RETROGRADE TRANSPORT OF NERVE GROWTH FACTOR IN LESIONED GOLDFISH RETINAL GANGLION CELLS[1]

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Abstract

Previous experiments have shown that nerve growth factor (NGF) enhances regeneration of goldfish optic nerve after local application of NGF at the site of the lesion. However, the site and mechanism of action of NGF are not yet known. One possibility is that NGF is taken up at the site of the lesion and retrogradely transported to the cell bodies of the retinal ganglion cells and thereby exerts its trophic effects. The present work was carried out to assess the role of retrograde transport of NGF in this enhanced regeneration of goldfish retinal ganglion cells. In intact retinal ganglion cells of the goldfish, ^{125}I-labeled NGF was found not to be retrogradely transported from the optic tectum to the retina, suggesting that retinal ganglion cells do not possess specific NGF receptors. However, if [^{125}I]NGF was injected at the site of an optic nerve lesion at the time of lesion, [^{125}I] NGF was retrogradely transported from the site of a lesion of the optic nerve to the cell body of retinal ganglion cells. The accumulated radioactivity was shown to be intact NGF by SDS-PAGE. The ability of NGF to decrease the time required for recovery of visual function was observed only when NGF was administered at the time of the injury. Likewise, no transport of [^{125}I]NGF was observed when it was injected at the crush site 16 hr or longer after crush. Thus, there is a temporal correlation between the ability of intact [^{125}I]NGF to be retrogradely transported from a lesion site to the retina and the regenerative effect of NGF. Autoradiography showed that the [^{125}I]NGF accumulated only in retinal ganglion cells. The transport of NGF in the lesioned goldfish visual system was not specific for NGF in that other proteins (cytochrome c, bovine serum albumin) were transported equally well. Likewise, transport of [^{125}I]NGF was not prevented by concomitant administration of excess unlabeled NGF. The retrograde transport of [^{125}I]NGF therefore was not selective and did not appear to be mediated by specific NGF receptors in this system. This nonspecific transport of [^{125}I]NGF did not occur in the axotomized spinal motor neurons in the neonatal or adult rat or in the newt. However, receptor-mediated transport is seen in lesioned sensory neurons in both species. These results suggest that if NGF can gain access to the inside of the cell bodies of goldfish retinal ganglion cells, it is capable of exerting positive effects on these neurons despite the lack of observable receptors for NGF. Perhaps one reason why the visual system of goldfish regenerates so successfully is that it is capable of retrogradely transporting a variety of proteins, possibly including endogenous trophic factors.

The goldfish visual system has provided a good model for studying the mechanisms underlying the regenerative process in the central nervous system (Sperry, 1944;

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Jacobson, 1970; Grafstein, 1975; Landreth and Agranoff, 1979). After optic nerve lesion, the retinal ganglion cell undergoes a series of morphological and biochemical changes (Murray and Grafstein, 1969; Murray and Forman, 1971; Grafstein, 1978; Agranoff et al., 1980). These changes are followed by a vigorous axonal outgrowth from the regenerating retina. The new fibers eventually terminate in specific target areas in the contralateral tectum and contribute to a functional recovery of the visual system (Gaze, 1970; Jacobson, 1970; Grafstein, 1978).

It has been demonstrated that nerve growth factor (NGF) has a profound effect on neuronal survival and

neurite outgrowth in embryonic and adult sympathetic and in embryonic sensory ganglia (for reviews see Levi-Montalcini and Angeletti, 1968; Varon, 1975; Bradshaw, 1978; Thoenen and Barde, 1980). Recent studies have also shown that NGF promotes regeneration of optic nerve axons in lower vertebrates. For example, after optic nerve lesion in the newt, the number of regenerating axons per cross-section of nerve is increased significantly in the NGF-treated animals as compared to controls, and the retinal ganglion cell body responses to axotomy are both accelerated and intensified by NGF treatment (Turner and Glaze, 1977; Turner et al., 1978; 1982; Turner and Delaney, 1979). In goldfish, NGF, if administered at the time of lesioning, decreases (20 to 40%) the time required for recovery of the startle reaction to a light stimulus after an optic nerve crush (Yip and Grafstein, 1982). The number of regenerating axons at various distances from the lesion site is approximately doubled in these animals. In addition, retinal ganglion cells treated with NGF prior to explantation can be shown to significantly increase neurite outgrowth in tissue culture (Turner et al., 1981, 1982). These results, together with the demonstration of immunoreactive sites for NGF in the goldfish brain (Benowitz and Shashoua, 1979) and the finding that golfdfish brain extract possesses NGF-like activity (Weis, 1968; Benowitz and Greene, 1979), suggest that an NGF-like substance may play a role in the regeneration of goldfish optic axons. However, the site and mechanism of action of NGF in the regenerating process are not known. One hypothesis is that NGF is taken up at the site of lesion and retrogradely transported to the perikarya of the retinal ganglion cells (Yip and Grafstein, 1982). Previous studies in mammalian systems have shown that NGF is taken up, through a specific receptor-mediated process, in adrenergic and sensory nerve terminals and is transported retrogradely to the cell bodies (Hendry et al., 1974a, b; Stöckel and Thoenen, 1975a, b; Johnson et al., 1978). The NGF reaching the perikarya in this manner is largely responsible for its biological effects (Hendry, 1975; Levi-Montalcini et al., 1975; Paravicini et al., 1975; Goedert et al., 1979).

In the present study, we attempted to demonstrate the retrograde transport of NGF to goldfish retinal ganglion cells. We found no evidence that NGF is retrogradely transported in the normal goldfish optic system. However, an injection of [^{125}I]NGF into a crushed optic nerve, at the time of the lesion, led to intracellular labeling of the retinal ganglion cells. The specificity of retrograde axonal transport of NGF in goldfish optic nerve was also examined and compared to the retrograde transport of NGF by axotomized motor and sensory neurons in newts and rats. Much of the data have been reported previously in abstract form (Yip and Grafstein, 1981; Yip and Johnson, 1982).

Materials and Methods

Preparation of iodinated NGF. The 2.5 S subunit of NGF, purified from adult male mouse submaxillary glands according to the method of Bocchini and Angeletti (1969), was used in all retrograde transport experiments. NGF was labeled by the lactoperoxidase method (Mar-

chalonis, 1969). The reaction was done at room temperature (23°C) with the following mixtures: 10 μl (1 mCi; Amersham) of Na^{125}I, 10 μl of NGF (10 μg), 6 μl of lactoperoxidase (160 to 200 mg/ml), and 25 μl of a 1:10^3 dilution of H$_2$O$_2$ (30%) in 0.2 M sodium phosphate buffer, pH 6.0. The reaction was terminated after 10 min by the addition of 150 μl of 0.05 M phosphate buffer, pH 7.5, containing 1 M NaCl and 0.1 M NaI. The reaction volume was adjusted to 600 μl with a buffer composed of Hanks' balanced salt solution buffered with 0.2 M HEPES and supplemented with 0.2% bovine serum albumin (BSA) and 0.1% protamine sulfate, pH 7.4. The amount of incorporation of Na^{125}I into NGF was 70 to 96%. Specific activities ranged from 60 to 80 μCi/μg. The reaction mixture was passed through a P-100 column to remove the unreacted iodine when used for NGF binding assay. The biological activity of each iodinated NGF preparation was verified by demonstrating retrograde transport after injection into the anterior eye chamber of a rat. Radioactivity in the ipsilateral and contralateral superior cervical ganglia (SCG) was determined 16 hr after the injection. All preparations produced ipsilateral:contralateral ratios of greater than five. [^{125}I]NGF was used routinely within 1 to 2 days after preparation. Equimolar concentrations of cytochrome *c* and BSA were iodinated under the same conditions.

Experimental animals and surgical procedures. Goldfish, *Carassus auratus*, 10 to 12 cm in body length were obtained from Ozark Fisheries, Stoutland, MO, and maintained at 20 ± 1°C in 10- to 15-gallon tanks. Adult newts, *Triturus viridescens* (Nasco, Fort Atkinson, WI), weighing 1.5 to 2 gm were used in some experiments. For surgical procedures, both goldfish and newts were anesthetized in a dilute (0.04%) tricaine methanesulfonate solution (Sigma Chemical Co., St. Louis, MO). In the goldfish experiments, the right optic nerve was crushed with jeweler's forceps at the back of the orbit, 3 to 4 mm from the eyeball. In the newt experiments, the right sciatic nerve was crushed at the level of midthigh. Testing for the effects of NGF on regeneration of optic nerve axons was carried out by determining the time of recovery of the startle reaction as described by Edwards et al. (1981) and Yip and Grafstein (1982).

Ten-day-old (30 to 40 gm) and adult (200 to 250 gm) Sprague-Dawley rats (Sasco Breeders, Omaha, NE) were housed in solid bottom cages, maintained on a 12-hr light-dark cycle, and were given food and water *ad libitum*. Rats were anesthetized with an intraperitoneal injection of chloral hydrate, 350 mg/kg. The crush of the right sciatic nerve was performed at the tendon of obturator internus.

Microinjection procedures. All injections were administered with a micropipette (tip diameter approximately 10 μm) attached to a Hamilton syringe. The tip of the micropipette was positioned and lowered into the crush site using a micromanipulator. A single injection of 1 to 4 μl (1 to 5 × 10^6 cpm/μl) of [^{125}I]NGF, [^{125}I]BSA or [^{125}I] cytochrome *c* was given in the optic nerve of the goldfish or in the sciatic nerve of the rat and the newt. [^{125}I]NGF was introduced into the nerve using mechanically generated pressure. Several injections of 2 to 6 μl were made into the goldfish tectum. The tip of the micropipette was

lowered approximately 0.25 to 0.5 mm into the tectal tissue. After treatment, goldfish and newts were kept in a moist chamber for 30 to 45 min before they were returned to water.

Tissue preparation for radioisotope counting. Animals were killed at different times after injection. Goldfish were dark-adapted for 30 to 45 min before decapitation. The eyes were rapidly enucleated in a partially dark room. The corneas were cut away and the retinas were carefully removed under dim dissecting light. The retina was washed three times in phosphate-buffered saline, ph 7.4, and placed in a 12 × 75 mm tube containing 1 ml of 10% buffered formalin.

The rats were perfused with isotonic saline followed by neutral buffered formalin. The lumbar spinal cord was exposed and postfixed in 10% formalin for 1 to 2 days. The rat dorsal root ganglia (DRG) (L_4 to S_1) on both sides and the corresponding spinal cord segment were dissected out for study. In the newt, spinal ganglia 15, 16, and 17 and the corresponding spinal cord segment were also removed. The unoperated contralateral DRG or spinal ganglia and that half of the spinal cord were used as controls. All tissues were counted using a Beckman model 4000 gamma counter. The radioactivity measured in the appropriate retina or DRG is contaminated by background radioactivity due to incorporation of blood-bone radioisotope. Because this background should affect both sides of the tissue equally, the amount of background radioactivity was measured in the retina or DRG contralateral to the injection. The molecular weights of the injected and transported proteins were determined by conventional SDS-PAGE and autoradiography.

Autoradiography. For autoradiographic studies of the localization of [^{125}I]NGF in goldfish retina, the retina was fixed in 10% formalin, embedded in Paraplast, sectioned at 8 μm, and mounted on glass slides. The slides were dewaxed and dried, then dipped in Kodak NTB-2 emulsion diluted 1:1 with H_2O. After the slides were exposed for 4 to 7 days at 4°C, they were developed and counterstained with toluidine blue for light microscopy.

Results

Retrograde transport of [^{125}I]NGF from optic tectum. Experiments were conducted to determine whether NGF is retrogradely transported from its target organ, the tectum, to the retinal ganglion cells. Goldfish were injected with [^{125}I]NGF in the left tectum and killed 2, 4, 6, 8, 10, and 16 hr later. The right retina, which provides the sensory innervation to the left tectum, was removed and counted for radioactivity. Very few counts, < 200 cpm, appeared in either retina, as shown in Figure 1, and there was no significant difference between the [^{125}I] NGF-injected and the noninjected retinas at any of the time points. Similar results were obtained in two other experiments.

Retrograde transport of [^{125}I]NGF in crushed optic nerve. In order to determine whether NGF was retrogradely transported to the retinal ganglion cells when applied to the optic nerve lesion site, goldfish optic nerves were crushed unilaterally and microinjected with [^{125}I] NGF at the lesion site at the time of crush. After a single
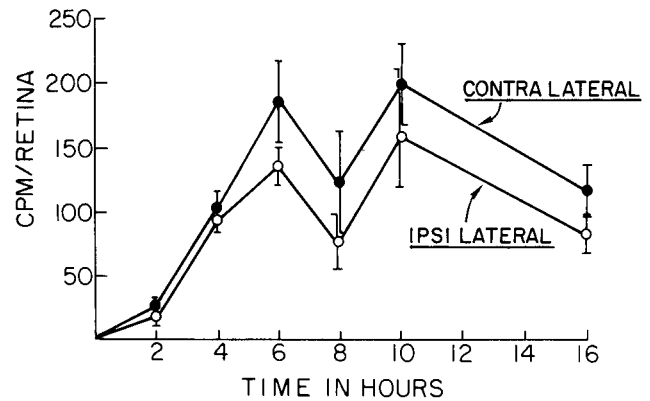

Figure 1. Time course of accumulation of radioactivity (mean ± SEM) in the goldfish retina after unilateral injection of [^{125}I] NGF (20×10^6 cpm) in the optic tectum ($n = 8$). The differences between ipsilateral (noninjected) and contralateral retinas (injected) are not statistically significant at any time point.
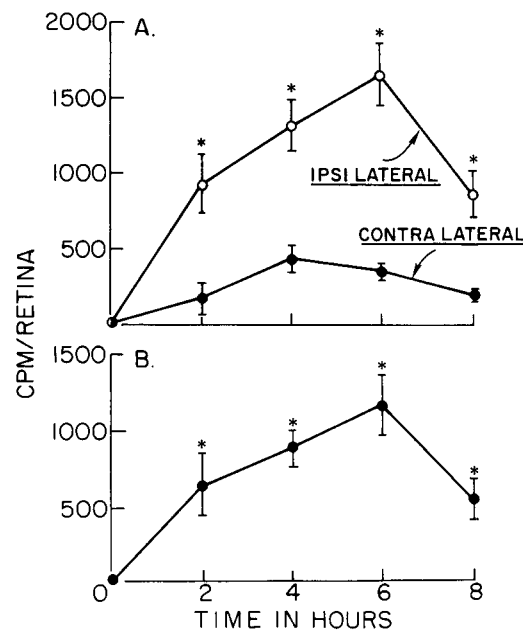

Figure 2. A, Time course of accumulation of radioactivity in the goldfish retina following injection of [^{125}I]NGF (3 to 4 × 10^6 cpm) into the crushed optic nerve at the time of lesion. All values for the [^{125}I]NGF-injected retinas are significantly (*) higher than for the corresponding noninjected retinas ($p < 0.05$ to 0.001). B, Difference between ipsilateral and contralateral retinas as shown in A.

injection of [^{125}I]NGF into the crushed optic nerve, there was a statistically significant difference between the amount of radioactivity accumulated in the retinas on the injected sides and the amount on the noninjected sides ($p < 0.05$ to 0.001) at all time points (2, 4, 6, and 8 hr) (Fig. 2). Accumulation of [^{125}I]NGF increased at a linear rate and reached a maximum 6 hr after the injection (Fig. 2). In the sham-operated group, [^{125}I]NGF was injected into the uncrushed optic nerve at the site where the nerve crush was placed in the animals already described; no significant retrograde transport of [^{125}I]NGF to the ipsilateral retina was observed (Fig. 3). This time course experiment in sham-operated animals was re-
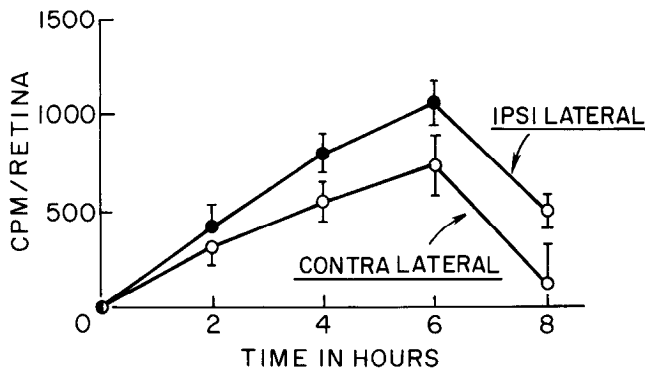
Figure 3. Time course of accumulation of radioactivity in the goldfish retina after injection of [^{125}I]NGF (3 to 4 × 10^6 cpm) in the sham-operated optic nerve. For each *time point*, the accumulation of [^{125}I]NGF radioactivity in the ipsilateral (injected) retinas is not significantly different from that of contralateral (noninjected) retinas.

peated and, again, no significant difference in the amount of radioactivity between the ipsilateral and contralateral retinas was observed. Other examples of the lack of transport in sham-operated animals are shown in Figure 5 and Table I.

To determine whether the observed transport of radioactive NGF was localized to the retinal ganglion cells, an autoradiographic examination of retinas from animals treated previously with local injection of [^{125}I]NGF to the crush was performed. As shown in Figure 4, grains were localized to a small number of labeled retinal ganglion cells from the injected side. Although no attempt has been made to determine precisely the number of labeled cells in the goldfish retina, we estimate that approximately 10% of the retinal ganglion cells were labeled. The most heavily labeled cells were found close to the margin of the retina. The majority of the silver grains were concentrated over the cytoplasm of the labeled neurons with very little labeling in the nucleus. No labeled cells were seen in retinas from the contralateral side of the same animals; nor were labeled cells found in retinas from sham-operated animals that had received an injection of equal amount of [^{125}I]NGF at the corresponding time points (not shown). These data indicate that the accumulation of radioactivity in the ipsilateral retina after application of [^{125}I]NGF to the lesion site represents retrograde transport to the retinal ganglion cells.

To determine the chemical nature of the accumulated radioactive material, homogenates of retinas in which optic nerves had been injected with [^{125}I]NGF 6 hr previously were chromatographed by SDS-PAGE and then the dried gels were autoradiographed. A single radioactive species migrating at approximately $M_r = 13,000$ was seen, indicating that the major accumulated species is NGF itself, rather than iodine or low molecular weight fragments of NGF. Densitometric tracing from the radiogram of a [^{125}I]NGF-injected retina showed a single peak which can be superimposed with [^{125}I]NGF standard (Fig. 5).

Correlation between the regenerative effects of NGF, its ability to be retrogradely transported, and the time of NGF administration. Previous experiments have suggested

that NGF may be effective only if it is given at the time of injury (Yip and Grafstein, 1981, 1982). The effects of NGF on the recovery time of visual function after optic nerve crush were examined when NGF was administered at different time intervals before or after the lesion. The time required for recovery of visual function in goldfish was determined by testing for the startle reaction (Edwards et al., 1981; Grafstein et al., 1982). It has previously been shown that the decreased time required for recovery of visual function correlated well with the increased distance of axonal outgrowth and the number of regenerating axons as revealed by histological examination (Yip and Grafstein, 1982). It was found that a single injection of NGF (900 Biological Units) was effective in decreasing by 42% of the mean recovery time when given at the time of injury. However, treatment with NGF either 12 or 48 hr before the lesion or 24 or 72 hr after the lesion had no effect on regeneration (Fig. 6). Experiments were then performed to investigate the ability of NGF to be transported at or after the time of lesion. Optic nerves were injected with [^{125}I]NGF 16 or 24 hr after crushing. The retinas were removed and counted for radioactivity 6 hr after the injection. As was seen previously (Fig. 2), a significant amount of [^{125}I]NGF accumulated in the ipsilateral retina when injected at the time of crush (Fig. 7). There was no significant difference in the accumulation of radioactivity between the retinas of the injected and noninjected sides, however, when [^{125}I]NGF was injected either 16 or 24 hr after crush. This indicates that the uptake and retrograde transport of [^{125}I]NGF in the goldfish optic nerve occurs if NGF is applied at the lesion site at the time of crush, but not if placed 16 or 24 hr after the crush. Thus, the regeneration-promoting effect of NGF was observable only when retrograde transport of [^{125}I]NGF could also be demonstrated, i.e, at the time of lesion.

Effect of a second lesion on the retrograde transport of [^{125}I]NGF. The failure to observe retrograde transport of [^{125}I]NGF when injected 16 or 24 hr after the lesion may have been due either to a decreased accessibility of [^{125}I]NGF at the crush site at these times or to a failure of the transport apparatus after injury. In order to distinguish between these possibilities, a second lesion was made at the same crush site 24 or 48 hr after the first lesion (0 hr). [^{125}I]NGF was administered at the time of second lesion. Table I summarizes the results of such studies. A significant accumulation of [^{125}I]NGF was observed in the ipsilateral retina only when injected at the time of lesion, but not when injected 24 or 48 hr after lesion as had been shown in the previous experiment (Fig. 7). However, if a second lesion was made either 24 or 48 hr after the initial lesion and [^{125}I]NGF was injected at the time of the second lesion, a significant amount of radioactivity ($p < 0.001$) accumulated in the ipsilateral retinas. This suggests that by making the second lesion at the same crush site, [^{125}I]NGF can again gain access to the newly axotomized nerve and thus can be retrogradely transported to the retinal ganglion cells.

Specificity of the retrograde transport in retinal ganglion cells. The specificity of this transport mechanism for NGF was examined by studying the ability of cytochrome *c* to be transported in the optic nerve under
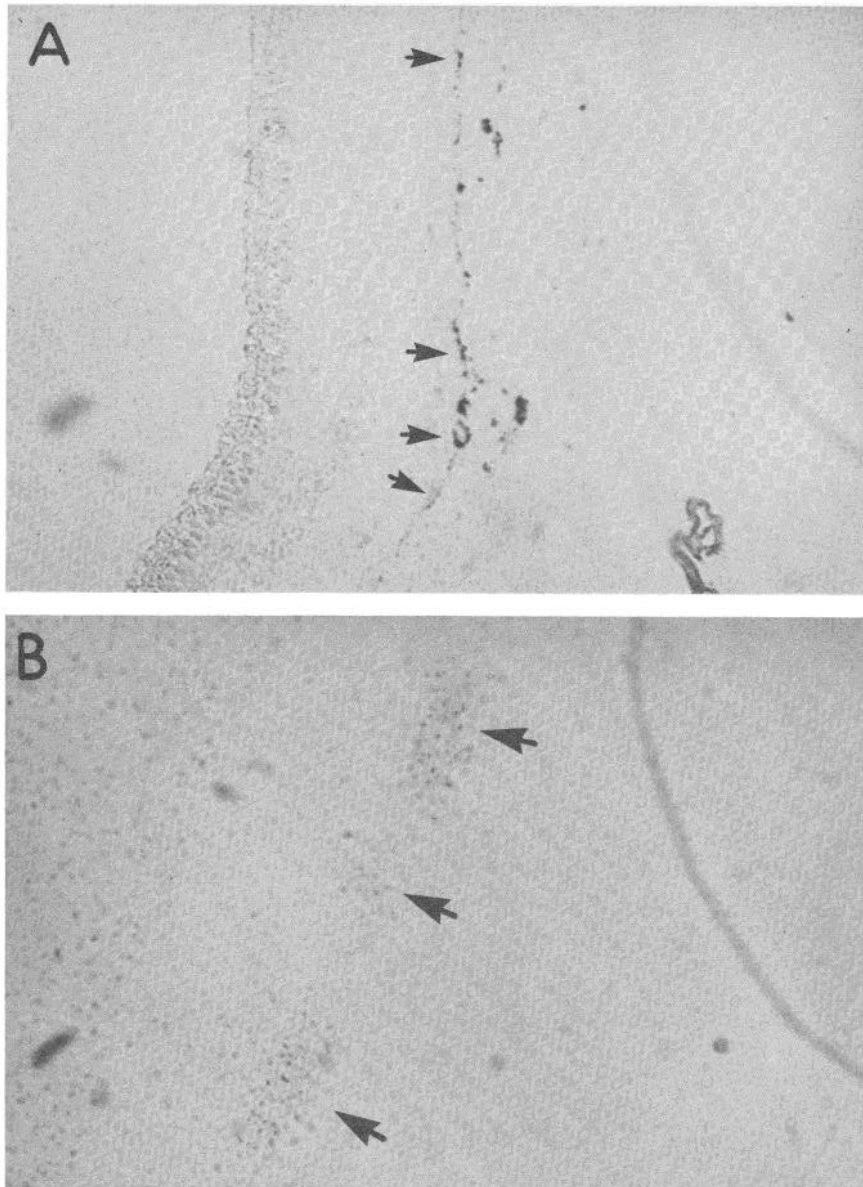
Figure 4. *A*, Brightfield autoradiograph of a goldfish retina 6 hr after injection of [^{125}I]NGF into the crushed optic nerve at the time of lesion. [^{125}I]NGF-labeled neurons were restricted to the retinal ganglion cell layer (indicated by *arrows*). No labeled cells can be found in the contralateral retina. Magnification × 200. *B*, Higher magnification of labeled retinal ganglion cells overlaid by silver grains. This indicates accumulation of [^{125}I]NGF in the cell body. Magnification × 1000.

identical experimental conditions. Cytochrome *c*, a commonly used negative control for NGF studies, has the same molecular weight and isoelectric point as NGF. Cytochrome *c* was iodinated in the same manner as NGF. Administration of [^{125}I]cytochrome *c* to a crush site in the goldfish optic nerve resulted in the accumulation of retrogradely transported radioactivity in a manner very similar to NGF. The results in Figure 8*A* show a statistically significant ($p < 0.001$) transport of cytochrome *c*. As had previously been shown with [^{125}I]NGF (Fig. 7), analysis of the radioactive material by SDS-PAGE indicated that intact [^{125}I]cytochrome *c* was the molecular species accumulating in the retina (not shown). In contrast, as has been previously reported (Hendry et al.,

1974a, b), when the same solutions were injected into the anterior eye chambers on rats, only [^{125}I]NGF was retrogradely transported back to the SCG, indicating specific receptor-mediated uptake had occurred (Fig. 8*B*). The lack of specificity of the transport system in the goldfish retinal ganglion cells can also be demonstrated by showing that [^{125}I]BSA was retrogradely transported when applied to a crushed optic nerve at the time of lesion (Fig. 9).

To determine whether retrograde transport of NGF in goldfish optic nerve is mediated through binding to membrane receptors, we evaluated the effect of the addition of a large excess of nonradioactive NGF on the transport of labeled NGF. The result, as illustrated in Figure 8*A*,
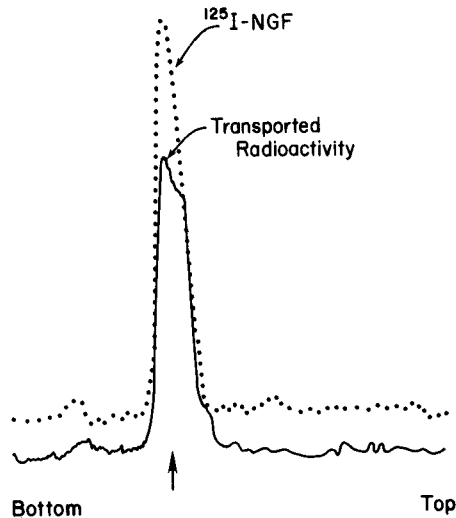
Figure 5. Densitometer tracings of autoradiograms of SDS-PAGE (15%) gels of iodinated NGF (^{125}I-NGF, *dotted line*) injected into an optic nerve lesion and of an extract of the retina 6 hr after injection of the labeled NGF (*solid line*).
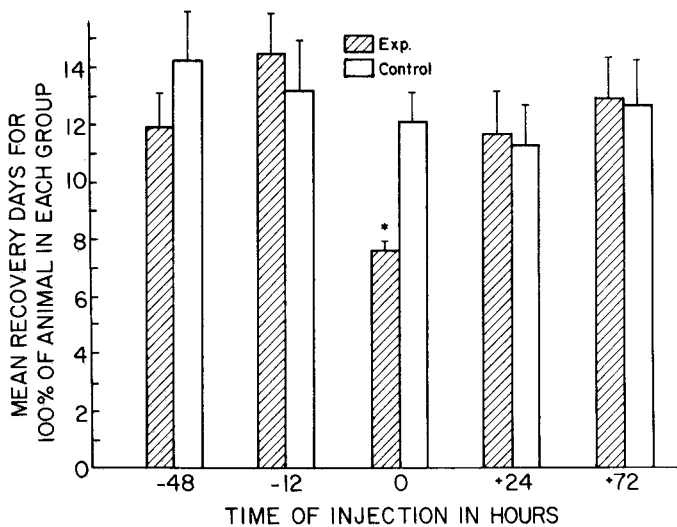

Figure 6. Mean recovery times for recovery of startle reaction (mean and SEM for 10 animals/group) with a single injection of 7 S NGF (900 Biological Units) or injection vehicle (0.05 M Tris buffer). Injections were made at 12 and 48 hr before the time of crush, or 24 and 72 hr after the nerve crush. Decrease in recovery time is statistically significant (* $p < 0.001$) only in the group which had NGF administered at the time of crush.

indicates that a large excess of nonradioactive NGF is unable to compete with the [^{125}I]NGF and to effectively block its transport to the goldfish retina. In contrast, an excess quantity of nonradioactive NGF blocked the transport of labeled NGF to the rat SCG (Fig. 8B).

Retrograde transport of [^{125}I]NGF in axotomized motor neurons. In order to determine whether the retrograde transport of NGF in axotomized axons is confined to the goldfish retinal ganglion cells or whether this phenomenon is common to all neurons, we investigated the retrograde transport of NGF in axotomized motor and sensory neurons in the sciatic nerve of the adult and neonatal rats. The time course, 4, 6, 8, and 10 hr of [^{125}I]NGF accumulation in the lower lumbar spinal cord (L_4 to S_1)

and the corresponding DRG was studied after unilateral injection of [^{125}I]NGF into the crushed sciatic nerve at the time of crush. There was a significant accumulation of [^{125}I]NGF in the L_4 and L_5 DRG on the injected side, as compared to the noninjected side, 6 hr after the labeled NGF was administered (Fig. 10). The radioactivity
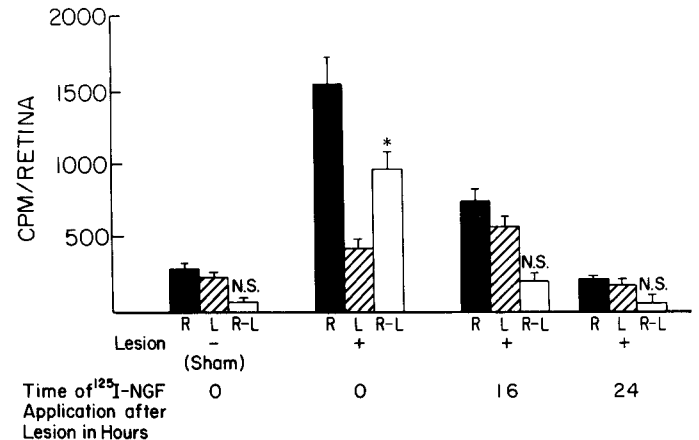

Figure 7. Administration of [^{125}I]NGF as a single local injection at the time of nerve crush (*+0*) or sham operation (*-0*), 16 or 24 hr after the crush (*+16*, *+24*). Accumulation of radioactivity in the retina is statistically significant (*; $p < 0.001$) only when [^{125}I]NGF was injected at the time of crush. *R*, right, injected side; *L*, left, noninjected side; *R-L*, difference between injected and noninjected sides.

TABLE I

Effect of a second lesion on the retrograde transport of [^{125}I]NGF in goldfish retinal ganglion cell

[^{125}I]NGF (3 × 10^6 cpm) was injected at the crush site with or without a second lesion at the same site, 24 or 48 hr after the first lesion (0 hr). Retinas were dissected 6 hr after the addition of [^{125}I] NGF. Radioactivity was determined as described under "Materials and Methods." The values given represent means ± SEM of five to six animals.

Treatment	Accumulated Radioactivity	
	Left	Right[a]
	cpm/retina	
No lesion at 0 hr		4 ± 58
+	160 ± 31	18
Injection at 0 hr		
1st lesion at 0 hr		
+	211 ± 491	1337 ± 172[b]
Injection at 0 hr		
1st lesion at 0 hr		
+	170 ± 37	147 ± 44
Injection at 24 hr		
1st lesion at 0 hr		
+	194 ± 54	1252 ± 282[b]
2nd lesion and injection at 24 hr		
1st lesion at 0 hr		
+	153 ± 45	256 ± 104
Injection at 48 hr		
1st lesion at 0 hr		
+	156 ± 46	916 ± 81[b]
2nd lesion and injection at 48 hr		

[a] Lesioned side.
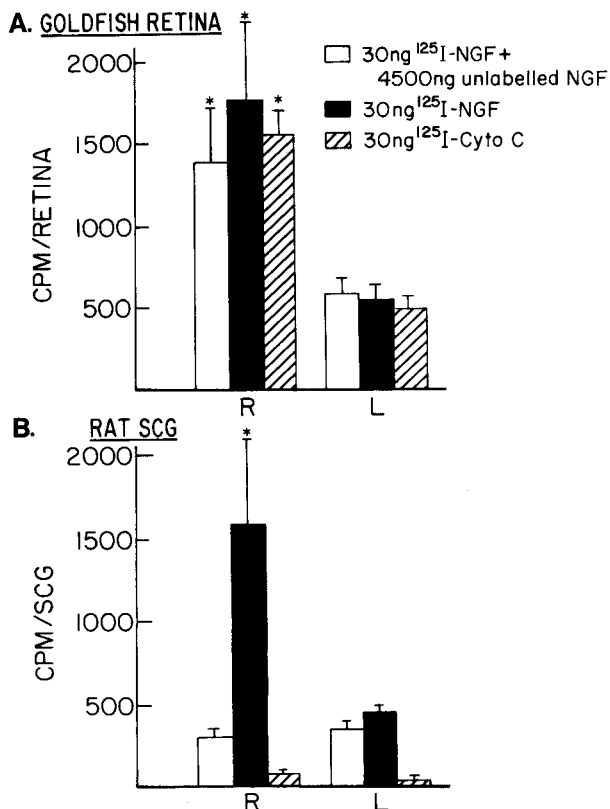[b] Significantly ($p < 0.001$) higher than control (left side).

A. GOLDFISH RETINA

B. RAT SCG

Figure 8. A Administration of [^{125}I]cytochrome c (3 × 10^6 cpm) or [^{125}I]NGF (3 to 4 × 10^6 cpm) in the presence or absence of excess unlabeled NGF to a crushed goldfish optic nerve. Significant ($p < 0.001$) accumulation of retrogradely transported radioactivity is denoted by an *asterisk. B*, Accumulation of radioactivity in the SCG, following an injection of [^{125}I]NGF into the anterior eye chamber of the rat.
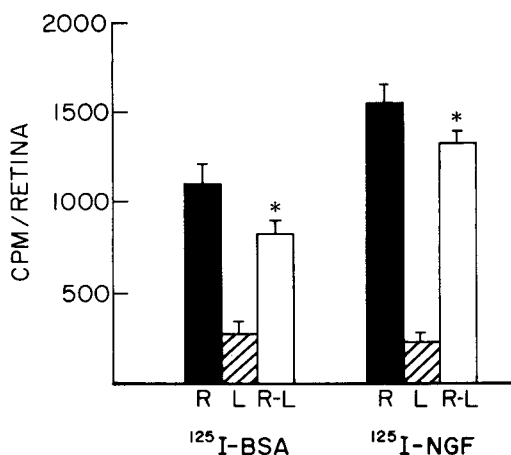

Figure 9. Administration of [^{125}I]BSA (1.1 × 10^6 cpm) to a crushed goldfish optic nerve. Accumulation of retrogradely transported radioactivity in the retina is statistically significant (*; $p < 0.01$). R, right, injected side; L, left, noninjected side; R-L, difference between injected and noninjected sides.

reached a maximum at 8 hr after injection and declined thereafter. However, in the spinal cord segments, no statistically significant difference between the amount of radioactivity accumulated on the injected and noninjected side was observed during the entire time course.
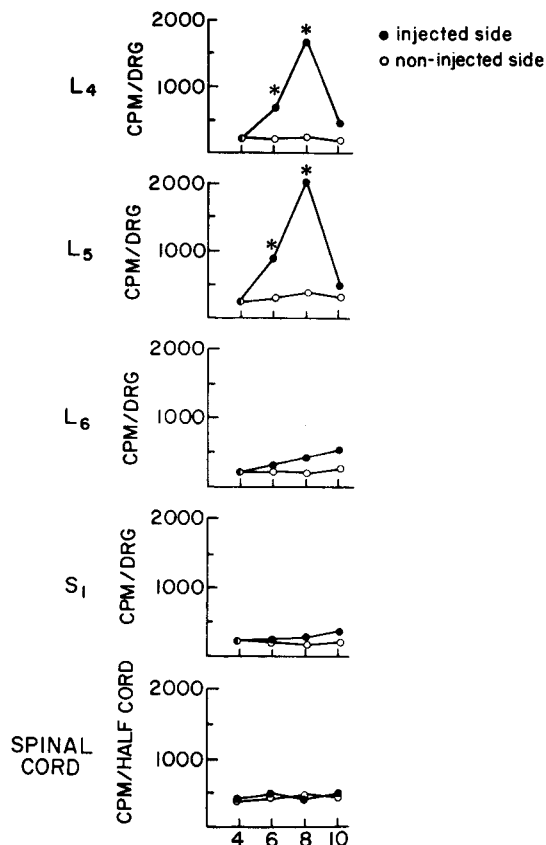

Figure 10. Time course of accumulation of radioactivity in the rat DRG (L$_4$ to S$_1$) and spinal cord after injection of [^{125}I] NGF (5 × 10^6 cpm) in the crushed sciatic nerve at the time of lesion. *Asterisks* indicate a significant difference between the values of injected and noninjected sides ($p < 0.001$).

Similar results were obtained in neonatal rats (data not shown).

Several recent papers have demonstrated that NGF has a stimulatory effect on the regeneration of transected retinal ganglion cells of the newt, *Triturus viridescens* (Turner and Glaze, 1977; Turner and Delaney, 1979). Experiments were performed to determine whether it is a general property for all axotomized neurons in the lower vertebrates to retrogradely transport NGF (i.e., whether these neurons are less discriminating in what they transport). [^{125}I]NGF was unilaterally injected into a crushed sciatic nerve of the newt at the time of lesion. Accumulation of ^{125}I radioactivity in the spinal ganglia 15, 16, and 17, the main contribution to the sciatic nerve of the newt, and the respective spinal cord segment was studied 2, 4, 6, and 8 hr after the injection. The data showed (Fig. 11) a result similar to that of the rat sciatic nerve; that is, the difference between the [^{125}I]NGF accumulated in the DRG (spinal ganglia 16 and 17 in the newt) of the injected and noninjected side showed a statistically significant difference ($p < 0.01$) and there was no significant accumulation of ^{125}I radioactivity in the spinal cord segments (Fig. 11). Thus, after injection of [^{125}I]NGF into a crushed sciatic nerve of either rat or newt, there is a preferential accumulation of radioactivity in the sensory neurons, but not motor neurons, on the injected side. In the presence of a large quantity of nonradioactive NGF, accumulation of [^{125}I]NGF was not
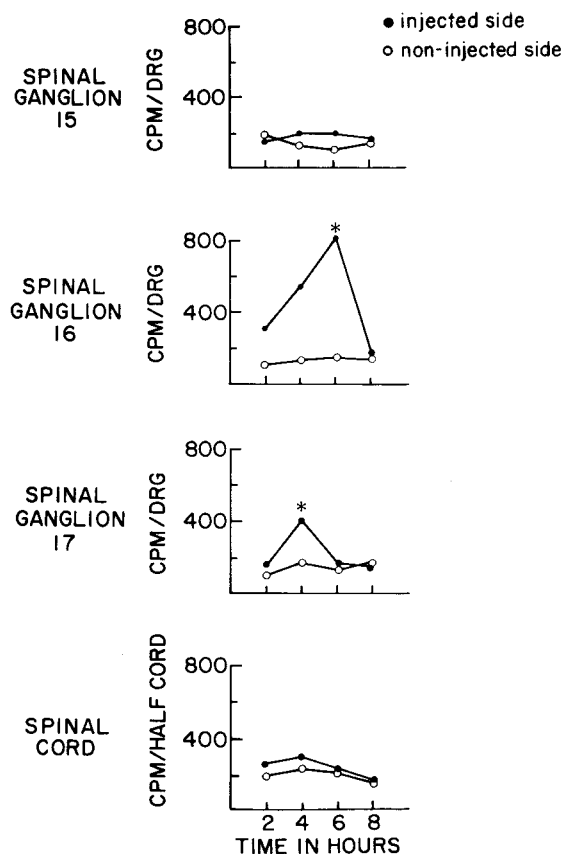
Figure 11. Time course of accumulation of radioactivity in the newt spinal ganglia (15 to 17) and spinal cord after injection of [^{125}I]NGF in the crushed sciatic nerve at the time of lesion. *Asterisks* indicate a significant difference ($p < 0.01$ to 0.001) between the values of injected and noninjected sides.

Figure 12. Accumulation of radioactivity in the rat DRG (L$_4$ to L$_6$) and spinal cord after injection of [^{125}I]NGF (4 × 10^6 cpm), [^{125}I]cytochrome c (3 × 10^6 cpm), or [^{125}I]NGF (4 × 10^6 cpm) in the presence of excess unlabeled NGF. Significant transport of radioactivity (*; $p < 0.01$ to 0.001) was seen only with [^{125}I]NGF alone and only in L$_4$,L$_5$ DRG. *R*, right, injected side; *L*, left, noninjected side; *R-L*, difference between injected and noninjected sides.

observed and [^{125}I]cytochrome c was not transported to the DRG of either rat (Fig. 12) or newt (Fig. 13) indicating that retrograde transport of NGF by the peripheral sensory neurons from a crush site is receptor mediated and specific for NGF.

Discussion

Our results show that no measurable amount of ^{125}I-labeled NGF is retrogradely transported from the optic nerve terminals in the intact goldfish optic tectum to the cell bodies in the retina. In addition, we have been unable to detect binding of [^{125}I]NGF to membrane from goldfish tectum and retina (unpublished data). Our results are consistent with the finding that [^{125}I]NGF did not bind to the retinal ganglion cells in tissue culture (M. E. Schwab and J. E. Turner, personal communication). Hence, at the limits of detection of the method, there is no evidence for specific NGF receptors in the goldfish retinotectal pathway.

Previous observations have demonstrated the stimulatory effect of NGF on the regenerative capacity of the crushed goldfish optic nerve (Turner et al., 1981, 1982; Yip and Grafstein, 1982). It has been suggested that this enhancement may be mediated through the uptake of NGF at the site of lesion and retrograde transport of NGF to the cell bodies of the retinal ganglion cells (Yip and Grafstein, 1982). The present experiments have pro-
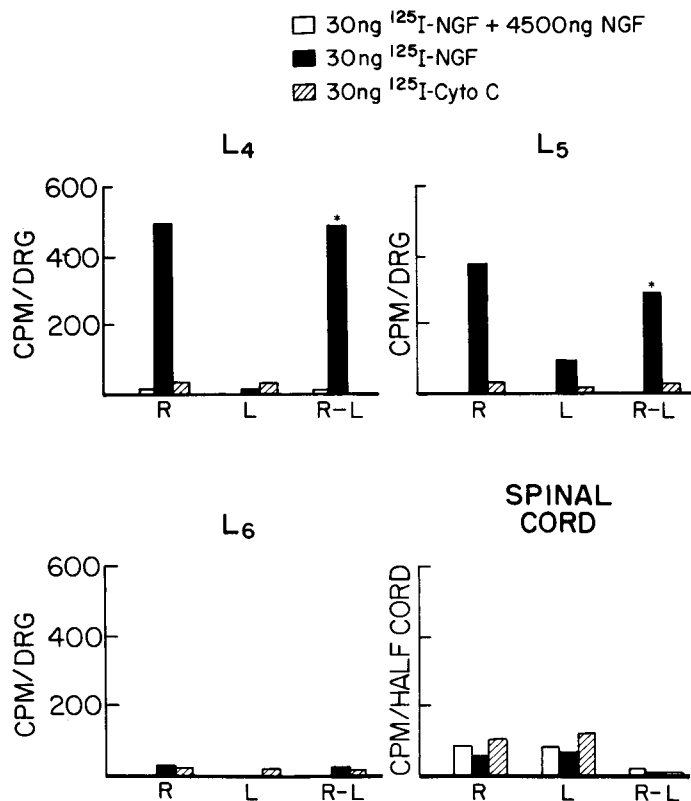
vided evidence for the presence of such a retrograde axonal transport in the axotomized goldfish optic nerve. This evidence is based on the observations that there was a preferential accumulation of ^{125}I radioactivity in the goldfish retina on the injected side after a local microinjection of [^{125}I]NGF into the crushed optic nerve. Administration of NGF in sham-operated animals did not result in the accumulation of significant amounts of radioactivity in the retinas on the injected side. The small but statistically insignificant difference shown in Figure 3 was probably because of the damage of some of the optic axons produced by the process of injection. Several identical experiments consistently showed that there was no difference between the injected and the noninjected retinas (see Fig. 7 and Table I) in the sham-operated animals.

The most direct evidence for axonal transport to the retinal ganglion cell was provided by the autoradiographic studies which revealed the presence of intensely labeled retinal ganglion cells on the injected side. It is interesting to note that most of the heavily labeled cells were located near the outer border of the retina. The presence of radioactivity over a small number of cells could be explained by the ability of different populations of retinal ganglion cells to take up NGF. In goldfish, it is known that the retinal ganglion cells are replaced by
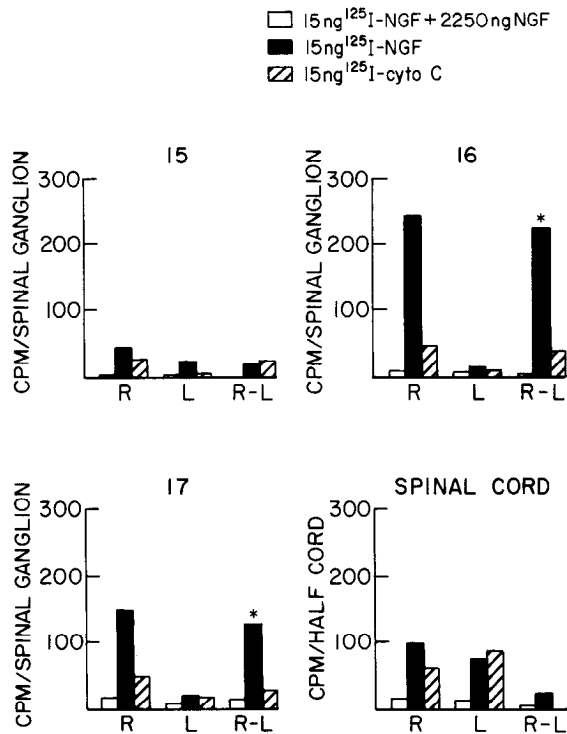
□ 15 ng ^{125}I-NGF + 2250 ng NGF
■ 15 ng ^{125}I-NGF
▨ 15 ng ^{125}I-cyto C

Figure 13. Accumulation of radioactivity in the newt spinal ganglia (15 to 17) and spinal cord after injection of [^{125}I]NGF (4 × 10^6 cpm), [^{125}I]cytochrome c (3 × 10^6 cpm), or [^{125}I]NGF (4 × 10^6 cpm) in the presence of unlabeled NGF. Significant transport of radioactivity (∗; $p < 0.05$ to 0.001) was seen again only with [^{125}I]NGF alone and only in spinal ganglia 16 and 17. R, right, injected side; L, left, noninjected side; R-L, difference between injected and noninjected sides.

new cells throughout life and the newly added cells are placed along the margin of the retina (Johns, 1977; Johns and Easter, 1977; Maier, 1978; Sharma and Ungar, 1980). It is possible that NGF is preferentially taken up by these "juvenile" retinal ganglion cells. The absence of any labeled cells on the noninjected side and the sham-operated retinas indicated that accumulation of radioactivity in the cells depends on the retrograde axonal transport of NGF and is not due to the accumulation of blood-borne [^{125}I]NGF by the cell bodies. The observation that the radioactivity of the transported molecule in the retina comigrates with authentic [^{125}I]NGF strongly suggests that the transported radioactive compound is, in fact, [^{125}I]NGF, rather than a fragment of NGF or free iodine.

Previous studies (Yip and Grafstein, 1981, 1982) have suggested that NGF is effective in enhancing goldfish optic nerve regeneration only when it is applied at the time of lesion. It the present study, NGF had no effect on recovery of visual function when it was administered before or after the crush. ^{125}I-labeled NGF applied 16 or 24 hr after the lesion was not retrogradely transported and did not accumulate in the retinal ganglion cells. It has been observed by Kao et al. (1977) that the cut ends of the severed nerve fibers seal as early as 3 hr after the transection. It is most likely that 16 or 24 hr after the lesion, NGF cannot gain access to the inside of axons and, hence, is not taken up and retrogradely transported

by the retinal ganglion cells. This hypothesis is further supported by the finding that after making a second lesion at the same site 24 or 48 hr later, and injecting the labeled NGF at the time of the second lesion, retrograde transport can again be observed.

The transport and the accumulation of NGF in the goldfish visual system does not appear to be a specific receptor-mediated process as is seen in the mammalian sympathetic and sensory neurons. Both cytochrome c and BSA were retrogradely transported as efficiently as was NGF by the axotomized goldfish retinal ganglion cells. In contrast, cytochrome c and BSA are not transported in mammalian sympathetic neurons (Hendry et al., 1974b), a finding we have confirmed in this study. This lack of specificity may indicate that injured retinal ganglion cells of goldfish are capable of picking up macromolecules indiscriminately from the environment. Among the macromolecules that we have tested in this study (NGF, cytochrome c, and BSA), and only NGF was shown to have a stimulatory effect on optic nerve regeneration (Yip and Grafstein, 1982; Turner et al., 1982). Goldfish retinal ganglion cells, which appear to be indiscriminant in what they transport, may be better able to acquire useful trophic factors that facilitate nerve regeneration. This could partially explain why optic nerve regeneration is so remarkably successful in goldfish. In contrast, NGF does not seem to have any effect on optic nerve regeneration in rats (H. K. Yip and E. M. Johnson, preliminary result). Furthermore, the retrograde transport of NGF in the goldfish optic nerve does not seem to be receptor mediated. This can be deduced from the observation that impairment of retrograde transport of NGF was not observed after an addition of a large excess of unlabeled NGF. This is consistent with the results from the binding assays in which we failed to demonstrate any specific NGF receptors in the goldfish retina and tectum (data not shown). These results are again in contrast to the findings that the uptake of NGF and the subsequent axonal transport by the mammalian sympathetic and sensory terminals are mediated through surface membrane receptors.

We have also demonstrated that this phenomenon of retrograde transport of NGF in goldfish retina does not occur in the axotomized rat and newt motor neurons. However, the axotomized sensory neurons of both the rat and the newt exhibit the ability to transport NGF retrogradely. The results indicate that the transport of NGF from a crush site is receptor mediated; that is, the receptors themselves presumably are being retrogradely transported toward the cell body. The similarity of the findings in DRG of both rat and newt suggests that this may be a general property of the sensory neurons.

It is very interesting that sensory neurons in newt also display the ability to selectively retrogradely transport NGF as is seen in birds and mammals (Thoenen and Barde, 1980). Previous studies have demonstrated that NGF has a stimulatory effect on the developing and regenerating systems in the lower vertebrates. Radeva and Taxi (1975) have shown that NGF stimulated the functional maturation of the newt (*Triturus cristatus*) nervous system. Robinson and Allenby (1974) demonstrated a stimulatory effect of NGF on hindlimb regen-

eration in *Xenopus laevis*. It remains to be shown that all of these effects in the lower vertebrates are mediated by a common mechanism.

In summary, the present study has provided evidence that NGF is not retrogradely transported in intact goldfish retinal ganglion cells. Only when the optic axons are crushed is NGF retrogradely transported from the lesion site to the retinal ganglion cells. The transport mechanism is not specific for NGF and is not mediated by surface receptors. However, this transport appears to be biologically significant because exogenously administered NGF can promote regeneration of optic nerves in lower vertebrates. These results suggest, at least in some cases, that if NGF can reach the internal environment of neurons which lack NGF receptors, it can stimulate the regeneration of those cells.

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