The Journal of Neuroscience, May 1, 1996, 16(9):2995–3008

# Retrograde Transport of Neurotrophins from the Eye to the Brain in Chick Embryos: Roles of the p75$^{NTR}$ and trkB Receptors

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The receptors involved in retrograde transport of neurotrophins from the retina to the isthmo-optic nucleus (ION) of chick embryos were characterized using antibodies to the p75 neurotrophin receptor and trkB receptors. Survival of neurons in the ION has been shown previously to be regulated by target-derived trophic factors with survival promoted or inhibited by ocular injection of brain-derived neurotrophic factor (BDNF) or nerve growth factor (NGF), respectively. In the present paper, we show that during the period of target dependence, these neurons express trkB and p75 neurotrophin receptor but not trkA or trkC mRNAs. We also show that BDNF and NT-3 were transported efficiently at low doses, whereas NGF was transported significantly only at higher doses. The transport of BDNF and NT-3 was reduced by high concentrations of NGF or by antibodies to either trkB or the p75 neurotrophin receptor. Thus both receptors help mediate retrograde transport of these neurotrophins. Ocular injection of the comparatively specific trk inhibitor K252a did not reduce transport of exogenous BDNF, but did induce significant neuronal death in the ION, which could not be prevented by co-injection of BDNF. Thus, transport of BDNF alone does not generate a trophic signal at the cell body when axonal trkB is inactivated. In summary, our results indicate that both p75 neurotrophin and trkB receptors can mediate internalization and retrograde transport of BDNF, but activation of trkB seems to be essential for the survival-promoting actions of this neurotrophin.

*Key words: BDNF; NT-3; NGF; NGF receptor; development; visual system; eye; retina; trkB; cell death; axonal transport;* in situ *hybridization; K252a; colchicine*

The survival of neurons depends on the retrograde transport of trophic signals (Purves, 1988; Barde, 1989; Oppenheim, 1991). Neurons die when this transport is interrupted (Cowan, 1970; Hendry et al., 1974; Johnson et al., 1978; Schwab and Thoenen, 1983; Catsicas and Clarke, 1987). The retrograde transport of nerve growth factor (NGF)-like molecules (collectively termed neurotrophins) plays an important role in the conveyance of trophic signals from the target to the cell body (Hendry et al., 1974; Korsching and Thoenen, 1983; Palmatier et al., 1984). Neurotrophins bind to receptors of two types, the so-called low-affinity receptor, a 75 kDa molecule (p75$^{NTR}$), and trk-family tyrosine kinase receptors (trkA, trkB, and trkC). Trk receptors seem to be sufficient for signal transduction at the cell body (for reviews, see Meakin and Shooter, 1992; Barbacid, 1994; Bothwell, 1995). The p75$^{NTR}$ receptor may signal independently of the trk receptors (Rabizadeh et al., 1993; Dobrowsky et al., 1994), or it may interact with the trk receptor to "present" the neurotrophin, to internalize and transport it, to increase ligand specificity, and/or to facilitate signaling (Hempstead et al., 1991; Kaplan et al., 1991; Meakin and Shooter, 1992; Barker and Shooter, 1994; Chao, 1994; Hantzopoulos et al., 1994; Mahadeo et al., 1994; Chao and Hempstead, 1995).

It is currently not certain which receptors mediate the internalization and retrograde transport of neurotrophins (Hosang and Shooter, 1987; Johnson et al., 1987, 1989; Chao, 1994; Kahle et al., 1994; Curtis et al., 1995). Both p75$^{NTR}$ and trkA are transported retrogradely (Johnson et al., 1987; Loy et al., 1994; Ehlers et al., 1995). Previous studies on adult and postnatal animals have implicated p75$^{NTR}$ as well as trk receptors as mediators of neurotrophin transport (DiStefano et al., 1992; Yan et al., 1993; Curtis et al., 1995). These studies were performed after the period of normal developmental cell death, when neurons depend less acutely on their target and express lower levels of the p75$^{NTR}$ receptor.

To determine which of the receptors are responsible for the retrograde transport of neurotrophins during the period of cell death and acute target dependence, we examined receptor expression and transport of neurotrophins by neurons of the isthmo-optic nucleus (ION) in chick embryos. The ION neurons require a target-derived neurotrophic factor (O'Leary and Cowan, 1984; Clarke, 1992). This factor may be brain-derived neurotrophic factor (BDNF), because ION neurons respond to BDNF (von Bartheld et al., 1994; Primi and Clarke, in press) and BDNF is produced in the retina of chick embryos (Herzog et al., 1994).

Received Nov. 13, 1995; revised Jan. 19, 1996; accepted Jan. 25, 1996.

This research was supported by National Institutes of Health Grants HD 29177 (C.S.v.B.), MH 48200 (L.F.R.), and NS 30305 (M.B.), and by the Helen Hay Whitney Foundation (D.O.C.). R.W. is supported by a fellowship from the International Committee, Karolinska Institute, Sweden, and L.F.R. is an investigator of the Howard Hughes Medical Institute. We thank Dr. Ronald Lindsay (Regeneron, Tarrytown, NY) for the gift of BDNF and NT-3, and Drs. Hideaki Tanaka and Gisela Weskamp for the gift of p75 antibodies. Basic FGF was kindly provided by Chiron Corporation. We also thank Drs. Yoshito Kinoshita, Bill Mobley, and Frank Peale for technical advice. Dr. Peter Clarke kindly shared unpublished results.

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Neurotrophins are transported from the retinal target to the ION, which transiently expresses $p75^{NTR}$ receptors (von Bartheld et al., 1991, 1994). We now show that both trkB and $p75^{NTR}$ contribute to the retrograde transport of BDNF and NT-3. The transport of BDNF alone is not sufficient for trophic signaling at the cell body; trkB has to be activated.

A preliminary account of our study has been published previously (von Bartheld et al., 1993).

## MATERIALS AND METHODS

### *Animals*

Fertilized chicken eggs (White Leghorn) were obtained from a local supplier and incubated in a force-draft incubator at 38°C. A total of ~1800 chick embryos and 15 hatched chicks (P1–5) were used. All embryos were staged according to Hamburger and Hamilton (1951); they are referred to as days of incubation or embryonic days. Hatched chicks were held in brooders with food and water *ad libitum*. All experimental procedures were approved by the local animal care committee and were conducted in compliance with the Policy on the Use of Animals in Neuroscience Research (Society for Neuroscience).

### In situ *hybridization of trkA, trkB, and trkC mRNAs*

*In situ* hybridization was performed on frozen sections through the IONs of 9-, 16-, and 18-d-old chick embryos and hatchling chicks (P1) with probes for chicken trkA, trkB, and trkC, as described previously (von Bartheld et al., 1995). For trkB and trkC, probes were used that recognize the kinase-containing domain as well as probes that do not distinguish between kinase-containing and truncated forms of the receptor (for details and control procedures, see Williams et al., 1995). In short, embryos were frozen over liquid nitrogen and stored at −80°C until used. Serial transverse sections (10 μm) through the head (E9, E16) or brain (E18, P1) were cut on a cryostat and thaw-mounted onto poly-L-lysine-coated slides (50 μg/ml). The sections were air-dried and stored at −80°C before use. Synthetic oligonucleotide probes (Scandinavian Gene Synthesis, Köping, Sweden) complementary to isolated chicken trkA, trkB, and trkC cDNAs were labeled at the 3′-end with deoxyadenosine 5′-[α-$^{35}$S]thiotriphosphate (Amersham, Arlington Heights, IL) to a specific activity of ~$1 \times 10^9$ cpm/μg, using terminal deoxynucleotidyl transferase (Promega, Madison, WI). The probes were purified on Nensorb columns (DuPont NEN, Wilmington, DE) before use. Hybridization was performed at 42°C for ~15 hr in a humidified chamber with 100 μl of hybridization cocktail containing 50% formamide, 4× SSC, 10% dextran sulfate, 0.5 mg/ml yeast tRNA, 0.06 M dithiothreitol, and 0.1 mg/ml sonicated salmon sperm DNA. After hybridization, the slides were washed four times for 15 min each in 1× SSC with 0.05% sarcosyl included in the first wash, washed three times for 15 min each in 0.5× SSC at 55°C, and washed twice for 1 min each in cold, RNase-free water. The sections were dehydrated in ethanol, air-dried, and coated with Kodak NTB-2 photographic emulsion. After ~6 weeks, the emulsion was developed and fixed, and the sections were counterstained lightly with cresyl violet.

### *Sources of antibodies, inhibitors, neurotrophins, and iodination procedure*

Antibodies specific for chicken $p75^{NTR}$ were kindly provided by Gisela Weskamp (ChEX antibody; Weskamp and Reichardt, 1991) and Hideaki Tanaka (M7902; Tanaka et al., 1989). Antibody to chicken trkB (R22781) and Fab fragments of this antibody were generated as described below (also see Lefcort et al., 1994). Control rabbit IgG and Fab were obtained from Jackson ImmunoResearch Labs (West Grove, PA), K252a from Kamiya Biomedical Company (Thousand Oaks, CA), and cytochrome C (from chicken heart) and monensin from Sigma (St. Louis, MO). Mouse NGF was prepared according to Mobley et al. (1976). BDNF and NT-3 were kindly provided by Dr. Ronald Lindsay (Regeneron, Tarrytown, NY). Insulin-like growth factor-1 (IGF-1) was purchased from Chemicon International (Temecula, CA), and basic fibroblast growth factor (bFGF) was kindly provided by Chiron Corporation (Emeryville, CA). Trophic factors and cytochrome C were radioiodinated with lactoperoxidase (Marchalonis, 1969; Sutter et al., 1979). Specific activities were 56–112 cpm/pg NGF, 83.6–125 cpm/pg BDNF, 73.9–129 cpm/pg NT-3, 41.1–70.3 cpm/pg cytochrome C, 128.7 cpm/pg IGF-1, and 102.7 cpm/pg bFGF. Peptides were used within 5 weeks after iodination. Because some iodination procedures can impair the biological activity of BDNF (Rodriguez-Tebar and Barde, 1988; Rosenfeld et al., 1993; but see DiStefano et al., 1992; Escandon et al., 1993), iodinated BDNF was tested in a dorsal root ganglion (DRG) cell survival assay at 100-2000 pg/ml (conditions as described below); it retained 83–91% of its activity up to 4 weeks after iodination, compared with native BDNF.

### *Intraocular injections and co-injection procedures*

Chicken eggs were windowed on the day before injections. Immediately preceding injections, a hole was cut in the chorioallantoic membrane with sterile microscissors. The eye nearest to the window (usually the right eye) was held in place with sterile surgical forceps, and the solution of 3–10 μl was injected into the vitreous using a Hamilton syringe (model 705; 10 or 25 μl) or an insulin syringe (28G1/2) (Becton Dickinson, Rutherford, NJ). Colchicine was dissolved in sterile PBS, pH 7.4, and injected at a final concentration of 6–8 μg/ml in the eye. Radioiodinated neurotrophins contained 1 mg/ml bovine serum albumin. Antibody (IgG) solutions were filter-sterilized and injected at a concentration of 70–90 μg/ml. Control and trkB Fabs were co-injected at a concentration of 1 mg/ml. K252a (1.8 μg) was co-injected at a concentration of 28 μg/ml, which causes degeneration of ~35% of the neurons in the contralateral ION within 48 hr. Cold cytochrome C or cold NGF was injected at a concentration of 23 μg/ml in the eye. Intraocular injections of hatchlings were performed with similar procedures. In about half of the transport experiments, 0.6–0.9 μg cytochrome C, 10 μg normal rabbit IgG, or 60 μg normal rabbit Fab were co-injected with the $^{125}$I-labeled neurotrophin as a control for subsequent procedures involving co-injection experiments. Co-injection of cytochrome C or normal rabbit IgG/Fab did not alter the transport efficiencies (data not shown).

### *Tissue processing and cell counts after injections of colchicine, K252a, or antibodies*

Animals injected with colchicine at ages E11.5, 12, 13, 14, 15, 16.5, 17, 18.5, 19, 20, P1, P2, and P3 were allowed to survive for 48 hr. Embryos injected at E13 with K252a (1.8 μg), trkB IgG (10 μg), Fab fragments of trkB IgG (60 μg), $p75^{NTR}$ antibodies (ChEX, 10 μg), a combination of $p75^{NTR}$ antibody (10 μg) and Fab fragments of trkB IgG (60 μg), or normal rabbit IgG (10 μg) or Fab (60 μg) were allowed to survive to E16 or E17. Animals were killed by decapitation, and heads were fixed by immersion in Methacarn's fixative. The brains were dissected after staging (Hamburger and Hamilton, 1951), dehydrated in a graded alcohol series, and embedded in paraffin. The isthmic region of the brain containing the two IONs was serially sectioned in the transverse plane at 10 μm. Every fourth section was collected and stained with thionin, and all ION neurons in which a nucleolus was visible were counted (Clarke, 1993). Slides were number-coded and counted blind as to treatment to avoid examiner bias. Statistical significance was determined by unpaired *t* test.

### *Tissue processing and quantification of autoradiographic signals*

After survival times of 2, 6, 8, 18, 20, 41, 54, or 64 hr, animals injected with [$^{125}$I]neurotrophin were killed with an overdose of Nembutal and perfused intracardially with 4% paraformaldehyde. The injected eyes and the eyes from the contralateral (control) side were dissected immediately and counted individually in a gamma counter (Gamma 5500, Beckman, Fullerton, CA). Some of the injected eyes were processed for autoradiography 2, 6, or 20 hr after injection. After staging of embryos (Hamburger and Hamilton, 1951), the brains were dissected and dehydrated in a graded alcohol series, and the radioactivity in the dehydrated midbrain was counted in a gamma counter. The brains were embedded in paraffin. The isthmic region of the brain containing the right and left ION was sectioned serially in the transverse plane at 10 μm. Serial sections were collected on five separate sets of slides. The first one was exposed for 5 d on X-ray film; the second, third, and fourth were coated with photographic emulsion (Kodak NTB), exposed for 3.5, 5.5, or 7.5 weeks at 4°C in the dark, developed, and counterstained with thionin. The fifth set was kept for reference. Slides were number-coded and analyzed under dark-field and bright-field illumination on a Nikon microscope (Optiphot 2) using 40× planachromat objectives (0.65 numerical aperture). The number of grains was counted over randomly chosen neurons in the ION from sections through the central part of the ION, blind as to treatment to avoid examiner bias. The counts were averaged and compared with counts from other sections and exposure times. The number of grains/400

$\mu m^2$ was counted in representative sections through the ION. To relate directly the grain counts over ION neurons with the counts per minute of the midbrain, 11 animals were co-injected with 9 $\mu$g monensin. This dose abolishes anterograde transport (von Bartheld et al., 1996) without affecting retrograde transport. Grain density over ION neurons was plotted as a function of the amount of radioactivity present in the eye at the time the animals were killed rather than the amount injected, because the amount delivered initially to the eye can only be estimated because of variability in syringe performance and leakage from the eye during injection.

### *SDS-PAGE autoradiography*

Two embryos were injected with [$^{125}$I]NT-3 into one eye at E14 and survived for 24 hr. They were killed with an overdose of Nembutal and perfused with PBS, and the radioactivity in the eyes was counted in a gamma counter. The IONs were dissected, lysed, homogenized, and boiled in 1:1 SDS loading buffer. A 15% polyacrylamide gel was loaded with 30–50 $\mu$l of the ION samples and with samples containing native [$^{125}$I]NT-3 diluted to 10–50 cpm/$\mu$l. The gel was run for 3 hr, stained with Coomassie blue, vacuum-dried, exposed on X-ray film for 3 months, and analyzed on a laser-scanning densitometer.

### *Effects of p75$^{NTR}$ antibody (ChEX) on binding of neurotrophins to the p75$^{NTR}$ receptor*

Binding assays were performed on transfected L cells as described (Vale and Shooter, 1985; Weskamp and Reichardt, 1991). In brief, L cells expressing chicken p75$^{NTR}$ (ChNL cells) were suspended in binding buffer at $10^6$ cells/ml and incubated with ChEX antibody or normal rabbit IgG at 75 $\mu$g/ml for 60 min at 4°C. Iodinated neurotrophins (NGF, BDNF, or NT-3) were added to a final concentration of 26 ng/ml and incubated for another 60 min at 4°C. Cell-bound radioactivity was collected by rapid sedimentation of the cells in a sucrose gradient for 3 min at 4°C (Vale and Shooter, 1985). The tubes were frozen immediately after centrifugation on dry ice, and the bottom (cell-bound radioactivity) and the rest of each tube (unbound radioactivity) were counted separately. Specific binding was determined as the difference in binding of [$^{125}$I]-neurotrophin in the presence versus absence of 1000-fold unlabeled NGF. All determinations were performed in duplicate or triplicate, and data are presented as the mean ± SEM from at least two independent experiments.

### *trkB Antibody: generation and characterization*

*Generation.* Antibodies specific for the extracellular domain of chicken trkB were generated using techniques similar to those described previously (Clary et al., 1994). In short, full-length trkB was cloned from an E8 chick library. Using PCR, the extracellular domain was amplified and tagged at the C terminus with six histidines plus the myc epitope (GGC-GAG-CAG-AAG-CTG-ATC-TCC-GAG-GAG-GAC-CTG). The tagged extracellular domain was cloned into a replicating COS (CV-1, origin, SV-40) cell expression vector (pMT23, courtesy of Dr. Gordon Wong, Genetics Institute, Cambridge, MA). The protein expressed by transfected COS-7 cells was purified by using $Zn^{2+}$-affinity chromatography and fast protein liquid chromatography; the identity of the protein was confirmed and then used as an immunogen to raise rabbit polyclonal antisera. Immunoblotting and immunocytochemistry confirmed that the trkB antibody recognizes chick trkB, but neither chick trkA nor chick trkC expressed in COS cells (data not shown). TrkB IgG was digested with papain agarose beads (Sigma) to isolate monovalent Fab fragments (Harlow and Lane, 1988).

*DRG survival assays.* DRGs from E7.0–7.5 chicken embryos were dissected, dissociated, and plated at 500 neurons/well. Cultures were treated with 0.1–0.5 ng/ml BDNF, NGF, or no trophic factor in F12 medium on substrata coated with poly-L-ornithine and laminin. TrkB IgG or nonimmune (control) rabbit IgG was applied at 55 or 375 $\mu$g/ml final concentration. TrkB Fabs or nonimmune (control) rabbit Fabs were applied at 50 or 250 $\mu$g/ml. Additional control cultures were treated with BDNF or NGF alone or with no factor. Cultures were examined after 24 hr, and all neurons with a process exceeding two cell diameters were counted, blind as to treatment to avoid examiner bias. Statistical significance was determined by unpaired *t* test.

*Binding.* Approximately 70 IONs were dissected from 13-d-old chick embryos. The cells were dissociated with trypsin and preincubated for 3 hr, yielding ~$2.5 \times 10^6$ cells. Aliquots of 50,000 cells were incubated in a volume of 50 $\mu$l on ice with or without excess cold BDNF (4 $\mu$g/ml final concentration) or trkB IgG (100 $\mu$g/ml final concentration) for 45 min and then for 2 hr with increasing concentrations of [$^{125}$I]BDNF, ranging from $1 \times 10^{-11}$ to $1 \times 10^{-10}$ M (high-affinity binding) and $1 \times 10^{-10}$ to $1 \times 10^{-8}$ M (low-affinity binding, Sutter et al., 1979; Rodriguez-Tebar and Barde, 1988). Cell-bound radioactivity was collected as described above. Specific binding was calculated by subtracting nonspecific binding from the total counts. The amount of bound radioactivity (femtomoles of BDNF dimers bound/$10^6$ cells) was plotted as a function of free BDNF.

## RESULTS

### Neurons in the ION express trkB mRNA but not trkA or trkC mRNAs

The p75$^{NTR}$ receptor as well as trk receptors may play a role in the retrograde transport of neurotrophins. To determine which trk receptors are expressed in the ION and thus may be involved in retrograde transport and trophic effects within the ION, *in situ* hybridization with chicken trk probes was performed on sections through the ION of chicken embryos at ages from E9 to P1 (E16 data shown in Fig. 1). For trkB and trkC transcripts, which exist in forms encoding a trk domain as well as truncated forms lacking this domain, probes were applied that selectively identify the kinase-specific forms in addition to probes that do not distinguish between the two forms. The ION expresses full-length trkB mRNA (Fig. 1*C*). Neurons are labeled homogeneously throughout the ION (compare Fig. 1, *A* and *C*). Compared with E16, trkB mRNA was expressed at significantly lower levels at E9 and P1 (data not shown). Neither trkA nor trkC mRNAs could be detected at any of the ages examined (Fig. 1*B,D*). The ION does not seem to express either full-length or truncated trkC, because neither the nonspecific trkC probe (not shown) nor the kinase-specific probe (Fig. 1*D*) labeled the ION at levels that could be detected by *in situ* hybridization.

### ION neurons require retrograde axonal transport during the third week of incubation

The survival of ION neurons depends acutely on their target during a distinct period of development (O'Leary and Cowan, 1984; Catsicas and Clarke, 1987) that coincides with maximal expression of p75$^{NTR}$ receptor mRNA (von Bartheld et al., 1994). To determine the time course and extent to which interruption of retrograde axonal transport affects the survival of ION neurons, colchicine was injected intraocularly in chick embryos (E11.5–E20) and hatchlings (P1–P3). Effects on neuronal survival were assayed 48 hr after each injection. Between E12 and E16, colchicine injection progressively reduced survival in the ION to ~10%. Sensitivity to colchicine was maximal between E15 and E19 (Fig. 2*A,C*); in animals sacrificed at E16, neuronal counts in the ipsilateral ION were lower (up to ~40%) when compared with normal, age-matched control IONs, indicating that at this age the ipsilateral ION was affected by the treatment with colchicine. Sensitivity to colchicine vanished abruptly with injections after E19 (Fig. 2*B,C*), rendering the ION independent of retrograde axonal transport in the hatchling (at least during the 48 hr time period examined). These results define a distinct period of acute dependence of the ION of the chick on retrograde axonal transport between E13 and E19 (Fig. 2*C*). The initial survival of many ION neurons after colchicine injections is not attributable to a delay in the interruption of axonal transport, because colchicine immediately blocks the transport of [$^{125}$I]BDNF and [$^{125}$I]NT-3 (data not shown).

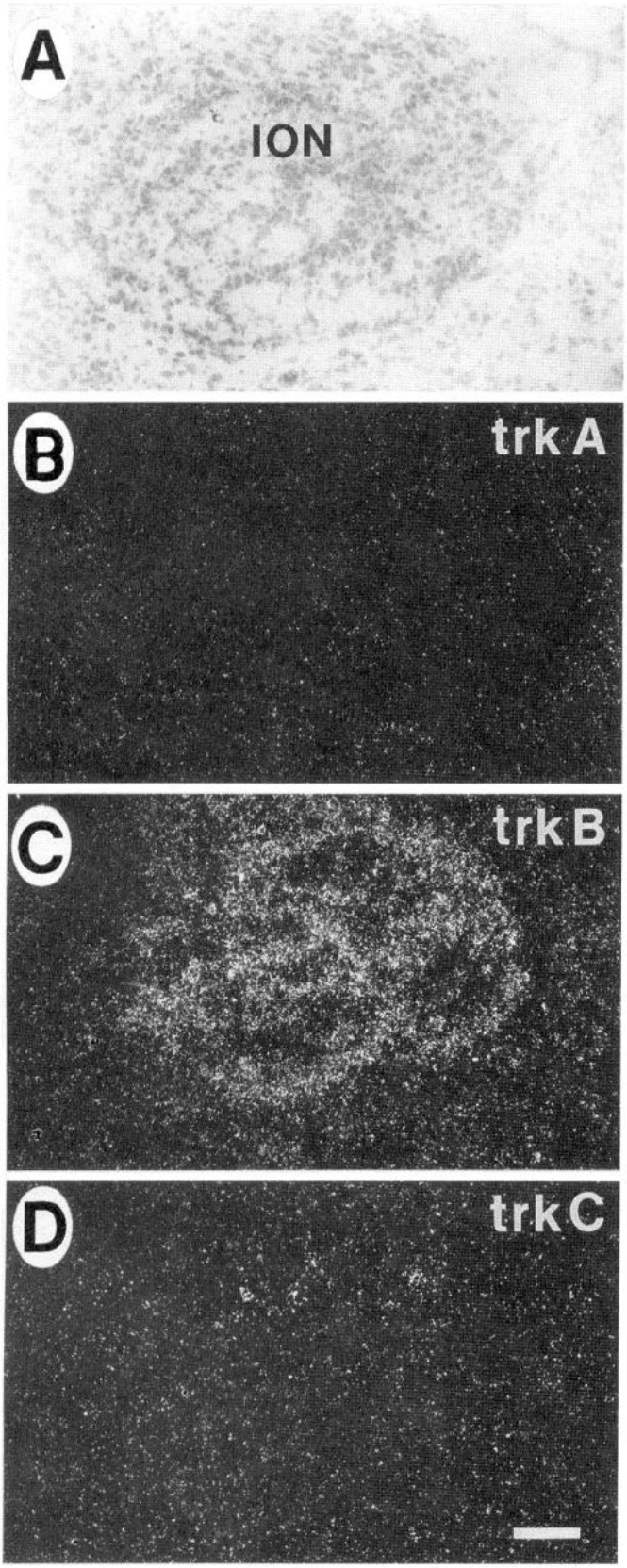


*Figure 1.* Expression of trk mRNAs in the isthmo-optic nucleus (*ION*) of 16-d-old chick embryos. *A*, Nissl-stained section through the ION. *B*, Lack of expression of trkA mRNA in the ION. *C*, Abundant expression of trkB mRNA. This section was hybridized with a probe that recognizes the sequence for the kinase domain. *D*, Lack of expression of trkC mRNA. Neither the truncated (not shown) nor the full length trkC are expressed in the ION at levels that could be detected by *in situ* hybridization. Scale bar (shown in *D*): 100 μm.

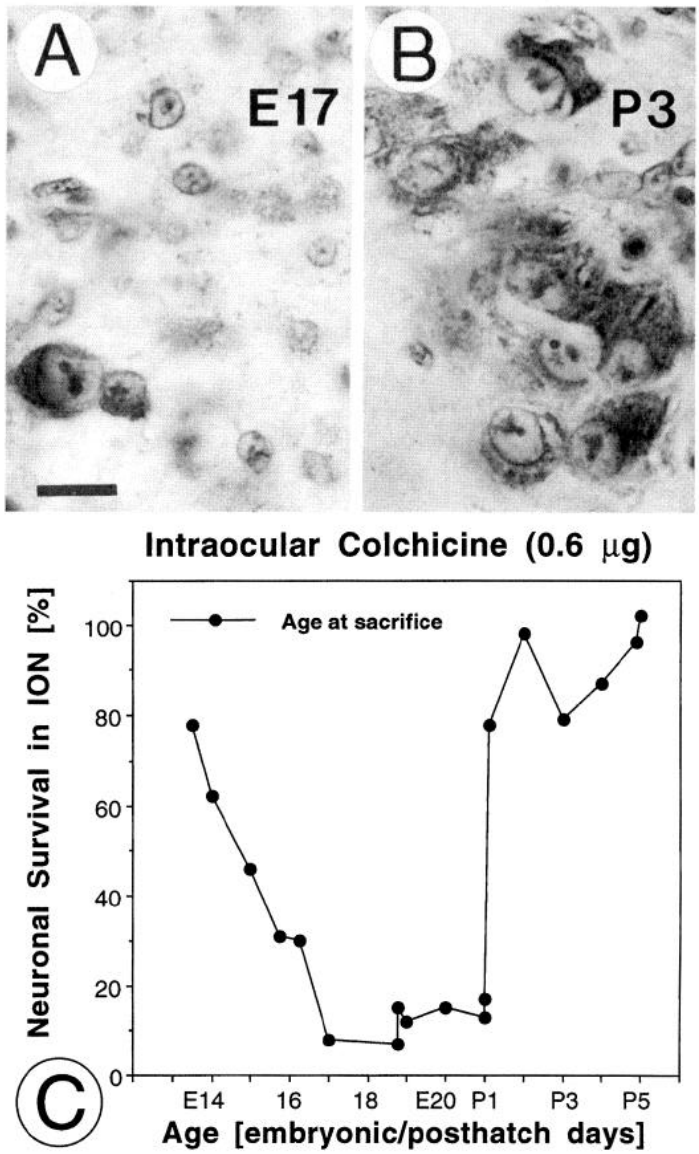


*Figure 2.* Effects of intraocular colchicine on the survival of neurons in the ION between embryonic day 11.5 (E11.5) and 5 d posthatch (P5). *A*, Nissl-stained section through the ION at *E17*. Most ION neurons die after injection of colchicine at E15. *B*, Section through the ION at *P3*. Very few neurons are affected by colchicine injections at P1. Scale bar (shown in *A*): 10 μm. *C*, Developmental profile of sensitivity of ION neurons to colchicine. The survival of ION neurons was assessed 48 hr after injection of colchicine. The percentage of surviving neurons (relative to the ipsilateral control ION) is plotted as a function of age. Note the gradual increase in sensitivity between E12 and E16, and the abrupt loss of sensitivity after injections at E19. The following average number of neurons was counted in the experimental ION of each age group: E13.5–14.0 ($n$ = 2), 10,121; E15 ($n$ = 1), 7167; E16 ($n$ = 2), 3813; E17 ($n$ = 1), 751; E18.5–19.0 ($n$ = 3), 1142; E20 ($n$ = 1), 1440; E20.5-P1 ($n$ = 3), 3401; P2 ($n$ = 1), 11,350; P3–P4 ($n$ = 2), 8476; P5 ($n$ = 2), 9864.

## Normal transport of neurotrophins from the eye to the ION

Neurotrophins are potential endogenous survival-promoting molecules whose transport was inhibited by colchicine. Results in Figure 3 show that during the period of colchicine sensitivity, BDNF and NT-3 are transported retrogradely from the retina to the ION in 12- to 17-d-old chick embryos (Fig. 3*B,C*). Weak but significant transport of NGF was also seen at the dose used in the experiment (Fig. 3*A*). For quantitative analysis of retrograde

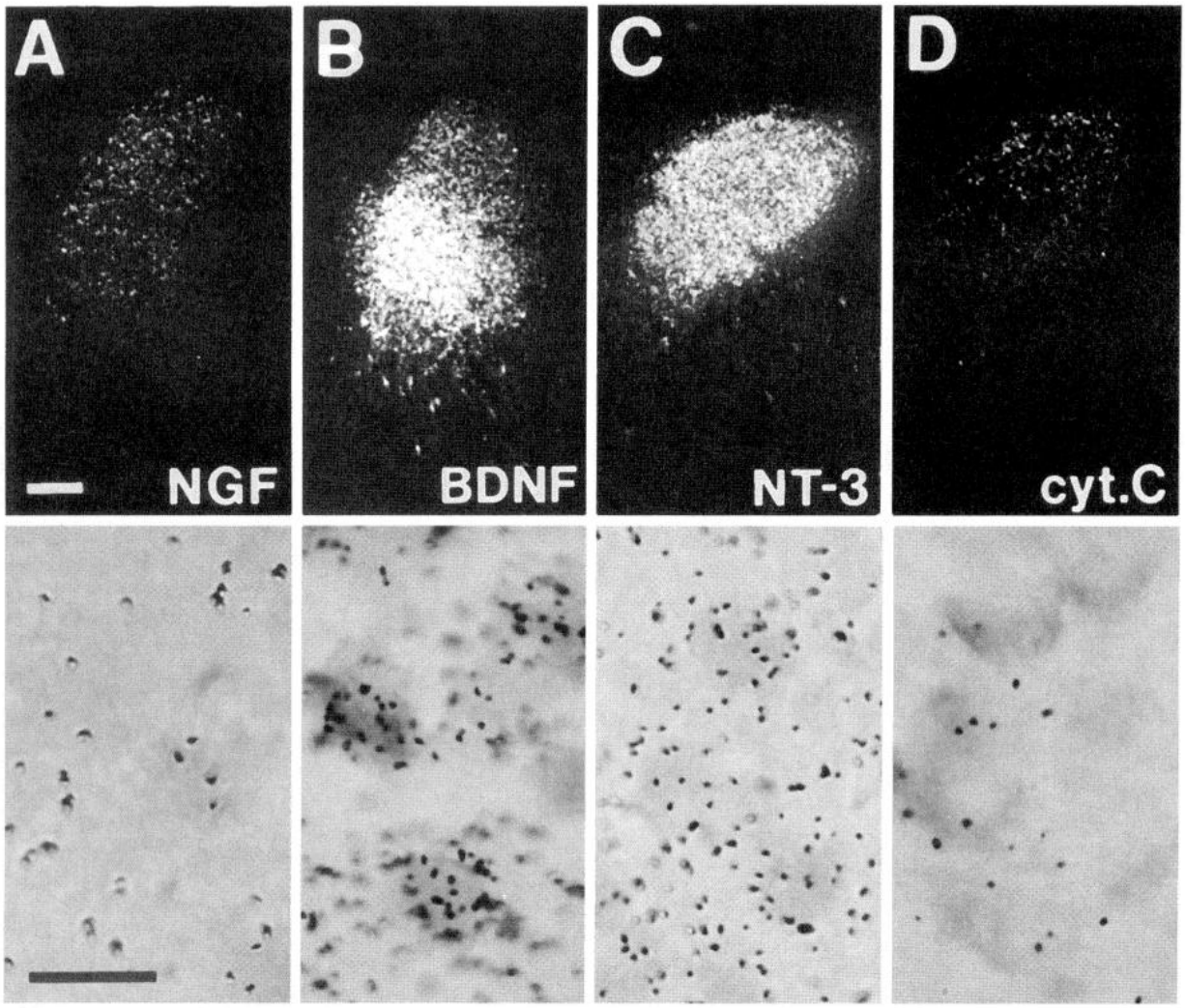


*Figure 3.* Retrograde transport of $^{125}$I-labeled neurotrophins and cytochrome C (*cyt.C*) from the eye to the ION in 15-d-old chick embryos (E15). The *upper panels* show dark-field images of the ION; the *lower panels* show bright-field views at higher magnification. Comparable amounts (~60 ng) of radioiodinated *NGF* (*A*), *BDNF* (*B*), *NT-3* (*C*), or *cyt.C* (*D*) were injected into the eye with doses of 17–20 ng remaining in the eye at the time animals were killed (= 20 hr after injection). Sections were processed for autoradiography. Note the robust transport of BDNF and NT-3 (*B*, *C*), compared with the weak transport of NGF (*A*) and faint transport of cyt.C (*D*). Several labeled ectopic ION neurons are visible in the *upper panel* of *B*. Scale bars: (*upper*) 200 μm; (*lower*) 10 μm.

transport, we focused on the age E15, when the dependence of the ION on retrograde transport is substantial (Fig. 2*C*).

*Clearance of neurotrophins from the eye*

To determine the distribution and time course of clearance of BDNF and NT-3 within the eye, the amount and distribution of these neurotrophins were measured at various times (Fig. 4*A*). The amount of radioactivity decreased to ~30% after 20 hr and 15% after 40 hr. Radiolabeled neurotrophins did not accumulate to significant amounts in the contralateral eye after intraocular injections. The average ratio of radioactivity was (191.4 ± 15.8):1 (SEM, $n$ = 44) for the experimental eye/control eye 20 hr after injections. At this time, 42 ± 3.5% (SEM, $n$ = 32) of the total radioactivity in the injected eye was in the retina. Within the retina, neurotrophins bound preferentially to the neuropil of the inner plexiform layer (data not shown).

*Grain accumulation and transport speed*

Grains were counted over ION neurons at various times after injection of iodinated BDNF or NT-3 to determine when maximal amounts of retrogradely transported BDNF and NT-3 accumulated in the ION (Fig. 4*B*). Transport of NT-3 was apparent at 6 hr and robust at 8 hr, but transport of BDNF was significantly lower than NT-3 at 8 hr. The pathway between the retina and the ION measures ~6 mm in the E15 chick embryo (Crossland, 1985); accordingly, the transport speed exceeded 1 mm/hr for NT-3, which is similar to the transport speeds of NGF reported previously (2–5 mm/hr) (Johnson et al., 1978; Grafstein and Forman, 1980; Schwab and Thoenen, 1983). At 18–20 hr, BDNF and NT-3 were transported heavily to the ION, and with similar efficiencies. At 54 hr ($n$ = 4), the amount of radiolabeled NT-3 was reduced significantly to ~60% of the 20 hr value. The 20 hr time point was used for the quantitative analysis of retrograde transport, consistent with previous studies (Johnson et al., 1978; DiStefano et al., 1992; Curtis et al., 1995). With all three neurotrophins, the distribution of autoradiographic grains was largely restricted to the cell bodies and axons of ION neurons in the midbrain tegmentum. Neurons within the ION (orthotopic ION neurons) as well as ectopic ION neurons (surrounding the ION) were labeled. There was no indication that different subpopulations or subdivisions of neurons were labeled in the ION by BDNF or NT-3, respectively.

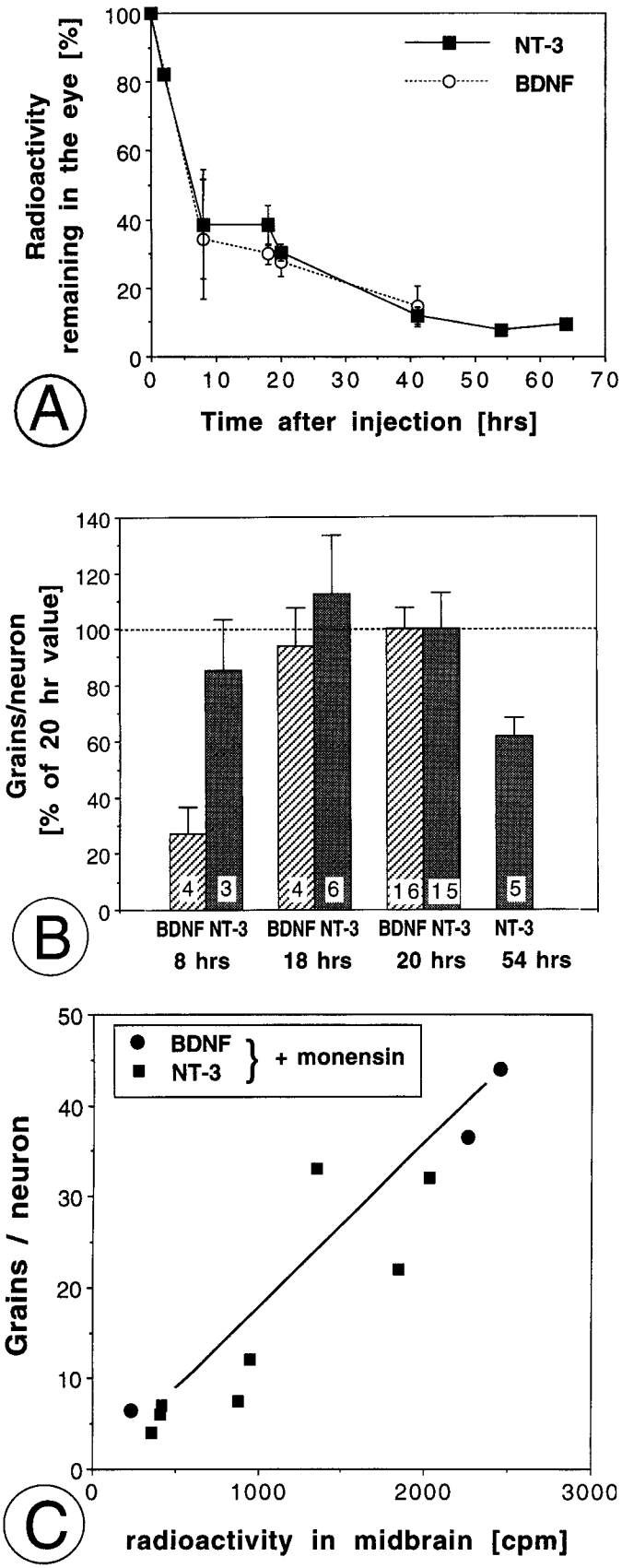


*Figure 4.* Time courses and quantification of radiolabeled BDNF and NT-3 in the eye and the ION. *A*, Clearance of radiolabeled BDNF and NT-3 from the eye of 15-d-old chick embryos after intraocular injection. Error bars = SEM. *B*, Accumulation of $^{125}$I-labeled BDNF and NT-3 in the ION after intraocular injection in 15-d-old chick embryos is shown at different times (20 hr time point, *stippled line* = 100%). Note that NT-3 accumulates faster than BDNF. Error bars: SEM. *Number in bars* = number of animals examined. *C*, The number of autoradiographic grains/neuron in the ION correlates in a linear fashion with the amount of radioactivity in the midbrain when the drug monensin is co-injected with the radiolabeled neurotrophin in the eye. Monensin abolishes the anterograde transport (by retinal ganglion cell axons) to the optic tectum.

Occasionally, a labeled neuron was observed in the ION *ipsilateral* to the injected eye, proving the existence of an intact transport mechanism in the very small number of ipsilaterally projecting ION neurons (Clarke and Cowan, 1976). There was no indication for release of radioactivity to the area surrounding the ION.

### Quantification of the retrograde transport of neurotrophins

Approximately 80–90% of the total radioactivity in the midbrain was not transported retrogradely to the ION, but rather anterogradely, by retinal ganglion cells, to the stratum opticum and layers a–g of the stratum griseum et fibrosum superficiale of the optic tectum (von Bartheld et al., 1996). Co-injection of monensin (Hammerschlag and Stone, 1982) completely abolished anterograde transport (von Bartheld et al., 1996), without affecting retrograde transport (data not shown). This allowed us to determine the amount of radioactivity in the ION (counts per minute), to correlate this amount with the grain counts (Fig. 4*C*), and thus to estimate the relative contributions of the radioactive ION (retrograde transport) and of the radioactive optic tectum (anterograde transport) to the total radioactivity in the midbrain. Knowing the specific activities of the [$^{125}$I]neurotrophins (125.5 cpm/pg BDNF; 126.6 cpm/pg for NT-3), we calculated that in our experiments ~140 pg of neurotrophins maximally accumulated in the midbrain after transport. As we do not know the rate of turnover of neurotrophins in the ION, we calculated the amount that accumulated at the maximal time (20 hr), which is less than the total amount transported. Because ~85% of the radioactive neurotrophins accumulated in the superficial layers of the optic tectum, ~20 pg (= 0.8 fmol) was accounted for by transport to the ION. Neurotrophin dimers have a MW of ~26,000. Accordingly, the maximal number of [$^{125}$I]neurotrophin dimer molecules that accumulated after retrograde transport is $6 \times 10^{23}/2.6 \times 10^4/10^{12}$ per pg = $4.6 \times 10^8$ per 20 pg. It follows that with 14,000 neurons in the ION at this age, each ION neuron transported and accumulated an average of 32,860 $^{125}$I-labeled dimer molecules. The maximal amount measured in the ION (10–20 pg = 0.4–0.8 fmol) is comparable with values reported for transport of neurotrophins to the DRG (0.6–1.8 fmol) (DiStefano et al., 1992) and to motor neurons (0.05–0.3 fmol) (Yan et al., 1993).

To evaluate the effects of antibodies to p75$^{NTR}$ or trkB receptors on retrograde transport, it was necessary to establish that transport can be measured reliably as a function of the amount injected into the target. Therefore, a dose–response curve was plotted for the transport of the neurotrophins NGF, BDNF, and NT-3 in the E15 embryo, with doses between 1 and 60 ng in the eye (Fig. 5*A*), equivalent to intraocular concentrations of ~15–900 ng/ml exogenous neurotrophin. Transport of BDNF and NT-3 saturated at ~30–50 ng (90–150 ng at the time of injection = 1.4–2.3 μg/ml), which is similar to the saturating concentration for NGF transport to the superior cervical ganglion (~1.5 μg/ml) (Johnson et al., 1978; Dumas et al., 1979). Significant transport of NGF was not detected with doses of <30 ng/ml. NGF was transported as efficiently as

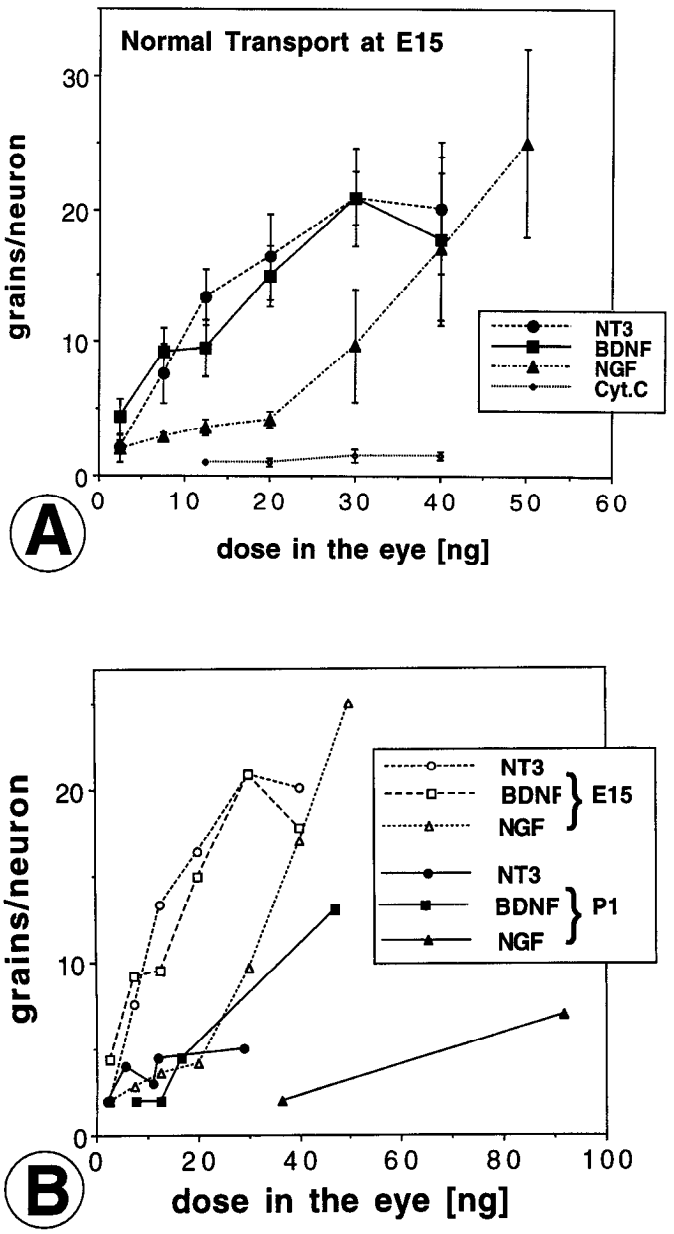


*Figure 5.* Dose–response curves of retrograde transport of neurotrophins and cytochrome C from the eye to the ION in 15-d-old chick embryos (*A*) and hatchling chicks (*B*). The average number of autoradiographic grains/ION neuron is plotted as a function of the amount in the injected eye at the time the animal is killed. *A*, Average specific activities for these experiments were 126.6 cpm/pg (NT-3), 125.5 cpm/pg (BDNF), 74.8 cpm/pg (NGF), and 57.9 cpm/pg (Cyt.C). BDNF and NT-3 are transported with significantly higher efficiencies than NGF in the lower dose range, but not at higher doses. These differences are not attributable to differences in the specific activities, because grain densities correlate in a linear fashion with the amount of radioactivity up to 30 grains/neuron (data not shown). Each data point is the mean of three to nine experiments. Error bars = SEM. *B*, Retrograde transport of $^{125}$I-labeled neurotrophins from the eye to the ION is reduced significantly in hatchlings (P1, *black symbols*) compared with 15-d-old embryos (E15, *open symbols*). Open symbols: averages; black symbols: values from individual experiments (NT-3, $n = 5$; BDNF, $n = 4$; NGF, $n = 2$).

BDNF or NT-3 only when higher, nonphysiological doses of neurotrophins were applied (Fig. 5*A*). A maximum of ~140 pg neurotrophin accumulated in the brain (or ~0.03% of the amount injected).

## Developmental regulation of retrograde transport of neurotrophins

ION neurons depend on their target during a distinct time period in the third week of incubation (Catsicas and Clarke, 1987) and are acutely sensitive to interruption of retrograde transport between E13 and E19 (Fig. 2*C*). To determine whether retrograde transport of neurotrophins decreases after this sensitive time period, $^{125}$I-labeled neurotrophins were injected in the eye of hatchling chicks (P1), and the animals were allowed to survive 24 hr, which was 4 hr more than that for the embryos to make up for the increasing distance (~0.2–0.3 mm/d) (Crossland, 1985) between the retina and the ION (E15: 5.6 mm; P1: 6.5 mm). The transport of all three neurotrophins was reduced significantly in the hatchling chick compared with the E15 embryo (Fig. 5*B*). The transport efficiencies were similar for NGF and BDNF/NT-3 in the hatchling (Fig. 5*B*). It is unlikely that the reduction of transport is a consequence of the slightly decreased concentration of neurotrophins in the eye (attributable to growth of the eye between E15 and P1), because anterograde transport of neurotrophins from the retina to the tectum was not reduced in the hatchling chick (C.S. von Bartheld, unpublished observations). The reduction of retrograde transport correlates with the decrease in expression of $p75^{NTR}$ (von Bartheld et al., 1994) as well as trkB receptor (present study).

## Control procedures

### *Specificity*

To determine whether the retrograde transport of BDNF and NT-3 is specific, two other growth factors (IGF-1 and bFGF) and cytochrome C, a molecule with similar weight and charge, were radioiodinated and injected intraocularly. Neither of these accumulated in the ION as shown for [$^{125}$I]cytochrome C, which is transported at levels barely above the threshold of detection and shows no increase with higher doses (Figs. 3*D*, 5*A*). Thus, the uptake/transport mechanism for neurotrophins seems to be specific.

### *Homologous competition*

To determine whether the internalization and/or transport of neurotrophins saturates in the manner of a receptor-mediated process, [$^{125}$I]neurotrophins were co-injected with an excess of 10- to 220-fold cold homologous factor. For the estimated concentrations of NGF ($n = 4$), BDNF ($n = 5$), and NT-3 ($n = 6$), see the legend to Figure 6. Excess cold homologous factor significantly reduced the retrograde transport of iodinated BDNF (Fig. 6*B*) and, to a lesser but still significant degree, of iodinated NT-3 (Fig. 6*C*) and NGF (Fig. 6*A*). These data indicate that the large majority of BDNF, and a major fraction of NT-3 and NGF, are transported retrogradely by a specific transport and/or a receptor-mediated uptake mechanism.

### *Lack of degradation*

To determine whether the fact that NT-3 seemed to be less sensitive to excess cold homologous factor than BDNF could be accounted for by transport of degradation products of iodinated NT-3 rather than the intact molecule, the homogenized protein extract of two IONs was run on a standard SDS-PAGE after intraocular injection of ~30 ng of [$^{125}$I]NT-3. After autoradiographic exposure of 12 weeks, the only visible band (>95% of the radioactivity according to the densitometric analysis) co-migrated with the native [$^{125}$I]NT-3 at ~14 kDa

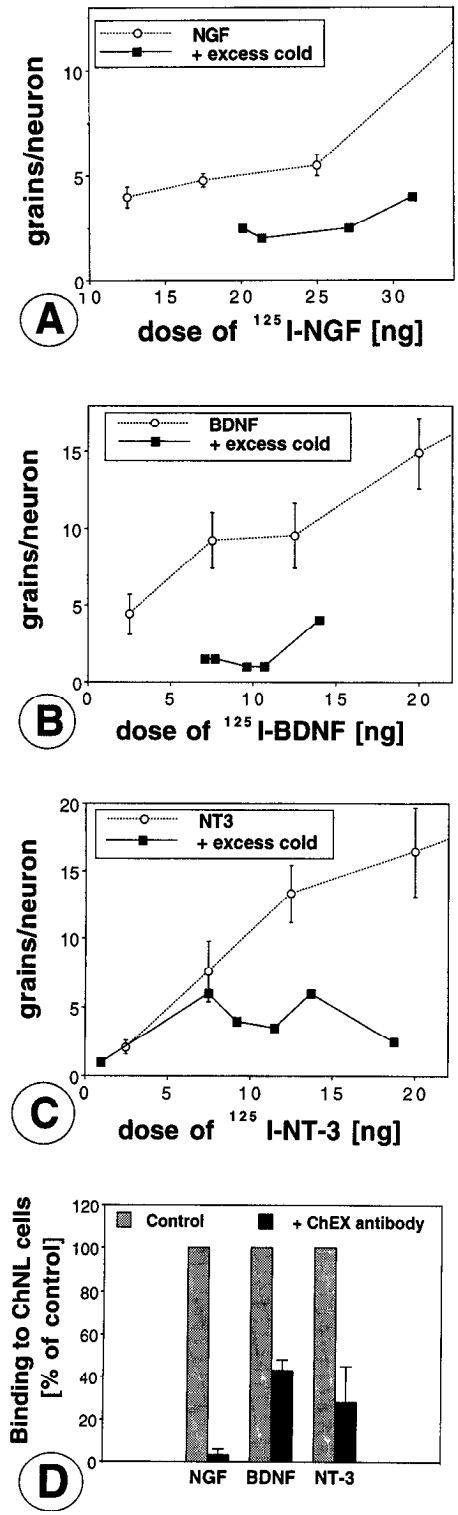


*Figure 6.* Competition of retrograde transport of neurotrophins to the ION by excess cold homologous factor (*A–C*) and effects of $p75^{NTR}$ antibody (ChEX) on neurotrophin binding (*D*). *A*, Significant reduction in the retrograde transport of [$^{125}$I]NGF with co-injection of excess cold (30- to 45-fold) NGF. The estimated concentrations for hot and excess cold NGF, respectively, are $2.8 \times 10^{-8}$ M and $1.3 \times 10^{-6}$ M; $3.1 \times 10^{-8}$ M and $1.2 \times 10^{-6}$ M; $3.1 \times 10^{-8}$ M and $1.3 \times 10^{-6}$ M; $4.2 \times 10^{-8}$ M and $1.3 \times 10^{-6}$ M. *B*, Significant reduction in the retrograde transport of [$^{125}$I]BDNF with co-injection of excess cold (10- to 220-fold) BDNF. The estimated concentrations for the hot and excess cold BDNF, respectively, are $8.4 \times 10^{-9}$ M and $1 \times 10^{-7}$ M; $8.6 \times 10^{-9}$ M and $1.1 \times 10^{-6}$ M; $1.1 \times 10^{-8}$ M and $1.9 \times 10^{-6}$ M; $1.2 \times 10^{-8}$ M and $2.5 \times 10^{-6}$ M; $1.6 \times 10^{-8}$ M and $1.9 \times 10^{-6}$ M. *C*, Significant reduction in the retrograde transport of [$^{125}$I]NT-3 with co-injection of excess cold (90- to 170-fold) NT-3. The estimated concentrations for the hot and excess cold NT-3, respectively, are $1.2 \times 10^{-9}$ M and $1.9 \times 10^{-7}$ M; $8.7 \times 10^{-9}$ M and $1.5 \times 10^{-6}$ M; $1.1 \times 10^{-8}$ M and $1.7 \times 10^{-6}$ M; $1.3 \times 10^{-8}$ M and $1.7 \times 10^{-6}$ M; $1.6 \times 10^{-8}$ M and $1.5 \times 10^{-6}$ M; $2.2 \times 10^{-8}$ M and $3.8 \times 10^{-6}$ M. *D*, Effects of ChEX ($p75^{NTR}$) antibody (75 μg/ml) on the binding of NGF, BDNF, and NT-3 (26 ng/ml) to ChNL cells, a fibroblastic cell line expressing $p75^{NTR}$. Data were obtained in duplicate or triplicate from two to three independent experiments. Nonspecific binding was determined by incubation with 1000-fold excess cold NGF; controls show specific binding in the presence of normal rabbit IgG (75 μg/ml). Bars: SEM. Note that ChEX antibody abolishes NGF-binding and significantly reduces binding of BDNF and NT-3.

(data not shown). Thus, intact and potentially functional [$^{125}$I]NT-3 accumulated in the ION.

## Excess cold NGF substantially reduces the transport of [$^{125}$I]BDNF

NGF enhances developmental cell death in the ION, possibly because of the interference of NGF with the binding of BDNF to the $p75^{NTR}$ receptor (von Bartheld et al., 1994). To determine whether excess cold NGF affected the transport of [$^{125}$I]BDNF, cold NGF was co-injected with [$^{125}$I]BDNF. Results in Figure 7*A* show that co-injection of a 50-fold excess of cold NGF (~1 μg) significantly reduced the transport of [$^{125}$I]BDNF. Similarly, a 50-fold excess of cold NGF also reduced the retrograde transport of [$^{125}$I]NT-3 (Fig. 7*C*).

## Antibodies to the $p75^{NTR}$ receptor reduce the retrograde transport of BDNF and NT-3

The heterologous competition experiments indicated that $p75^{NTR}$ receptors may be involved in the transport of neurotrophins. To test this hypothesis directly, antibodies to the chicken $p75^{NTR}$ receptor (ChEX) that block binding of neurotrophins were co-injected. The ChEX antibody nearly abolishes binding of NGF (Weskamp and Reichardt, 1991) and substantially reduces binding of BDNF and NT-3 to $p75^{NTR}$ expressing L cells at a concentration of 75 μg/ml (Fig. 6*D*). The ChEX antibody seems to be more effective in preventing binding of NGF to the $p75^{NTR}$ receptor than NT-3 or BDNF, indicating that different residues in $p75^{NTR}$ mediate interactions with the different neurotrophins. This is consistent with the observation that different residues of each of the neurotrophins dominate the epitope for $p75^{NTR}$ binding (Urfer et al., 1994).

The ChEX antibody was injected intraocularly in E14/15 chick embryos at an estimated final concentration of 70 μg/ml in the eye. This concentration of antibody induces a significant enhancement of cell death in the ION (von Bartheld et al., 1994) (Fig. 8*C*). When co-injected with [$^{125}$I]BDNF, the ChEX antibody significantly reduced the retrograde transport of [$^{125}$I]BDNF by ION neurons (Fig. 7*B*). Control experiments with irrelevant rabbit IgG injected at the same concentration (70 μg/ml) did not alter the retrograde transport of [$^{125}$I]BDNF. As an additional control, another antibody (M7902) against the $p75^{NTR}$ receptor was co-injected, using a similar concentration. Antibody 7902 does not prevent the binding of neurotrophins to the $p75^{NTR}$ receptor, but rather increases binding of neurotrophins to this receptor (Bothwell, 1995). The M7902 antibody had no consistent effect on the retrograde transport of [$^{125}$I]BDNF to the ION (data not shown). Co-injection of ChEX antibodies (5.0–7.5 μg/eye) with [$^{125}$I]NT-3 also reduced the retrograde transport of this neurotrophin (Fig.

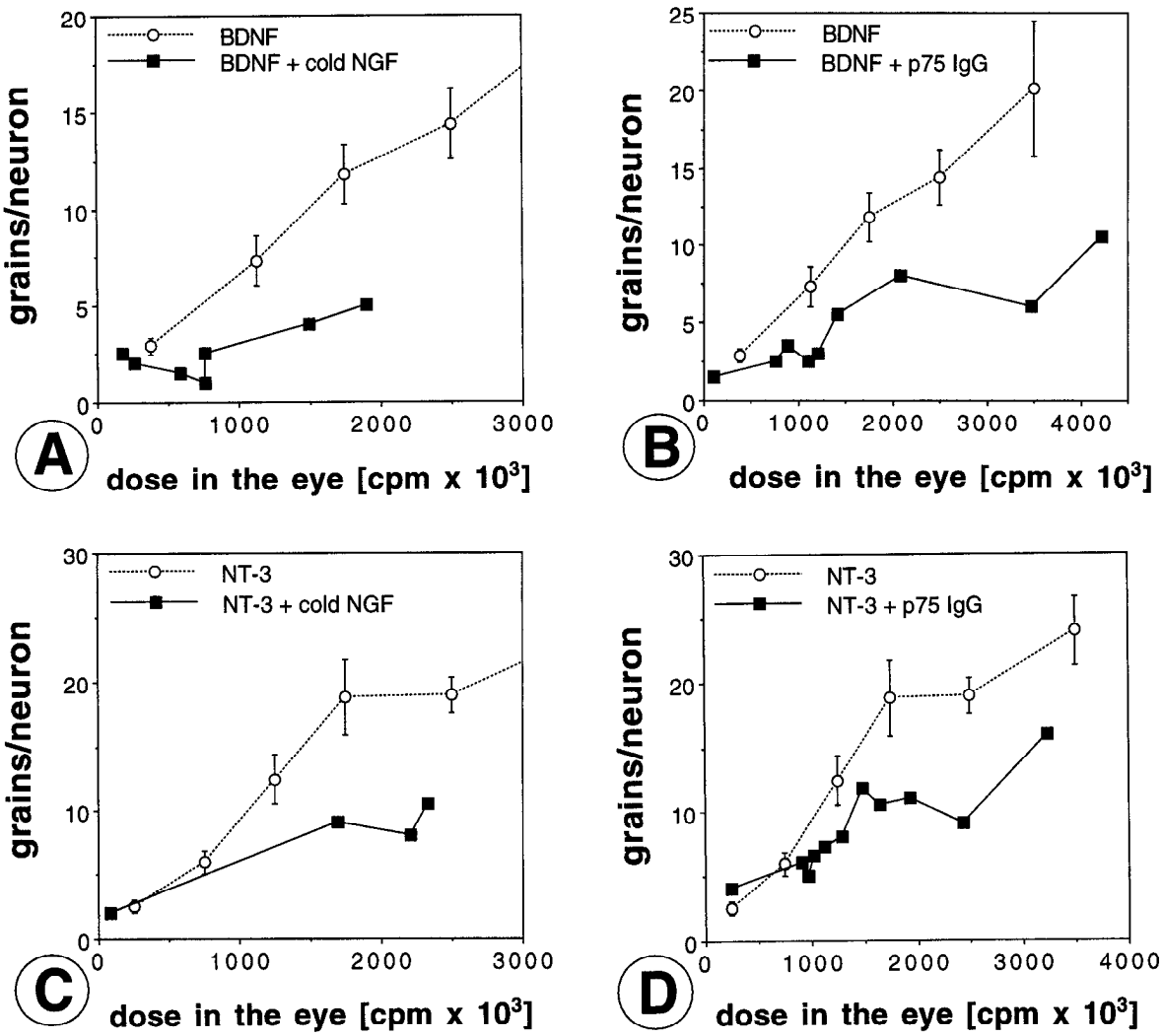


*Figure 7.* Effects of co-injection of 50-fold excess NGF (*A*, *C*) or 70 μg/ml ChEX IgG (anti-$p75^{NTR}$ receptor) (*B*, *D*) on the retrograde transport of [$^{125}$I]BDNF (*A*, *B*) and [$^{125}$I]NT-3 (*C*, *D*) from the eye to the ION at 15 d of incubation. The average number of autoradiographic grains/ION neuron is plotted as a function of the dose of $^{125}$I-labeled neurotrophin in the eye 20 hr after injection. At the time of injection, the estimated concentrations for the [$^{125}$I]BDNF and the excess cold NGF, respectively, were $1.2 \times 10^{-9}$ M and $2.3 \times 10^{-7}$ M; $1.7 \times 10^{-9}$ M and $1.2 \times 10^{-6}$ M; $3.8 \times 10^{-9}$ M and $0.9 \times 10^{-6}$ M; $5.0 \times 10^{-9}$ M and $0.6 \times 10^{-6}$ M; $1.0 \times 10^{-8}$ M and $0.6 \times 10^{-6}$ M; $1.0 \times 10^{-8}$ M and $0.9 \times 10^{-6}$ M; $2.4 \times 10^{-8}$ M and $0.6 \times 10^{-6}$ M. The concentrations for [$^{125}$I]NT-3 and excess cold NGF, respectively, were $0.6 \times 10^{-9}$ M and $2.3 \times 10^{-7}$ M; $1.3 \times 10^{-8}$ M and $2.9 \times 10^{-7}$ M; $2.1 \times 10^{-8}$ M and $0.6 \times 10^{-6}$ M; $2.8 \times 10^{-8}$ M and $2.9 \times 10^{-7}$ M. Excess cold NGF (*A*) or co-injection of antibody against the $p75^{NTR}$ receptor (*B*) reduces the transport of BDNF significantly. Co-injection of cold NGF (*C*) or $p75^{NTR}$ antibody (*D*) also causes a reduction of NT-3 transport.

7*D*). Thus, results in this section implicate $p75^{NTR}$ in transport of both BDNF and NT-3.

### Effects of trkB antibodies on the retrograde transport of BDNF

To compare the possible contributions of $p75^{NTR}$ receptors and trkB receptors to the retrograde transport of neurotrophins, effects of an antibody to chicken trkB were examined. This antibody recognizes chicken trkB but not chicken trkA or trkC in antigen blots and immunoprecipitation assays (data not shown). Properties of the trkB antibody were first characterized on BDNF-responsive DRG neurons. TrkB antibody (IgG) did not reduce the survival of BDNF-treated DRG neurons (Fig. 8*A*). The possibility that the bivalent trkB antibody (IgG) may activate the trkB receptor was tested in a DRG survival assay. TrkB IgG increased the survival of DRG neurons in the absence of neurotrophins (Fig. 8*A*), presumably because the bivalent IgG dimerizes and activates trkB, as has been observed previously for bivalent antibodies to trkA and trkC (Clary et al., 1994; Lefcort et al., in press). To avoid complications resulting from dimerization of trkB, we prepared monovalent anti-trkB Fab fragments. We found that 50–250 μg/ml of the Fabs significantly reduced, in a dose-dependent fashion, the survival of BDNF-treated but not NGF-treated DRG neurons (Fig. 8*B*). This indicates that the trkB Fabs block binding of BDNF to trkB but not binding of NGF to trkA.

When tested in equilibrium experiments on ION neurons *in vitro*, 100 μg/ml trkB IgG reduced binding of BDNF at 1, 2, and $5 \times 10^{-11}$ M (range of high-affinity binding) by ~45% (data not shown), but not at 1 and $3 \times 10^{-10}$ M, 1 and $3 \times 10^{-9}$ M, or $1 \times 10^{-8}$ M (range of low-affinity binding) (Rodriguez-Tebar and Barde, 1988). The same concentration of trkB IgG reduced the survival of ION neurons *in vivo* after intraocular injection by only 9% (Fig. 8*C*), possibly because of the intrinsic activity of the trkB IgG. When trkB IgG was co-injected in the eye with [$^{125}$I]BDNF to achieve an estimated concentration of 100 μg/ml (similar to

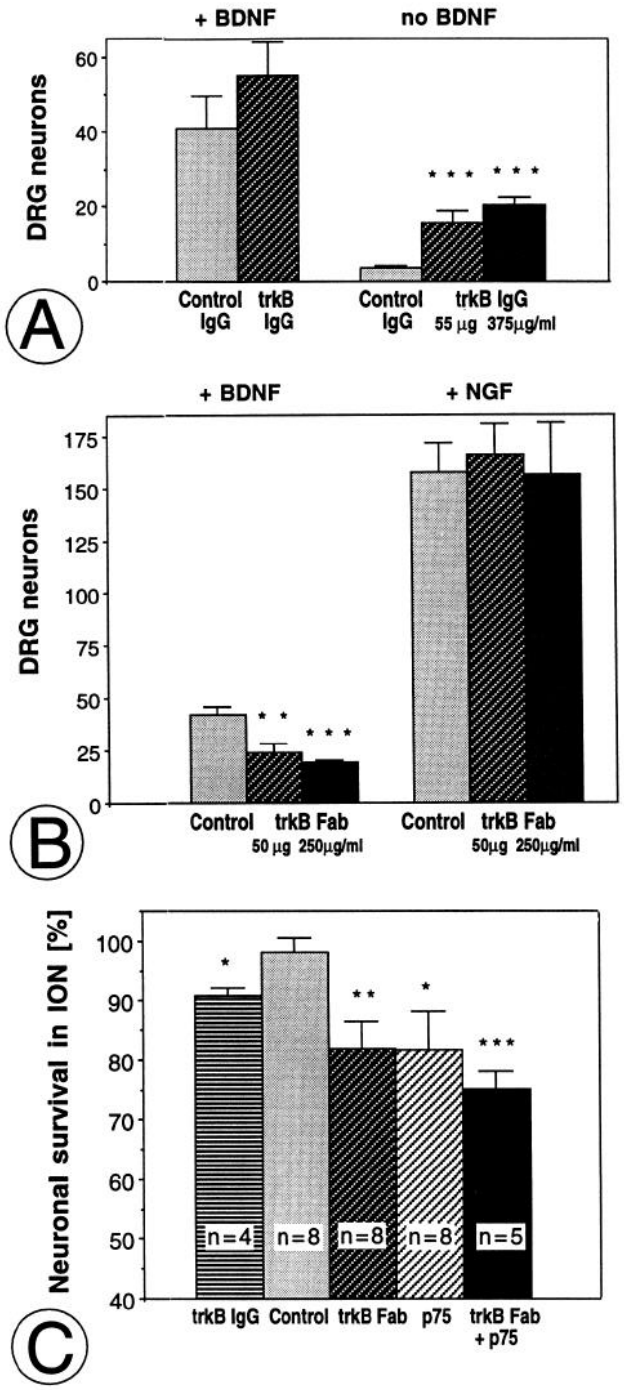


*Figure 8.* Effects of trkB IgG (*A*) and trkB Fabs (*B*) on the survival of DRG neurons *in vitro* and ION *in vivo* (*C*). *A*, TrkB IgG does not reduce the survival of BDNF-treated DRG neurons, but supports DRG neurons in the absence of BDNF (or other trophic factors). *B*, TrkB Fabs reduce the survival of BDNF-treated DRG neurons, but not the survival of NGF-treated DRG neurons. *C*, TrkB IgG enhances cell death in the ION marginally. TrkB Fabs and $p75^{NTR}$ (ChEX) antibodies have a similar effect (20%) on enhancement of cell death in the ION. The effects of trkB Fab and ChEX are not additive. Normal rabbit IgG or Fabs were used at the same concentrations as the trkB antibodies for the control experiments (only some data shown). There was no detectable effect of normal IgG or normal Fabs on the survival of DRG or ION neurons. All error bars: SEM (2–10 determinations). Significance levels (vs control) were determined by unpaired *t* test (*, $p < 0.025$; **, $p < 0.01$; ***, $p < 0.005$).

that used with the ChEX $p75^{NTR}$ antibody), there was little or no effect on the transport of [$^{125}$I]BDNF (Fig. 9*A*). When the combined effect of trkB IgG and $p75^{NTR}$ (ChEX) antibodies on the transport of [$^{125}$I]BDNF was tested with co-injection of 100 µg/ml trkB antibody and 100 µg/ml ChEX antibody, the rate of transport of BDNF was reduced to the same degree as seen after co-injection with ChEX antibody alone (data not shown).

Monovalent trkB Fab fragments (at an estimated intraocular concentration of 1 mg/ml) enhanced developmental cell death in the ION significantly, by ~20% after injection at E13 (Fig. 8*C*). Higher doses of the trkB Fabs in the eye resulted in systemic effects (substantial cell death bilaterally in the ION, data not shown). The effects of trkB Fab and ChEX (which each enhanced cell death in the ION by ~20%) were not additive (Fig. 8*C*). When trkB Fabs, at 1 mg/ml, were co-injected in the eye with $^{125}$I-labeled BDNF, the transport of BDNF was significantly reduced at lower doses of BDNF; no effect on the transport was seen with higher doses of BDNF (Fig. 9*B*). The lack of inhibition at higher BDNF concentrations may result from a relatively low affinity of interaction of trkB Fabs, allowing competitive displacement by BDNF or, alternatively, may indicate that $p75^{NTR}$ mediates most of the transport at higher BDNF concentrations.

### Activation of trkB is required for signaling but not for retrograde transport of BDNF

To determine whether retrograde transport of the survival-promoting actions of target-derived BDNF requires trkB activation, we examined effects of local application in the eye of K252a, a trk inhibitor that inactivates trkB (Knusel and Hefti, 1992). K252a significantly enhances normal developmental cell death in the experimental but not control ION at an intraocular concentration of 28 µg/ml (Fig. 9*C*) (P.G.H. Clarke, personal communication). To test whether activation of trkB is required for internalization and/or retrograde transport of BDNF, [$^{125}$I]BDNF was co-injected in the eye with K252a (at a final concentration of 28 µg/ml). K252a had no significant effect on the retrograde transport of BDNF (Fig. 9*D*). The lack of an effect of K252a on retrograde transport of BDNF allowed us to ask whether the ION neurons could be rescued from the effect of K252a in the target (inactivation of axonal trkB) by transport of BDNF from the retina to the cell bodies in the ION. Co-injection of K252a and BDNF did not rescue ION neurons from cell death (Fig. 9*C*). The survival and transport data together indicate that activation of trkB is not necessary for the retrograde transport of BDNF, but that activated trkB derived from the target field is required for the survival of ION neurons. Apparently, transport of BDNF to the cell body is not sufficient for trophic signaling without activation of trkB.

## DISCUSSION

### Internalization, transport, and signaling of neurotrophins

The nature of the functional form of the neurotrophin receptor has been much debated. It may consist of homodimers of trk receptors (Jing et al., 1992), or it may require an interaction of trk receptors with the $p75^{NTR}$ receptor (Hempstead et al., 1991; Weskamp and Reichardt, 1991; Mahadeo et al., 1994). Trk receptors are essential for signaling, whereas the $p75^{NTR}$ receptor seems to have a modulatory influence on neurons (for review, see Meakin and Shooter, 1992; Chao, 1994; Bothwell, 1995). Initial models envisioned simultaneous binding of neurotrophins by trk and $p75^{NTR}$ receptors (Bothwell, 1991;

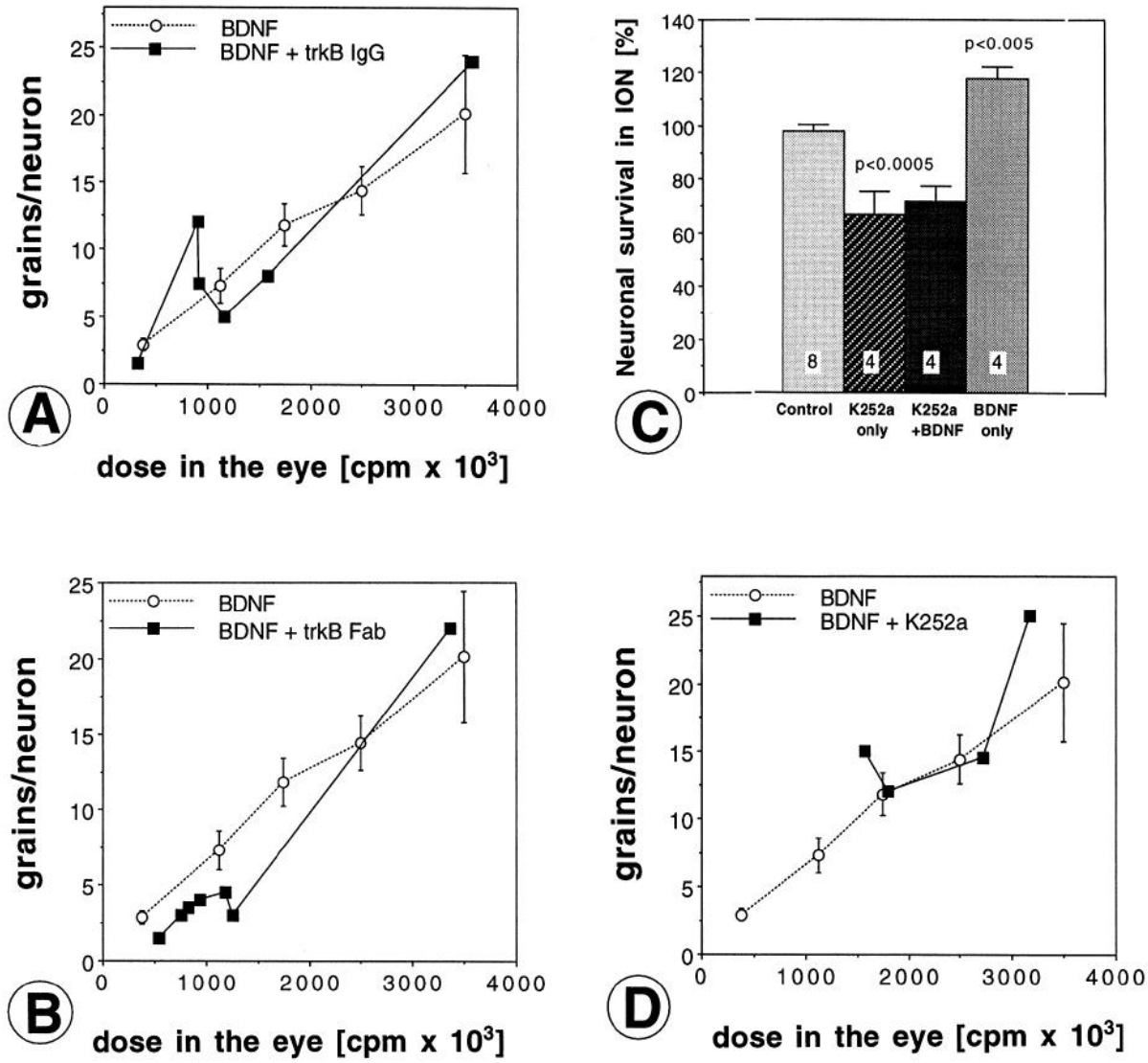


*Figure 9.* Effects of trkB IgG and trkB Fabs on the retrograde transport of BDNF to the ION (*A*, *B*) and the effect of the trk inhibitor K252a on the survival of ION neurons (*C*) and the transport of BDNF (*D*). *A*, Co-injection of trkB IgG (100 μg/ml) does not reduce the retrograde transport of BDNF. *B*, Co-injection of trkB Fab fragments (1 mg/ml) reduces the transport of BDNF in the lower (physiological) dose range. *C*, The trk inhibitor K252a (intraocular concentration: 28 μg/ml) significantly enhances neuronal death in the ION. Co-injection of 200 ng BDNF with K252a in the eye does not rescue the ION neurons from cell death. Injection of BDNF alone has been shown previously to increase the survival of ION neurons by 20% (von Bartheld et al., 1994). *Numbers in bars* = numbers of animals examined. *D*, K252a does not reduce the retrograde transport of BDNF to the ION. All bars = SEM.

Hempstead et al., 1991). Alternative models now propose that $p75^{NTR}$ may "present" the neurotrophin to the trk receptor (Ibañez et al., 1992; Jing et al., 1992; Barker and Shooter, 1994; Chao, 1994), a concept initially proposed by Johnson et al. (1988), or that $p75^{NTR}$ may alter the conformation and/or activate the trk receptor (Mahadeo et al., 1994; Bothwell, 1995). Increasing evidence suggests that the ratio of $p75^{NTR}$ to trk receptors is crucial for high-affinity binding of neurotrophins (Battleman et al., 1993; Chao, 1994; Clary and Reichardt, 1994; Mahadeo et al., 1994; Chao and Hempstead, 1995).

Neurotrophins are produced in the target; the signal therefore must be conveyed from the axon terminus to the cell body over a considerable distance. Little is known about internalization of neurotrophins at the axon terminal and mechanisms of transport. We have examined the transport of neurotrophins from the axon terminals to the cell bodies in the ION. The ION provides an advantageous system because it consists of a homogeneous cell population that expresses only trkB and $p75^{NTR}$ receptors.

### Dependence of the ION on retrograde transport

The ION acutely requires its target during development from E13 to E18 (Catsicas and Clarke, 1987), extending slightly beyond the period of naturally occurring cell death (E13–17) (Clarke et al., 1976). During this period, intraocular colchicine causes the degeneration of many ION neurons within 24–48 hr (Blaser and Clarke, 1992). Our study shows that the onset of transport dependence of ION neurons is gradual (E12–15) and that sensitivity to interruption of transport is lost abruptly at E19. BDNF as well as NGF and NT-3 are transported from the retina to the ION cell bodies, but ION neurons respond to BDNF more than NT-3 with increased survival, whereas NGF enhances cell death (von Bartheld et al., 1994). ION neurons express full-length trkB in addition to the $p75^{NTR}$ receptor. Other trk

receptors are not expressed in the ION at levels that can be detected by *in situ* hybridization (Fig. 1).

### Normal transport of neurotrophins to the ION at E15

It is remarkable that the retrograde transport of the different neurotrophins exhibits different dose–response curves. NGF is transported to the ION very poorly at lower doses, but is transported as efficiently as BDNF and NT-3 at higher doses. This result shows that transport characteristics of neurotrophins cannot be extrapolated from one, possibly nonphysiological (pharmacological) dose range. For meaningful conclusions, it is necessary to establish dose–response relationships of neurotrophin transport in their entire range, including physiological doses. For example, the functionally irrelevant transport of NGF in motor neurons may occur only at higher (pharmacological) doses (Yan et al., 1988, 1993; DiStefano et al., 1992). Dose-dependency of retrograde transport may contribute to the specificity of neurotrophin action *in vivo* (Korsching, 1993).

### $p75^{NTR}$ receptor is involved in the mechanism of retrograde transport of BDNF

We have shown that the retrograde transport of BDNF and NT-3 from the retina to the ION can be reduced by co-injection of 50-fold excess NGF or by co-injection of antibody against $p75^{NTR}$. Similar conclusions have been reached for the transport of NT-4 in mammalian neurons (Anderson et al., 1995; Curtis et al., 1995). These results implicate the $p75^{NTR}$ receptor in the internalization and/or retrograde transport of trkB ligands, because NGF competes with the binding of BDNF and NT-3 to $p75^{NTR}$ but not trkB (until a 1000-fold higher concentration is reached) (Rodriguez-Tebar et al., 1990, 1992). Apparently, NGF can interfere with binding of these neurotrophins to the $p75^{NTR}$ receptor (cf. DiStefano et al., 1992; Yan et al., 1993). Consistent with this interpretation, Dechant et al. (1993) showed that NGF competes with BDNF for binding to chicken DRG neurons expressing $p75^{NTR}$, but not in the cell line A293 that expresses trkB but lacks $p75^{NTR}$. $p75^{NTR}$ may not always be necessary for BDNF transport, as indicated by the transport of BDNF in some adult neural circuits apparently lacking significant $p75^{NTR}$ expression (DiStefano et al., 1992; Anderson et al., 1995). Sensory neurons from $p75^{NTR}$ *knockout* mice did not differ in their survival response with BDNF or NT-3 compared with wild-type neurons (Davies et al., 1993). These *in vitro* data, however, do not exclude the possibility that $p75^{NTR}$ may have an important function for transport *in vivo*, a function that would not be apparent using sensory neurons in a survival assay *in vitro*.

Does NGF utilize $p75^{NTR}$ for transport? Curtis et al. (1995) reported that axonal transport of NGF, unlike NT-4 transport, seemed not to require $p75^{NTR}$ in trkA-expressing neurons. In PC12 cells, trkA (regardless of trkA activity) is essential for internalization of NGF, and $p75^{NTR}$ is not necessary (Kahle et al., 1994); however, $p75^{NTR}$ is capable of internalizing NGF in 33B glioma cells, which do not express trkA (Kahle and Hertel, 1992). The involvement of $p75^{NTR}$ in NGF transport is also shown for neurons. The monoclonal antibody 192 increases the affinity of NGF binding to $p75^{NTR}$ (Chandler et al., 1984), and co-injection of this antibody significantly increases NGF transport to the superior cervical ganglion *in vivo* (Taniuchi and Johnson, 1985). TrkA and trkC receptors, the preferred receptors for NGF and NT-3, respectively, are not expressed in the ION neurons. Nevertheless, NGF and NT-3 are transported retrogradely from the retina to the ION. Apparently, NGF can be internalized and transported (at high concentrations) as efficiently as the neurotrophins BDNF and NT-3. This result is in agreement with the retrograde transport of NGF in postnatal motoneurons that, like the ION, also lack trkA receptor. In fact, the efficiency of transport of NGF in motoneurons exceeds that of BDNF (Yan et al., 1993), at least when high doses of neurotrophins are applied. Interestingly, NGF was not transported efficiently by ION neurons at lower doses. The sigmoidal nature of the neurotrophin transport dose–response function is consistent with the recent proposal that neurotrophin binding induces a conformational change in the $p75^{NTR}$ receptor dimer, which results in an increased affinity for neurotrophins (Bothwell, 1995). According to this model, BDNF effectively triggers a conformational change at low concentrations, whereas NGF does so only at much higher concentrations. This suggests that a conformational change of $p75^{NTR}$ may be an essential step in $p75^{NTR}$ internalization or transport.

### Are trk receptors involved in internalization/transport of neurotrophins?

Trophic signals are transported from the axon terminals to the cell body. Although activation of trk (presumably trkB in the case of the ION) is essential, it is not clear in what form the trophic signal is transported. $p75^{NTR}$ and trkB receptors are believed to be expressed in sensory neurons in a ratio of ~10–15:1 (Rodriguez-Tebar and Barde, 1988; Meakin and Shooter, 1992), and a similar ratio seems likely for the ION, considering the labeling intensities for $p75^{NTR}$ mRNA (von Bartheld et al., 1991) and trkB mRNA (present study). Consistent with this, our transport data indicate that a substantial fraction of BDNF binds to $p75^{NTR}$ during transport, whereas another possibly smaller fraction of BDNF binds to trkB, as assessed by the inhibitory effects of anti-$p75^{NTR}$ IgG and anti-trkB Fabs. How could the $p75^{NTR}$ and the trkB receptor interact during transport and/or signaling? BDNF may be passed on from $p75^{NTR}$ to trkB during transport ("presentation" model) (Jing et al., 1992; Barker and Shooter, 1994; Chao, 1994), or trkB may be activated by BDNF-bound, dimerized $p75^{NTR}$ (conformation-change model) (Bothwell, 1995). Another possible interpretation of our data, which we cannot exclude, is that survival of ION neurons requires trophic signals emanating independently from trkB and $p75^{NTR}$ receptors.

In summary, our study shows that trkB and $p75^{NTR}$ are major carriers of BDNF from the axon terminus to the cell body. The contribution of $p75^{NTR}$ to the internalization and/or transport of BDNF is functionally relevant, but $p75^{NTR}$ alone is not sufficient for trophic signaling. Inactivation of the trkB receptor in the terminals with K252a has little effect on transport; nevertheless, it induces a significant increase in cell death in the ION. Apparently, the retrograde transport of BDNF via $p75^{NTR}$ receptors and/or inactivated trkB receptors is not sufficient for trophic action, whereas activation of axonal trkB (mediated to some extent via $p75^{NTR}$) is required for trophic signaling.

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