Expression of trkA, trkB, and trkC in Injured and Regenerating Retinal Ganglion Cells of Adult Rats

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Purpose. To investigate changes in percentage of tyrosine kinase (trk)A-, trkB-, and trkC-immunopositive ($^+$) retinal ganglion cells (RGCs) at various times after optic nerve (ON) axotomy; the proportion of RGCs regenerating axons into peripheral nerve (PN) grafts that are trkA$^+$, trkB$^+$, and trkC$^+$; whether intravitreal PN-ON implants affect trk immunoreactivity; and the levels of trk mRNAs in ON-injured retinas.

Methods. The ON was transected intraorbitally. Proportions of trkA$^+$, trkB$^+$, and trkC$^+$ RGCs and levels of trk mRNAs were studied by using immunocytochemistry and Northern blot methods, respectively, in injured and RGC-regenerating retinas.

Results. In normal retinas, only small numbers of trkB$^+$ and trkC$^+$, but not trkA$^+$, RGCs were seen. The optic fiber layer was intensively immunolabeled with trkB. After ON injury, the proportions of trkA$^+$, trkB$^+$, and trkC$^+$ RGCs rapidly increased and reached their peaks by 3 to 5 days. During the next 3 weeks, the proportion of trkA$^+$ or trkB$^+$ RGCs gradually decreased, but the proportion of trkC$^+$ RGCs remained high. Intravitreal implants of PN+ON segments transiently but significantly suppressed injury-induced increases in all these trk$^+$ RGC proportions for approximately 5 days. In contrast, 3 days after ON injury, quantitative retinal expression of trkA mRNA, and to a lesser extent trkC mRNA, was downregulated, whereas trkB mRNA expression remained unaffected. Higher proportions of trkA$^+$ and trkB$^+$ RGCs and higher levels of all trk mRNAs were seen in regenerating RGCs and retinas, respectively.

Conclusions. This study provides a kinetic analysis of expression of trk in RGCs and retinas after ON injury and during regeneration. (*Invest Ophthalmol Vis Sci.* 2002;43:1954–1964)

The functions of neurotrophic factors (NTFs) in the mammalian nervous systems are diverse, encompassing neurogenesis, growth, differentiation, survival, neurite outgrowth and axon regeneration.[1–11] Neurotrophin (NT) is one of the NTF families that has been extensively studied and well characterized. Members of the NT family include nerve growth factor (NGF),[12] brain-derived neurotrophic factor (BDNF),[1] NT-3,[13,14] and NT-4/5.[15] NTs exert their biological actions by binding to their cognate tyrosine kinase (trk) proto-oncogene trk receptors that include trkA, trkB, and trkC. The process from receptor–ligand binding to eventual exertion of biological actions is complex, and the mechanisms regulating the numerous NT functions are yet to be defined. Basically, biological signaling involves receptor-mediated homodimerization, autophosphorylation of tyrosyl residues, and activation of executive molecules.[3] Although there is some overlap of ligand–receptor specificity among different NTs,[15–18] each NT binds with high affinity to its specific trk receptor[19]: NGF binds trkA,[20] BDNF and NT-4/5 bind trkB,[15,18] and NT-3 binds trkC.[21] All NTs also bind with similar low affinity to the p75 receptor.[3,22] In addition, there are different forms or variants of trkB and trkC. Some of them may not have biological functions, because they do not have the catalytic kinase domain.[23,24]

Mammalian visual systems have been used for the study of NTF functions for many years. TrkA, trkB, trkC, and p75 receptors are expressed in developing retinas[25–31]; however, the related information in the adult retinas, especially after injury and during axon regeneration, is limited. Only trkB[32–34] and a very low level of trkA[28] was reported, whereas trkC remained negative in adult retinas.[33] These observations are compatible with numerous reports on the protective effect of the trkB ligands BDNF/NT-4/5 and negative effects of the trkA ligand NGF and the trkC ligand NT-3 on the survival of developing and adult retinal ganglion cells (RGCs).[4,5,7,35–41]

The loss of adult neurons and failure to regrow axons after injury are common phenomena in the mammalian central nervous system (CNS) and presents a great challenge for neuroscientists. Optic nerve (ON) transection causes loss of more than 90% of RGCs by 2 weeks after transection in adult rodents. The RGCs start to die 4 to 5 days after initial ON injury, and the peak cell death occurs 5 to 10 days after injury.[39,42] Loss of RGCs also occurs in certain pathologic conditions, such as glaucoma, optic neuritis, and ischemic optic neuropathy. Intravitreal application of appropriate NTs[43,37–40] or a peripheral nerve (PN) segment[44–46] has been shown to rescue RGCs and/or enhance neurite outgrowth under various conditions. However, of various intravitreal applications using NGF, BDNF, NT-3, NT-4/5, FGF, glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and PN segment implant, only CNTF or a PN segment promotes long-distance axon regeneration in injured RGCs.[6,8,45,47,48] To gain more information on the response of trk receptors to eye injury, improve our understanding of neuroprotection pathways and the potential use of NTs to ameliorate RGC loss under these debilitating conditions, and enhance axonal regeneration after injury, we examined the changes in trkA-, trkB-, and trkC-immunopositive ($^+$) RGCs and levels of these trk mRNAs in whole retinas after injury. To determine whether the RGCs that can regenerate possess unique trk receptor properties, we also compared expression of trkA, trkB, and trkC in PN-ON grafted (providing an RGC axon regeneration environment) eyes against injury-only eyes.

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METHODS

Experimental animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eighty-five young adult Sprague-Dawley rats (200–300 g) were used in the study. All surgical procedures were performed in animals under intraperitoneal ketamine (80 mg/kg) and xylazine (8 mg/kg) anesthesia.

Experimental Models

Normal Retinas. Two eyes of a normal animal were used for examination of $trkA^+$, $trkB^+$, and $trkC^+$ labeling in normal adult retinas. In an attempt to prelabel all RGCs and to determine the percentages of $trkA^+$, $trkB^+$, and $trkC^+$ RGCs among the whole RGC population, in three rats, 6% Fluoro-Gold (Fluorochrome, Englewood, CO) in distilled water was applied to both superior colliculi (SCs). These rats were allowed to survive for another 3 days for retrograde transport of Fluoro-Gold and subsequent retrograde labeling of RGCs. To apply Fluoro-Gold to the SCs, two small holes ($\sim 2 \times 2$ mm) were drilled in the left and right frontal bones just rostral to the coronal suture. Neural tissues below and caudal to the craniotomy were removed by aspiration until both SCs were exposed. A pulled glass micropipette was used to inject 6% Fluoro-Gold into different parts of the SCs. Then a piece of gelfoam soaked with the 6% Fluoro-Gold was placed on the surface of each SC. To optimize retrograde labeling, care was taken to ensure that the tip of the micropipette remained relatively superficial, because the axons of RGCs terminate in the upper layers of the SC.

Retinas after ON Injury. Intraorbital transection of the left ON was performed on 45 rats to study the proportions of $trkA^+$, $trkB^+$, and $trkC^+$ RGCs after ON injury. The left ON was exposed through a posterior temporal intraorbital approach. The dural sheath of the ON was longitudinally opened with a 27.5-gauge needle. Complete transection of the ON was made by removing a 1-mm segment of the ON inside the dura with a pair of iridectomy scissors, leaving a 0.5-mm stump attached to the optic disc. The removed ON segments were sometimes used for intraocular insertion. Care was taken to avoid damage to the ophthalmic artery, which is located on the inferomedial dural sheath of the ON in rats.[49] To mimic the supply of NTF, autologous PN and/or ON segments were implanted in the vitreous chamber of the injured eye in some animals immediately after ON transection. PN segments (1 mm) were obtained from the peroneal nerve of the left hindlimb. Previous studies have shown that this intravitreal implant approach increases the number of surviving and regenerating RGCs.[6,44–48]

Experimental animals were allowed to survive for 3, 5, 9, 14, 28, and 60 days after ON axotomy. Generally, 2 days before animals were killed, the transected ON site was reaccessed and 6% Fluoro-Gold soaked in a piece of gelfoam was applied to the injury site behind the optic disc to label surviving RGCs. In animals with 3 days' survival time, Fluoro-Gold was applied at the time of ON lesion. Animals were perfused with saline followed by 4% paraformaldehyde in 0.1 M PBS. The retinas were removed and postfixed in the same fixative for 1 hour, then transferred to 30% sucrose in PBS overnight at 4°C. Parasagittal cryostat sections at 10-μm thickness were obtained for immunocytochemical staining and fluorescence microscopy.

RGC-Regenerating Retinas. Fifteen rats were used in the study of $trkA^+$, $trkB^+$, and $trkC^+$ labeling in RGC-regenerating retinas. Animals received a 1.5-cm long autologous PN-onto-ON (PN-ON) bridging graft. The PN graft was obtained from the peroneal branch of the left sciatic nerve and attached with a 10-0 suture onto the proximal stump of the intraorbitally transected ON. The PN grafts provided a suitable environment for RGC axons to regenerate.[6,8,10,46,48,50] To better expose the ON for PN-ON grafting, an additional transverse cut was made on the dural sheath after the ON transection procedure. Care was again taken to avoid damage to the ophthalmic artery during the procedure. The distal part of the PN graft was placed on the skull under the scalp.

Surgically altered animals were divided into four groups. Group 1 received a PN-ON graft only and served as the control. Groups 2 and 3 received a PN-ON graft plus an intravitreal implant of a PN or ON segment (1 mm), respectively. Group 4 received a PN-ON graft plus an intravitreal implant of both PN and ON segments (1 mm each). The survival time was 4 weeks. This time was chosen, because previous results show that the number of regenerating RGCs reaches the highest level 4 weeks after PN-ON transplantation.[48] Three days before animals were killed, 6% Fluoro-Gold was injected into the distal end of the PN graft to label RGCs that had regenerated axons to the site of dye application. Animal perfusion and immunoprocessing were the same as in retinas subjected to ON injury only.

Retinas for Studies of Expression of trkA, trkB, and trkC mRNA. Twenty-one rats were used in the studies of expression of trkA, trkB, and trkC mRNA in injured and RGC-regenerating retinas. Procedures for ON transection and transplantation of an autologous PN graft onto ON were the same as in the studies of proportion of $trkA^+$, $trkB^+$, and $trkC^+$ RGCs. Expression of each trk mRNA was studied in whole retinas ($n = 10$) of ON-injured rats at postlesion day (PLD) 3. This time point was chosen because initial results revealed that the changes in proportion of $trkA^+$, $trkB^+$, and $trkC^+$ RGCs reached most detectable levels at PLDs 3 to 5. Nonlesioned left eyes of the rats served as control. In the study of RGC regenerating retinas, survival time for the animals with PN-ON autografts was 4 weeks ($n = 11$).

Immunocytochemistry

Parasagittal cryostat sections of the retinas were stained using immunofluorescence procedures. Briefly, sections were blocked with normal goat serum (NGS) and bovine serum albumin followed by incubation with primary rabbit anti-trkA (a gift of Louis Reichardt; dilution 1:5000) and anti-trkB (Amgen, Thousand Oaks, CA; recognizes both full-length and truncated forms; dilution 1:4000), or anti-trkC (Amgen; recognizes both full-length and truncated forms; dilution 1:4000) antibodies overnight. Retinal sections treated with carrier solution without primary antibody served as the negative control. After three washes, biotinylated secondary goat anti-rabbit IgG (dilution 1:250; Vector Laboratories, Burlingame, CA) in PBS supplemented with NGS was added, and the sections were incubated for 1 hour. Trk staining was detected with fluorescent avidin-Texas red (dilution 1:100; Vector), which was chosen to minimize interference with Fluoro-Gold labeling (excitatory wave-length 595 to 604 nm versus 350 to 395 nm). Whether trk^+ cells in the ganglion cell layer (GCL) were RGCs was determined by detection using different filters for Fluoro-Gold and Texas red in the same field. A cell was counted as a trk^+ RGC if it had an immunopositive cell body and contained Fluoro-Gold labeling.

Examination of the Proportions of $trkA^+$, $trkB^+$, and $trkC^+$ RGCs

For detailed analysis of results of the different experimental interventions, six sections per animal and three to five animals per time point were used in three to four immunostaining analyses for each trk study. In rats with survival time of no more than 2 weeks, 4 fields from both central and peripheral parts of each section were chosen for analysis. Because the number of surviving RGCs was low in rats with longer survival time, such as 28 and 60 days after ON lesion, whole retinal sections were used for analysis. Counting of both Fluoro-Gold and Texas red (trk^+)–labeled RGCs was performed in the fields, and the percentage of Texas red–stained trk^+ RGCs versus Fluoro-Gold–labeled RGCs at each time point was then determined. Although sagittal sections may not be accurate for quantifying the number of RGCs, they provide accurate proportional analysis.

Northern Blot

Three plasmids were used to obtain the templates: (1) pKS-rTrkA (1.5 kb) plasmid containing a 1.5-kb *Eco*RI cDNA fragment of trk A (Regeneron Pharmaceuticals, Tarrytown, NY); (2) pKS-rTrkB/ED (665-969) plasmid containing a 0.3-kb *Xho*I cDNA fragment of trk B (Regeneron);

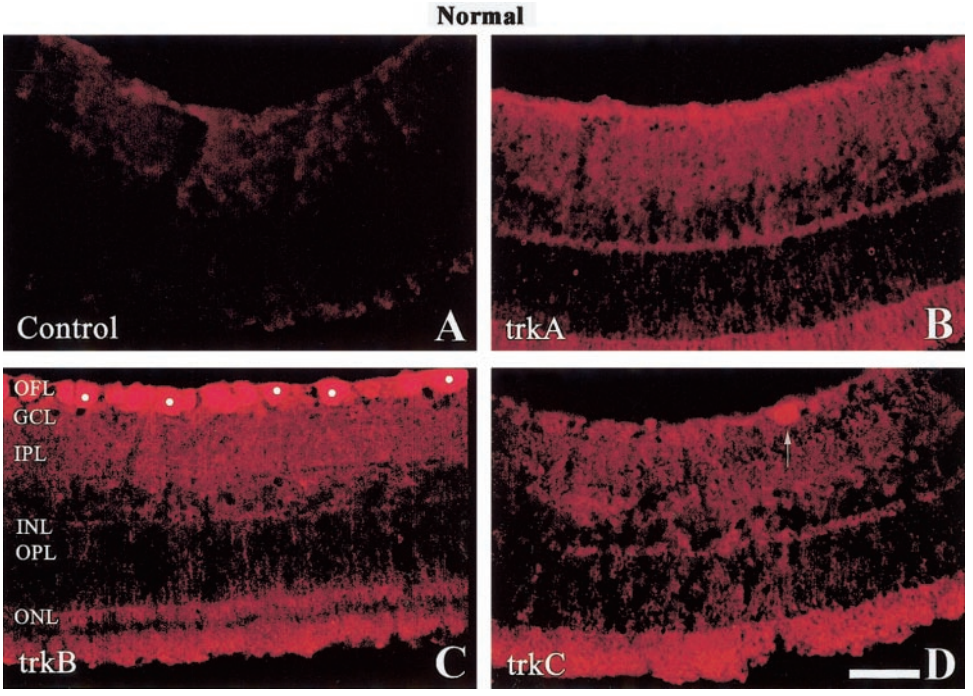

FIGURE 1. Fluorescence photomicrographs of cryostat parasagittal sections of normal retinas with avidin–Texas red staining for trkA, trkB, and trkC. No positive staining was observed in the retinal sections with the omission of primary antibody (negative control, **A**) and with trkA antibody (**B**). Intensive trkB labeling was observed on the axon bundles (some are indicated by *white dots*) in the OFL, which consists of both axons of RGCs and processes of Müller cells (**C**). The number of $trkB^+$ (**C**) and $trkC^+$ (**D**) cells in the GCL was low. *Arrow*: one $trkC^+$ cell (**D**). Scale bar, 50 μm.

and (3) pKS-rTrkC/ED (−9–302: first antisense [AS]) plasmid containing a 0.3-kb *Sac*I cDNA fragment of trk C (Regeneron).

Isolation of RNA. Eyeballs were dissected in cold phosphate buffer and quickly frozen in liquid nitrogen. They were stored at −80°C until use. Total RNA was extracted from the retinas by extraction reagent (TRIzol; Gibco, Grand Island, NY), according to the manufacturer's instructions. After extraction, the isolated RNA samples were analyzed spectrophotometrically at 260 and 280 nm. mRNA was then extracted by a kit (Oligotex; Qiagen, Valencia, CA) and optical density measured.

Preparation of ^{32}P-Labeled DNA Probes. DNA probes were labeled with a DNA labeling kit (MegaPrime; Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions, with minor modification. Briefly, 100 ng of linearized cDNA template, 10 μL [α^{32}P] dCTP (Amersham), and 4 μL enzyme (1 U/μL DNA polymerase I Klenow fragment) were added. Probes were labeled at 37°C for 20 minutes and purified by spin column (MicroSpin G-50; Amersham). After denaturing, the probes were added to the hybridization buffer.

Northern Blot Analysis. mRNA (3–4 μg) from each time point was loaded onto 1.2% agarose-formaldehyde gel. After electrophoresis, samples were transferred to nylon membrane sheets (Hybond-N^+; Amersham). The membrane was baked at 80°C for 2 minutes and stored at −80°C until use. On the day of hybridization, the membrane was first prehybridized at 65°C in hybridization buffer (ExpressHyb; Clontech, Palo Alto, CA) for 0.5 hour. Denatured [α^{32}P] dCTP labeled probe was then added to the fresh hybridization buffer. After incubation for 1 hour, the membrane was washed with 2× SSC plus 0.05% SDS three times within 40 minutes at room temperature followed by another washing with 0.1× SSC plus 0.1% SDS twice for another 40 minutes at 50°C. After washing, the membrane was wrapped by plastic wrap and exposed in a phosphorescence imaging cassette (Molecular Dynamics, Sunnyvale, CA) for 1 to 2 nights. Northern blot images were read by a phosphorescence imaging scanner, and data were analyzed with the accompanying software (ImageQuant; Molecular Dynamics). The relative mRNA levels of trkA, trkB, and trkC receptors were normalized in comparison with β-actin mRNA levels. The membrane was also exposed to x-ray film that was developed and fixed for permanent record. For rehybridization, the membrane was stripped by soaking in boiling 0.5% SDS to remove hybridized probes. This procedure for rehybridization does not affect quantification analysis if multiple stripping is avoided.

Exclusion of Possible Involvement of Ischemic Injury

We routinely perform and thus have extensive experience in ON transection and PN-ON grafting procedures.[6,8,44–46,48] Successful execution of these procedures renders no obvious bleeding around the eyeball and ON, and blood supply to the retina is not disrupted.[51] We confirmed the retinal blood supply immediately after ON transection and PN-ON grafting procedure, by use of a method published elsewhere.[50] Briefly, the left pupil was dilated with topical application of atropine. A drop of saline was place on the cornea just after the surgical procedures, a coverslip applied, and the retina examined with the aid of a dissecting microscope to ascertain the state of the retinal blood supply. No temporary blanching was observed in the successfully performed surgeries (no obvious bleeding during the surgery). In this study, adverse events (excessive bleeding around the eyeball or ON) rarely occurred during the operative procedure, and the animal was discarded if such an event occurred.

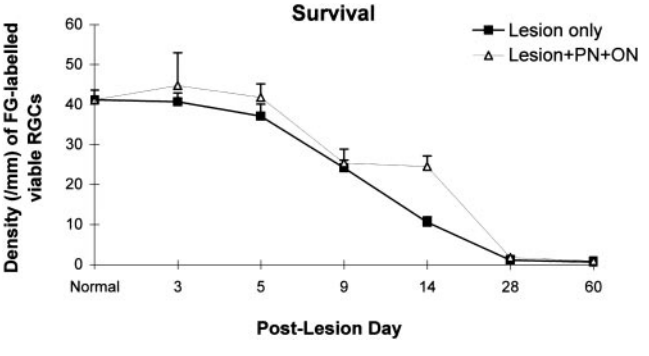

FIGURE 2. Time course of density of Fluoro-Gold–labeled surviving RGCs with SD after ON axotomy, with or without intravitreal implant of PN+ON segments.

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by the Dunnett and Bonferroni tests. The Dunnett test was used to compare the mean data of groups with ON injury against the same control group, whereas the Bonferroni test was used for intragroup comparisons.

RESULTS

TrkA, TrkB, and TrkC Staining in Normal Retinas

Control retinal sections without the primary antibody contained no obvious staining when viewed under the fluorescence microscope (Fig. 1A). There were very few, if any, $trkA^+$ cells (Fig. 1B) in normal retinas, which yielded a normal percentage of $trkA^+$ RGCs of approximately 0%. Numerous $trkB^+$ cells were observed in the GCL of normal retinas (Fig. 1C), but Fluoro-Gold–labeled $trkB^+$ RGCs accounted for only a small proportion (8.9%) of these $trkB^+$ cells. TrkC expression in the normal retinas was weak, and the average proportion of $trkC^+$ RGCs was low (5.5%; Fig. 1D).

Intensive and persistent $trkB^+$ labeling in the optic fiber layer (OFL), which consists of both RGC axons and Müller cell processes, was seen (Fig. 1C) in both normal and ON-injured animals. The axons converged toward the ON head. The thickness and intensity of $trkB^+$ staining increased toward the ON head, and $trkB^+$ labeling persisted as long as there were viable axons (at least 4 weeks after ON lesion). After ON injury, there was a progressive reduction in the number of surviving RGCs. Thus, the thickness of OFL gradually decreased over the next 2 to 3 weeks. A corresponding gradual reduction in the area of $trkB^+$ labeling was also observed during this period.

$TrkB^+$ labeling appeared to predominate on the axons of RGCs rather than on Müller cell processes in the OFL. This is suggested by our short-term Fluoro-Gold application study (data not shown here). Owing to the short time (15–18 hours) given for retrograde transport of Fluoro-Gold, much of the dye was still in the axons of the OFL on its way to the RGC somata. Thus, labeling of RGCs was still faint, but the axons of RGCs in the OFL were clearly shown. At this early time point, the intensive $trkB^+$ staining in the OFL precisely overlapped the Fluoro-Gold–labeled axon bundles.

Time Course of RGC Survival after ON Injury

The density of Fluoro-Gold–labeled viable RGCs in the three normal control animals was the highest among all rats examined. The average number of Fluoro-Gold–labeled surviving RGCs in the parasagittal sections of normal retinas was 41.1/mm (Fig. 2). Because RGCs only start to die at PLD 4 to 5 days after ON axotomy,[39,42] the viable RGC density in animals with survival times of 3 or 5 days after ON injury was very close to normal (40.7 and 37.1/mm, respectively; Fig. 2). As expected, the density of surviving RGCs started to decrease sharply after PLD 5. By PLD 9, nearly half of RGCs had died (viable RGC density, 24.2/mm); by PLD 14, approximately 75% of the RGCs were lost (viable RGC density, 10.7/mm); by PLD 28, no more than 5% (viable RGC density 1.15/mm) of RGCs had survived; and by PLD 60, almost all RGCs had died (0.67/mm; Fig. 2).

Intravitreal implant of PN+ON segments had a small trophic effect on RGC survival. The density of Fluoro-Gold–labeled RGCs was higher in the animals intravitreally implanted with PN+ON segments than the injury-only ones at all corresponding time points examined (44.7/mm PLD 3; 41.8/mm, PLD 5; 25.4/mm, PLD 9; Fig. 2). The difference between the two experimental conditions became more obvious and reached a statistically significant level at PLD 14 (24.5/mm vs. 10.7/mm; Bonferroni, $P < 0.01$). The differences remained throughout the rest of the examination period (1.78/mm vs. 1.15/mm, PLD 28; 0.94/mm vs. 0.67/mm, PLD 60). Note that regardless of any protection supplied by intravitreal implants, most RGCs had died by 2 months after ON injury.

Studies of Trk^+ RGCs

The time course of the level of $trkA^+$, $trkB^+$, and $trkC^+$ RGCs after ON injury, with or without intravitreal implant of PN+ON segments, is summarized in Figure 3. ON injury induced a rapid increase in the percentage of trk^+ RGCs in all three trk receptors. Such increases were dramatically suppressed by intravitreal implantation of PN+ON segments within the time frame of PLD 3 to 5 (Fig. 3).

$TrkA^+$ RGCs. The $trkA^+$ RGCs in normal rats were increased to 41.4% by ON injury at PLD 3 and reached a peak of 60.2% at PLD 5 (Fig. 3, top). Both levels are significantly higher than the normal control (0%; Dunnett, $P < 0.001$). The injury-induced increase in the proportion of $trkA^+$ RGCs was transient, and the proportion decreased sharply after 5 days. The average proportion of $trkA^+$ RGCs decreased to 21% by PLD 9. The proportion further decreased to the very low level of 2% by PLD 14 and remained at that low level until PLD 28 (1.3%). By PLD 60, most RGCs had died, but a high proportion (66.8%) of the RGCs with long-term survival expressed trkA receptor. This level is significantly higher than the trkA normal control (Dunnett, $P < 0.001$).

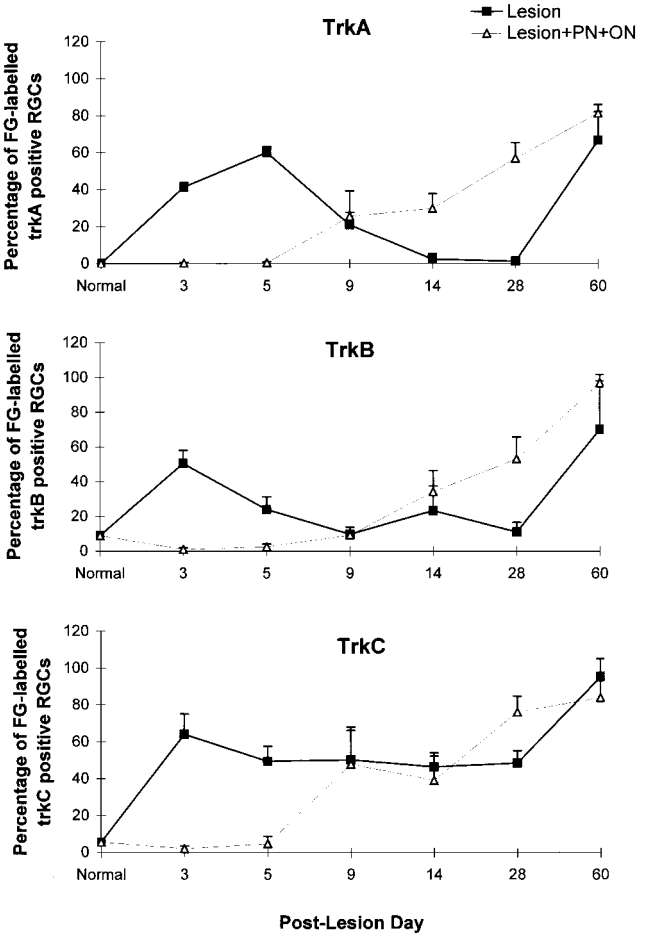

FIGURE 3. Time course of average percentage of $trkA^+$ (*top*), $trkB^+$ (*middle*), and $trkC^+$ (*bottom*) RGCs with SD after ON lesion and lesion plus intravitreal implant of PN and ON.

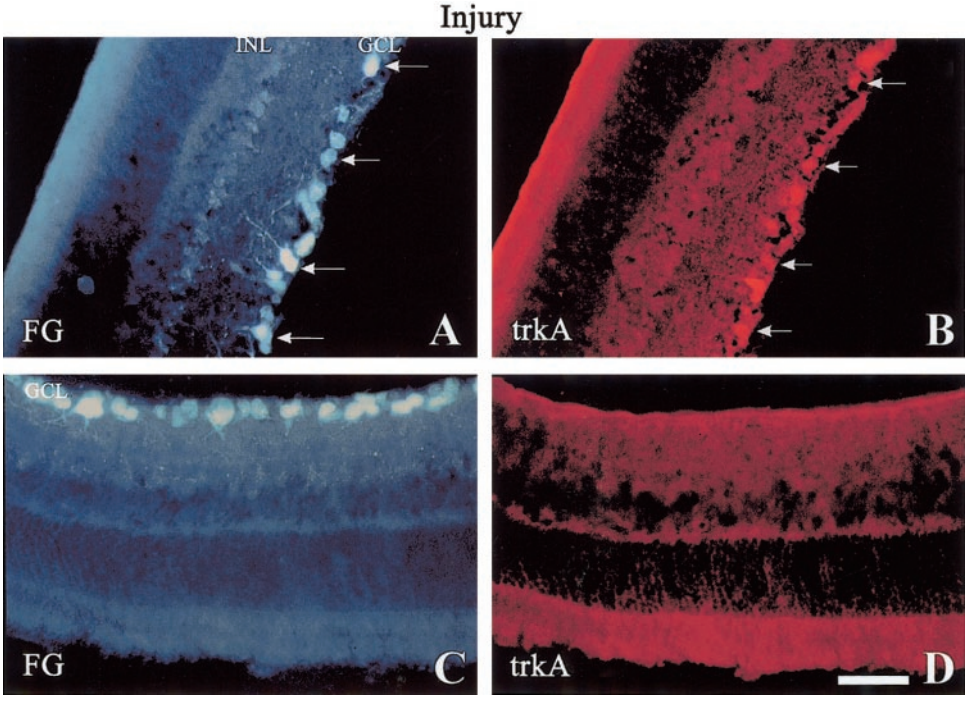

FIGURE 4. Fluorescence photomicrographs of Fluoro-Gold–labeled RGCs (**A**, **C**) and avidin-Texas red trkA$^+$ cells (**B**, **D**) in PLD 3 retinas. (**A**, **B**) are the same field from a lesion-only retina; (**C**, **D**) are the same field from a retina with lesion plus intravitreal implant of PN+ON. Some of Fluoro-Gold–labeled (**A**) trkA$^+$ (**B**) RGCs are indicated by *arrows*. After intravitreal implant of PN and ON segments, no RGCs were trkA$^+$ (**D**). Scale bar, 50 μm.

After intravitreal implant of PN+ON segments, the increase in the percentage of trkA$^+$ RGCs observed in the ON-injury-only animals was dramatically suppressed from 41.4% to 0.26% at PLD 3 and from 60.2% to 0.53% at PLD 5 (Fig. 3). All levels were significantly lower (Bonferroni, $P < 0.001$) than in injury-only animals at the corresponding time points. Figure 4 shows the characteristics of ON injury–induced increase (Figs. 4A, 4B) and implant-related suppression (Figs. 4C, 4D) in Fluoro-Gold–labeled (Figs. 4A, 4C) trkA$^+$ (Figs. 4B, 4D) RGCs in the same double-labeled fields 3 days after ON injury.

The implant-related suppression was temporary, and the percentage of trkA$^+$ RGCs began to increase substantially after PLD 5 (Fig. 3). By PLD 28, the level (57%) of trkA$^+$ RGCs in the PN-ON-implanted animals was close to peak value (60.2%) observed in the ON-injury-only animals at PLD 5 (Fig. 3). As in injury-only animals, the average proportion of trkA$^+$ RGCs in long-term surviving RGCs (PLD 60) was also high in the intravitreal PN+ON implanted animals, reaching 81.5% 2 months after the injury.

The proportions of trkA$^+$ regenerating RGCs 4 weeks after the ON injury with or without intravitreal implants are shown in Figure 5. The percentage of trkA$^+$ RGCs clearly increased in regenerating RGCs (19.9%) 4 weeks after ON transection and PN graft (Fig. 5). This percentage is significantly higher than the normal control (0%; Dunnett, $P < 0.001$) and the injury-only percentage at the same time point (1.3%; Bonferroni, $P < 0.01$). Intravitreal implant of a PN or ON segment substantially increased the proportion of trkA$^+$ regenerating RGCs (28.6% and 33.5%, respectively; Fig. 5). Compared with the corresponding percentage in animals with the PN graft only (19.9%), the differences failed to reach a statistically significant level (Bonferroni, $P > 0.05$). However, the effects of both PN and ON intravitreal segments appeared to be additive, because implant of PN+ON segments yielded a much higher level (47.8%; Fig. 5). This percentage is significantly higher (Bonferroni, $P < 0.05$) than the corresponding percentage in PN-graft–only animals. The characteristics of trkA labeling in RGC regenerating retinas in animals with intravitreal implants are shown in Figures 6A and 6B.

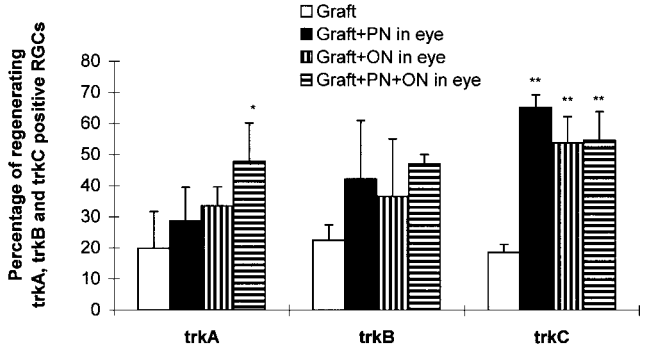

FIGURE 5. The average percentages and SDs of trkA$^+$, trkB$^+$, and trkC$^+$ regenerating RGCs 4 weeks after ON lesion and PN graft, with or without intravitreal implant of PN and/or ON.

A time point of PLD 3 was chosen to analyze trkA mRNA expression in the retina, because changes in trk receptors were most obvious at this time after ON injury. Northern hybridization analysis showed that trkA mRNA expression level was low in retinas at PLD 3 (Figs. 7 and 8). The reduction in trkA mRNA expression was quite striking; the level decreased approximately fourfold when compared to normal (Fig. 8). Note that this is in contrast with our observation of the proportion of trkA$^+$ RGCs at PLD 3.

The expression levels of trkA mRNAs in regenerating retinas 4 weeks after PN-ON transplantation were substantially higher than in both normal and injured retinas (Figs. 7, 8). The magnitude of the increase for all trks was approximately twofold when compared with normal retinas. Because trkA mRNA level in whole retinas decreased soon after ON injury, the high level of trkA mRNA expression in RGC-regenerating retinas provided a striking contrast (seven times higher than in ON-injury-only retinas).

TrkB$^+$ RGCs. ON injury also dramatically increased the proportion of trkB$^+$ RGCs, which quickly reached a peak of 50.4% at PLD 3. This percentage is significantly higher than the trkB normal control (8.9%; Dunnett, $P < 0.05$). The injury-induced increase in trkB$^+$ RGCs also appeared to be transient

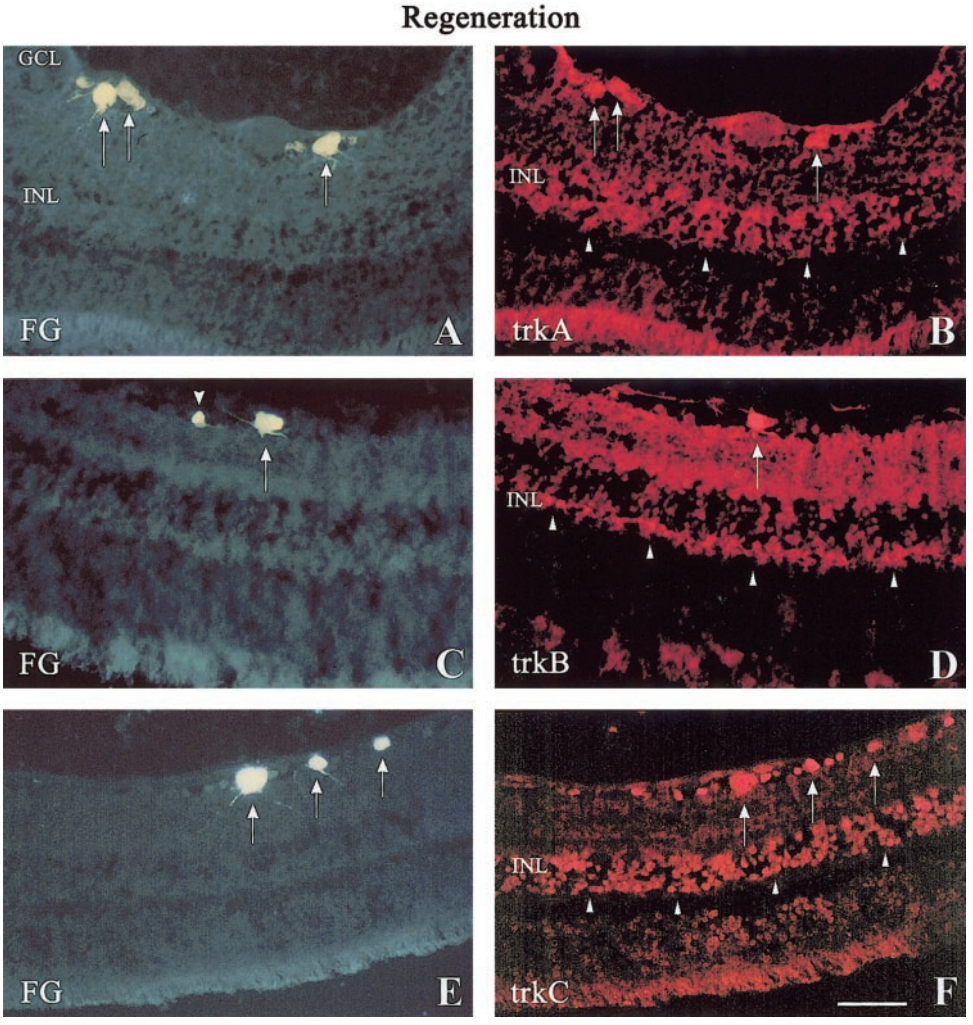

FIGURE 6. Fluorescence photomicrographs of Fluoro-Gold–labeled regenerating RGCs (**A**, **C**, **E**) and avidin-Texas red–stained $trkA^+$, $trkB^+$, and $trkC^+$ cells (**B**, **D**, **F**) in retinas with intravitreal implants of PN and ON 4 weeks after the ON injury. *Arrows*: Fluoro-Gold–labeled regenerating (**A**) $trkA^+$ (**B**) RGCs. The INL appears to be $trkA^+$. One Fluoro-Gold–labeled regenerating RGC is $trkB^+$ (**C**, **D**, *arrow*); the other regenerating RGC (**C**, *arrowhead*) is $trkB^-$. INL is also $trkB^+$ (**D**, *arrowheads*). *Arrows*: Fluoro-Gold–labeled regenerating RGCs (**E**) that are $trkC^+$ (**F**). The INL was strongly stained with trkC (**F**, *arrowheads*). Scale bar, 50 μm.

(Fig. 3). The average percentage of $trkB^+$ RGCs decreased from the peak at PLD 3 to 23.9% at PLD 5 and farther to 9.7% at PLD 9. Then the value fluctuated for the remainder of the month (Fig. 3). In the long-term surviving RGCs (PLD 60), the percentage of $trkB^+$ RGCs was 70.1%, also significantly higher than the trkB normal control (Dunnett, $P < 0.001$).

After intravitreal implant of PN+ON segments, the expression of trkB in RGCs was significantly suppressed compared to the ON-injury-only animals; and the percentage of $trkB^+$ RGCs decreased to 0.94% at PLD 3 (Fig. 3). This value was significantly lower (Bonferroni, $P < 0.001$) than the value in injury-only animals at the same time point. By PLD 5, the level was at was 2.53%. Note that the proportions at PLD 3 and PLD 5 were even lower than the trkB normal control level (data not shown). Figure 9 shows the characteristics of ON injury-induced increase (Figs. 9A, 9B) and implant-related suppression (Figs. 9C, 9D) in Fluoro-Gold–labeled (Figs. 9A, 9C) $trkB^+$ (Figs. 9B, 9D) RGCs in the same double-labeled fields 3 days after ON injury.

The suppression of trkB expression by PN+ON implant also appeared to be transient. The proportion of $trkB^+$ RGCs increased to 9.2% at PLD 9, 34.3% at PLD 14, and farther to 53.2% at PLD 28. The percentage at PLD 28 was close to the peak that occurred soon after ON transection in injury-only animals. In the few long-term surviving RGCs (PLD 60), the proportion reached 95.9% (Fig. 3).

The percentage of $trkB^+$ RGCs among regenerating RGCs 4 weeks after ON transection and PN graft was 22.5% (Fig. 5). This percentage is not significantly higher than the $trkB^+$ normal control (8.9%). Intravitreal implant of PN or ON also increased the proportion of $trkB^+$ regenerating RGCs (42.2% and 36.6%, respectively; Fig. 5). The increased levels are significantly higher than the $trkB^+$ normal control (Dunnett, $P < 0.05$). In addition, the percentage in PN-implanted animals is also significantly higher than the injury-only percentage at the same time point (10.9%; Bonferroni, $P < 0.05$). Probably owing to the high level of variation in these groups of implant recipients, the increases are not significantly higher when compared with the level (22.5%) in PN-graft–only animals (Bonferroni, $P > 0.05$). The effects of PN and ON implant did not appear to be synergistic, because the percentage of $trkB^+$ regenerating RGCs after intravitreal PN and ON implant reached only a marginally higher level (47.1%, Fig. 5) than the percentages when PN and ON grafts were applied individually. This level is still not significantly higher (Bonferroni, $P > 0.05$) than the PN graft-only level (22.5%), but it is significantly higher (Bonferroni, $P < 0.05$) than the ON-injury-only level (10.9%) at the same time point.

Northern hybridization analysis showed no appreciable change in expression of trkB mRNA in the whole retinas at PLD 3 (Figs. 7, 8). This is also in contrast with our observation of the proportion of $trkB^+$ RGCs at PLD 3. The expression level of trkB mRNAs in regenerating retinas 4 weeks after PN-ON transplantation was substantially higher, however, than both normal and ON-injury-only retinas (Figs. 7, 8). The magnitude of the

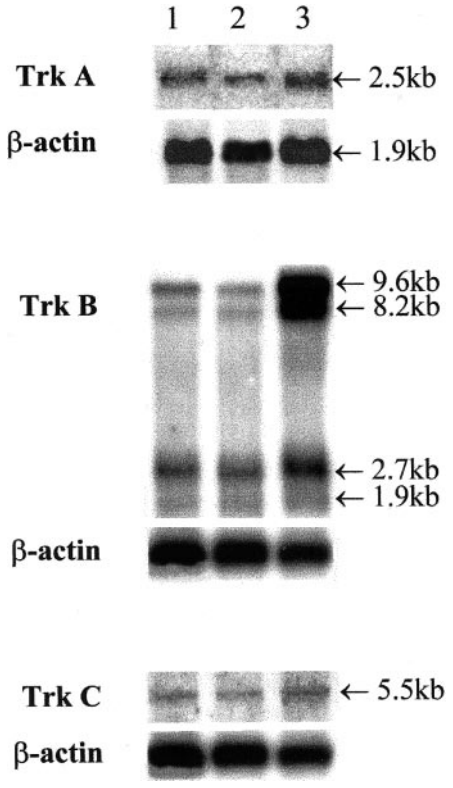

FIGURE 7. Quantitative analysis of trkA, trkB, and trkC mRNA levels in the adult retinas by Northern blot hybridization. *Lane 1*: nonlesioned control retinas; *lane 2*: ON-transected retinas; and *lane 3*: PN-grafted regenerating retinas.

increase in trkB mRNA expression is approximately twofold when compared with normal or ON-injury-only retinas.

TrkC$^+$ RGCs. ON injury also increased the proportion of trkC$^+$ RGCs. The extent and persistence of this injury-induced increase was the most profound among the three trk receptors. The response of trkC expression to ON injury was rapidly initiated, and the proportion of trkC$^+$ RGCs soon reached a peak of 64% by PLD 3. The percentage decreased marginally to 49.3% by PLD 5 and maintained this high level throughout the remainder of the month (Fig. 3). The proportion of trkC$^+$ RGCs among long-term surviving RGCs, similar to trkA$^+$ and trkB$^+$ RCSs, was high (95%) 2 months after the injury. When compared with the trkC normal control (5.5%), the proportions of trkC$^+$ RGCs at all these time points after ON injury are significantly higher (Dunnett, $P < 0.01$).

After intravitreal implant of PN+ON segments, the percentage of trkC$^+$ RGCs decreased to a mere 2.04% at PLD 3 and 4.65% at PLD 5 when compared to the ON-injury-only animals (Fig. 3). These percentages are significantly lower (Bonferroni, $P < 0.001$) than their counterparts in injury-only animals at the correspondent time points. They are also lower than the trkC normal control. Figure 10 shows the characteristics of the ON injury-induced increase (Figs. 10A, 10B) and implant-related suppression (Figs. 10C, 10D) in Fluoro-Gold–labeled (Figs. 10A, 10C) trkC$^+$ (Figs. 10B, 10D) RGCs in the same double-labeled fields 3 days after ON injury.

Among the three trk receptors, expression of trkC appeared to resume the lesion-induced increase most promptly after the transient suppression by the intravitreal implant. The proportion of trkC$^+$ RGCs increased to 47.7% by PLD 9 and maintained a similar level (39.1%, PLD 14) before reaching 76.1% by PLD 28 (Fig. 3). The latter was even higher than the peak that occurred soon after ON axotomy in the injury-only animals. As occurred in trkA$^+$ and trkB$^+$ labeling, the average proportion of trkC$^+$ RGCs in the long-term surviving RGCs was very high after the intravitreal PN+ON implant, reaching a 83.9% 2 months after the injury.

Among the regenerating RGCs, the proportion of trkC$^+$ cells was 18.6% 4 weeks after ON transection and PN attachment (Fig. 5). This value is significantly higher (Dunnett, $P < 0.01$) than the trkC$^+$ normal control (5.5%) but significantly lower ($P < 0.01$) than the injury-only level of 48.4% at PLD 28. The effect of intravitreal implant of PN and/or ON segment on the proportion of trkC$^+$ regenerating RGCs was most profound. Intravitreal implant of PN or ON alone increased the proportion to 65.2% and 53.8%, respectively (Fig. 5). These proportions are significantly higher than both the trkC$^+$ normal control (Dunnett, $P < 0.001$) and the PN graft-only percentage of trkC$^+$ regenerating RGCs (Bonferroni, $P < 0.001$ for PN implant and $P < 0.05$ for ON implant). However, when compared with the injury-only level (10.9%) at PLD 28, the difference is not statistically significant. The PN+ON implant approach yielded a proportion of 54.7% (Fig. 5), a level not significantly different from the two percentages obtained when PN or ON was applied alone.

Northern hybridization analysis showed that expression of trkC mRNA decreased slightly in the whole retinas at PLD 3 (Figs. 7, 8). This is also in contrast with our observation of the proportion of trkC$^+$ RGCs at PLD 3. The expression level of trkC mRNAs in RGC-regenerating retinas 4 weeks after PN-ON transplantation was also higher than in both normal and ON-injury-only retinas (Figs. 7, 8). The magnitude of the increase in

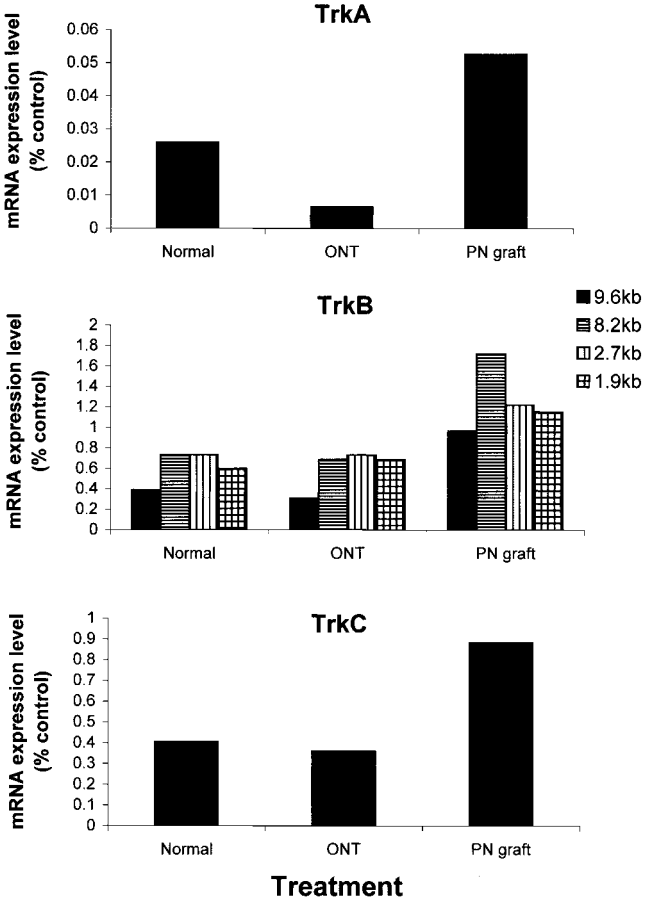

FIGURE 8. Expression levels of trkA, trkB, and trkC transcripts indicated as the percentage of their optical density normalized with the corresponding optical density of an internal control of β-actin mRNA.

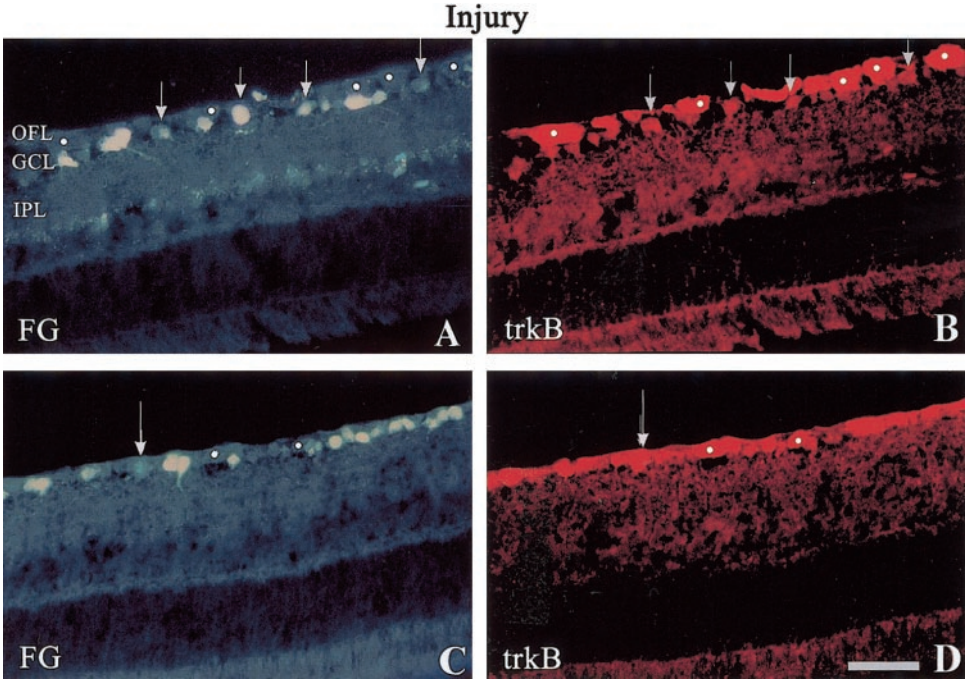

FIGURE 9. Fluorescence photomicrographs of Fluoro-Gold–labeled RGCs (**A**, **C**) and avidin-Texas red trkB$^+$ cells (**B**, **D**) in PLD 3 retinas. (**A**, **B**) Same field in a lesion-only retina; (**C**, **D**) same field in a retina with lesion plus intravitreal implant of PN+ON. *Arrows*: Fluoro-Gold–labeled (**A**, **C**) trkB$^+$ (**B**, **D**) RGCs. Intensive Fluoro-Gold–negative but trkB-positive immunolabeling was localized, probably in the OFL (indicated by *white dots*). After intravitreal implant of PN and ON segments, the number of trkB$^+$ RGCs was low; only 1 Fluoro-Gold–labeled RGC was trkB$^+$ in this field (**C**, **D**, *arrow*). Scale bar, 50 μm.

expression of trkC mRNA was more than twofold when compared with normal or ON-injury-only retinas (Fig. 8).

Density of Regenerating RGCs

When PN tissue was transplanted onto the transected ON, RGC axons regrew. At 28 days, the density of regenerating RGCs was 0.74/mm (Fig. 11). When PN-ON grafts were combined with intravitreal implants of PN and/or ON, all regenerating RGC densities were higher (2.31/mm for PN implant, 1.79/mm for ON implant, and 2.33/mm for PN+ON implant). The densities in both PN and PN-ON implant recipients were significantly higher (Dunnett, $P < 0.01$) than the graft-only density. Note that surviving RGC levels in injury-only animals and animals with injury plus intravitreal implants of PN-ON at the 28-day time point were 1.15 and 1.78/mm, respectively (Fig. 2). All regenerating RGC densities in intravitreal implant recipients were higher than those surviving RGC densities at the same time point (Fig. 2, 11), with the two levels in PN and PN+ON implant recipients being significantly higher (Bonferroni, $P < 0.05$). Thus, PN graft appeared not just to provide an environment for RGC axons to regenerate but also to promote RGC survival.

Long-Term Surviving Retinas

Some common phenomena were observed in animals with relatively long cell survival times (28 and 60 days, with or

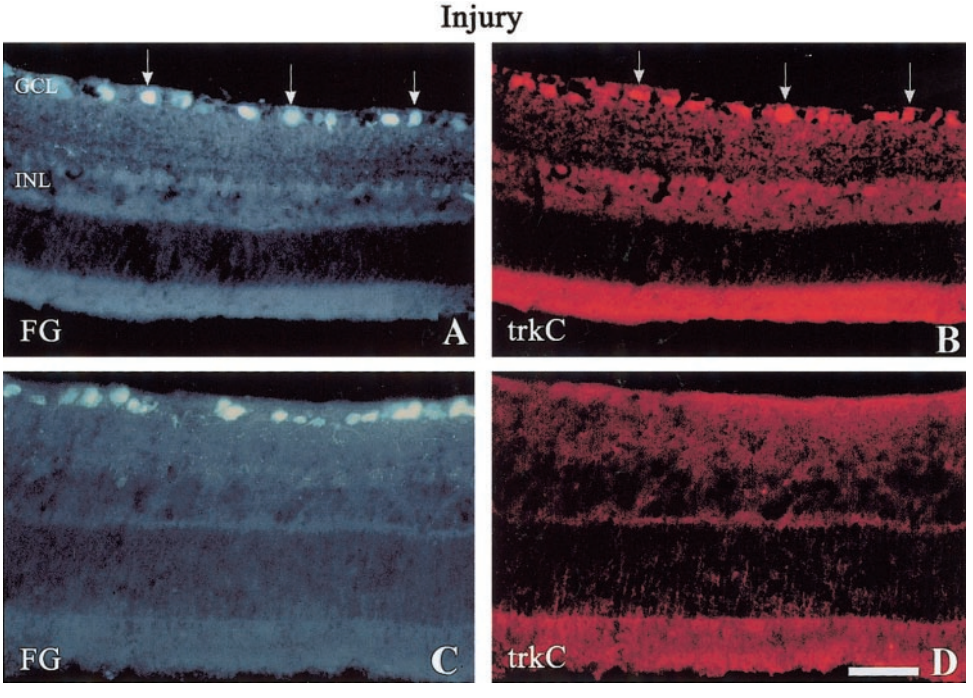

FIGURE 10. Fluorescence photomicrographs of Fluoro-Gold–labeled RGCs (**A**, **C**) and avidin-Texas red trkC$^+$ cells (**B**, **D**) in PLD 3 retinas. (**A**, **B**) Same field in a lesion-only retina; (**C**, **D**) same field in a retina with lesion plus intravitreal implant of PN and ON. *Arrows*: Fluoro-Gold–labeled (**A**) trkC$^+$ (**B**) RGCs. GCL is trkC$^-$ (**D**) after intravitreal implant of PN and ON segments. Scale bar, 50 μm.

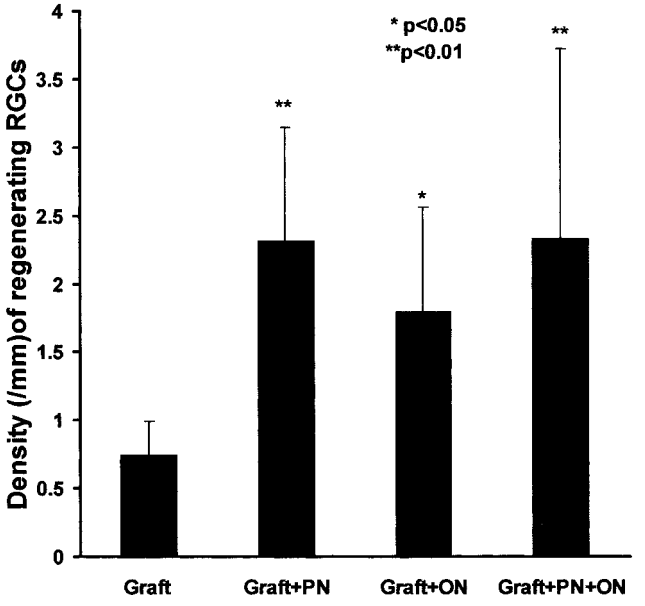

FIGURE 11. The average density of regenerating RGCs under different conditions 28 days after PN-ON grafting. Comparisons were made against the graft-only control (Dunnett test).

without PN graft): The majority of trkA$^+$, trkB$^+$, and trkC$^+$ ON-injury-only or regenerating RGCs may be type I RGCs, because the somata of these cells were large (Fig. 6, arrows). Widespread expression of trkA, and particularly of trkC, was seen in the inner nuclear layer (INL; arrowheads in Fig. 6B and 6F), and trkB was also seen in the inner nuclear layer (INL; arrowheads in Fig. 6D).

DISCUSSION

In this study we demonstrated that (1) a high level of trkB protein was present in the OFL of normal adult rat retinas whereas the proportions of trkA$^+$, trkB$^+$, and trkC$^+$ RGCs were low; (2) RGCs responded quickly to ON injury and became trkA$^+$, trkB$^+$, and trkC$^+$, a phenomenon that was transiently blocked by intravitreal implant of PN+ON; (3) there was a higher proportion of trkA$^+$ or trkB$^+$ RGCs with regenerating axons compared with injury-only RGCs at the same time point (PLD 28); (4) in contrast to the rapid increases in the proportion of trkA$^+$, trkB$^+$, and trkC$^+$ staining in injured RGCs soon after ON axotomy, mRNA expression of trkA, and to a lesser extent trkC, was downregulated in the whole retinas; and (5) mRNA levels for all trks substantially increased in RGC-regenerating retinas.

Normal Retinas

There was little, if any, trkA expression in the GCL of normal adult rat retinas. This is consistent with a previous study in which trkA was only weakly expressed on sparsely distributed large RGCs in adult rats.[28] Similar observation of weak trkA staining was also obtained in neonatal rat RGCs (Hu B, Tang LS, Cui Q, So K-F, Yip HK, manuscript submitted). Numerous trkB$^+$ cells were seen in normal retinas, but the number of trkB$^+$ Fluoro-Gold–labeled RGCs was quite low. This observation is supported by neonatal rat studies in which the number of trkB$^+$ RGCs decreased as the visual system matured (Hu B, Tang LS, Cui Q, So K-F, Yip HK, manuscript submitted). TrkC expression was evident in normal retinas, but the proportion of trkC$^+$ RGCs was low. This is also supported by neonatal rat studies in which the expression of trkC in RGCs decreased as the visual system matured (Hu B, Tang LS, Cui Q, So K-F, Yip HK, manuscript submitted).

The observation that retinal OFL is heavily labeled with trkB receptors suggests that trkB may be axonally transported and stored in the axons. Axonal transport of trkB receptors may occur in both directions: They may either be produced in the somata of the RGCs and anterogradely transported to the axons,[52] or they may be picked up at nerve terminals and retrogradely transported.[53,54] The presence of a high level of trkB at the RGC axon may help to explain the widely reported trophic actions of its ligands BDNF and NT-4/5 on neonatal and adult RGC survival and neurite growth,[5,35,37–41] because the large amount of trkB receptors in and/or on the axons can be readily recruited to orchestrate biological actions when BDNF and NT-4/5 become available. Note that the signal transduction through internalization of exogenous NTs, with their cognate receptors on the axons and retrograde transport of the receptor–ligand complex, can be conducted efficiently and rapidly to the somata.[54]

ON-Injured Retinas

We routinely perform ON transection and PN grafting procedures.[6,8,44,45,48] Animals used in this study did not have obvious bleeding around the eyeball and ON during surgical procedure, and retinal blood supply was confirmed immediately after the PN-ON procedure. Thus, it is unlikely that ischemic retinal injury occurred in this study.

ON axotomy had a dramatic effect on trkA, trkB, and trkC immunoreactivity in RGCs, such as that which occurred after ON injury, when high proportions (approximately 60%) of RGCs became positive for trkA, trkB, and trkC. Thus a substantial amount of injured RGCs possessed at least two types of trk receptors at this time point. Among the three trks, trkC was most profoundly affected by ON injury. Because the number of surviving RGCs was only slightly reduced at PLD 5,[39] the rapid and significant increase in the percentages of trkA$^+$, trkB$^+$, and trkC$^+$ RGCs within PLDs 3 to 5 reflected a real change in the trkA, trkB, and trkC properties of the injured RGCs and was not a result of selective removal of different populations of RGCs.

The transient but complete blockade of the ON injury-induced increase in trkA$^+$, trkB$^+$, and trkC$^+$ RGCs by intravitreal implant of PN+ON segments also reflected a real change in expression of these trks. It remains unknown why intravitreal implants dramatically suppressed the expression of all three trks in RGCs. However, this is consistent with a recent observation in SC-ablated neonatal rats in which intravitreal application of NT-4/5 reduced trkB immunolabeling in RGCs (Spalding KL, Cui Q, and Harvey AR, manuscript in preparation). In addition, similar downregulation of trkB expression by BDNF has been observed in other neuronal populations.[55,56]

The change in the proportion of identified (Fluoro-Gold–labeled) RGCs that were positive for trkA, trkB, or trkC after PLD 5 was complicated by probable differences in the viability of injured RGCs, expressing different levels and/or types of trks and was also influenced by the continuous and dramatic decline in the total number of surviving RGCs. The percentages of the observed trkA$^+$, trkB$^+$ and trkC$^+$ RGCs may thus reflect a combined outcome of a direct effect on the expression of trkA, trkB, and trkC of ON injury and intravitreal implant and on the selective survival of RGCs. Although a high proportion of trkA$^+$, trkB$^+$ or trkC$^+$ RGCs was seen in animals with long-term survival, the real number of surviving RGCs was very low 4 weeks after ON axotomy.

During the period of RGC death (PLD 5–28), the proportion of trkA$^+$ RGCs decreased dramatically, suggesting that trkA$^+$ RGCs were more vulnerable to ON injury. However, when a trophic supply was present (intravitreal implant of PN+ON),

all proportions of $trkA^+$, $trkB^+$, and $trkC^+$ RGCs continued to increase during this period, indicating a favorable selection of $trkA^+$, $trkB^+$, and $trkC^+$ RGCs for survival when trk receptors were activated. The protective action of the trks and intravitreal implant on RGC survival was minor and short-lived; a large proportion of RGCs died at PLD 28, regardless of the trk property or presence of a putative trophic supply.

The low proportion of $trkB^+$ RGCs from approximately 1 week after ON injury may help to explain the absence of the long-term survival effect of BDNF and NT-4/5 in adult RGCs.[51,57] It may also be due to (1) the change in the balance of full-length versus truncated forms of trkB receptors in the retinas and/or RGCs, because it is well known that different forms of trkB receptors are present in adult rat retinas and the ON[34]; (2) the change in the ratio of the three trk receptors; and (3) the receptor or signal pathway that predominates under different conditions. Available evidence suggests that each trk carries distinctive signaling properties and may contain additional factor-binding sequences that favor alternative pathways.[58]

Notwithstanding the presence of trkA and especially trkC receptors in injured RGCs, a minimal influence of their respective ligands NGF and NT-3 on RGC survival and neurite outgrowth has been observed.[5,6,35,39,40] This indicates that binding of a NT with its cognate high-affinity receptor trk may not always lead to a biological action. Although the mechanisms underlying the failure of functional action remain to be elucidated, one explanation for the failure in the retina may be incomplete signal transduction through malfunctioning trkC. Different forms of trkC (full-length or truncated) have been found, and at least one variant does not have the biological catalytic domain.[24]

RGC-Regenerating Retinas

The proportions of $trkA^+$, $trkB^+$, and $trkC^+$ RGCs were high among regenerating RGCs. Although intravitreal implants of a PN and/or ON segment resulted in further increases in the proportions of $trkA^+$, $trkB^+$, and $trkC^+$ regenerating RGCs, this result may not be solely derived from a direct increase in the proportion of trks in RGCs, because the implants may also have had a protective effect on the survival of $trkA^+$, $trkB^+$, and $trkC^+$ RGCs, thus providing an increased pool of $trkA^+$, $trkB^+$, and $trkC^+$ RGCs to be recruited for regeneration. Axonal regeneration of injured RGCs appeared more likely to occur in $trkA^+$ and $trkB^+$ RGCs, in that higher percentages of regenerating RGCs were $trkA^+$ and $trkB^+$.

Molecular Studies

Our molecular studies in ON-injury-only retinas suggest that variable expressions of trk mRNAs exist among different cellular populations of the retinas. Although the proportion of $trkA^+$, $trkB^+$ and $trkC^+$ RGCs was increased soon after ON injury, the mRNA levels of all three trks in the whole retinas did not follow this trend. In fact, the levels of mRNA for trkA and trkC went in the opposite direction. The unaffected trkB mRNA level after ON injury is in agreement with a recent report that mechanical eye injury does not alter the mRNA level of trkB in developing and adult rat retinas.[59] In regenerating retinas, all trk mRNA levels increased with the increase in trkA, with trkC mRNA levels being most significant. This is in parallel with our results on proportions of $trkA^+$, $trkB^+$, and $trkC^+$ regenerating RGCs, and also with a recent finding in which trkA mRNA expression was shown to be increased in the GCL during axon regeneration in tench.[60] Note here that RGCs account for only a small cellular proportion in the whole retina; thus, a change in trkA, trkB, or trkC expression in RGCs alone would not necessarily significantly influence the overall expression level in whole retina. Because the $trkA^+$ RGCs increased as its mRNA expression in the whole retina decreased at PLD 3, the downregulation of expression of trkA mRNA presumably reflected a decrease in the non-GCL part of the retina.

Future Studies

Currently, we are focusing on the expression status of trkA, trkB, and trkC in different subtypes of RGCs and the extent of colocalization of trks on the same RGC in vitro. These studies will provide further information for developing clinical strategies to rescue RGCs under pathologic conditions. The extent to which trkA, trkB, and trkC receptors are involved in the axonal regeneration process should be further investigated.

Acknowledgments

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