gene in adult sensory neurons. *Eur J Neurosci* 2:389–396, 1990
45. Kaplan DR, Martin-Zanca D, Parada LF: Tyrosine phosphorylation and tyrosine kinase activity of the *trk* proto-oncogene product induced by NGF. *Nature* 350:158–160, 1991
46. Miller FD, Speelman A, Mathew TC, Fabian J, Chang E, Pozniak C, Toma JG: Nerve growth factor derived from terminals selectively increases the ratio of p75 to trkA NGF receptors on mature sympathetic neurons. *Dev Biol* 161:206–217, 1994
47. Verge VMK, Merlio J-P, Grondin J, Ernfors P, Persson H, Riopelle RJ, Hokfelt T, Richardson PM: Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF receptor mRNA in primary sensory neurons: responses to injury and infusion of NGF. *J Neurosci* 12:4011–4022, 1992
48. Reinhardt RR, Chin E, Zhang B, Roth RA, Bondy CA: Selective coexpression of insulin receptor-related receptor (IRR) and TRK in NGF-sensitive neurons. *J Neurosci* 14:4674–4683, 1994
49. Recio-Pinto E, Lang FF, Ishii DN: Insulin and insulin-like growth factor II permit nerve growth factor binding and the neurite formation response in cultured human neuroblastoma cells. *Proc Natl Acad Sci U S A* 81:2562–2566, 1984
50. Ishii DN: Implication of insulin-like growth factors in the pathogenesis of diabetic neuropathy. *Brain Res Rev* 20:47–67, 1995
51. Lee K-F, Davies AM, Jaenisch R: p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. *Development* 120:1027–1033, 1994
52. Lee K-F, Li E, Huber LJ, Landis SC, Sharpe AH, Chao MV, Jaenisch R: Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. *Cell* 69:737–749, 1992

Author Queries (please see Q in margin and underlined text)

Q1: Edits to last part of sentence (“and, by treating...”) okay?
Q2: Please define NFL and NFM.
Q3: This is the only occurrence in the paper of $p75^{LNTR}$ (inclusion of the “L”). As meant?
Q4: Please supply city and state of Jackson Immunoresearch.
Q5: Sentence okay? Original was incomplete.
Q6: In Figure 4, B γ-PPT has 3 marks in the lower left hand corner that look like check marks. Would you like to explain these marks on the figure? If not, should they be removed?
Q7: Please provide first names for everyone listed in acknowledgments.
Ref. 1: Please provide the names of the authors for chapter 64