Ectopic *trkA* Expression Mediates a NGF Survival Response in NGF-Independent Sensory Neurons but not in Parasympathetic Neurons

Timothy E. Allsopp, Michelle Robinson, Sean Wyatt, and Alun M. Davies

School of Biological and Medical Sciences, Bute Medical Buildings, St. Andrews University, St. Andrews, Fife KY16 9AJ, United Kingdom

Abstract. We have investigated the role of *trkA*, the tyrosine kinase NGF receptor, in mediating the survival response of embryonic neurons to NGF. Embryonic trigeminal mesencephalic (TMN) neurons, which normally survive in the presence of brain-derived neurotrophic factor (BDNF) but not NGF, become NGF-responsive when microinjected with an expression vector containing *trkA* cDNA. In contrast, microinjection of ciliary neurotrophic factor (CNTF)-dependent embryonic ciliary neurons with the same construct does not result in the acquisition of NGF responsiveness by these neurons despite de novo expression of *trkA* mRNA and protein. The failure of *trkA* to result in an NGF-promoted survival response in ciliary neurons is not due to absence of the low-affinity NGF receptor, p75, in these neurons. Quantitative RT/PCR and immunocytochemistry showed that TMN and ciliary neurons both express p75 mRNA and protein. These findings not only provide the first direct experimental demonstration of *trkA* mediating a physiological response in an appropriate cell type, namely NGF-promoted survival of embryonic neurons, but indicate that not all neurons are able to respond to a *trkA*-mediated signal transduction event.

Work on NGF has substantiated the proposal that the survival of developing neurons depends on the supply of neurotrophic factors from their target fields and that the limited production of these factors results in the death of superfluous neurons (Davies, 1988*b*; Barde, 1989). NGF is synthesized in the target fields of NGF-dependent neurons during development (Davies et al., 1987*a*; Korsching and Thoenen, 1988) in relation to the final innervation density (Harper and Davies, 1990) and experimental manipulation of the availability of NGF shortly after these neurons innervate their targets influences the number of neurons that survive (Levi-Montalcini and Angeletti, 1968; Johnson et al., 1982).

NGF is a member of an homologous family of neurotrophic factors that comprises brain-derived neurotrophic factor (BDNF)[1] (Barde et al., 1982; Leibrock et al., 1989), neurotrophic factor-3 (Hohn et al., 1990; Maisonpierre et al., 1990; Ernfors et al., 1990; Rosenthal et al., 1990; Jones and Reichardt, 1990), *Xenopus* NT-4 (Hallbook et al., 1991), and mammalian NT-4/5 (Berkemeier et al., 1991; Ip et al., 1992*a*). The available evidence suggests that each of these factors promotes the survival of a distinctive set of embryonic neurons. For example, NGF promotes the survival of sympathetic neurons (Chun and Patterson, 1977; Greene, 1977), certain populations of sensory neurons (Davies and Lindsay, 1985), and basal forebrain cholinergic neurons (Hatanaka et al., 1988), whereas BDNF promotes the survival of NGF-insensitive sensory neurons (Davies et al., 1986*b*; Lindsay et al., 1985), retinal ganglion cells (Johnson et al., 1986), and the dopaminergic neurons of the substantia nigra (Hyman et al., 1991). In addition to members of the NGF family of small basic homodimeric proteins, several unrelated proteins are capable of promoting the survival of various neurons at particular stages of their development. Of these proteins, ciliary neurotrophic factor (CNTF), a small acidic protein, is the most extensively studied (Barbin et al., 1984; Lin et al., 1989; Stockli et al., 1989). This factor promotes the survival of a wide variety of embryonic neurons, including many of those supported by members of the NGF family of neurotrophic factors (Barbin et al., 1984; Ip et al., 1991; Buj-Bello, A., and A. M. Davies, unpublished findings).

Two kinds of transmembrane glycoproteins are receptors for members of the NGF family of neurotrophic factors: p75 (Chao et al., 1986; Johnson et al., 1986; Radeke et al., 1987) and members of the *trk* family of tyrosine kinases

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Address all correspondence to T. Allsopp, School of Biological and Medical Sciences, Bute Medical Buildings, University of St. Andrews, St. Andrews, Fife KY16 9AJ, Scotland.

[1] *Abbreviations used in this paper*: BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; DMTG, dorsomedial trigeminal ganglion; HIHS, heat-inactivated horse serum; RT/PCR, reverse transcription/PCR; TMN, trigeminal mesencephalic nucleus.

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(Kaplan et al., 1991*a*,*b*; Klein et al., 1991*a*,*b*, 1992; Nebreda et al., 1991; Berkemeier et al., 1991; Cordon et al., 1991; Glass et al., 1991; Hempstead et al., 1991; Lamballe et al., 1991; Meakin et al., 1992; Soppet et al., 1991; Squinto et al., 1991). Whereas p75 binds NGF, BDNF, and NT-3 with equal low-affinity (Sutter et al., 1979; Rodriguez-Tébar and Barde, 1990; Rodriguez-Tébar et al., 1992), *trk* tyrosine kinases exhibit a greater degree of specificity: *trkA* binds NGF, NT-3, and NT-4/5; *trkB* binds BDNF, NT-3, and NT-4/5 and *trkC* binds NT-3.

The demonstration that neurotrophic factors promote rapid transphosphorylation of *trk* tyrosine kinases (Kaplan et al., 1991*a*, *b*; Klein et al., 1991*a*, *b*; Soppet et al., 1991) and elicit a response from oocytes and cell lines transfected with *trk* cDNAs (Cordon et al., 1991; Glass et al., 1991; Lamballe et al., 1991; Loeb et al., 1991; Nebreda et al., 1991; Squinto et al., 1991) suggests that these tyrosine kinase receptors are directly involved in mediating the action of neurotrophic factor receptors. Although p75 is probably not a functional receptor on its own (Hempstead et al., 1989), several findings suggest that p75 is required for certain responses to neurotrophic factors. Membrane fusion and cell transfection experiments suggest that both p75 and *trk* have to be present for the formation of specific, functional, high-affinity NGF receptors (Hempstead et al., 1989, 1991; Matsushima and Bogenmann, 1990; Pleasure et al., 1990). PC12 cells transfected with chimeric receptors consisting of the extracellular domain of the EGF receptor and the transmembrane and intracellular domains of p75 extend neurites in response to EGF (Yan et al., 1991). Antisense p75 oligonucleotides interfere with kidney morphogenesis (Sariola et al., 1991) and retard an early maturational change in developing sensory neurons (Wright et al., 1992). A null mutation of the p75 gene in mice associated with a marked sensory deficit in homozygotes that is due to loss of cutaneous sensory nerve fibres (Lee et al., 1992). In contrast to these studies, prevention of NGF binding to p75 by either anti-p75 antiserum (Weskamp and Reichardt, 1991) or mutation of the NGF protein (Ibanez et al., 1992) does not apparently interfere with the action of NGF. Also, transfection of *trkA*, *trkB*, or *trkC* into 3T3 fibroblasts (a cell type lacking p75) results in the proliferation of these cells in response to neurotrophic factors (Cordon et al., 1991; Glass et al., 1991; Klein et al., 1992; Lamballe et al., 1991) suggesting that p75 is not essential for signal transduction.

To date, all cell transfection experiments using the *trk* family of neurotrophic factor receptors have been carried out in oocytes, PC12 pheochromocytoma cells and fibroblast cell lines. Although these studies have provided valuable data on the function and potential specificity of *trk* tyrosine kinase receptors, the mechanism of neurotrophic factor signal transduction leading to survival in neurons may differ from signal transduction associated with mitogenesis or differentiation in physiologically irrelevant cell types. For these reasons we have studied the role of *trkA* in mediating an appropriate cellular response to NGF in an appropriate cell type at the relevant stage of development. We have used two NGF-insensitive populations of cranial neurons at the stage when they are dependent on neurotrophic factors for survival. During the phase of naturally occurring neuronal death, the survival of the neural crest-derived proprioceptive neurons of the trigeminal mesencephalic nucleus (TMN) is promoted by BDNF (Davies et al., 1986*a*, *b*) and a muscle-derived factor (Davies, 1986) that may be NT-3 (Hohn et al., 1990). At the same stage of development, the survival of the neural crest-derived parasympathetic neurons of the ciliary ganglion is promoted by CNTF (Barbin et al., 1984). NGF does not, however, promote the survival of either TMN (Davies et al., 1987*b*) or ciliary (Helfand et al., 1976; Rohrer and Sommer, 1982) neurons in dissociated culture at this stage.

We have shown that microinjection of a *trkA* expression vector into two different populations of NGF-insensitive neurons leads to an NGF-promoted survival response from only one of these despite the expression of *trkA* and p75 mRNA and protein in both populations. Our results directly demonstrate that *trkA* mediates the survival response of embryonic neurons to NGF, but show that not all neurotrophic factor-dependent neurons are able to survive in response to NGF even if they express both components of the NGF receptor.

Materials and Methods

Materials

Purified recombinant human NGF, human BDNF, and rat CNTF were gifts of Gene Burton, John Winslow, and Dave Shelton (Genentech Inc., San Francisco, CA). These factors were used at a final concentration of 2 ng/ml, which is the maximally effective concentration of BDNF and CNTF for supporting the survival of E10 TMN and ciliary neurons, respectively, and is the maximally effective concentration of NGF for supporting the survival of the NGF-dependent dorsomedial trigeminal ganglion (DMTG) neurons (data not shown). A full-length rat *trkA* cDNA was a gift of Susan Meakin, Philip Barker, and Eric Shooter (Stanford University, Stanford, CA). Anti-chicken p75 antiserum was a gift of Gisela Weskamp (University of California, San Francisco, CA). An anti-*trkA* antiserum that recognizes the COOH-terminal receptor kinase domain (Kozma et al., 1988) was a gift of Aviva Tolkovsky (Oxford University, Oxford). Anti-chicken 70-kD neurofilament antiserum was a gift of Diana Moss (Liverpool University, Liverpool, UK). PCR primers were synthesized on an Applied Biosystems PCR-Mate DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). Poly-ornithine and EHS laminin were purchased from Sigma Chemical Co. (St. Louis, MO). All tissue culture dishes and reagents were obtained from GIBCO BRL (Gaithersburg, MD).

Cell Culture

Fertile white Leghorn chicken eggs were incubated at 38°C in a forced-draft incubator. After 10 d of incubation (E10), ciliary ganglia and the midline part of the TMN were dissected from the embryos using electrolytically sharpened tungsten needles (Davies, 1988*a*). After incubation with 0.1% trypsin in calcium and magnesium-free HBBS for 9 min at 37°C, the dissected tissue was washed twice in Ham's F12 medium plus 10% heat-inactivated horse serum (HIHS) and was dissociated by gentle trituration using a siliconised, fire-polished Pasteur pipette. Nonneuronal cells were removed by differential sedimentation through a precooled column of Ham's F14 medium containing 10% HIHS (Davies, 1988*a*). The column fractions containing the neurons were centrifuged at 2,000 *g* for 5 min and the neurons were plated in 60-mm-diameter tissue culture dishes that had been coated with polyornithine (0.5 μg/ml in 0.15 M borate buffer, pH 8.7, overnight) and laminin (20 μg/ml in F14 medium, 4 h). The cells were cultured in 5 ml of Ham's F14 medium supplemented with 10% HIHS, 5% heat-inactivated FCS (HIFCS), penicillin (60 mg/l), streptomycin (100 mg/l), and 24 mM $NaHCO_3$, with or without neurotrophic factors, at 37°C in a humidified 4% CO_2 incubator.

Cell Microinjection

Before injection, the neurons were washed (3 × 3 ml washes over 10 min) with warm F12 medium supplemented with 10% HIHS plus 5% HIFCS and were placed in this medium without neurotrophic factors on the stage of a Nikon Diaphot inverted microscope (Nikon Inc., Melville, NY). The microscope stage and a Narishige micromanipulator (Narishige USA, Inc., Greenvale, NY) that was attached to the microscope were enclosed within a humidified incubator chamber that was maintained at 37°C. Cell injection pipettes (GD-1; Narishige) were pulled on a Campden Instruments moving coil puller. All neurons within an area that was marked on the inside of the culture dish were pressure-injected with either the sense or antisense pCMX-*trkA* construct (rat *trkA* cDNA subcloned into the XbaI site of the pCMX expression vector in both orientations with respect to the constitutively active CMV promoter) at a concentration of 100 μg/ml in PBS. Each neuron was injected until slight cell swelling was observed, which usually took place within a few seconds. More than 95% of the neurons that received intra-nuclear injection were still surviving 1 h after injection. Neurons within a separate marked area of the same culture dishes served as uninjected control cells. After injection, the neurons were washed (3 × 3 ml washes over 10 min) with warm F14 culture medium and cell counts were carried out to determine the initial sizes of the injected and uninjected populations. The numbers of neurons surviving 24 and 48 h after injection were counted and are expressed as a percentage of the postinjection number.

Immunocytochemistry

Cells were stained for *trkA* protein between 24 and 48 h after injection as follows. The cultures were washed twice with warm serum-free F14 medium and were fixed with 4% paraformaldehyde for 30 min at room temperature. After washing twice with PBS, the cells were permeabilized with 0.3% Triton X-100 in PBS for 20 min and were incubated with 10% HIHS in PBS for a further 30 min (all at room temperature). The dish area surrounding the neurons to be stained was dried and 150–200 μl of anti-*trk* antiserum (1:400 dilution in PBS plus 1% HIHS) was applied for 60 min at room temperature. After washing with PBS (3 × 5 ml over 10 min), the neurons were incubated with Texas red–conjugated goat anti-rabbit IgG antibody (1:750 dilution in PBS plus 1% HIHS) for 60 min at room temperature. This secondary antiserum had been extensively absorbed to remove cross-species reacting antibodies. Finally, the neurons were washed with PBS and were mounted in Citifluor beneath glass coverslips. Immunofluorescent cells were viewed with a Nikon diaphot microscope equipped with epifluorescence and were photographed using Kodak T-Max 400 film (Eastman Kodak Co., Rochester, NY). No immunofluorescence was observed in cultures that were stained with second antisera alone.

Cell-surface expression of p75 was detected using a rabbit anti–chicken p75 antiserum (gift of Gisela Weskamp). Paraformaldehyde-fixed cultures were incubated for 1 h at room temperature with 5 μg/ml of this antiserum in PBS plus 1% normal rabbit serum. After washing, the cultures were incubated for 60 min with a 1:100 dilution of FITC-conjugated goat anti–rabbit IgG secondary antibody. After washing with PBS, the cultures were photographed as described above. No immunofluorescence was observed in cultures that were stained with second antisera alone.

Measurement of p75 mRNA in TMN and Ciliary Neurons

A quantitative reverse transcription/PCR (RT/PCR) technique was used to determine if TMN and ciliary neurons express p75 mRNA. This technique is based on the co-reverse transcription and co-amplification of p75 mRNA and a slightly larger p75 RNA standard. Because the target mRNA and control RNA are present in the same reverse transcription and PCR amplification reactions and use the same primers, the effect of the numerous variables that influence the efficiency of amplification is nullified; the relative amounts of the amplified products reflect the relative concentrations of the p75 mRNA and p75 control RNA in the original reaction mixture.

Differential sedimentation (Davies, 1986) was used to purify neurons (>98% enrichment) from dissociated cell suspensions of the TMN and ciliary ganglia. Total RNA (Chomczynski and Sacchi, 1987) was reverse transcribed using GIBCO BRL Superscript enzyme in the supplied buffer plus 0.5 mM dNTPs and 10 μM random hexanucleotides for 45 min at 37°C. 10-μl aliquots were added to 50-μl PCR reactions containing NBL Taq DNA polymerase in the supplied buffer adjusted to 1.85 mM $MgCl_2$ plus 0.1 mM dNTPs and 6 μl of ^{32}P-labeled oligonucleotide primers. The primers were: (5′) 5′-CCTGTGTACTGCTCTATCCTGG-3′ and (3′) 5′-TTGTTCTGCTTGCAGCTGTTCC-3′. These hybridize 100 bp apart in the chicken p75 sequence (Large et al., 1989). These primers were synthesized on an Applied Biosystems DNA synthesizer and were labeled at their 5′ ends in a 40-μl reaction containing 4 μl of 10 × T4 polynucleotide kinase buffer (0.5 M TRIS, pH 7.6, 100 mM $MgCl_2$, 1 mM spermidine, 50 mM DTT, 1 mM EDTA, pH 8), 2 μg of each primer, 30 μl γ^{32}P-ATP (3,000 Ci/mmol, 10 mCi/ml) and 4.5 μl of T4 polynucleotide kinase (10 U/μl). The reaction was mixed thoroughly and incubated at 37°C for 45 min, after which a further 5 μl of polynucleotide kinase were added, followed by a further 45-min incubation at 37°C. The labeled primers were purified from the reaction mixture using the Stratech Mermaid kit. 135 μl of high-salt binding solution (supplied with the kit) were added to the reaction followed by 50 μl of Mermaid glassfog. This was mixed thoroughly and left for 15 min at room temperature to permit the primers to bind to the glassfog. The mixture was microfuged briefly to sediment the glassfog plus bound primers. After washing the glassfog twice with the solution provided, the primers were eluted into 250 μl of water by incubation at 60°C for 10 min. 6 μl of this solution were used per PCR reaction. p75 cDNA was amplified by eight cycles of 94°C for 60 s, 54°C for 90 s, and 72°C for 45 s followed by 15 cycles of 91°C for 60 s, 52°C for 120 s, and 72°C for 120 s. There was a final 72°C incubation for 10 min.

The above conditions are optimal for reverse transcription and amplification of 25–50 fg of p75 gene run-off transcripts such that the rate of reaction does not plateau. PCR products were analyzed after electrophoresis of reaction aliquots on 7% acrylamide/TBE gels and autoradiography. The level of p75 mRNA in the extracted RNA was quantified by co-amplifying with standard run-off transcripts from the p75 cDNA (gift of Tom Large) which have an additional 4 bp insert between the primer annealing sites. 50 fg standard transcripts were added to RNA from approximately 50 E10 TMN and 50 ciliary neurons. Densitometry was used to determine the level of p75 mRNA by comparing the relative intensities of the p75 mRNA and p75 RNA standard bands.

To compare the relative level of p75 mRNA in TMN and ciliary neurons, the level of the mRNA encoding the ubiquitous, constitutively expressed L27 ribosomal protein was also measured in RNA samples from TMN and ciliary neurons by a quantitative PCR amplification technique. The level of p75 mRNA in samples could then be expressed relative to the level of L27 mRNA in these samples. The experimental error of this method was consistently smaller than that of relating the level of p75 mRNA to the number of neurons in cell pellets determined by haemocytometry. To determine the level of L27 mRNA, 10-μl aliquots of reverse transcribed neuronal total RNA were added to 50-μl PCR reactions containing NBL Taq DNA polymerase in the supplied buffer plus 5 μl of the following labeled primers: (5′) 5′-GGCTGTCATCGTGAACAT-3′ and (3′) 5′-CTTCGCTATCTTCTTCTTGCC-3′. These hybridize 127 bp apart in the chicken L27 sequence (Lebeau et al., 1991) and were labeled as described for the p75 primers. L27 cDNA was amplified by eight cycles of 94°C for 60 s, 60°C for 105 s, and 72°C for 45 s followed by 17 cycles of 91°C for 60 s, 58°C for 180 s, and 72°C for 180 s. There was a final 72°C incubation for 10 min.

The above conditions are optimal for reverse transcription and amplification of 500 fg of L27 gene run-off transcripts such that the rate of reaction does not plateau. PCR products were analyzed after electrophoresis of reaction aliquots on 7% acrylamide/TBE gels and autoradiography. The level of L27 mRNA in the extracted RNA was quantified by co-amplifying with standard run-off transcripts from the L27 cDNA which have an additional 4-bp insert between the primer annealing sites. 360 bp of L27 cDNA was obtained by reverse transcription of total RNA from E11 chicken dorsal root ganglia and subsequent PCR amplification using primers based on the published L27 cDNA sequence (Lebeau et al., 1991). 500-fg standard transcripts were added to varying amounts of total RNA from E10 TMN and ciliary neurons. Samples in which mRNA and standard bands were of similar intensity were used to calculate L27 mRNA in each RNA sample. Autoradiographs were scanned with a Personal Densitometer (Molecular Dynamics Inc., Kent, England). The levels of p75 mRNA and L27 mRNA were determined by calculating the ratios between the amplification products of these mRNAs and their corresponding RNA standard amplification products and multiplying the known amounts of the initial RNA standards by these ratios. The results are expressed as the quotient of the amount of p75 mRNA and the amount of L27 mRNA in RNA extracted from TMN or ciliary neurons.

Measurement of trkA mRNA in Injected TMN and Ciliary Neurons

A RT/PCR technique was also used to determine if TMN and ciliary neurons express *trkA* mRNA after injection with the pCMX-*trkA* construct. Total RNA extracted from injected neurons was reverse transcribed as described above. The level of *trkA* mRNA was quantified by co-amplifying with a run-off transcript from a rat *trkA* cDNA that had an additional 3-bp insert between the primer annealing sites. 50 fg of standard transcripts were added to RNA from approximately equal numbers of injected E10 TMN and ciliary neurons. The PCR products were analyzed by electrophoresis of reaction products on 7% acrylamide/TBE gels and autoradiography. The primers for *trkA* were: forward 5'-CGTCATGGCTGCTTTTATGG-3' and reverse 5'-ACTGGCGAGAAGGAGACAG-3'. The cycling conditions were: 60 s at 94°C, 60 s at 55°C, 60 s at 72°C for 22 cycles. The relative levels of *trkA* mRNA in injected ciliary and TMN neurons were assessed by comparing ratios of standard RNA to *trkA* mRNA amplification products in RNA extracted from these neurons.

Results

To investigate the role of *trkA* in mediating the survival response of embryonic neurons to NGF, we microinjected the NGF-insensitive TMN and ciliary ganglion neurons with the pCMX vector containing *trkA* cDNA to see if *trkA* expression would permit these neurons to respond appropriately to NGF. Experiments were carried out at the tenth day of embryonic development (E10) when the survival of these neurons depends on BDNF and CNTF, respectively. To devise a suitable injection protocol for the experiments, we studied the rate at which E10 TMN and ciliary neurons die in culture when deprived of neurotrophic factors. When cultured in the absence of nonneuronal cells in medium without added neurotrophic factors, >80% of these neurons died within 12 h. Similarly, if TMN and ciliary neurons were grown for 12 h with BDNF and CNTF, respectively, and deprived of these factors by extensive washing they also died rapidly. In these circumstances, 10% or less of the neurons survived 48 h after neurotrophic factor deprivation (data not shown). The finding that neurons could be initially maintained in culture with neurotrophic factors and died rapidly after their removal was particularly advantageous for microinjection experiments. This facilitated the preparation of large numbers of healthy neurons that were well attached to the culture substratum for microinjection. It also provided a very sensitive assay for ability of *trkA* to rescue neurons growing with NGF because virtually all of the neurons in control cultures died 48 h after neurotrophic factor deprivation.

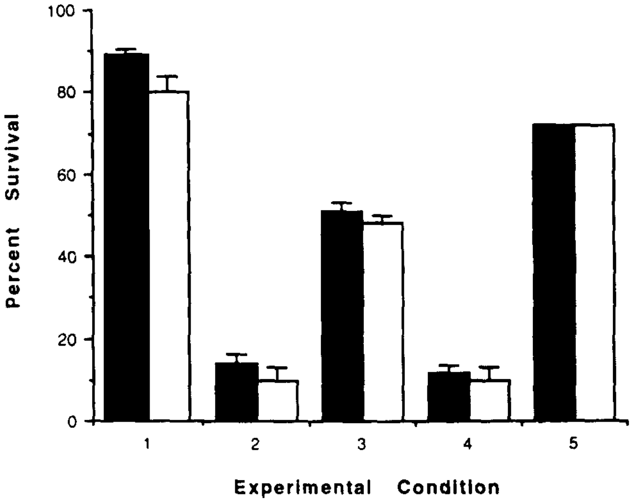

Figure 1. E10 TMN neurons survive with NGF after injection of *trkA* vector. Bar chart of the percent survival of E10 TMN neurons. After 12 h of incubation with saturating levels of BDNF (2 ng/ml), the neurons were deprived of BDNF by extensive washing. The neurons were then treated in one of several ways: resupplemented with BDNF (column set *1*), grown without neurotrophic factor (column set 2), microinjected with the pCMX expression vector containing *trkA* cDNA in the sense orientation and grown with 2 ng/ml NGF (column set *3*), microinjected with the pCMX expression vector containing *trkA* cDNA in the antisense orientation and grown with 2 ng/ml NGF (column set *4*), or microinjected with the pCMX expression vector containing *trkA* cDNA in the sense orientation and grown with 2 ng/ml BDNF (column set *5*). Neuronal survival was assessed 24 (■) and 48 (□) h after initial BDNF deprivation and is expressed as a percentage of the number of neurons in the corresponding cultures at the time of BDNF deprivation. The mean (bar height) and standard error (error bars) of the results from five experiments are shown (~1,300 injected neurons).

TMN Neurons Survive in Response to NGF After Injection with the pCMX/trkA Expression Vector

When TMN neurons were grown for 12 h in the presence of BDNF and deprived of this factor by extensive washing, only 10% of the neurons survived for the next 48 h in NGF-supplemented cultures compared with 80% in cultures re-supplemented with BDNF (Fig. 1). The small number of neurons surviving with 2 ng/ml NGF was not significantly different from the number surviving in NGF-free culture medium (data not shown). Furthermore, concentrations of NGF up to 5 μg/ml did not promote the survival of TMN neurons above this low background level (data not shown). When TMN neurons were injected with pCMX/*trkA* expression vector immediately after BDNF deprivation and grown with 2 ng/ml NGF, almost 50% of the neurons survived for the next 48 h. This response of injected neurons to NGF was not due to some non-specific effect of the injection because neurons that were injected with the pCMX vector that had the *trkA* cDNA subcloned in the antisense orientation did not show an enhanced survival response when grown with NGF (Fig. 1). Approximately 70% of the neurons injected with the *trk* expression vector survived when grown in medium re-supplemented with BDNF (Fig. 1). This showed that microinjection and *trk* expression were not in themselves detrimental to TMN neurons. Despite this, however, we were never able to achieve >50% survival of injected TMN neurons cultured with NGF (five experiments; ~1,300 injected neurons). Exposure of pCMX/*trkA*-injected TMN neurons to super-saturating levels of NGF (2 μg/ml) failed to increase the number of surviving neurons (data not shown), suggesting that sub-optimal receptor occupancy could not account for the lower survival response of these cells to NGF.

To demonstrate that *trkA* protein is synthesized in TMN neurons from the injected *trkA* cDNA template, we used an antiserum that recognizes the kinase domain of this protein (Kozma et al., 1988). This antibody was raised against the carboxy-terminal kinase domain of human *trkA* and immunoprecipitates from membrane extracts of sympathetic neurons a 140-kD phosphotyrosine glycoprotein that binds iodinated NGF (A. Tolkovsky, personal communication). The majority of pCMX/*trkA*-injected TMN neurons exhibited intense staining of the cell body and distinct, though faint, staining of neurites 12 or 24 h after injection (Fig. 2). Uninjected neurons and neurons injected with vector alone or vector with *trkA* cDNA subcloned in the antisense orientation exhibited only very faint immunoreactivity restricted to the cell body. Although there was some variation in staining intensity of the cell body among injected neurons, the

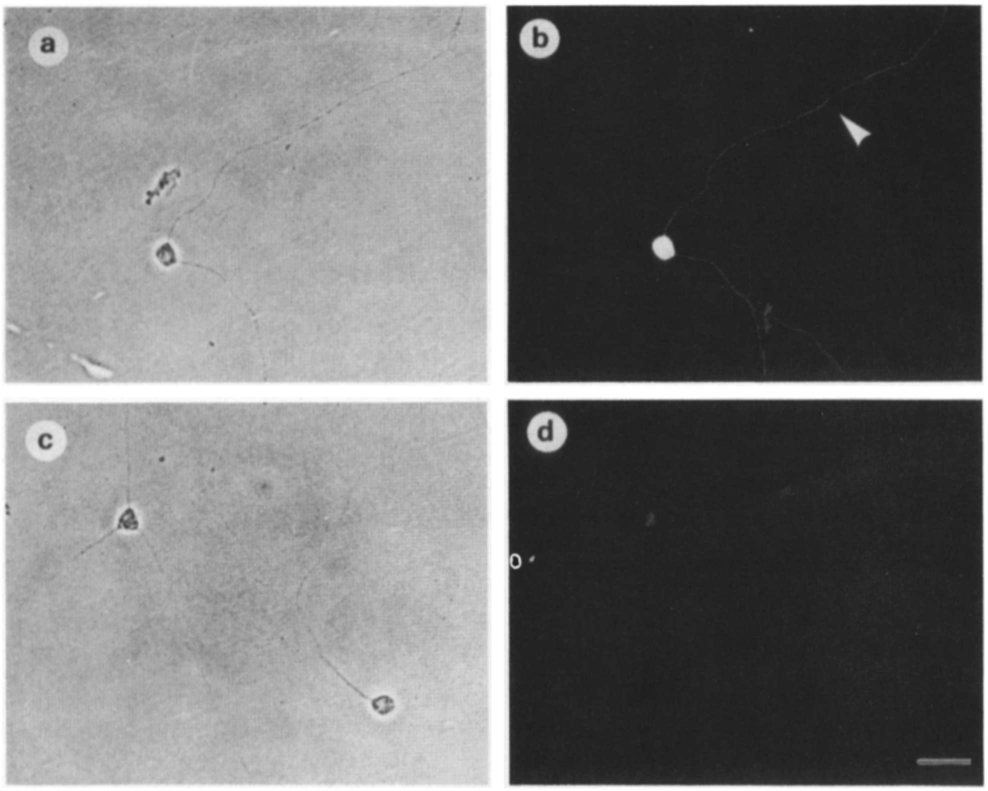

Figure 2. TMN neurons injected with the pCMX/*trkA* expression vector synthesize *trkA* protein. Phase contrast (*a* and *c*) and corresponding fluorescent images (*b* and *d*) of E10 TMN neurons. The neurons were either injected with the pCMX/*trkA* expression vector and grown with 2 ng/ml NGF (*a* and *b*), or remained uninjected and grown with 2 ng/ml BDNF (*c* and *d*) for 24 h. The cells were then fixed, permeabilized and stained for *trkA* protein using an antiserum that recognizes the kinase domain of the protein. The injected TMN neurons exhibited intense staining of the cell body (*b*) and distinct, though faint, staining of neurites (*b*; *white arrowhead*). Some of the uninjected neurons exhibited a very faint immunoreactivity restricted to the cell body (*d*). The staining intensity of all injected neurons was much greater than the very low level of staining observed in uninjected neurons. Neither injected nor uninjected neurons were stained by secondary antibody alone. Bar, 100 μm.

staining intensity of all injected neurons was clearly greater than the very low level of staining observed in uninjected neurons. Additionally, uninjected neurons never displayed neurite staining. At the resolution of our microscopy, however, we were not able to ascertain whether *trkA* staining was localized predominantly to the plasma membrane, as would be required for NGF binding. Neither injected nor uninjected neurons were stained by secondary antibody alone. It is likely that the very low level of immunoreactivity in uninjected neurons may have been due to cross-reactivity of the antiserum with the highly conserved kinase domains of other *trk* tyrosine kinase family members.

Because only pCMX/*trkA*-injected neurons exhibited *trkA* immunoreactivity in neurites, we were able to compare the proportion of pCMX/*trkA*-injected neurons that showed positive neurite staining when grown in the presence of either NGF or BDNF. Table I shows that the majority of TMN neurons showed neurite staining when grown under either condition.

Ciliary Neurons Do Not Survive in Response to NGF After Injection with the pCMX/trkA Expression Vector

When ciliary neurons were grown for 12 h with CNTF and deprived of this factor by extensive washing, <10% of the neurons survived for the next 48 h in NGF-supplemented cultures compared with 60% surviving in cultures resupplemented with CNTF (Fig. 3). As in TMN cultures, the very small number of ciliary neurons surviving with 2 ng/ml NGF was not significantly different from the number surviving in NGF-free culture medium. In contrast to TMN neurons, NGF failed to rescue ciliary neurons injected with the *trkA* expression vector immediately after CNTF deprivation. There was negligible difference in the number of surviving injected and uninjected ciliary neurons grown with 2 ng/ml NGF for 48 h. The lack of an NGF survival response from pCMX/*trkA*-injected ciliary neurons was not due to the trauma of injection or to some nonspecific detrimental effect

Table I. Similar Percentages of TMN and Ciliary Neurons Express trkA Immunostaining in Neurites After Injection with the pCMX/trkA Expression Vector

	Factor	Percent survival	Percent with stained neurites
TMN neurons	BDNF	79 ± 5	72 ± 11
	NGF	63 ± 13	70 ± 4
Ciliary neurons	CNTF	81 ± 7	61 ± 6
	NGF	17 ± 2	43 ± 13

TMN and ciliary neurons were grown for 12 h with BDNF and CNTF, respectively, before being deprived and injected with the *trkA*/pCMX expression vector. The neurons were then grown for a further 24 h with either the same neurotrophic factor or NGF. The surviving neurons were fixed and processed for immunocytochemistry using the anti-*trkA* antiserum. Neurons scored positive for *trkA* immunostaining if they displayed neurite staining. The number of neurite-stained neurons is expressed as a percentage of the surviving neuron population. The neurons surviving are expressed as a percentage of the starting neuronal population at the time of injection. Data are compiled from two independent experiments each of which was set up in duplicate; the mean ± standard deviation are shown.

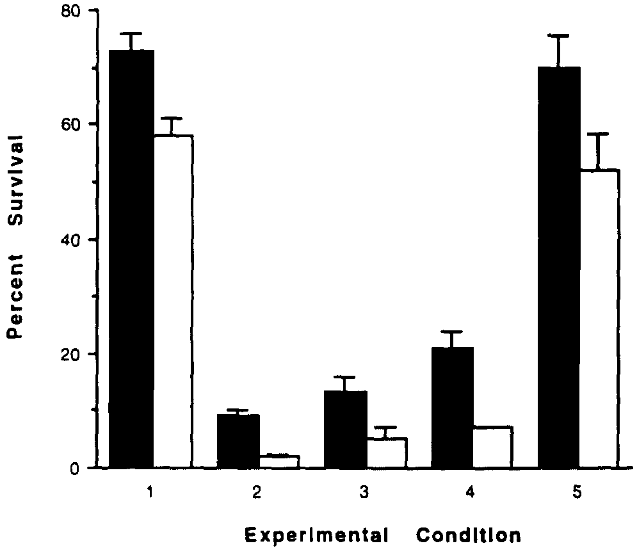

Figure 3. Ciliary neurons do not survive with NGF after injection with the pCMX/*trkA* expression vector. Bar chart of the percent survival of E10 ciliary neurons. After 12 h incubation with saturating levels of CNTF (3 ng/ml), the neurons were deprived of CNTF by extensive washing. The neurons were then treated in one of several ways: resupplemented with CNTF (column set *1*), grown without neurotrophic factor (column set *2*), microinjected with the pCMX expression vector containing *trkA* in the sense orientation and grown with 2 ng/ml NGF (column set *3*), microinjected with the pCMX expression vector containing *trkA* in the antisense orientation and grown with 2 ng/ml NGF (column set *4*), or microinjected with the pCMX expression vector containing *trkA* in the sense orientation and grown with 3 ng/ml CNTF (column set *5*). Neuronal survival was assessed 24 (■) and 48 (□) h after initial CNTF deprivation and is expressed as a percentage of the number of neurons in the corresponding cultures at the time of CNTF deprivation. The mean (bar height) and standard error (error bars) of the results are from two experiments (~800 injected neurons).

of the pCMX/*trkA* construct in ciliary neurons because pCMX/*trkA* injection did not affect the survival response of ciliary neurons to CNTF; virtually the same proportion of injected and uninjected ciliary neurons survived for 48 h in medium containing CNTF (Fig. 3).

pCMX/trkA-injected TMN and Ciliary Neurons Express Rat trkA mRNA and Protein

To determine if *trkA*-expressing TMN and ciliary neurons differ in their ability to express *trkA* mRNA after injection with the pCMX/*trkA* vector, we used RT/PCR amplification of *trkA* mRNA from TMN and ciliary neurons that had been injected with the pCMX/*trkA* expression vector and maintained in NGF for 24 h. PCR primers were designed according to the published rat *trkA* cDNA sequence (Meakin et al., 1992). RT/PCR was performed on aliquots of RNA extracted from equal numbers of injected ciliary and TMN neurons and the efficiency of the RT/PCR reactions was monitored by the inclusion of a known amount of a slightly smaller *trkA* RNA transcript. Fig. 4 (lanes *3* and *5*) shows that there was no obvious difference in the ratio between the amplification products of *trkA* mRNA and *trkA* RNA standard, indicating that *trkA* mRNA was transcribed with equal efficiency from the injected pCMX/*trkA* vector in both ciliary and TMN neurons. No *trkA* mRNA was detected in uninjected E10 chicken ciliary and TMN neurons maintained with CNTF or BDNF (Fig. 4, lanes *4* and *6*) nor in E10 chicken DMTG neurons which respond to NGF (lane *7*). This shows that the primers are selective for mammalian rather than chicken *trkA* under the stringent amplification conditions used.

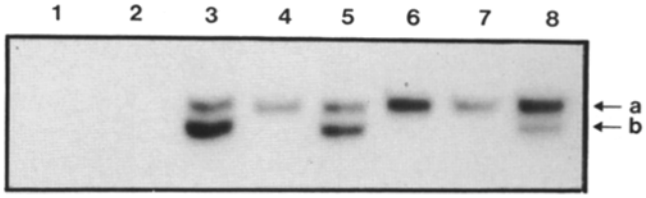

Figure 4. TMN and Ciliary neurons injected with the pCMX/*trkA* expression vector express equivalent amounts of *trkA* mRNA. Autoradiograph of PCR amplification products electrophosed on a 7% nondenaturing polyacrylamide gel. Total RNA from equal numbers of injected and uninjected E10 TMN and ciliary neurons grown for 24 h with NGF was reverse transcribed and a 79-bp fragment of *trkA* cDNA was amplified by PCR using end-labeled *trkA*-specific primers (*arrow b*). In each case 50 fg of a *trkA* cRNA control template with a 3-bp insert between primer annealing sites was added to reverse transcription reactions prior to amplification to generate an 82-bp fragment (*arrow a*). Lane *1*, no reverse transcriptase, ciliary neuron RNA; lane *2*, no RNA added; lane *3*, RNA from injected ciliary neurons; lane *4*, RNA from uninjected ciliary neurons; lane *5*, RNA from injected TMN neurons; lane *6*, RNA from uninjected TMN neurons; lane *7*, RNA from an equivalent number of freshly isolated and purified E10 chicken DMTG neurons; lane *8*, RNA from 25 times more freshly isolated and purifed E14 mouse trigeminal neurons. No products were detected in total RNA extracted from injected neurons in the absence of reverse transcriptase (not shown).

To compare the relative levels of *trkA* mRNA in pCMX/*trkA*-injected chicken neurons with the endogenous levels of *trkA* mRNA in neurons that normally depend on NGF for survival, we estimated the relative level of *trkA* mRNA in E14 mouse trigeminal neurons, >90% of which depend on NGF for survival (Buchman and Davies, 1993). We were able to use the same PCR primers as used for rat *trkA*, because the mouse and rat *trkA* nucleotide sequences are identical in the primer annealing sites (Martin-Zanca et al., 1990; Meakin et al., 1992). When RNA from pCMX/*trkA*-injected ciliary and TMN neurons was amplified alongside RNA from a 25-fold greater number of purified E14 trigeminal neurons, the *trkA* mRNA signal was greater in RNA from injected chicken neurons compared with RNA from mouse trigeminal neurons (Fig. 4; compare lanes *3* and *5* with lane *8*). Thus, *trkA* mRNA is expressed from injected pCMX/*trkA* template in ciliary and TMN neurons at a level that is at least 25 times greater than in NGF-dependent trigeminal neurons.

To determine if the lack of an NGF survival response from pCMX/*trkA*-injected ciliary neurons was due to failure of *trkA* protein expression in these neurons, we used immunocytochemistry to determine if *trkA* protein was translated from the rat *trkA* mRNA template expressed in pCMX/*trkA*-injected ciliary neurons (Fig. 5). As in the case of pCMX/*trkA*-injected TMN neurons, the majority of pCMX/*trkA*-injected ciliary neurons exhibited intense staining of the

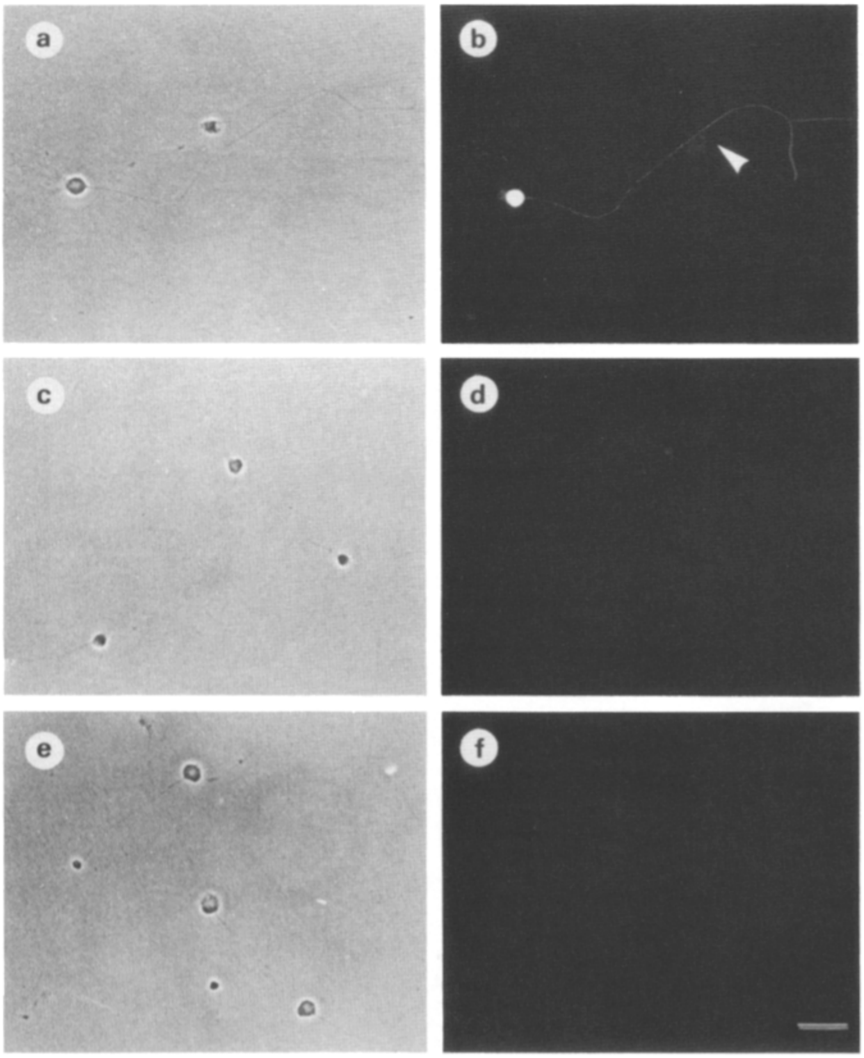

Figure 5. Ciliary neurons express *trkA* protein following injection despite showing no NGF survival response. Phase contrast (*a*, *c*, and *e*) and corresponding fluorescent images (*b*, *d*, and *f*) of E10 ciliary neurons. The neurons were either injected with the pCMX/*trkA* expression vector and grown for 24 h with 3 ng/ml CNTF (*a* and *b*) or remained uninjected and grown for 24 h with 3 ng/ml CNTF (*c–f*). The neurons were then fixed, permeabilized and stained for *trkA* protein (*a–d*) or exposed to Texas red–conjugated second antibody alone (*e* and *f*). The injected ciliary neurons exhibited intense staining of the cell body (*b*) and distinct, though faint, staining of neurites (*white arrowhead*), identical to the staining pattern observed for TMN neurons injected with the pCMX/*trkA* expression vector (see Fig. 2 *b*). Some uninjected neurons exhibited very faint immunoreactivity restricted to the cell body (*d*). As for TMN neurons injected with the pCMX/*trkA* expression vector, the staining intensity of all ciliary neurons injected with the pCMX/*trkA* expression vector was much greater than the low level of staining observed in uninjected neurons. Neither uninjected neurons (*f*) nor injected neurons (data not shown) were stained by secondary antibody alone. Bar, 100 μm.

cell body and faint staining of neurites when grown for 24 h with CNTF after injection. As for injected TMN neurons, we were unable to ascertain whether *trkA* was localized predominantly to the plasma membrane of injected ciliary neurons. Furthermore, there may have been quantitative differences in the level of *trkA* protein expression in injected ciliary and TMN neurons that we could not resolve by fluorescence microscopy. As with uninjected TMN neurons, uninjected ciliary neurons never displayed neurite staining and exhibited only very faint staining of their cell bodies with the anti-*trkA* tyrosine kinase antiserum. Neither injected nor uninjected ciliary neurons were stained by secondary antibody alone. Although only a minority of pCMX/*trkA*-injected ciliary neurons survived in NGF after 24 h, many of these (43% ± 13) showed visible neurite staining (Table I). There was no significant difference in the percentage of pCMX/*trkA*-injected TMN neurons with positive neurite staining grown in the presence of BDNF compared with the percentage of pCMX/*trkA*-injected ciliary neurons with positive neurite staining grown in the presence of CNTF (72 ± 11 and 61 ± 6, respectively). A small difference was observed in the number of neurite-stained ciliary neurons grown with CNTF versus NGF (61% ± 6 versus 43% ± 13, respectively). This was probably a reflection of the reduced viability of pCMX/*trkA*-injected ciliary neurons grown in the presence of NGF as these neurons die rapidly under these conditions.

TMN and Ciliary Neurons Express p75 mRNA and Protein

To investigate if the lack of an NGF survival response from pCMX/*trkA*-injected ciliary neurons was due to the absence of the low-affinity NGF receptor, p75, we used RT/PCR and immunocytochemistry to determine if p75 mRNA and p75 protein are expressed in ciliary neurons. Using PCR primers based on the chicken p75 sequence (Large et al., 1989), preliminary studies generated a specific p75 amplification product from reverse transcribed RNA extracted from E10 ciliary neurons that had been purified to >95% neurons by differential sedimentation (Davies et al., 1986*b*). Detailed quantitative comparisons in the absolute levels of p75 mRNA between ciliary and TMN neurons were then undertaken. For these studies, total RNA was extracted from samples of purified ciliary and TMN neurons whose numbers had been estimated by haemocytometry. To further correct for any differences in cell number between ciliary and TMN neuron samples, the level of the ubiquitous, constitutively expressed L27 ribosomal protein mRNA was measured in these samples by quantitative RT/PCR amplification. RT/PCR reactions were carried out on aliquots of extracted RNA that were estimated to have the same numbers of ciliary and TMN neurons (Fig. 6). These results clearly showed that p75 mRNA

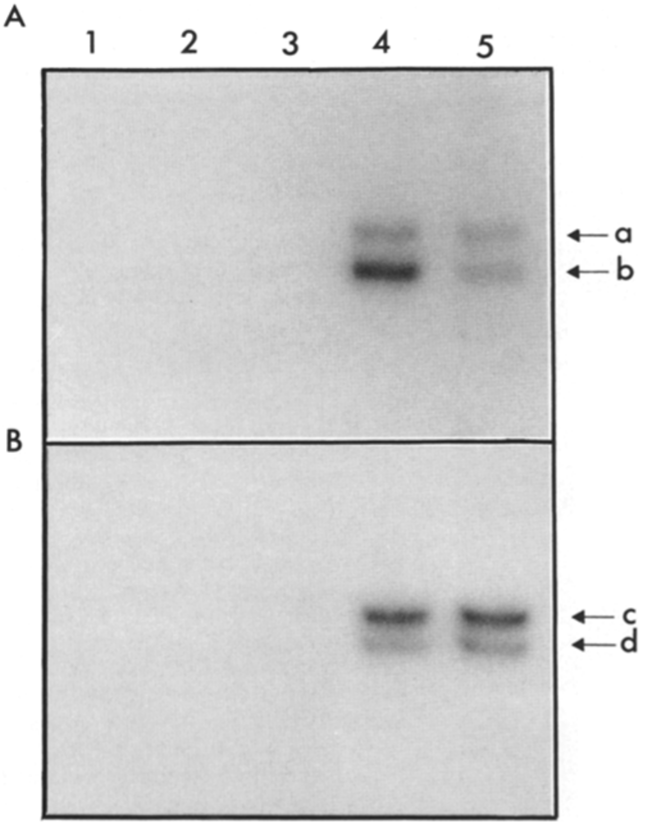

Figure 6. TMN and Ciliary neurons express p75 mRNA. Autoradiographs of PCR amplification products electrophosed on a 7% nondenaturing polyacrylamide gel. Total RNA from approximately equal numbers of E10 TMN and ciliary neurons was reverse transcribed using end-labeled p75-specific primers (*A*) and an 88-bp fragment of p75 cDNA was amplified by PCR (*arrow b*). For quantitative RT/PCR, 25 fg of a p75 cRNA control template was added to extracted RNA prior to reverse transcription and amplification. This control cRNA had an additional 4-bp insert between the primer annealing sites, generating a 92-bp fragment (*arrow a*). Lane *1*, no reverse transcriptase (TMN neuron RNA); lane *2*, no reverse transcriptase (ciliary neuron RNA); lane *3*, no RNA added; lane *4*, TMN neuron RNA; lane *5*, ciliary neuron RNA. To verify that equal amounts of total RNA from each tissue were added to the initial reverse transcription reactions, RT/PCR was used to measure the amount of mRNA for the L27 ribosomal protein using L27-specific end-labeled primers (*B*). This generated a 127-bp fragment (*arrow d*). These reactions included 500 fg of an L27 cRNA control template that had a 4-bp insert between the primer annealing sites and yielded a 131-bp fragment (*arrow c*).

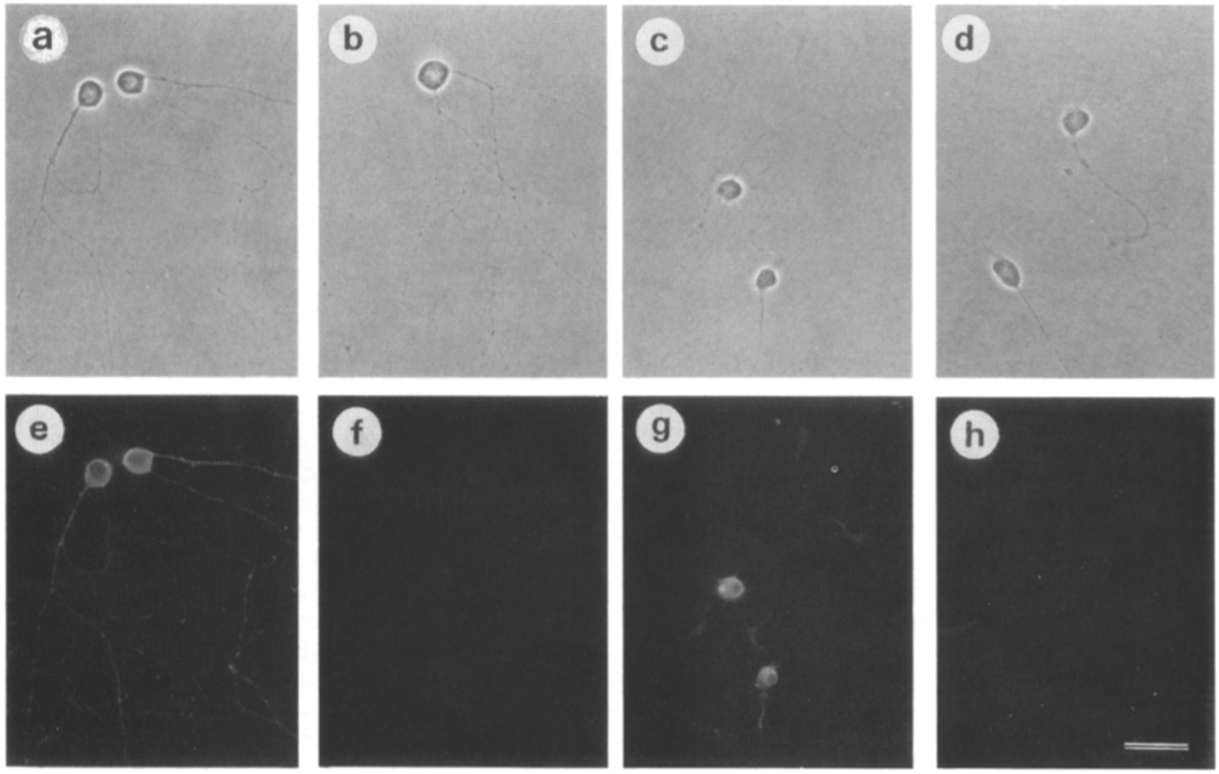

Figure 7. TMN and Ciliary neurons express p75 protein. Phase contrast (*a–d*) and corresponding fluorescent images (*e–h*) of E10 TMN neurons (*a*, *b*, *e*, and *f*) and E10 ciliary neurons (*c*, *d*, *g*, and *h*) immuno-stained for p75. After the neurons were grown for 36 h in either BDNF (TMN neurons) or CNTF (ciliary neurons), they were fixed in 4% formaldehyde for 30 min. They were then washed extensively and were incubated with either anti-p75 antiserum (1:1000 in PBS) followed by anti-rabbit Texas red conjugate (*e* and *g*) or exposed to anti-rabbit Texas red conjugate alone (*f* and *h*). Bar, 75 μm.

was present in both ciliary and TMN neurons. The ratio between the level of p75 mRNA and L27 mRNA in TMN neurons was 0.276 ± 0.044 (mean ± SEM, $n = 5$ separate assays of different TMN neuron preparations) and the ratio between the level of p75 mRNA and L27 mRNA in ciliary neurons was 0.085 ± 0.006 (mean ± SEM, $n = 5$ separate assays of different ciliary neuron preparations). Thus, the level of p75 mRNA was approximately threefold higher in TMN neurons than in ciliary neurons.

Staining of cultured TMN and ciliary neurons with a rabbit polyclonal antiserum that recognizes an epitope in the extracellular domain of the chicken p75 receptor (gift of Gisela Weskamp) showed that both kinds of neurons expressed this protein at the cell surface. The cell bodies and neurites of both kinds of neurons were stained by this antiserum. The staining of neurites was punctate in places, with the cell body displaying an intense peripheral ring of fluorescence characteristic of membrane staining. Neurons incubated with secondary antibody alone were unlabeled (Fig. 7).

Discussion

We have shown that the survival of NGF-insensitive, BDNF-dependent TMN neurons can be promoted by NGF after *trkA* is synthesized in these neurons from an injected *trkA* cDNA template. This is the first direct experimental evidence of *trkA* mediating an appropriate cellular response to NGF in an appropriate cell type, that is, NGF-promoted survival of embryonic neurons. Previous work has shown that NGF causes proliferation of 3T3 fibroblasts transfected with *trkA* cDNA (Cordon et al., 1991) and promotes meiotic maturation of oocytes injected with *trkA* mRNA (Nebreda et al., 1991). Both of these inappropriate cellular responses to NGF are preceded by transphosphorylation of *trkA*. This indicates that activation of the tyrosine kinase domain of *trkA* in these nonneuronal cells is capable of activating second messenger pathways culminating in responses that are otherwise elicited by other factors (FGF in the case of fibroblasts and progesterone in the case of oocytes). *trkA* transfection also restores NGF responsiveness to a mutant PC12 pheochromocytoma cell line (Loeb et al., 1991). Our findings indicate that TMN neurons possess the intracellular components that are capable of transducing a *trkA*-mediated NGF signal into a survival response. Possibly these are the same as or similar to those activated by BDNF in TMN neurons. It is unclear, however, why not quite as many *trkA*-injected TMN neurons survive with NGF as survive with BDNF. This might be due to incomplete activation or reduced expression of an essential effector for NGF signaling.

Given the ability of NGF to promote a variety of cellular responses in diverse types of non-neuronal cells expressing *trkA*, it was surprising to find that NGF failed to promote the survival of embryonic ciliary neurons injected with the pCMX/*trkA* expression vector during the stage of neurotrophic factor dependence. This was not due to failure of *trkA* mRNA and protein expression in ciliary neurons. RT/PCR analysis demonstrated that pCMX/*trkA*-injected ciliary neurons expressed as much *trkA* mRNA as pCMX/*trkA*-injected TMN neurons and that the level of *trkA* mRNA in these injected neurons was >25-fold higher than the level of endogenous *trkA* mRNA in NGF-dependent sensory neurons. Furthermore, immunocytochemistry showed *trkA* immunoreactivity on the cell body and neurites of pCMX/*trkA*-injected ciliary neurons. The faint staining of only the cell body of uninjected TMN and ciliary neurons with the anti-*trkA* antiserum was probably due to either cross-reaction with other tyrosine kinases or reactivity against endogenous *trk* species. Irrespective of the reason for the low level of staining in uninjected neurons, injected TMN and ciliary neurons clearly expressed greatly elevated levels of rat *trkA* protein. There was no significant difference in the percentage of pCMX/*trkA*-injected ciliary neurons growing with CNTF and the percentage of pCMX/*trkA*-injected TMN neurons growing with BDNF that possessed anti-*trkA*-stained neurites. This suggests that the translation of the *trkA* mRNA and the distribution of the receptor protein was similar for both kinds of neuron. Neither the injection procedure nor the expression of *trkA* protein in ciliary neurons was detrimental to the neurons because pCMX/ *trkA*-injected, *trkA*-expressing ciliary neurons survived in culture medium containing CNTF. We were unable to demonstrate if the rat *trkA* expressed in injected TMN and ciliary neurons could be activated by NGF because the number of injected neurons was too small to permit biochemical demonstration of NGF-induced transphosphorylation. The ineffectiveness of NGF in promoting the survival of *trkA*-expressing ciliary neurons cannot be attributed to the absence of the low-affinity NGF receptor, p75, because both p75 mRNA and p75 protein are expressed in ciliary neurons. Although quantitative RT/PCR showed that the level of p75 mRNA in ciliary neurons is about one third that in TMN neurons, no obvious differences in the intensity of p75 immunoreactivity were observed among these neurons. Thus, despite the expression of both *trkA* and p75, ciliary neurons remained refractory to NGF.

Why do ciliary neurons that express NGF receptors fail to respond to NGF? Ciliary neurons may be unusual in that they only possess the signal transduction and intracellular components for surviving in response to cytokines. In contrast to many kinds of embryonic neurons, ciliary neurons do not survive in response to any members of the NGF family of neurotrophic factors (Helfand et al., 1976; Lindsay et al., 1985; Rohrer and Sommer, 1982; Davies et al., 1993). Instead, these neurons are supported by CNTF (Barbin et al., 1984) which acts via a different set of cell surface receptors to those used by the NGF family of neurotrophic factors (Davis et al., 1991; Ip et al., 1992*b*; Squinto et al., 1990). The CNTF receptor complex is thought to consist of three components. A glycosylphosphotidylinositol-linked extracellular protein (CNTFR α subunit) that is homologous with one of the two components of the interleukin-6 (IL-6) receptor (Davis et al., 1991), a 130-kD transmembrane glycoprotein (gp130) that is similar to the other component of the IL-6 receptor and a third component that might be related to a gp130 homologue that binds leukemia inhibitory factor (Ip et al., 1992*b*). Binding of CNTF to responsive cells leads to tyrosine phosphorylation of gp130 and the putative gp130-like component of the CNTF receptor complex, although these receptor components do not possess intrinsic tyrosine kinase activity. Phosphorylation of these receptor components does not occur in fibroblasts and PC12 cells in response to a variety of factors (including NGF, BDNF, NT-3, PDGF, and FGF) that utilize receptor tyrosine kinases (Ip et al., 1992*b*), suggesting that these phosphorylations may be specific for the signal transduction pathways activated by

CNTF and LIF. It has yet to be ascertained, however, what steps occur subsequent to gp130 phosphorylation in ciliary neurons that lead to the survival of these cells.

It is possible that the intracellular signal cascade leading to survival in ciliary neurons has some components in common with the signal cascade leading to survival in neurons that are supported by members of the NGF family of neurotrophic factors. The introduction of a constitutively active oncogenic form of *ras* p21 protein into ciliary neurons promotes their survival and neurite outgrowth in the absence of CNTF (Borasio et al., 1989). This suggests that downstream components of a signal cascade from *ras* p21 are present in ciliary neurons. However, function-blocking antibodies to $p21^{c\text{-}ras}$ do not block the survival-promoting effects of CNTF, suggesting that endogenous $p21^{c\text{-}ras}$ is not involved in CNTF signal transduction leading to survival. $p21^{c\text{-}ras}$ is essential for the signal cascade in PC12 cells that is initiated by NGF-mediated activation of *trkA* and involves MAP kinases and the kinase *raf-1* (Wood et al., 1992; Thomas et al., 1992; Meng Sheng and Green, 1992; Ohmich et al., 1992). Furthermore, recent work has shown that $p21^{c\text{-}ras}$ mediates the survival response of nodose and dorsal root ganglion neurons to BDNF and NGF, respectively (Ng and Shooter, 1992; Borasio et al., 1993). Our demonstration that ectopic expression of *trkA* in ciliary neurons does not confer NGF responsiveness suggests that activated *trkA* in ciliary neurons does not couple with guanine–nucleotide exchange factors for $p21^{c\text{-}ras}$. Perhaps ciliary neurons lack the specific SH2 domain-containing adapter molecules to link phosphorylated *trkA* receptors to the exchange factors (Egan et al., 1993). Ciliary neurons might therefore be a useful experimental model for elucidating the molecular mechanism of *ras* activation by *trk* tyrosine receptor kinases in embryonic neurons.

The recent finding that NGF-promoted survival of sympathetic neurons does not require functional $p21^{c\text{-}ras}$ (Borasio et al., 1993) suggests that $p21^{c\text{-}ras}$ is not an essential component of the signal cascade leading to survival in all NGF-dependent neurons. This finding together with our current study suggests that downstream of the initial signal transduction event there are multiple intracellular signal cascades leading to survival in embryonic neurons.

Ciliary neurons also differ from neurons that depend on members of the NGF family of neurotrophic factors in possessing a cell death mechanism that is insensitive to the proto-oncogene *bcl-2*. Over-expression of *bcl-2* prevents cell death in BDNF-deprived TMN neurons and NGF-deprived DMTG neurons, but does not rescue CNTF-deprived ciliary neurons (Allsopp et al., 1993). Thus, the difference in the cell death pathways in these neurons may provide an explanation for the inability of NGF to prevent cell death in ciliary neurons expressing *trkA*.

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