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Retrograde Transport and Steady-State Distribution of ^{125}I-Nerve Growth Factor in Rat Sympathetic Neurons in Compartmented Cultures

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We have used compartmented cultures of rat sympathetic neurons to quantitatively examine the retrograde transport of ^{125}I-nerve growth factor (NGF) supplied to distal axons and to characterize the cellular events that maintain steady-state levels of NGF in cell bodies. In cultures allowed to reach steady-state ^{125}I-NGF transport, cell bodies contained only 5–30% of the total neuron-associated ^{125}I-NGF, whereas 70–95% remained associated with the distal axons. This was true over an 8 pM to 1.5 nM ^{125}I-NGF concentration range, indicating that saturation of high affinity receptors could not account for the large fraction of ^{125}I-NGF remaining in axons. Dissociation assays indicated that 85% of ^{125}I-NGF associated with distal axons was surface-bound. At steady-state, only 2–25% of the distal axon-associated ^{125}I-NGF was retrogradely transported each hour, with higher transport rates associated with younger cultures and lower ^{125}I-NGF concentrations. The velocity of ^{125}I-NGF retrograde transport was estimated at 10–20 mm/hr. However, as in a previous report, almost no ^{125}I-NGF transport was observed during the first hour after ^{125}I-NGF administration, indicating a significant lag between receptor binding and loading onto the retrograde transport system. During ^{125}I-NGF transport through axons spanning an intermediate compartment in five-compartment cultures, little or no ^{125}I-NGF was degraded or released from the axons. After transport, ^{125}I-NGF was degraded with a half-life of 3 hr. In summary, although some cellular events promoted NGF accumulation in cell bodies, distal axons represented by far the principal site of NGF-receptor interaction at steady-state as a result of a low retrograde transport rate.

Key words: nerve growth factor; retrograde transport; sympathetic neurons; axon; degradation; endocytosis; retroendocytosis

Neurotrophic factors are internalized by axons and retrogradely transported to cell bodies. This was first demonstrated by the finding that ^{125}I-nerve growth factor (NGF) injected into the eye is delivered to neuronal cell bodies of the sympathetic superior cervical ganglion (SCG) by axons innervating the iris (Hendry et al., 1974a,b; Stöckel et al., 1974; Stöckel and Thoenen, 1975; Johnson et al., 1978). Later it was confirmed that endogenous NGF produced in targets of sympathetic axons is retrogradely transported and accumulates to high levels in sympathetic ganglia (Korsching and Thoenen, 1983a,b, 1988; Palmatier et al., 1984; Nagata et al., 1987). NGF is mostly intact after its transport (Hendry et al., 1974a; Stöckel et al., 1974, 1976; Johnson et al., 1978; Dumas et al., 1979; Ure et al., 1994). These observations have been interpreted as suggesting that NGF directly participates in signaling in cell bodies, likely as part of a transported ligand–receptor complex. This vesicular transport hypothesis of NGF signaling is supported by correlations between NGF retrograde transport and changes in enzyme activity and gene expression that occur in cell bodies (Paravicini et al., 1975; Stöckel and Thoenen, 1975; Kessler and Black, 1979; Goedert et al., 1981; Miller et al., 1994).

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The vesicular transport hypothesis of NGF signaling has been interpreted as implying that NGF should be concentrated in the cell bodies of responsive neurons. In calculations of transport velocity an assumption is made that NGF enters the transport pathway rapidly after binding to receptors on axon terminals and that transport rates are limited only by the velocity of the transport mechanism (Hendry et al., 1974a,b; Johnson et al., 1978). It also is assumed that NGF is neither degraded nor released intact while en route to cell bodies. However, some NGF is contained in lysosomal organelles in axons (Claude et al., 1982a; Bernd and Greene, 1983), and the possibility that it may be degraded or released before reaching cell bodies has not been tested.

Rigorous tests of these assumptions have been difficult to perform in retrograde transport models *in vivo*. In the present study we have examined these assumptions, using compartmented cultures of sympathetic neurons given ^{125}I-NGF on their distal axons, which allowed determination of the amount of NGF associated with distal axons, the amount transported per hour to cell bodies, the amount accumulated by cell bodies at steady-state, and the location where NGF or its degradation products are released.

MATERIALS AND METHODS

Materials. Newborn Sprague Dawley rats were supplied by the University of Alberta Farm. Trypsin was obtained from Calbiochem (La Jolla, CA). L15CO_2 culture medium was obtained from Life Technologies (Grand Island, NY). Adult rat serum was prepared from whole blood supplied by Lab Animal Services, University of Alberta. Rat tail collagen was prepared by the method of Hawrot and Patterson (1979). 2.5S NGF was purchased from Cedarlane Laboratories (Hornby, Ontario). Teflon dividers were purchased from Tyler Research Instruments (Edmonton, Alberta). Na^{125}I was purchased from Amersham (Oakville, Ontario).

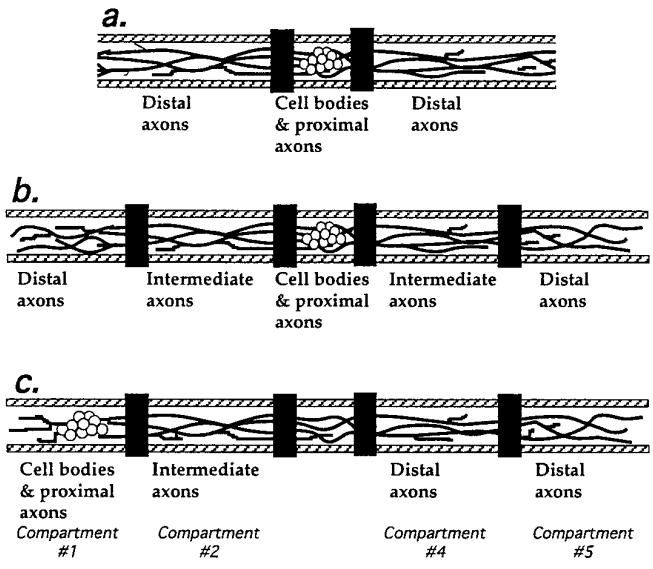

Figure 1. Compartmented culture designs. Illustrations of individual tracks (20 tracks per culture) are shown from three different designs of compartmented cultures used in the present study. Teflon septa separate compartments. Distal axon compartments, in which ^{125}I-NGF was always applied, were separated from cell body/proximal axon compartments by distances of the following: *a*, 1 mm; *b*, 6 mm; *c*, 8 mm. Illustrations are not to scale.

PD-10 Sephadex G-25M columns were purchased from Pharmacia Biotech (Baie d'Urfé, Quebec). Reagents for which suppliers are not stated were obtained from Sigma (St. Louis, MO).

Compartmented cultures. Sympathetic neurons were isolated from the superior cervical ganglia of newborn rats by 0.1% (w/v) trypsin incubation and mechanical dissociation, as previously described (Campenot et al., 1991). The standard culture medium was L15CO_2 supplemented with the prescribed additives and 6% methylcellulose. Compartmented cultures were constructed as previously described (Campenot, 1992). Briefly, rat tail collagen was air-dried onto 35 mm culture dishes, and then parallel scratches were made in the substrate. Teflon dividers of either three-compartment or five-compartment design were seated onto the collagen tracks with silicon vacuum grease. Cell suspension was plated into a single compartment of each culture (2000–3000 neurons/culture), and axons extended into adjacent compartments containing NGF. For the first 6 d after plating, medium additives in the cell body/proximal axon compartment included 2.5% rat serum, 1 mg/ml ascorbic acid, 10–200 ng/ml NGF, and 10 μM cytosine arabinoside. Thereafter, cell body compartments received only rat serum and ascorbic acid. Axon compartments received only NGF as a medium additive at concentrations of 10–200 ng/ml throughout the entire culture period. Medium was changed every 4–6 d. Cultures were maintained in a 5% CO_2 atmosphere at 37°C.

Radioiodination of NGF. NGF was radioiodinated by the lactoperoxidase method. The following ingredients were mixed at room temperature for 1 hr: 3–5 μl (1.5 mCi) of Na^{125}I, 10 μl (10 μg) of NGF, 37 μl of 0.5 M potassium phosphate buffer, pH 7.4, 10 μl of 33 μg/ml lactoperoxidase, and 10 μl of 0.003% H_2O_2. After the first 30 min of incubation, an additional 10 μl of H_2O_2 was added. The reaction was terminated with 5 μl of β-mercaptoethanol and 415 μl of 1 mg/ml BSA in potassium phosphate buffer. Specific activities and labeling efficiencies were calculated after acid precipitation (20% trichloroacetic) of an aliquot of the reaction mixture. Free iodine was separated from ^{125}I-NGF with Sephadex G-25M gel filtration columns. Radioactivity was measured with a Wallac 1470 gamma counter. Specific activities averaged 151 cpm/picogram (pg), and ^{125}I-NGF was used within 3 weeks of iodination. Molar concentrations of NGF were based on a molecular weight of 26,000.

Retrograde transport assay and sample analysis. Three different designs of compartmented cultures were used, based on two models of Teflon dividers. The cultures differed principally by the number of compartments through which axons could unidirectionally extend (Fig. 1). All retrograde transport assays were performed by applying ^{125}I-NGF to distal axons in compartments most distal to the cell body compartment and incubating at 37°C to allow time for the neurons to retrogradely transport ^{125}I-NGF. Radioactivity from cell body/proximal axon compartments (transported ^{125}I-NGF) and, in some experiments, from the intermediate axon compartments (5-compartment cultures) was quantified after the incubation. Medium was collected and combined with a cold PBS rinse; then cell extracts were made by using reducing sample buffer containing 1.3% sodium dodecyl sulfate. Total transported ^{125}I-NGF was the combined radioactivity from cell bodies/proximal axons plus the medium bathing them. Background diffusion of ^{125}I-NGF under the Teflon divider was very low. For example, when ^{125}I-NGF was applied at a concentration of 1500 cpm/μl (10 ng/ml) to both side compartments of three-compartment dishes lacking cells, typically radioactivity accumulated in the center compartment at a rate of 10 cpm/hr (0.06 pg/hr). After a 24 hr incubation in these mock cultures, the final ^{125}I-NGF concentration in center compartments resulting from background diffusion was typically ~1 pM. To compete the uptake of ^{125}I-NGF directly by the cell bodies, we included 8 nM NGF in the medium bathing cell bodies/proximal axons in all experiments. The presence of 8 nM NGF in this compartment was found not to affect the retrograde transport rate. For example, in one experiment 10.4% of the axonal ^{125}I-NGF was transported per hour (transport rate) when 8 nM NGF was included in the cell body compartment, whereas the transport rate was 9.8%/hr when NGF was absent from this compartment. The fraction of cellular ^{125}I-NGF found in cell bodies/proximal axons also was unaffected by including NGF in the cell body compartment. Results from a second, similar experiment were consistent with these conclusions.

The amount of ^{125}I-NGF associated with or dissociated from distal axons was determined after the overnight ^{125}I-NGF incubation. Axons were rinsed with cold PBS for 20–30 sec (or in some experiments not rinsed), and then either (1) fresh medium containing 1 μg/ml NGF (except 1 experiment, using 0.4 μg/ml) was added back to the compartments in dissociation experiments, or (2) the axons were harvested immediately to determine axon-associated ^{125}I-NGF. All axons were harvested eventually by vigorous trituration with only water, which reduced the release of nonspecifically bound ^{125}I-NGF from the collagen substrate, as compared with detergent extraction. The 20–30 sec rinse before axon harvesting or chasing was found in a separate experiment not to reduce levels of axon-associated ^{125}I-NGF, as compared with no rinse. Also, there was no correlation between rinsing and relative transport rates, which also implied that significant amounts of ^{125}I-NGF did not dissociate from low affinity receptors on axons during a 20–30 sec rinse.

Specific transport, axon association, and dissociation were determined by subtracting nonspecific values from totals. Nonspecific values were determined by performing the same procedures in cultures in which a 100- to 200-fold higher concentration of unlabeled NGF was included in the ^{125}I-NGF incubation with distal axons. Typically, nonspecific transport accounted for <10% of total transport, whereas nonspecific association accounted for 40–50% of the total axon association. The decrease in free ^{125}I-NGF concentration in axon compartments caused by 24 hr of axonal uptake and transport was at most 22% but was most often <10%. Cell body/proximal axon ^{125}I-NGF at steady-state ranged from 1–96 pg, but it is not informative to make comparisons of absolute quantities between experiments because of the variability in neuronal number and axon growth between culture platings.

Kinetic analysis of degradation. Kinetic analysis was performed on the decay of cell-associated radioactivity. The pulse–chase protocol is described under Results. Cultures were used in which distal axons were axotomized at the end of the pulse, which eliminated interference because of the prolonged chase transport from distal axons. Cell-associated radioactivity at the various intervals was not measured in these cultures but, instead, was estimated by subtracting the cumulative release at each time point from the total amount of radioactivity released during the chase. This method could be used because the total cumulative release of radioactivity over at least 30 hr was a close estimate of the initial cell-associated NGF. That is, in cultures in which distal axons were left intact during the chase, the amount of radioactivity measured in cell bodies/proximal axons at the end of a 30 hr chase represented only 5% of the total center compartment radioactivity (medium + cell extract), some of which was attributable to nonspecific transport. In cultures in which chase transport was absent, residual cell body NGF was likely only ~2–3%. Then the estimated fraction of cell-associated ^{125}I-NGF was plotted as a function of time.

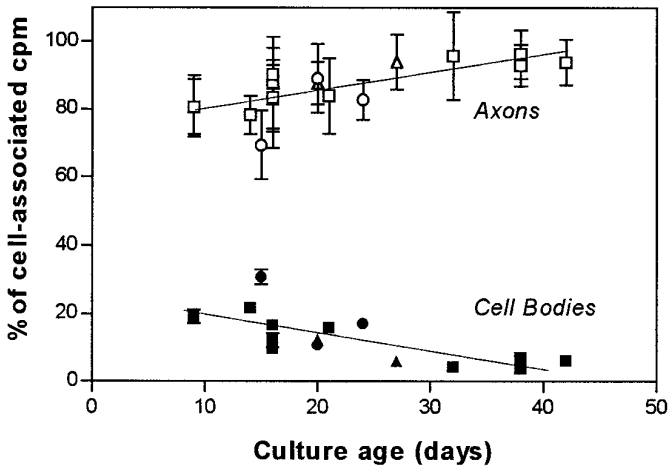

Figure 2. Relative distributions of cell-associated NGF. In three-compartment cultures of various ages, 0.2–40 ng/ml ^{125}I-NGF (8 pM to 1.5 nM) was supplied to distal axons for 15–24 hr, during which time some ^{125}I-NGF was retrogradely transported to cell bodies/proximal axons. Distal axons in some cultures additionally received ≥100-fold excess NGF for determination of nonspecific association/transport. After removing the culture medium and rinsing all compartments, we made cell extracts and quantified the radioactivity. Shown are relative proportions of ^{125}I-NGF radioactivity associated with distal axons or accumulated in cell bodies/proximal axons, with nonspecific values subtracted. Concentrations of applied ^{125}I-NGF are 0.2 ng/ml (*circles*); 3–20 ng/ml (*squares*); 40 ng/ml (*triangles*). Distal axon-associated ^{125}I-NGF is the sum of both side compartments. In each experiment two to four cultures were used per treatment group. Error bars ± SEM fall within symbols when not visible. Linear regressions were calculated from data from a total of 17 experiments.

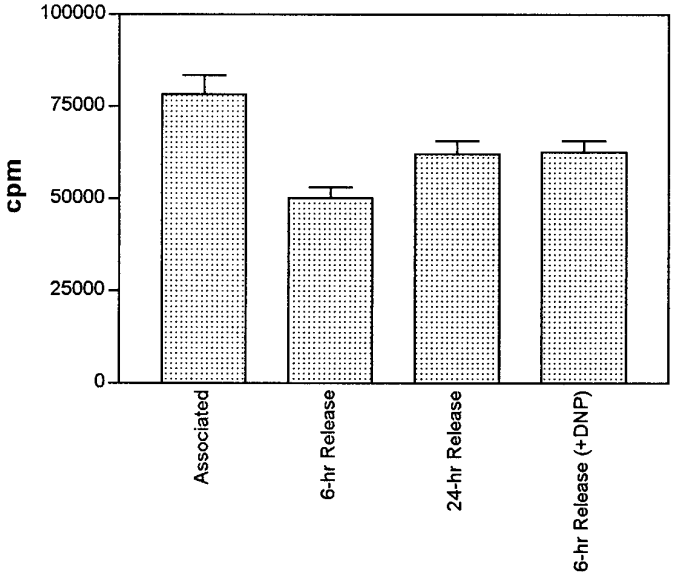

Figure 3. ^{125}I-NGF dissociation from distal axons. In 37-d-old three-compartment cultures, distal axons were supplied with 20 ng/ml ^{125}I-NGF, alone or with ≥100-fold excess NGF for 10 hr, after which cultures from each of these two groups were split into three subsequent groups. (1) Distal axons were harvested immediately (6 sides); (2) Axonal ^{125}I-NGF was chased with 0.4 μg/ml NGF for 6 hr and then for 18 hr at 37°C (6 sides), and the dissociated ^{125}I-NGF was collected after each chase; (3) Axonal ^{125}I-NGF was chased with 0.4 μg/ml NGF and 500 μM dinitrophenol (1000× dilution) for 6 hr at 37°C (4 sides), and the dissociated ^{125}I-NGF was collected. Specific values for ^{125}I-NGF association and dissociation are shown (means ± SEM). Two other experiments gave similar results.

RESULTS

NGF is associated mostly with distal axons at steady-state

The steady-state distribution of NGF in three-compartment cultures (shown in Fig. 1*a*) was investigated by bathing distal axons continuously in ^{125}I-NGF for 15–24 hr, during which time some of the ^{125}I-NGF was retrogradely transported to cell bodies and proximal axons. Previous results have shown that steady-state association with axons is reached within 2 hr (Hawrot, 1982), and steady-state accumulation of ^{125}I-NGF in cell bodies/proximal axons is reached in ~8 hr (Claude et al., 1982b). Also, most radioactivity in cell bodies/proximal axons is intact ^{125}I-NGF by SDS-PAGE analysis (Ure and Campenot, 1994). As shown in Figure 2, the ^{125}I-NGF that retrogradely accumulated in cell bodies/proximal axons at steady-state accounted for only 5–30% of the cellular ^{125}I-NGF, whereas the remainder was associated with distal axons. The distribution was slightly age-dependent, such that proportionally more ^{125}I-NGF became axon-associated in older cultures. Interestingly, the distribution was only weakly dependent on ^{125}I-NGF concentration, although the concentration of ^{125}I-NGF supplied to distal axons ranged from 0.2–40 ng/ml (8 pM to 1.5 nM). The highest cell body accumulation (30%) occurred in an experiment using 0.2 ng/ml ^{125}I-NGF, but in two other experiments with this concentration the cell body accumulation was not markedly higher than at higher ^{125}I-NGF concentrations. At 8 pM ^{125}I-NGF, most ^{125}I-NGF binding should be to high affinity receptors, based on reported receptor affinities from other neurons (Sutter et al., 1979; Godfrey and Shooter, 1986). Therefore, at this concentration the abundance of ^{125}I-NGF associated with distal axons could not have resulted from extensive binding to low affinity receptors that might not have participated in transport.

Axon-associated ^{125}I-NGF is mostly surface-bound

Dissociation assays at 37°C were performed to determine whether the distal axon ^{125}I-NGF was surface-bound or internalized and possibly en route to cell bodies. This method was used rather than acid wash (Buxser et al., 1990), because the latter technique seemed to fix axons to the substrate. After overnight incubations with 10–20 ng/ml ^{125}I-NGF, distal axons were chased at 37°C in the presence of ≥100-fold excess unlabeled NGF. All radioactivity released from axons was intact ^{125}I-NGF by SDS-PAGE analysis (data not shown). The warm chase was performed over two consecutive intervals of 6 and 18 hr. Figure 3 shows representative results from one of four experiments. From all experiments, over the total 24 hr chase 75–97% (mean = 85%) of the axon-associated ^{125}I-NGF was released, in similarity to other cells (Sutter et al., 1979; Landreth and Shooter, 1980). Most of the release occurred during the first chase interval, but ~20% occurred later, indicative of very slow dissociation of some of the ^{125}I-NGF. There was no indication that ^{125}I-NGF release varied with culture age (16- to 42-d-old cultures).

As has been suggested previously for PC12 cells (Eveleth and Bradshaw, 1988; Kasaian and Neet, 1988; Buxser et al., 1990), some of the warm-chased ^{125}I-NGF may have been released from an intracellular pool by retroendocytosis. Because retroendocytosis has been shown to be blocked by dinitrophenol (DNP) treatment (Marshall, 1985; Formisano et al., 1994), we included a group with DNP (0.5 mM) in the medium during the first 6 hr chase. DNP, which depletes ATP stores, is effective in our culture

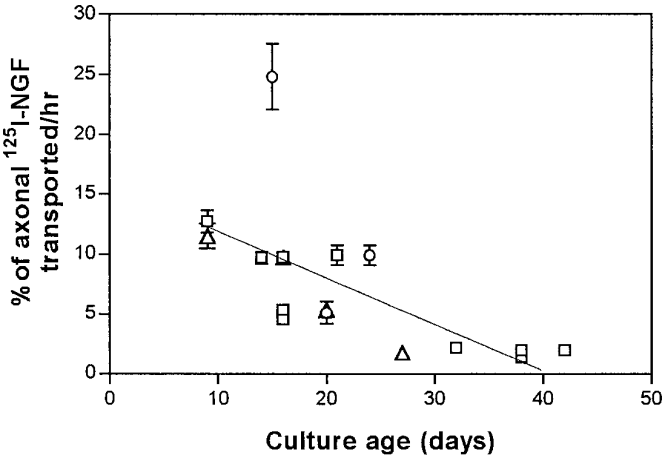

Figure 4. Rates of retrograde transport. Data are taken from experiments described in Figure 2. Transported ^{125}I-NGF was quantified by collecting all radioactivity from center compartments (medium + cell bodies/proximal axons) after the 15–24 hr incubations with 0.2–40 ng/ml ^{125}I-NGF. Total transport was divided by the transport interval and then compared with the amount of ^{125}I-NGF associated with distal axons at steady-state (100%) to determine the transport rate (%/hr). Concentrations of applied ^{125}I-NGF are 0.2 ng/ml (*circles*), 3–20 ng/ml (*squares*), and 40 ng/ml (*triangles*). Error bars ± SEM fall within symbols when not visible. A linear regression was calculated from data from a total of 17 experiments.

model in blocking retrograde transport of leukemia inhibitory factor (Ure et al., 1994). Three experiments were performed with 10–20 ng/ml ^{125}I-NGF, and a representative result is shown in Figure 3. DNP treatment did not reduce the amount of ^{125}I-NGF released from the axons and even slightly increased its release in two of three experiments, in similarity to previous reports (Olender and Stach, 1980, 1981; Stach and Wagner, 1982). These results suggest that axons did not retroendocytose ^{125}I-NGF and, in turn, suggest that ~85% of distal axon NGF was surface-bound, a portion of which was associated with very slowly dissociating (high affinity) sites, in similarity to a previous report (Godfrey and Shooter, 1986).

Rate of retrograde transport

To quantify the rate at which the axon-associated ^{125}I-NGF was delivered to cell bodies/proximal axons directly, we used additional data from the experiments described in Figure 2. Total NGF transport was calculated as the combined radioactivity collected from the cell bodies/proximal axons, representing intact NGF, and from the medium bathing them, representing products of NGF degradation released by the neurons during the incubation (Ure and Campenot, 1994). After 24 hr, >85% of the transported ^{125}I-NGF was degraded and released into the medium. The average transport rate was determined first by dividing total NGF transport by the period of transport, which averaged 20 hr (cpm/hr), and then expressing it as a percentage of NGF associated with distal axons (final units = %/hr). The rate determined by this method differed by ≤2%/hr, as compared with the rate calculated once steady-state transport was reached. This small discrepancy likely reflected the absence of ^{125}I-NGF transport within the first hour of ^{125}I-NGF application to distal axons (Claude et al., 1982b). When ^{125}I-NGF was supplied to distal axons for 15–24 hr, only 2–25% of the distal axon ^{125}I-NGF was transported to cell bodies each hour, with the rate declining as a function of culture age (Fig. 4). Expressed differently, no less than 4 hr was required for ^{125}I-NGF to be transported in an amount equivalent to the amount associated with axons at steady-state; in older cultures as long as 50 hr was required. In similarity to the ^{125}I-NGF distribution, the transport rate was not strongly dependent on the concentration of ^{125}I-NGF supplied to axons, which ranged from 0.2–40 ng/ml (8 pM to 1.5 nM). For example, in two experiments the transport rates in 4 ng/ml ^{125}I-NGF (0.15 nM) were only 1%/hr higher than in 40 ng/ml ^{125}I-NGF (1.5 nM). Using 0.2 ng/ml ^{125}I-NGF, at which the binding should be predominantly to high affinity receptors, we found that the transport rate in only one of three experiments differed markedly from transport rates at higher ^{125}I-NGF concentrations. These results are consistent with receptors of multiple affinities participating in NGF transport and suggest that transport occurred at a low rate even when ^{125}I-NGF associated mostly with high affinity receptors.

NGF is retrogradely transported at a velocity of 10–20 mm/hr

We have used the release of degraded ^{125}I-NGF from the cell bodies to estimate the velocity of NGF retrograde transport. This was accomplished by comparing the time course of release in three-compartment cultures with the time course in five-compartment cultures in which the NGF was transported an additional 5 mm through an intermediate axon compartment (Figs. 1*a*,*b*). We assumed that internalization and loading of NGF into the transport system as well as degradation and release of NGF from cell bodies occurred with the same time course in both types of cultures. Therefore, any delay in the appearance of degraded NGF from cell bodies in five-compartment cultures would represent the time required for transport through the extra 5 mm of axon. After ^{125}I-NGF was applied to distal axons, medium in the center compartments was exchanged at 15 min intervals. We observed that the first release of radioactivity from cell bodies was delayed in five-compartment cultures by only 15–30 min (1–2 intervals), as compared with three-compartment cultures (Fig. 5). Therefore, the transport velocity is estimated to have been 10–20 mm/hr (i.e., 5 mm/15–30 min). This is higher than the estimate of 2–3 mm/hr made for NGF retrograde transport in adult rat sympathetic neurons *in vivo* (Hendry et al., 1974a,b; Johnson et al., 1978), but it is similar to estimates of 7–13 mm/hr for NGF transport by sensory neurons (Stöckel et al., 1975; Yip and Johnson, 1986) and 12 mm/hr for dopamine β-hydroxylase transport in sympathetic axons of the sciatic nerve (Brimijoin and Helland, 1976).

Little or no ^{125}I-NGF is degraded or released from axons during retrograde transport

Some NGF in sympathetic axons (Claude et al., 1982a) and in neurites of PC12 cells (Bernd and Greene, 1983) is associated with lysosomal organelles. Moreover, a variety of evidence supports the possibility that degradative events might occur in axons (Broadwell, 1980; Doherty et al., 1990; Gatzinsky et al., 1991a,b; Renfrew and Hubbard, 1991; Overly et al., 1995). Also, release of previously internalized intact ^{125}I-NGF from PC12 cells has been reported (Eveleth and Bradshaw, 1988; Buxser et al., 1990). We addressed the possibility of en route release of breakdown products or intact NGF by investigating transport in five-compartment cultures shown in Figure 1*c*. In these cultures there are additional compartments interposed between the cell body/proximal axon compartment and the distal axon compartment in which ^{125}I-NGF was applied. One of these compartments is large, termed the

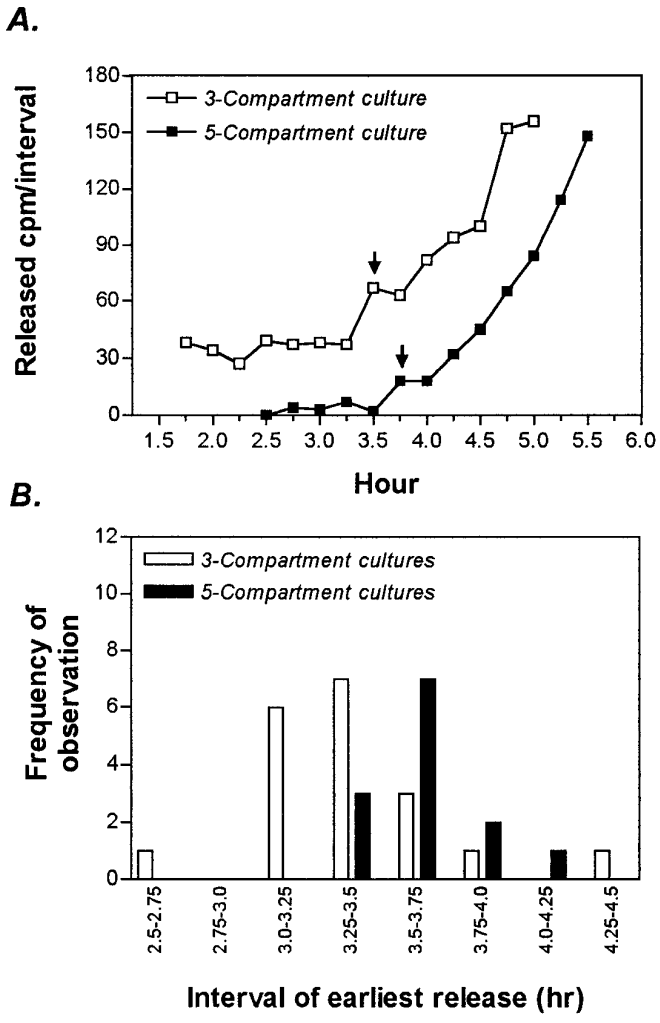

Figure 5. Velocity of retrograde transport. In both three- and five-compartment cultures (shown in Fig. 1*a,b*), distal axons were incubated with 10–65 ng/ml ^{125}I-NGF. At 15 min intervals, medium from the cell body/proximal axon compartment was exchanged, and the radioactivity in the medium aliquots was quantified. *A*, Shown are representative cultures in which the first appearance of radioactivity above background (representing release by the neurons) is marked by an *arrow*. *B*, Cumulative data show when radioactivity was first released from cell bodies/proximal axons. The 15–30 min delay in five-compartment cultures was attributed to transport across the intermediate axon compartment spanning 5 mm. Transport velocity was estimated as 5 mm/(0.25–0.5 hr) = 10–20 mm/hr.

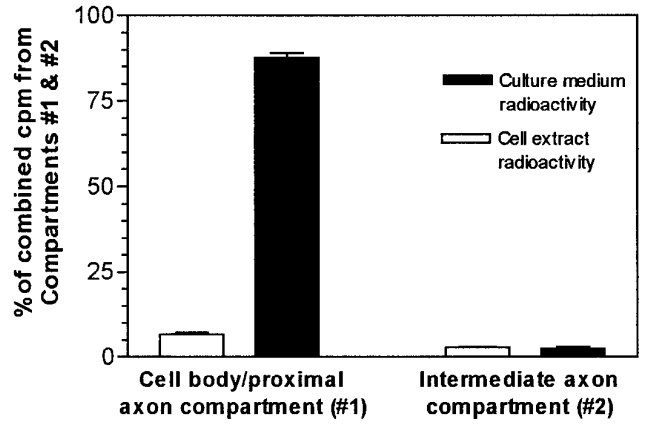

Figure 6. Lack of release of NGF or its degradation products by intermediate axons. In five-compartment cultures (shown in Fig. 1*c*), distal axons were incubated with 10–50 ng/ml ^{125}I-NGF for 22–40 hr, during which ^{125}I-NGF was retrogradely transported through intermediate axons and into cell bodies/proximal axons. Then radioactivity was collected from various fractions, as shown. Data are expressed as percentage of total combined radioactivity from both compartments. Shown are means ± SEM of 13 cultures.

intermediate axon compartment. We were able to determine whether any NGF being transported through the intermediate axon compartment was released en route to cell bodies. ^{125}I-NGF at 10–50 ng/ml was applied to distal axons (compartments 4 and 5) for 22–40 hr, after which all of the radioactivity transported into the cell body/proximal axon and intermediate axon compartments (1 and 2) was quantified. Mean results from 13 cultures are shown in Figure 6. Of all the radioactivity collected, only 3% was from the medium bathing intermediate axons, which was not significantly above background. Thus, little or no NGF or its breakdown products were released from axons while NGF was en route to cell bodies. In previous experiments we showed that cell bodies/proximal axons do not retain low molecular weight breakdown products of ^{125}I-NGF, which indicates that they are released quickly from the neurons (Ure and Campenot, 1994). Thus, it is unlikely that in the present experiments ^{125}I-NGF was degraded in intermediate axons and that the degraded ^{125}I-NGF was transported to cell bodies/proximal axons. Therefore, these results suggest that nearly all ^{125}I-NGF loaded onto the retrograde transport system was delivered intact to cell bodies.

NGF degradation after retrograde transport

The turnover of cell body/proximal axon NGF was investigated in three-compartment cultures, both under steady-state conditions and in pulse–chase experiments. Under steady-state conditions, radioactivity levels in cell bodies/proximal axons are relatively constant beyond ~10 hr (Claude et al., 1982b) and degraded ^{125}I-NGF is released into the medium at a linear rate (data not shown). Turnover rates can be determined by sampling the radioactivity released into the medium during this time and comparing the release with ^{125}I-NGF levels in the cell bodies/proximal axons at harvesting. From 10 three-compartment cultures (14–38 d old) we found, on average, that the release of degraded ^{125}I-NGF was equivalent to 39% of the cell body/proximal axon pool per hour, corresponding to an average turnover interval of 2.7 hr (range 1.6–4.3 hr). Similar rates have been observed in PC12 cells (Layer and Shooter, 1983) and in freshly dissociated sensory neurons (Sutter et al., 1979).

For the pulse–chase analysis of degradation rates, ^{125}I-NGF (50–200 ng/ml) was supplied to distal axons in 26- to 36-d-old cultures for 5 hr, during which time ^{125}I-NGF accumulated both in distal axons and in cell bodies/proximal axons. Then the ^{125}I-NGF-containing medium was exchanged with medium containing at least 200 ng/ml NGF. During the chase the medium bathing cell bodies/proximal axons was exchanged several times and the released radioactivity quantified. Because this radioactivity represents only degraded ^{125}I-NGF (Ure and Campenot, 1994), we were able to determine the time course of release of ^{125}I-NGF degradation products. Representative results from one of four experiments are shown in Figure 7*A* (chase-intact cultures). The degradation and release of ^{125}I-NGF breakdown products occurred with a half-life of 6.1 hr, which reflects not only the clearance of ^{125}I-NGF that was in cell bodies at the end of the pulse but also reflects retrograde transport and clearance of ^{125}I-NGF associated with distal axons at the end of the pulse.

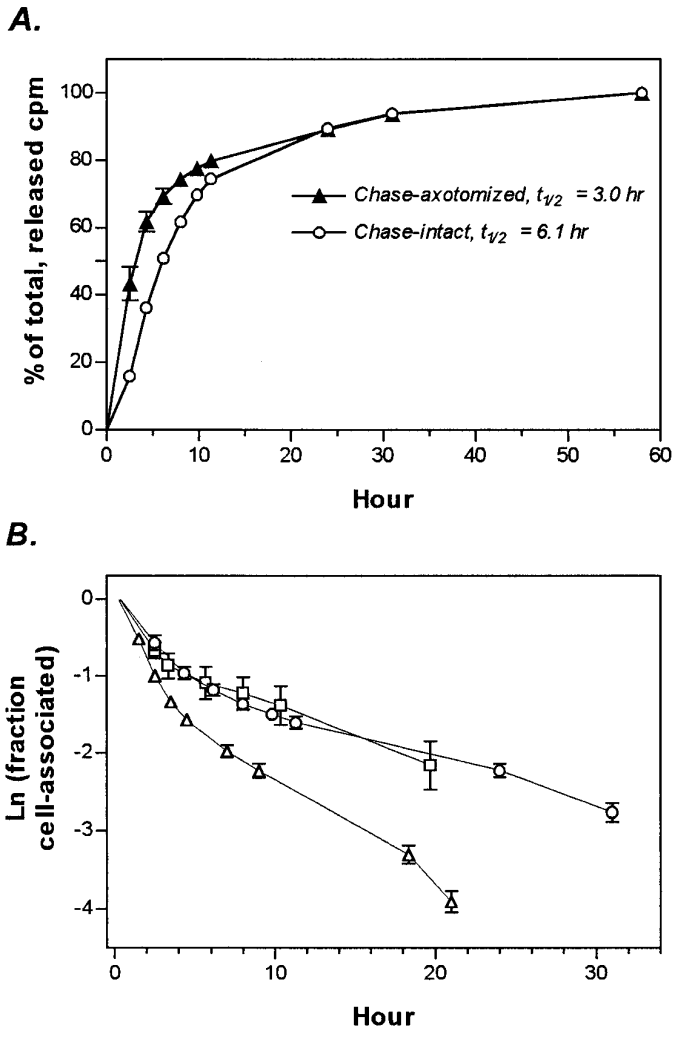

Figure 7. Time course of NGF degradation by pulse–chase analysis. *A*, Distal axons of 26-d-old three-compartment cultures were pulsed with 200 ng/ml ^{125}I-NGF for 5 hr, during which ^{125}I-NGF associated with distal axons and retrogradely accumulated in cell bodies/proximal axons. After the pulse, distal axons were left intact in some cultures (*circles*) or were removed by axotomy in other cultures (*triangles*), and the ^{125}I-NGF-containing medium was replaced with medium containing 200 ng/ml NGF. Radioactivity in the medium bathing cell bodies/proximal axons, representing ^{125}I-NGF degradation products, was quantified repetitively in the same cultures by medium exchange at the times shown. Data are expressed as percentages of the total cumulative release. Mean values from one of three experiments (±SEM; 3 cultures/group) are shown. *B*, Kinetic analysis of the estimated decay of ^{125}I-NGF from cell bodies/proximal axons from cultures in which distal axons were absent during the chase (see Materials and Methods). Results from three separate experiments, indicated by different symbols, are shown (9 cultures total).

The pulse–chase analysis also was performed in cultures in which distal axons were removed immediately after the 5 hr ^{125}I-NGF pulse (chase-axotomized cultures). Because there was no prolonged retrograde transport in these cultures, the accumulation of radioactivity in medium bathing cell bodies/proximal axons during the chase primarily reflected degradation of the ^{125}I-NGF that resided in cell bodies at the end of the pulse. A representative time course, from one of three experiments, is shown in Figure 7*A*. As expected, the half-maximal release of ^{125}I-NGF degradation products in the chase-axotomized cultures occurred sooner ($t_{1/2}$ = 3 hr) than in cultures in which distal axons were left intact during the chase ($t_{1/2}$ = 6.1 hr), although the pattern of clearance was similar in both types of cultures. From the 3 hr difference in half-maximal release we can conclude that, when the ^{125}I-NGF supply is removed from distal axons (in chase-intact cultures) and the axons are chased with NGF, significant amounts of ^{125}I-NGF continue to be retrogradely transported for no more than ~3 hr. After its delivery to cell bodies, ^{125}I-NGF is degraded with an average half-life of ~3 hr. A similar half-life for NGF *in vivo* has been reported previously (Korsching and Thoenen, 1985).

To determine whether all ^{125}I-NGF in cell bodies/proximal axons was degraded at a uniform rate or, alternatively, whether there was more than one rate of degradation, we performed a kinetic analysis (see Materials and Methods) of the data from the chase-axotomized cultures above. Results from all three experiments (total of 9 cultures) are shown in Figure 7*B*. In each experiment the decay in cell body ^{125}I-NGF was biphasic, suggesting that there were two distinct rates of ^{125}I-NGF degradation. Slopes from the early and late stages of ^{125}I-NGF turnover seemed to differ two- to threefold. These data suggest that cell bodies/proximal axons contained two or more functionally different pools of ^{125}I-NGF that were degraded at different rates, the net result being an average half-life of 3 hr.

DISCUSSION

Some cellular events promote NGF accumulation in cell bodies

Retrograde accumulation of trophic factor in cell bodies is believed to be important for retrograde signaling. Several aspects of the processing of NGF by sympathetic neurons, which we have investigated, promote accumulation of NGF in cell bodies. First, we observed that little or no NGF was degraded or released intact from axons during retrograde transport, which was not known from previous experiments. Degradation or release of intact NGF during transport, if it had been observed, seriously would have questioned views about the role of NGF transport.

A second aspect is the rapid transport velocity for NGF of 10–20 mm/hr. This velocity matches reported retrograde organelle velocities from a variety of axons (Forman et al., 1977; Smith and Cooper, 1981; Koles et al., 1982; Breuer et al., 1987; Abbate et al., 1991), which suggests that once NGF is loaded onto the transport mechanism it is optimally delivered to cell bodies. The several-fold, higher velocity that we observed, as compared with that in sympathetic neurons *in vivo* (Hendry et al., 1974a,b; Johnson et al., 1978), might suggest that the velocity of retrograde transport is faster in immature neurons used for culturing than in adult neurons used in the *in vivo* studies.

A third process favoring accumulation of NGF in cell bodies is a relatively slow degradation rate. As compared with the turnover of a wide variety of internalized ligands in non-neuronal cells, the 3 hr half-life for cell body NGF that we observed is relatively slow (Chen et al., 1982; Huang et al., 1982; Wakai et al., 1984; Davies et al., 1985; Fujii et al., 1986; Zoon et al., 1986; Roupas and Herington, 1987; Sorkin et al., 1991; Yanai et al., 1991; Auletta et al., 1992; Nielson, 1992; Pandey, 1992; Zapf et al., 1994). Interestingly, kinetic analysis suggested that the 3 hr average half-life is more likely the net result of two distinct rates of degradation. Basic FGF and TNF-α can be internalized and degraded at different rates, depending on the type of receptor to which they are bound (Pennica et al., 1992; Gleizes et al., 1995), so by analogy, perhaps the degradation rate for NGF is different, depending on whether NGF is bound to trkA or p75.

NGF transport is rate-limited upstream of the transport mechanism

We observed that no less than 4 hr was required for ^{125}I-NGF to be retrogradely transported in amounts equivalent to steady-state levels in distal axons. This interval corresponds to a maximum transport rate of 25%/hr. Periods of at least 10 hr (transport rate $\geq$ 10%/hr) were most common. Transport rates seemed to be only weakly dependent on the ^{125}I-NGF concentration, which ranged 200-fold from 8 pM to 1.5 nM, suggesting that receptors of more than one affinity were involved in NGF transport. This conclusion is consistent with previous findings *in vivo* (Dumas et al., 1979). Because virtually all axonal binding should be to high affinity receptors at 8 pM NGF on the basis of binding characteristics of other neurons (Sutter et al., 1979; Godfrey and Shooter, 1986) and because it has been shown previously that NGF bound to high affinity receptors in PC12 cells is internalized at a high rate (Bernd and Greene, 1984), we expected to observe higher transport rates than we did. The low transport rate observed at any concentration used did not reflect a low capacity of the transport mechanism, because previous results indicate that retrograde transport does not saturate until at least 4 nM NGF (Hawrot, 1982). The low transport rate also did not result from release of degraded or intact NGF while it was on the transport mechanism, as shown above. Therefore, we conclude that retrograde delivery of NGF to cell bodies was rate-limited before the shuttling of NGF-containing organelles along the microtubule-based transport mechanism. The finding that ~85% of the distal axon NGF was surface-bound at steady-state strongly suggests that internalization was rate-limiting. Theoretically, had the rate-limiting step occurred after internalization, then a large intra-axonal accumulation of NGF should have been observed.

We considered whether the transport rate was low because of a high fraction of binding to low affinity receptors that might not have been efficient at internalizing NGF (Bernd and Greene, 1984; Hosang and Shooter, 1987; Kasaian and Neet, 1988). This is an inadequate explanation for the transport rates observed at 8 pM NGF, because at this concentration very little of the binding should have been to low affinity receptors. Because p75 neurotrophin receptor binds NGF with mostly low affinity, the low transport rate at 8 pM NGF cannot be explained by excessive binding to p75. It is possible, however, that the slightly lower transport rates that were observed when using the highest NGF concentration (40 ng/ml) might have resulted from increased binding to p75, because several studies indicate that p75 internalizes and/or transports NGF at a low rate, if at all (Le Bivic et al., 1991; Kahle and Hertel, 1992; Kahle et al., 1994; Mahadeo et al., 1994; Curtis et al., 1995). Furthermore, augmented p75 binding might have been the reason for lower transport rates in older cultures. We have found that p75 mRNA levels increase with culture age in neurons, given a concentration of NGF similar to that used to maintain cultures in the present study (Ma et al., 1992).

As an explanation for the low transport rate, we hypothesize that a large fraction of the axon-bound NGF might have been bound to receptors that did not have immediate access to sites of internalization. That is, perhaps molecules necessary for efficient internalization are in limited supply or are compartmentalized in axons. For example, although it has been shown that receptors along sensory axons *in vivo* do internalize and retrogradely transport NGF (Richardson and Riopelle, 1984), it is possible that only receptors on growth cones internalize NGF at a high rate.

Transport velocity does not account for the delay in NGF transport

^{125}I-NGF first appears in cell bodies/proximal axons after a delay of ~1 hr after its application to distal axons (Claude et al., 1982b) (additional data not shown). This delay is not likely to result from a delay in ^{125}I-NGF binding to receptors on the axonal surface, because significant binding to distal axons occurs within 10 min of ^{125}I-NGF application (Hawrot, 1982). We can now consider whether transport velocity accounts for the delay. Because the transport velocity was found to be 10–20 mm/hr and because a distance of only 1 mm separates distal axon and cell body/proximal axon compartments, ^{125}I-NGF should have appeared in cell bodies/proximal axons within several minutes if it had been internalized immediately by axons and loaded onto the retrograde transport mechanism. Moreover, the transport mechanism should have been sufficiently rapid to transport a large quantity of ^{125}I-NGF (e.g., 1 axonal receptor load) within the first hour of incubation, because the longest distance over which ^{125}I-NGF had to be transported in, for example, 20-d-old cultures was ~20 mm, based on observed axon extension rates of 1 mm/d (Campenot, 1982). Assuming that the average transport rate measured after 4 hr of transport (data not shown) (see also Claude et al., 1982b) is similar to rates observed during earlier periods, our assays should have detected transport within several minutes. Therefore, we can now interpret that the lag in transport is not a reflection of the transport velocity. Instead, the lag might indicate that NGF-receptor complexes reside at the axon surface for a considerable period before being internalized, which is consistent with our observation of a large proportion of axonal ^{125}I-NGF at the surface. Also, NGF-containing organelles possibly could undergo a maturation step before loading onto the retrograde transport mechanism, which could contribute to the lag. Endosomal maturation has been described for epidermal growth factor (EGF) (Dunn and Hubbard, 1984; Schmid et al., 1988; Stoorvogel et al., 1991).

Distal axons as reservoirs of NGF

A surprising result from this study was that less NGF was maintained in cell bodies by retrograde transport than was associated with distal axons at steady-state. Given the importance of retrograde signaling to neurons and the important role that trophic factor transport is thought to play in this process, it could have been predicted that more ^{125}I-NGF would have accumulated in cell bodies. A relatively low level of cell body NGF is not an obvious interpretation from previous studies *in vivo* showing that NGF is more concentrated in sympathetic ganglia than in samples of target tissues (Korsching and Thoenen, 1983a, 1988; Nagata et al., 1987). However, in similarity to our findings, more recent studies *in vivo* report that at least for some sympathetic neurons NGF is extensively associated with axon terminals (Liu et al., 1996). As already discussed, several transport parameters are consistent with a role for NGF transport in retrograde signaling, but the larger amount of NGF associated with distal axons than in cell bodies suggests other important functional roles. Axonal NGF undoubtedly has local signaling functions, such as promoting axon growth, but additionally, axonal NGF might generate other types of retrograde signals that travel to cell bodies unaccompanied by NGF.

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