NGF-stimulated Retrograde Transport of trkA in the Mammalian Nervous System

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Abstract. The present study was designed to clarify the in vivo function of trkA as an NGF receptor in mammalian neurons. Using the rat sciatic nerve as a model system, we examined whether trkA is retrogradely transported and whether transport is influenced by physiological manipulations. Following nerve ligation, trkA protein accumulates distal to the ligation site as shown by Western blot analysis. The distally accumulating trkA species were tyrosine phosphorylated. The trkA retrograde transport and phosphorylation were enhanced by injecting an excess of NGF in the footpad and were abolished by blocking endogenous NGF with specific antibodies. These results provide evidence that, upon NGF binding, trkA is internalized and retrogradely transported in a phosphorylated state, possibly together with the neurotrophin. Furthermore, our results suggest that trkA is a primary retrograde NGF signal in mammalian neurons in vivo.

NGF, the prototype of a family of closely related polypeptide growth factors including brain-derived neurotrophic factor (BDNF)[1], neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), stimulates the differentiation, maintains the phenotype, and supports the survival of distinct populations of neurons (34–36, 57). In general, neurotrophins interact with two different types of cell surface receptors present on responsive neurons. The first described receptor corresponds to a 75- to 80-kD integral membrane glycoprotein ($p75^{NGFR}$) that binds all neurotrophins with equal affinity (9, 45). A second type of NGF receptor has been identified as the product of the proto-oncogene trkA, a member of the trk family of tyrosine kinase receptors (25, 28). The trkA gene product is a 140-kD integral membrane glycoprotein with tyrosine kinase activity (26, 39). Two trkA-related genes, trkB and trkC, were subsequently cloned and shown to encode high–affinity receptors for BDNF and NT-4/5 (51, 52) and NT-3 (21, 32), respectively. Although considerable cross talk exists between the neurotrophin ligands and their receptors, there is clear receptor preference for particular neurotrophins (21). The neurotrophin specificity of trkA may also be determined by the relative cellular expression levels of $p75^{NGFR}$ and trkA (1). Efforts to elucidate the signal transduction pathways used by NGF receptors have involved PC12 cells, primary neuron cultures, or fresh tissue sections. In these systems, NGF rapidly stimulates the tyrosine phosphorylation and activation of trkA (3, 26, 28, 29, 38, 62). The phosphorylation of trkA results in activation of several signaling proteins via phosphorylation, as well as the induction of immediate early genes (reviewed in reference 4 and 8). Stable neuronal differentiation of PC12 cells requires the trk-mediated tyrosine phosphorylation of SHC and PLC-1 and the activity of ras (53, 58, 63). The specificity of NGF-mediated responses in neuronal cells may be determined in part by distinctive targets of neurotrophic factor-induced tyrosine kinase activity, such as the recently identified *suc*-associated neurotrophic factor-induced tyrosine-phosphorylated target (SNT) protein (42).

Neuronal terminals, the sites of neurotrophin/receptor interaction, are often many centimeters away from the cell body, a condition that necessitates signal transduction via retrograde axonal transport (30). Retrograde messengers in the NGF signal transduction cascade (e.g., an activated receptor complex, an activated downstream kinase, or a second messenger molecule) are currently unknown. Exogenously administered [^{125}I]NGF is retrogradely transported in a specific saturable manner by sympathetic and sensory neurons (18, 54) as well as some central neurons (7, 49). Indeed, this technique has been used to identify novel neurotrophin-responsive populations of neurons (30), including cholinergic neurons of the basal forebrain (48). Although NGF itself is retrogradely transported, the

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1. *Abbreviations used in this paper*: BDNF, brain-derived neurotrophin-4/5, DRG, dorsal root ganglia; NT-3, neurotrophin-3; NT-4/5, neurotrophin-4/5; SNT, *suc*-associated neurotrophic factor-induced tyrosine-phosphorylated target.

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intracellular injection of NGF does not mimic its neurotrophic signal (19), demonstrating that NGF itself is not the intracellular retrograde signal and suggesting that some other receptor-generated messenger is retrogradely transported from the nerve terminal to the cell body.

It has been suggested that the retrograde transport of NGF is initiated by endocytic internalization of the ligand–receptor complex (30), presumably following binding to high-affinity receptors (2). The present study was designed to determine whether trkA is retrogradely transported and whether such transport is physiologically relevant to neurotrophin signal transduction. To address these questions, we performed sciatic nerve ligations in conjunction with immunoblot analysis of trkA in nerve segments distal to the ligation to determine whether this receptor accumulates in a way that suggests retrograde transport. To assess the physiological significance of the retrograde transport of trkA, we determined the effect of footpad injection of NGF and anti-NGF antibodies on levels of trkA in various segments of the sciatic nerve as well as the phosphorylation state of this receptor. The results of these studies indicate that trkA is retrogradely transported in adult mammalian axons and that retrogradely transported trkA is tyrosine phosphorylated. Blocking target-derived NGF with anti-NGF antibodies eliminates the retrograde transport and phosphorylation of trkA, whereas the saturation of sensory terminals with an excess of NGF enhances transport and phosphorylation. These experiments suggest that trkA is a primary retrograde NGF signal effector in vivo.

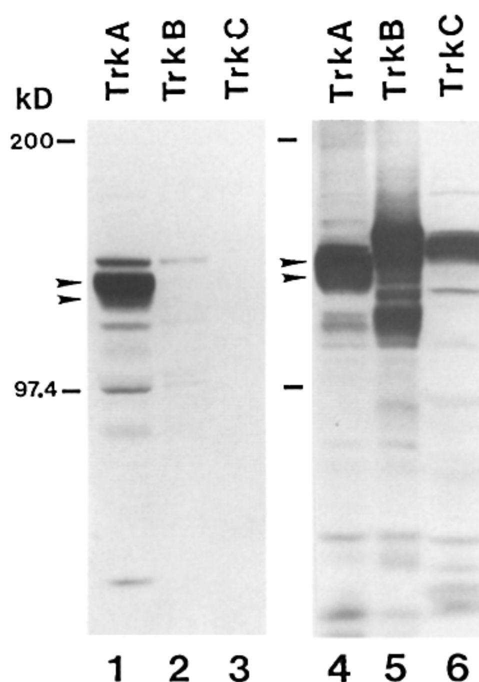

Figure 1. Specificity of trk antibodies. Lysates of Sf9 cells expressing trkA, trkB, or trkC were electrophoresed on SDS–polyacrylamide gels and transferred to nitrocellulose membrane. Membranes were probed with anti-trkA 488-507 (lanes *1–3*) or anti-pantrk 203 (lanes *4–6*) antibodies. Positions of prominent trkA proteins are indicated by double arrowheads.

Materials and Methods

trk Antisera

Anti-pantrk 203 antibody was generated against the COOH-terminal 14 amino acids of human trk (17). Antibodies against this peptide recognize trkA, trkB, and trkC (26, 28, 51, 59). The first specific trkA antibody was generated in rabbits using a peptide encoding amino acids 488–507 in the juxtamembrane region of human trkA (FMTLGGSSLSPTEGKGSGLE). This antibody is referred to here as anti-trkA 488–507. The specificity of trk antibodies was assessed using detergent lysates of Sf9 insect cells infected with recombinant baculovirus encoding human trkA (R. Stephens and D. Kaplan, unpublished observations), rat trkB (R. Stephens and D. Kaplan, unpublished observations), or rat trkC (P. Tsoulfas and L. Parada, unpublished observations). The anti-trkA 488-507 antibody specifically recognized a prominent 140-kD band and possibly a faint 160–180-kD band only in trkA-expressing Sf9 cell lysates (Fig. 1, lanes *1–3*). The anti-pantrk 203 antibody recognized prominent 140–150-kD bands in trkA-, trkB-, and trkC-expressing Sf9 cells, lysates, and possibly a faint 180-kD band only in trkA-expressing SF9 cell lysates (Fig. 1, lanes *4–6*). Antibodies to trk were used for immunoblotting as previously described (17, 26) at dilutions of 1:2,000 to 1:3,000 (Fig. 1). A second trkA-specific antibody used in the present study, referred to as RTA, was generated in rabbits using a fusion protein, purified from a baculovirus-Sf9 cell expression system, which corresponds to the entire NH_2-terminal domain of trkA (amino acids 1–416). The generation and specificity of this antibody has been previously described (6).

Sciatic Nerve Ligations and Footpad Injections

Adult male Sprague-Dawley rats were anesthetized with nitrous oxide/oxygen/ethrane at a 66:33:1 ratio, and the sciatic nerve was exposed. Two ligatures, 2 mm apart, were placed at the level of the mid sacroiliac synosteosis following sacroiliac disarticulation. Immediately after nerve ligation, injections of either saline (200 μl), NGF (80 μg in 200 μl PBS; Genentech, South San Francisco, CA) or anti-NGF antiserum (100 μl serum in 100 μl PBS) were made into the ipsilateral footpad. After 18 h, rats were killed by decapitation, and 8-mm nerve segments were taken at successive sites proximal and distal to the ligature. Nerve sections were immediately frozen on dry ice and stored for later preparation as described below. The previous experiment was repeated three times and all segments from all animals from each experiment were processed in the same blot.

Tissue Sample Preparation

Tissue was prepared by washing once with cold PBS and once with cold TBS and homogenizing in cold TBS with 1% NP-40, 10% glycerol, 1 mM PMSF (Sigma Chemical Co., St. Louis, MI), 10 μg/ml aprotinin (Boehringer Mannheim Corp., Indianapolis, IN), 1 μg/ml leupeptin (Boehringer Mannheim), and 500 μM orthovanadate (Sigma) with a probe sonicator for 30 s. Homogenates were spun at 10,000 rpm for 10 min at 4°C, and the supernatant was subjected to either immunoprecipitation or SDS-PAGE. Equal protein quantities were determined by the BCA method (Pierce Chemical Company, Rockford, IL) and by immunoblot analysis for myelin basic protein. Briefly, nerve segment homogenates were diluted serially and applied to a nitrocellulose membrane by vacuum suction on a dot blot apparatus (Bio-Rad Labs., Richmond, CA). Nitrocellulose membranes were then probed with the mouse monoclonal antibody SMI 99, directed against myelin basic protein (Sternberger Monoclonals, Inc., Baltimore, MD) followed by HRP-conjugated goat anti-mouse immunoglobulin (Kirkegaard & Perry Labs., Inc., Gaithersburg, MD), and immunoreactive spots were visualized with ECL (Amersham Corp., Arlington Heights, IL). Dilutions required to abolish visualization of myelin basic protein immunoreactivity were used to indicate equal lengths of nerve in different segments. Quantitative experimental comparisons between nerve segments were then performed only on samples on the same blot as described below.

Immunoblotting and Immunoprecipitation

For pantrk and trkA immunoblots, protein samples were separated by SDS-PAGE and transferred to nitrocellulose as described previously (14). Nitrocellulose membranes were blocked with 5% milk and incubated with pantrk and trkA antisera at a 1:2,500 dilution overnight at 4°C. Immunoreactive protein bands were visualized using HRP-linked goat anti-rab-

bit antisera with ECL (Amersham). For immunoprecipitation, 50–100 μl of supernatant from tissue homogenates (25–50 μg protein) were precleared with 30 μl of a 50% suspension of protein A–Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) containing 1% BSA in 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 5 mM EDTA. Supernatants from the precleared homogenates were incubated with 4 μl of rabbit pantrk antibody overnight at 4°C. Following antibody incubation, 30 μl of the protein A–Sepharose solution mentioned above was added, and the mixture was incubated for 1 h at 4°C. Protein A–Sepharose beads were then spun down and washed three times with 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 5 mM EDTA. Immunoprecipitated proteins were analyzed by SDS-PAGE and phosphotyrosine immunoblot using the mouse monoclonal antibody 4G10 (UBI, Lake Placid, NY) at a 1:1,000 dilution.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from adult rat dorsal root ganglia (DRG) by the RNAzol method (Tel Test, Inc., Freadswood, TX) (5), and 0.5 μg was denatured in PCR reaction buffer (Perkin-Elmer Cetus, Norwalk, CT) with 0.5 μl RNase Block (Stratagene Inc., La Jolla, CA) at 65°C for 3 min. RT was carried out in PCR reaction buffer with 28 pmol of antisense primer (5′-agccccagggatggcagatc-3′) and 1 μl M-MLV RT (GIBCO BRL, Gaithersburg, MD) with 2 mM of each deoxynucleotide triphosphate at 42°C for 1 h. Heteroduplexes were melted by incubation at 95°C for 5 h; 28 pmol of sense primer (5′-ccagcgggatctgcaacggc-3′) in PCR reaction buffer with 1 μl AmpliTaq polymerase (Perkin-Elmer Cetus) was added. PCR reactions were cycled 40× with annealing and polymerization temperatures of 58 and 72°C, respectively. Northern blotting total RNA from the DRG and ventral horn of an adult male Sprague-Dawley rat was isolated using the RNAzol B method as per manufacturer's instructions (Tel Test, Inc.) (5). Total RNA (15 μg), determined spectrophotometrically, was electrophoresed on a 1% agarose, 2.2 M formaldehyde gel, transferred to a Nytran (Schleichter & Schuell, Inc., Keene, NH) nylon membrane in 20× SSC overnight, and baked for 2 h at 80°C. For hybridization, a gel purified 1.45-kb rat trkA insert corresponding to 40 amino acids of the extracellular domain and extending through the 3′ untranslated region (40) was labeled with the random hexanucleotide priming method to a specific activity of $\sim 10^9$ cmp per μg and then diluted to 10^7 cpm per ml of hybridization buffer containing 500 mM sodium phosphate buffer (pH 7.2), 7% SDS, 1 mM EDTA, and 1% BSA. After preincubation in hybridization buffer without probe for 3 h at 65°C, the membrane was incubated overnight in hybridization buffer containing the labeled probe at 65°C. The next morning, the blot was washed once for 20 min at room temperature in 40 mM sodium phosphate buffer (pH 7.2), 5% SDS, 1 M EDTA, and 0.5% BSA, followed by two washes, one at room temperature for 20 min and one at 65°C for 10 min in 40 mM sodium phosphate buffer (pH 7.2), 1% SDS, and 1 mM EDTA. The membrane was then exposed for 1 wk at 70°C with intensifying screens and developed in an automatic film processor.

In Situ Hybridization

Riboprobes for rat trkA were transcribed from a 1.45-kb cDNA insert corresponding to the same region of trkA used for Northern hybridization subcloned into pBluescript KS+ (Stratagene) using the Promega transcription kit as per manufacturer's instructions (Promega, Madison, WI). Antisense riboprobes (900 bp) were transcribed with T7 RNA polymerase after linearization of the plasmid with ScaI, and 600-bp sense probes were transcribed using T3 RNA polymerase also after linearization of the plasmid with ScaI. Transcription reactions, alkaline hydrolysis of full-length probes, prehybridization, and hybridization steps were carried out as previously described (31). Thick sections (6 μM) of fresh frozen DRG from an adult male Sprague-Dawley rat were cut on a cryostat, thaw mounted onto Vectabond (Vector Laboratories, Inc., Burlingame, CA) subbed slides, and used for hybridization.

Results

Different Molecular Forms of trkA Are Present in Brain and in Neonatal and Adult Sensory Ganglia

To determine the presence of trkA protein species in rat brain, DRG, and sciatic nerve, we performed immunoblot analysis on anterior brain, DRG, and sciatic nerve tissue homogenates using a trkA-specific antibody (Fig. 2). This analysis revealed the presence of a 180-kD species in DRG (Fig. 2, *A*, *left* and *B*, lane *2*) and adult sciatic nerve (Fig. 3 *C*, lane *1*) and minor 140-kD and 110-kD species in adult rat DRG (Fig. 2 *B*, lane *2*). Similar analysis revealed trkA species in the 140- and 110-kD range in adult rat anterior brain homogenates (Figs. 2, *A* and *B*, lane *1*), as well as in PC12 cell lysates and in primary DRG cultures from E15 rat embryos (data not shown). Dilution of the primary antibody resulted in disappearance of the 110-, 140-, and 180-kD bands as did preincubation of the primary antibody with the immunogenic peptide (Fig. 2 *B*). Furthermore, immunoblot analysis of sciatic nerve homogenates with an additional trkA-specific antibody that recognizes an extracellular epitope likewise revealed a prominent

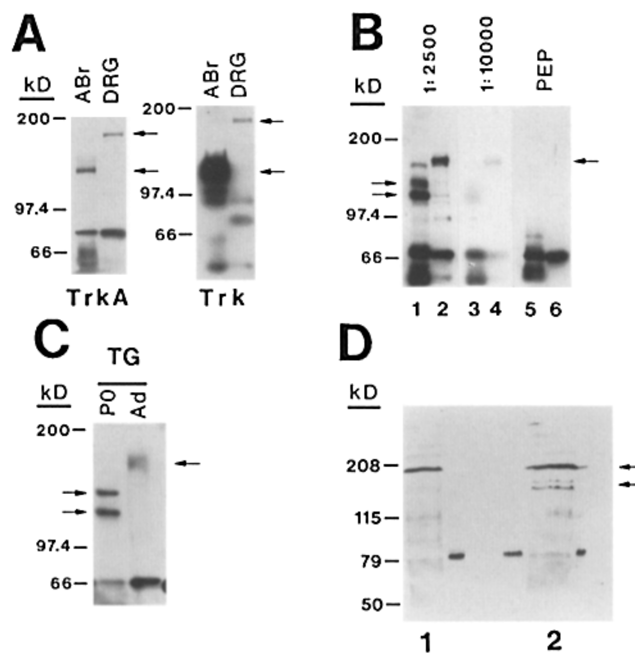

Figure 2. Immunoblot analysis of trkA species in newborn and adult rat sensory ganglia, sciatic nerve, and brain. In each case, 25 μg of total protein were loaded per lane. Positions of molecular mass markers (in kD) are shown. (*A*) Adult rat anterior brain (Bar), sciatic nerve, and DRG and newborn rat trigeminal ganglia were homogenized and subjected to immunoblot analysis using anti-trkA 488-507 or anti-pantrk 203 antibodies as described in Materials and Methods. ABr and adult rat DRG samples were analyzed with both anti-trkA 488-507 (*left*) and anti-pantrkA 203 (*right*). Arrows indicate 140- and 180-kD trkA species. (*B*) The specificity of the anti-trkA 488-507 antibody was tested by dilution of the antibody and by blocking with 50 μg/ml of the immunogenic peptide (lanes *1*, *3*, and *5* anterior brain; lanes *2*, *4*, and *6* DRG). (*C*) The presence of trkA in P0 and adult trigeminal ganglia was also determined by immunoblot analysis using the anti-trkA 488-507 antibody. Arrows in both *B* and *C* indicate 180-kD species in DRG (*left-pointing arrow*) and 140- and 110-kD trkA species in P0 and ABr (*right-pointing arrows*). The 110-kD species most likely represents under-glycosylated trkA (39). (*D*). The identity of the 180-kD trk species was further examined by immunoblot analysis of sciatic nerve homogenates. A prominent 180-kD species was observed in sciatic nerve (*arrow*), using either the anti-pantrk 203 antibody (lane *1*) or RTA anti-trkA antibody (lane *2*).

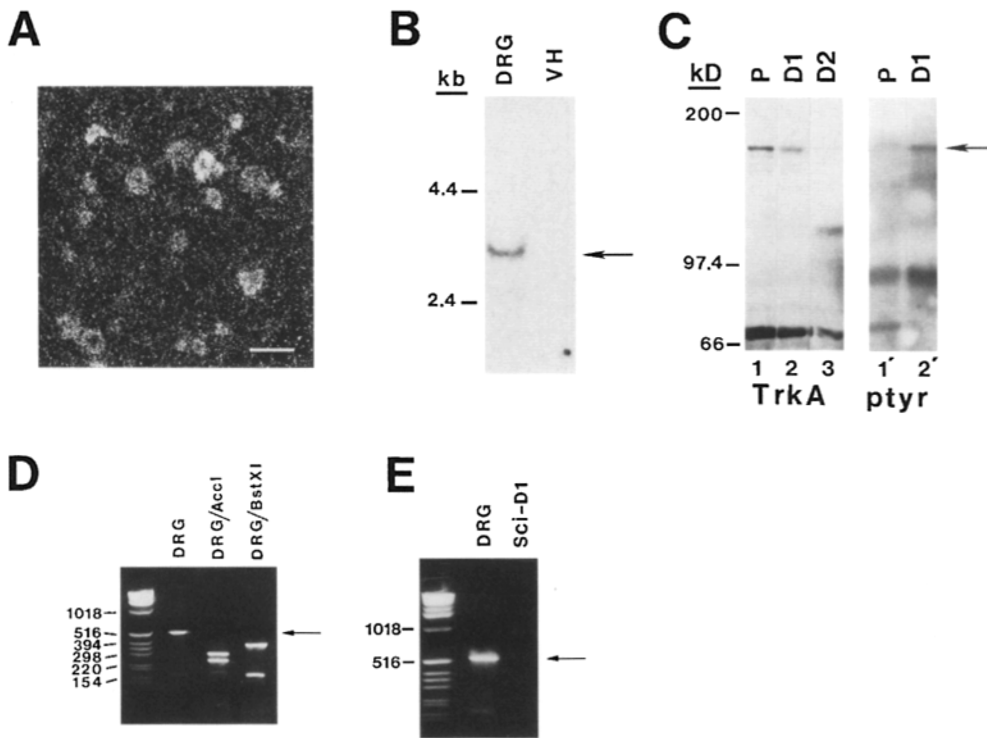

Figure 3. Source and retrograde transport of trkA in sciatic nerve sensory axons. (*A*) The trkA mRNA is present in adult rat DRG neurons. Sections of adult rat DRG were hybridized with a trkA-specific probe. The trkA mRNA is detectable in a large number of DRG neurons. (*B*) Source of trkA receptors present in the sciatic nerve. Total RNA (10 μg) from adult rat DRG and ventral horn was subjected to Northern analysis using a trkA-specific probe. The trkA mRNA is detectable in DRG, but not in ventral horn, as a single 3.5-kb band (*arrow*). Positions of 1.4- and 2.4-kb markers are shown. (*C*) Retrograde transport of phosphorylated trkA in the sciatic nerve. Segments of sciatic nerve immediately proximal to (*P*), and successively distal from (*D1*, immediately distal; *D2*, far distal), a ligation site were homogenized and subjected to trkA immunoblot analysis using the anti-trkA 488-507 antibody (lanes *1–3*) or immunoprecipitation with the anti-pantrk 203 antibody followed by immunoblot analysis using the 4G10 antiphosphotyrosine antibody (lanes *1'* and *2'*). The amount of protein loaded per lane (lanes *1–3*) and subjected to immunoprecipitation (lanes *1'* and *2'*) was normalized for nerve length, as described in Materials and Methods, and represented 10–20 μg in each case. Positions of molecular mass markers (in kD) are shown. Arrow indicates the position of trkA bands. (*D* and *E*) The trkA species present in sciatic nerve represents transported, and not de novo, expressed receptors. (*D*) Total RNA (0.5 μg) from adult DRG was subjected to RT-PCR using rat trkA-specific sense and antisense primers. The identity of the amplified 527-bp fragment (*arrow*) was confirmed by restriction digest with both AccI (with expected fragment sizes of 241 and 286 bp) or BstXI (with expected fragment sizes of 153 and 374 bp). (*E*) The 527-bp fragment (*arrow*) characterized in *D* was amplified from RT-PCR reactions from DRG total RNA but not from total RNA isolated from a nerve segment immediately distal to the ligation site (*D1*), using the same primers as in *D*. Bar, 50 μm.

180-kD trkA species (Fig. 2 *D*). Immunoblotting with a pantrk antibody that recognizes the terminal 14–amino acid epitope of the trkA molecule also revealed the presence of bands at 110 and 140 kD in anterior brain homogenates and 180 kD in DRG homogenates (Fig. 2 *A*, *right*). To determine whether the 180-kD form of trkA we observed was specific for a certain developmental stage of sensory neurons, we performed immunoblot analysis for trkA on trigeminal ganglia homogenates from newborn and adult rats. The trkA species present in newborn trigeminal ganglia migrates at 110 and 140 kD, whereas a single 180-kD trkA species is seen in adult trigeminal ganglia (Fig. 2 *C*). The 110-kD band is most likely the underglycosylated form of trk originally observed in trkA-expressing fibroblasts (39) also seen in PC12 cells and brain tissues (17, 29). These results raise the possibility of differential trkA processing in neonatal and adult sensory neurons.

trkA Is Expressed in Adult Rat DRG and Is Retrogradely Transported in Peripheral Nerve

To determine the source of trkA receptors in the sciatic nerve, we performed trkA in situ hybridization on sections of adult rat DRG as well as trkA Northern blot analysis on total RNA isolated from adult DRG and ventral horn. Using a trkA-specific probe, in situ hybridization revealed the presence of trkA mRNA in DRG neurons (Fig. 3 *A*). The trkA mRNA is present as a single 3.5-kb species in adult rat DRG (Fig. 3 *B*) and is absent from adult rat ventral horn (Fig. 3 *B*). To determine whether nerve sheath cells could be the source of trkA receptors present in sciatic nerve, we performed RT-PCR on total RNA isolated from normal adult rat sciatic nerve and a segment of nerve immediately distal to a ligation site. The latter experiment was designed to determine whether the trkA gene is upregulated in sheath cells in response to injury as has been reported for $p75^{NGFR}$ (56). A 527-bp species corresponding to the trkA PCR product was amplified from total DRG RNA but not from sciatic nerve RNA (Fig. 3 *E*). The identity of this 527-bp species was verified by restriction analysis (Fig. 3 *D*). The expression of the trkA gene in the adult DRG, but not in adult ventral horn or sciatic nerve, demonstrates that trkA present in the adult rat sciatic nerve must be derived from DRG neurons. To determine whether trkA is transported retrogradely in peripheral axons, sciatic nerve ligations were performed in the adult rat. At 18 h after ligation, small nerve segments (~8 mm) were taken at various sites proximal and distal to the

ligation site and processed for immunoprecipitation or immunoblot analysis. TrkA was seen in the proximal and immediately distal nerve segments but was present in only small amounts in far distal segments (Fig. 3 *C*, lanes *1–3*), suggesting that this protein is retrogradely transported. To determine the phosphorylation state of the trkA receptor in sciatic nerve segments, trk protein from the nerve segments just proximal and just distal to the ligation site were immunoprecipitated from the homogenates with an anti-pantrk antibody that recognizes all members of the trk receptor family. These immunoprecipitates were then resolved by SDS-PAGE and subjected to phosphotyrosine immunoblotting. A 180-kD tyrosine phosphorylated species was observed in both nerve segments, with significantly more of this species present in the immediately distal segment than in the immediately proximal segment (Fig. 3 *C*, lanes *1'* and *2'*). Note that the total amount of trkA in the proximal segment was actually somewhat higher than that in the immediately distal segment (Fig. 3 *C*, lanes *1* and *2*).

At the Nerve Terminal, NGF Stimulates and Anti-NGF Antibody Blocks the Retrograde Transport of trkA

To address the physiological significance of the retrograde transport of trkA, NGF stimulation and blocking experiments were performed. Sciatic nerve ligations were performed as described above, this time followed by injection in the footpad of either saline, NGF, or anti-NGF antibodies. Footpad injection of NGF enhanced the distal accumulation of trkA in the nerve (Fig. 4, *A* and *B*; segments D1 and D2, *top*). In contrast, footpad injection of anti-NGF antibodies reduced the distal accumulation of trkA (Fig. 4, *A* and *C*; segments D1 and D2, *top*). These data indicate that the retrograde transport of trkA is stimulated by NGF and blocked by anti-NGF antibodies delivered into the terminal field, consistent with a ligand binding-mediated mechanism of retrograde transport. To determine whether the increased retrograde transport of trkA in response to NGF represents an enhanced transport of active trkA tyrosine kinase, the phosphorylation state of trkA in peripheral nerve segments was assayed. The trk receptors were immunoprecipitated from sciatic nerve segments and subjected to phosphotyrosine immunoblot analysis. Following footpad injection of NGF, an increase in the accumulation of tyrosine phosphorylated trkA was observed as compared to levels of phosphorylated trkA in the saline-injected control (Fig. 4, *A* and *B*; segments D1 and D2, *bottom panels*). Conversely, anti-NGF antibody injection virtually abolished the distal accumulation of tyrosine phosphorylated trkA (Fig. 4, *A* and C; segments D1 and D2, *bottom panels*). These results demonstrate that the retrograde transport of tyrosine phosphorylated (and presumably enzymatically active) trkA is dependent on NGF present at the nerve terminal and suggest that this retrogradely transported trkA may serve as a signal from the terminal to the cell body.

Discussion

The present studies demonstrate that trkA expressed in adult rat DRG neurons is retrogradely transported in a tyrosine phosphorylated state and that the retrograde transport of trkA can be influenced by physiological manipulations of NGF in target fields. TrkA is present in adult rat DRG and sciatic nerve primarily as a 180-kD species. The migration of this species in polyacrylamide gels is different from that reported in PC12 cells (26), transfected NIH3T3 cells (28), or primary cultures of embryonic rat DRG neurons (Ehlers and Koliatsos, unpublished observations). However, higher molecular mass trk species of ~200 and 280 kD have been reported in PC12 cells (15). These trk species bind NGF, are recognized by anti-trk antibodies,

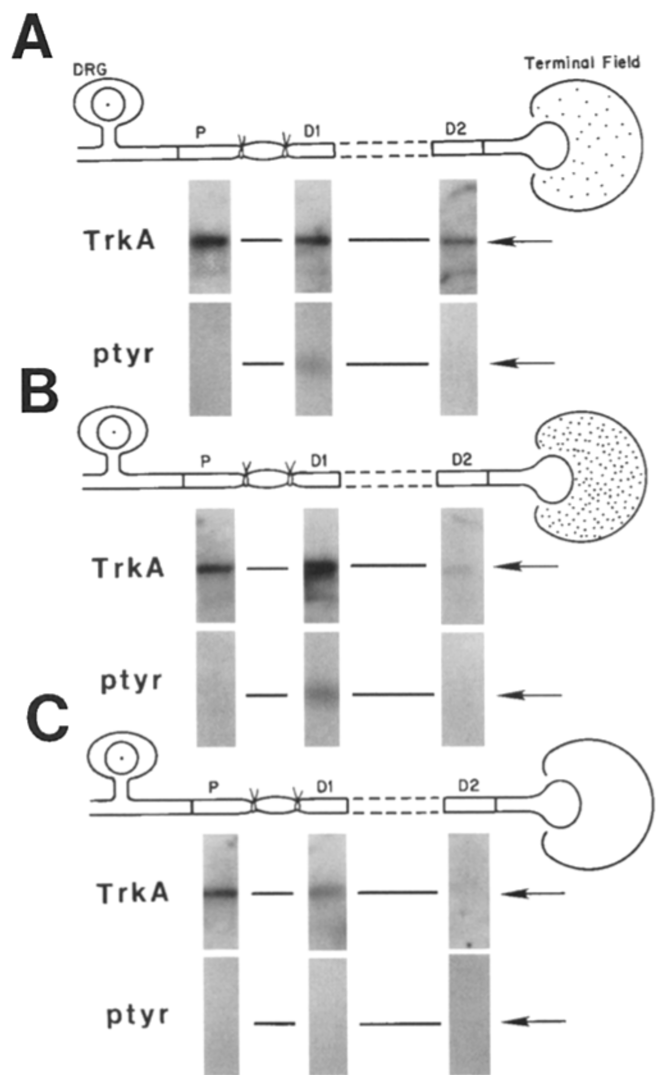

Figure 4. Retrograde transport of trkA is influenced by physiologically relevant manipulations. Proximal and distal segments of sciatic nerve following nerve ligation and footpad injection of either saline (*A*), NGF (*B*), or anti-NGF (*C*) antisera were homogenized and subjected either to trkA immunoblot analysis (A–C; *top*) or pantrk immunoprecipitation followed by immunoblot analysis using the 4G10 antiphosphotyrosine antibody (*A–C*; *bottom*). Each lane corresponds to the nerve segment illustrated above it (*P*, immediately proximal to ligature; *D1*, immediately distal to ligature; *D2*, far distal from ligature), and the amount of protein loaded or subjected to immunoprecipitation was normalized for nerve length as described in the Materials and Methods (10–20 μg in each case).

and are phosphorylated on tyrosine upon binding NGF (15). Evidence in the present study indicating that the observed 180-kD species is trkA includes: the recognition of a 180-kD species in adult rat DRG by three different antibodies directed against three different epitopes of the trkA molecule, the ability of the immunogenic peptide to abolish binding of the trkA-specific antibody to the 180-kD band, the presence of a 180-kD tyrosine phosphorylated species in pantrk immunoprecipitates, and the transport behavior of the 180-kD form of trkA in peripheral axons. Future studies will need to examine directly the ability of the 180-kD form of trkA to bind NGF by in vivo cross-linking experiments.

The existence of only one trkA mRNA species in adult rat DRG suggests that the 180-kD form of trkA is posttranslationally modified in a manner different from the 140-kD form. The 140-kD form of trkA itself is a highly glycosylated protein, proceeding from an 80-kD naked polypeptide through a 110-kD glycosylated intermediate to a 140-kD fully processed form (39). The 180-kD form of trkA can be precipitated by wheat germ lectin agarose from DRG homogenates (M. D. Ehlers and V. E. Koliatsos, unpublished observations), indicating that it is glycosylated as well. However, preliminary experiments have failed to demonstrate an appreciable decrease in the apparent molecular mass of the 180-kD trkA species following treatment with *N*-glycosidase F (M. D. Ehlers and V. E. Koliatsos, unpublished observations), raising the possibility that some other type of posttranslational modification, perhaps O-linked glycosylation, accounts for the increased apparent molecular mass. Smaller amounts of 110- and 140-kD trkA forms can be seen in trkA immunoblots from adult rat DRG. Whether these forms of trkA represent precursors to a fully processed 180-kD form or represent forms of trkA processed along a pathway different from that of the 180-kD form remains to be determined. These lower molecular mass trkA species may also play a role in trkA signal transduction in adult sensory axons and may, in fact, be retrogradely transported, but their relatively low abundance in adult sensory nerves precluded further analysis in this study. Although the 180-kD form of trkA is the predominant form of trkA in adult rat DRG, only the 110- and 140-kD forms of trkA are present in DRG from neonatal rats, suggesting that the posttranslational modification of trkA may be developmentally regulated.

NGF rapidly stimulates trkA tyrosine phosphorylation and activation of PLC-γ1 (27, 61), PI-3 kinase (43, 47), and SHC (46, 53, 55). These proteins subsequently induce the activation of ras, several serine/threonine kinases including the Erks (MAP kinases), Raf-1, B-Raf, and p90rsk, and various immediate early genes that lead to the characteristic biological effects of NGF (4, 8, 44, 58, 63). It is unknown whether an activated signaling molecule is produced at some point along this local signal transduction pathway at the nerve terminal, which is subsequently retrogradely transported to the perikarya. The fact that NGF itself is retrogradely transported in peripheral axons led to the initial hypothesis that the neurotrophin itself might act as its own intracellular signaling molecule (23). The later discovery that direct introduction of NGF into the cytoplasm of PC12 cells does not result in neuronal differentiation (19) suggested, however, that some other molecule with access to the cytoplasm must transduce the signal.

In this study, ligation of the sciatic nerve of adult rats leads to an accumulation of trkA protein in the immediately distal nerve segment. The paradigm of nerve ligation or crush with subsequent examination of the distal accumulation of axonal proteins has been previously used to demonstrate the retrograde transport of $p75^{NGFR}$ by immunocytochemical localization (24). The mechanism of retrograde transport is presumed to involve receptor internalization in an endocytic fashion (30). It has been reported that high-affinity NGF receptors internalize NGF in PC12 cells (2). In addition, the manipulation of target fields of neurons has been a time-honored practice in the study of target-derived trophic factors (10–13, 20), and, for a molecule to represent a neurotrophic signal, it must respond in a physiologically appropriate manner to events that occur at the target field. Indeed, the injection of exogenous NGF into the target field enhances the distal accumulation of trkA, whereas injection of anti-NGF antibodies greatly diminishes the distal accumulation of trkA in sciatic nerve axons. Thus, our findings are consistent with the idea that, upon binding NGF, trkA is endocytosed at the nerve terminal as a coated vesicle and subsequently delivered to the cell body by retrograde transport. The finding that trkA is retrogradely transported in adult rat peripheral axons suggests that trkA might be acting as a signaling molecule between the nerve terminal and neuronal cell body. Indeed, immunoprecipitation of trk species from nerve segments immediately proximal and distal to the ligation site followed by phosphotyrosine immunoblot analysis revealed that the trkA species in the distal segment was tyrosine phosphorylated, whereas the trkA species in the proximal segment was only weakly or not at all tyrosine phosphorylated. Studies in vitro have shown that tyrosine autophosphorylation of trkA occurs after NGF binding and is necessary for NGF signal transduction (26, 28, 37). Our results imply that retrogradely transported trkA is an active kinase and are consistent with the notion that trkA can act as a retrograde neurotrophic signal effector. The state of trkA as an active kinase in the axon suggests further that retrogradely transported trkA may continue to be bound to NGF. Interestingly, the retrograde transport of tyrosine phosphorylated trkA occurs in the absence of terminal field manipulation suggesting that NGF signaling is present, to at least some degree, in adult sensory neurons. The role of this steady-state transport of phosphorylated trkA in the survival or phenotypic maintenance of adult sensory neurons remains to be examined.

The injection of exogenous NGF into the target field increased the accumulation of tyrosine phosphorylated trkA in segments of sciatic nerve distal to a ligature, whereas injection of anti-NGF antibodies greatly diminished this accumulation. These findings support the idea that NGF binding to trkA at the nerve terminal leads to endocytosis and retrograde transport of an active tyrosine phosphorylated receptor. Whether additional signaling molecules, including $p75^{NGFR}$, play a role in the retrograde transport of trkA, NGF, or a trkA–NGF complex was not addressed specifically in this study. Several scenarios can be envisioned, including NGF transport via a trkA monomer, via

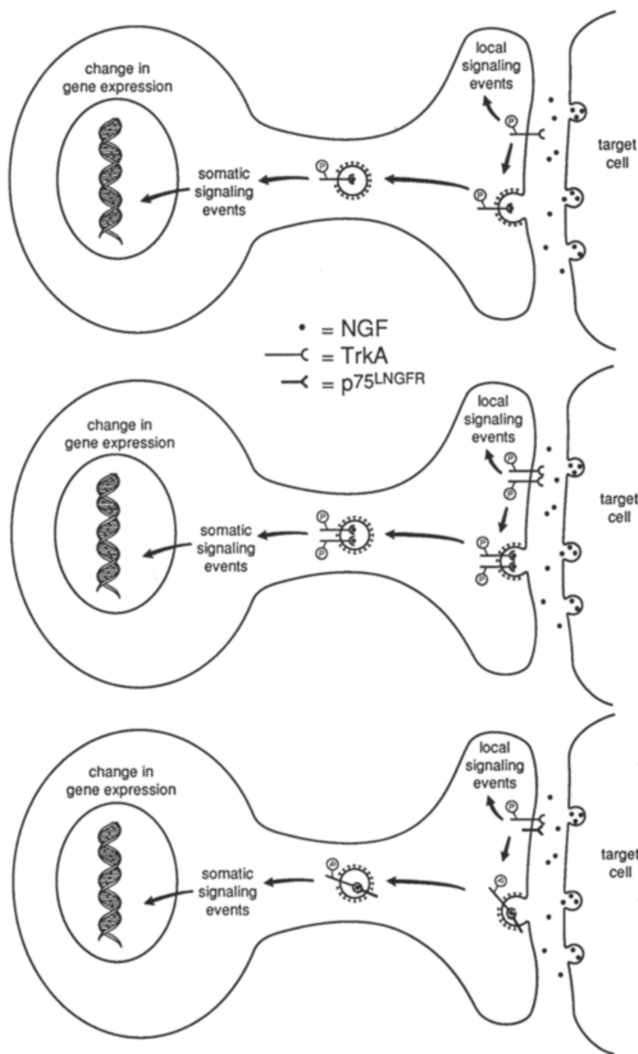

Figure 5. Proposed models for a retrograde NGF signaling complex. Data presented in the present study are consistent with a model whereby NGF first binds to trkA receptors at the nerve terminal, resulting in activation of the receptor by autophosphorylation. Receptor activation leads to local signaling events that may affect processes such as neurite outgrowth. The NGF/NGF receptor complex is then internalized through endocytosis and retrogradely transported up the axon. The exact nature of the retrogradely transported NGF signaling complex is not clear but may involve only trkA in monomeric (*top*) or dimeric (*middle*) form or may involve both trkA and the low-affinity NGF receptor, $p75^{NGFR}$ (*bottom*).

a trkA homodimer, or perhaps via a trkA/p75 heterodimer (Fig. 5) (16, 22). This last possibility is suggested by the known retrograde transport of $p75^{NGFR}$ (24). The demonstration of a necessary role of trkA in the above processes would require demonstrating that, in the absence of trkA, retrograde transport of NGF does not take place. The utilization of trkA null mice appears to be an ideal way to address the above question (50). However, the principal NGF-transporting sensory system (i.e., small axons that transmit pain and temperature) degenerates early in the life of these animals. Therefore, even if non-trkA receptors, such as $p75^{NGFR}$ play a role in mediating NGF transport, there would be no conduit (or uptake site) for NGF trafficking in these mice. Similar problems complicate the study of the role of $p75^{NGFR}$ in NGF/trkA retrograde transport using p75 null mice (33). Primary culture models of axonal transport using neurons from normal trkA null and $p75^{NGFR}$ null animals may be used to circumvent these problems.

The existence of $p75^{NGFR}$, along with the expression of truncated splice forms of trkB and trkC lacking the kinase domain (41, 59, 60) as well as biologically unresponsive full-length splice forms of trkC with amino acid inserts in the kinase domain, have added much complexity to the field of neurotrophin signal transduction. Further complications are introduced by the differential expression of these splice variants in different tissues and at different developmental stages (59, 60). The determination of the exact nature of the retrogradely transported neurotrophin/receptor complexes will be a crucial step in understanding the physiological significance of the various forms of neurotrophin receptors and their roles in neurotrophin signal transduction.

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