Published in final edited form as:

Exp Neurol. 2001 November ; 172(1): 81–91. doi:10.1006/exnr.2001.7795.

Neurotrophin Receptor TrkB Activation Is Not Required for the Postnatal Survival of Retinal Ganglion Cells *in Vivo*

Baerbel Rohrer^{*,1}, Matthew M. LaVail[†], Kevin R. Jones[‡], and Louis F. Reichardt^{*}

^{*}Howard Hughes Medical Institute, School of Medicine, University of California at San Francisco, San Francisco, California 94143

[†]Beckman Vision Center, School of Medicine, University of California at San Francisco, San Francisco, California 94143

[‡]MCD Biology, University of Colorado, Boulder, Colorado 29425

Abstract

During early postnatal development, apoptosis of retinal ganglion cells (RGCs) is regulated by target contact with the optic tectum. The neurotrophins BDNF and NT-4, but not NGF, prevent the apoptosis of retinal ganglion cells that is otherwise observed after target ablation or axotomy. Thus receptors activated by BDNF and NT-4 are candidates to mediate the early postnatal survival of RGCs. BDNF and NT-4, but not NGF, bind to all isoforms of the receptor TrkB, whether or not they contain a tyrosine kinase domain. To examine the roles of TrkB receptor isoforms in early postnatal survival, we compared RGC numbers in wild-type mice to those in a mutant lacking all isoforms of TrkB. Surprisingly, no reduction in RGCs was observed in the mutant at postnatal day 16, the latest age at which these animals are consistently viable, so TrkB signaling is not essential for target-dependent survival of these cells. In wild-type mice, RGCs also are lost gradually during adulthood, possibly due to oxidative stress. To determine whether TrkB signaling regulates this phase of RGC degeneration, RGC numbers were examined in a viable mutant of TrkB that expresses only about 25% the normal level of TrkB receptor kinase. Compared to controls, approximately 20% of the RGC were lost in mutant 3-month-old-animals. Thus, TrkB signaling is not required for survival of RGCs during the period of target-dependent survival, but does appear to reduce degeneration of RGCs in adult animals.

Keywords

trkB; retinal ganglion cell; optic nerve; myelination; neuroprotection

INTRODUCTION

Mouse retinal ganglion cells (RGC)² are born between E10 and E18 with the maximum rate of birth at E15. Cell death, on the other hand, occurs in two distinct phases. A first phase

¹To whom correspondence should be addressed at the Storm Eye Institute, Room 707, Medical University of South Carolina, 167 Ashley Avenue, Charleston, SC 29425. Fax: (843) 792-1723. rohrer@musc.edu.

²Abbreviations used: BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CNTF, ciliary neurotrophic factor; E, embryonic day; EM, electron microscopy; Het, heterozygous; KO, knockout; NGF, nerve growth factor; NT-3, neurotrophin

occurs before birth around E15.5 (20). This corresponds to the peak of RGC neurogenesis. A second wave of apoptosis occurs during the early postnatal period, with a maximum at P2. This correlates with the time of elaboration and focusing of retinal ganglion cell projections in the target area of the superior colliculus (SC) (52). In addition, RGCs are lost in a slow phase of age-related degeneration (47), possibly due to oxidative stress (6). Frade and Barde (20) reported that the early phase of cell death in mouse embryos is mediated by nerve growth factor (NGF) acting through its low-affinity receptor p75 (p75NTR), which is expressed at high levels in the developing ganglion cell layer and optic nerve. As the second phase of cell death correlates with the time of target innervation, it is generally thought that limiting amounts of neurotrophic factors in the target field control this phase of apoptosis. In support of this hypothesis, Ma *et al.* (29) have shown that BDNF injections into the SC of the intact hamster reduced the rate of RGC cell death during this phase. In addition, Cui and Harvey (13) demonstrated that neonatal RGCs, which normally undergo apoptosis if the target cells are removed, can be rescued by BDNF or neurotrophin 4 (NT-4) injections into the SC. Neurotrophins also influence apoptosis of adult RGCs. Axotomy-induced apoptosis of RGCs can be delayed by applying BDNF in both adult mouse and rats (15, 30, 31). BDNF is a survival factor for RGCs *in vitro* (32), it is expressed in the retinorecipient layers of the SC in hamster and rat (29, 35) and is retrogradely transported to the retina (36), suggesting that target-derived BDNF is a major source of the neurotrophin for RGCs. A major receptor for BDNF is the receptor tyrosine kinase TrkB. RGCs have been shown to express high levels of both full-length and truncated forms of TrkB (39, 44).

The neurotrophin receptor TrkB comes in two different isoforms, the full-length, kinase-containing isoform and two truncated isoforms, TrkB.T1 and TrkB.T2 (37). Activation of TrkB tyrosine kinase stimulates cell survival pathways by triggering PI-3 kinase and Erk kinase cascades (42). Signaling mechanisms of TrkB.T1 and TrkB.T2 are less certain. BDNF activation of TrkB.T1 or TrkB.T2 has been shown to increase the rate of acidic metabolite release from the cell, which is considered to be a common physiological consequence of many signaling pathways (3). Truncated isoforms of TrkB also can inhibit the formation of dimers of kinase-containing isoforms of TrkB, thereby modulating BDNF signaling (19). Yet the truncated receptors may not signal directly, but may instead regulate ligand availability by binding BDNF.

We have previously shown that *trkB*^{-/-} animals, which have a life span of approximately 3 weeks, develop structurally normal but small eyes (39). In these mice, the retinal layers develop normally although retinal cell migration and differentiation are delayed. Photoreceptor maturation is developmentally delayed, and synaptic signaling is severely impaired.

To test the hypothesis that TrkB kinase signaling is required for postnatal RGC survival, we took advantage of two lines of *trkB* mutant animals. The *trkB*^{-/-} mice, which were previously used for the photoreceptor study, are complete null mutants, lacking both the full-length and the truncated isoforms of TrkB (39). The *trkB* hypomorphic line also lacks the

3; NT-4, neurotrophin 4; P, postnatal day; p75NTR, p75 neurotrophin receptor; RGC, retinal ganglion cell; RT, room temperature; SC, superior colliculus; TrkB, tropomyosin-related kinase B; WT, wild-type.

truncated forms of TrkB, but still expresses small amounts of the kinase-containing isoform (49, 50). Contrary to our expectations, at P16 the lack of TrkB signaling in the *trkB* knockout mouse did not lead to an increase in cell death in the RGC layer, resulting in cell numbers indistinguishable from those of their wild-type littermates, but myelination in the optic nerve was reduced by 70%. Normal age-related adult RGC degeneration, however, appears to be accelerated in the *trkB* hypomorphic animals. As a result, by P90, hypermorphic animals have lost approximately 20% of their RGCs in comparison to age-matched controls.

MATERIALS AND METHODS

Animals

Two *trkB* mutant lines were analyzed, the *trkB* knockout mouse (*trkB*^{-/-} (39)) and a *trkB* hypomorphic line (*trkB*^{fbz/fbz} (49, 50)), as well as BDNF^{lacZ} heterozygous mice (5). In the null mutant, expression of both the full-length and the truncated isoforms of TrkB is abolished by deletion of the first coding exon (exon S), which contains the start site and the signal peptide for TrkB and is shared by all TrkB isoforms (39). This results in a complete absence of both the kinase and the nonkinase isoforms as confirmed by Northern and Western blotting (data not shown). *TrkB*^{-/-} homozygous mutants die within 3 weeks of birth and reach only approximately 45% of the wild-type body weight (P16 WT, 11.4 ± 1.28 g versus P16 *trkB*^{-/-}, 4.86 ± 1.22 g (39)). *TrkB* hypomorphic animals were created by inserting a rat *trkB* cDNA flanked by loxP sites and followed by an SV40 poly(A) sequence into the first coding exon. Homozygous *trkB*^{fbz/fbz} animals do not express any truncated isoforms of TrkB. Although the expression of full-length TrkB is reduced by approximately 75% in comparison to their littermates (49), animals were found to survive for 6 months and longer. In the BDNF^{lacZ} mice, the coding sequence of β -galactosidase was inserted into the coding exon of BDNF. As a result, cells that normally express the neurotrophin BDNF can be identified using the enzymatic activity of β -galactosidase or antibodies against the enzyme (5). Mice heterozygous for BDNF^{lacZ}, with no known deficit in the visual system, were used in these studies.

Immunohistochemistry

Animals were sacrificed by cervical dislocation, after which eyes were enucleated and immersion fixed in Carnoy's fixative for 2 h. Eyes were then dehydrated and embedded in paraffin in transverse orientation. Seven-micrometer sections were cut and dried onto poly-L-lysine-coated glass slides, and sections were dewaxed and rehydrated for immunohistochemistry. Nonspecific binding was blocked in 3% bovine serum albumin and 10% normal goat serum in Tris-buffered saline, pH 7.4, containing 0.4% Triton X-100. Primary antibodies were applied in blocking solution overnight at room temperature (RT), followed by either fluorophore-coupled or biotinylated secondary antibodies for 4 h at RT. The enzymatic protocol was completed by incubation in ABC reagents (Vectastain) and using diaminobenzidine as substrate. For fluorescence double-labeling, primary and secondary antibodies were applied together. Slides were mounted in Fluoromount-G (Southern Biotechnology Assoc., Inc.) and analyzed by confocal microscopy. Digital images were pseudocolored and superimposed using Adobe PhotoShop software. Sections labeled

with enzyme system techniques were dehydrated, embedded in Permount, and analyzed by light microscopy. Antibodies used in this study include a polyclonal antibody raised to the extracellular domain of TrkB (24) that stains the majority of RGCs in mouse retina (39), a monoclonal antibody specific for β -galactosidase (Promega), a rabbit polyclonal antibody to Brn3A (25), a transcription factor that stains the majority of mouse RGCs, and rabbit polyclonal antibodies (Swant, Switzerland) to two markers that, in the RGC layer, stain mainly displaced amacrine cells and very few retinal ganglion cells, parvalbumin and calbindin (22, 23). FITC-coupled anti-rabbit and Texas red-coupled anti-mouse antibodies were purchased from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA) and biotinylated anti-rabbit antibodies and the Vectastain kit from Vector Laboratories, Inc. (Burlingame, CA). Immunohistochemistry experiments were repeated on a minimum of five different eyes per genotype.

X-gal staining

Depending on the age of the animals, pups were deeply anesthetized either on ice or with avertin (30 mg/kg) and then were transcardially perfused with 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.4) for 5 min. Brains were removed immediately from the skull and the two hemispheres were separated, whereas the eyes were enucleated and the cornea pierced to allow fluid exchange. Both were washed extensively in PBS followed by substrate buffer (2 mmol/L $MgCl_2$, 0.02% NP-40, 0.01% sodium deoxycholate in PBS, pH 7.3) to preserve enzymatic activity. Substrate was then added to the substrate buffer (final concentrations: 5 mmol/L ferrocyanide, 5 mmol/L ferricyanide, and 1 mg/ml X-gal in DMSO) and tissues were incubated for up to 12 h at 37°C to detect β -galactosidase activity. Tissues were postfixed in 4% PFA, equilibrated in 30% sucrose (in PBS), and embedded in OCT. Fourteen-micrometer frozen sections were cut using a cryostat. Images were taken using a standard bright-field microscopy.

Anterograde DiI tracing

Pups (P0 and P6, four animals per age and genotype) were deeply anesthetized on ice. The eyelids were carefully pried apart with a blunt pair of forceps. Small amounts of DiI (10% in dimethylformamide) were delivered into the RGC layer, using a micropipette (43). The tip of the micropipette could be guided visually using the optics of the mouse eye. Four hours after dye delivery, animals were again deeply anesthetized on ice and transcardially perfused with 4% PFA (pH 7.4). The brain was mounted in 3.7% low-melting agar and sagittal sections (80 μm) were cut using a Vibratome. DiI-labeled arbors were photographed using epifluorescence illumination and a standard automatic photographic attachment. Images were digitized and assembled using Adobe PhotoShop software. The retinal localization of the DiI injection in each experimental animal was verified in retinal flat mounts.

Semithin section

Animals were deeply anesthetized with CO₂ and perfused with fixative (4% glutaraldehyde, 2% PFA in PBS, pH 7.4). After enucleation, eyes were hemisected in dorsoventral orientation through the optic nerve and the superior and inferior oblique muscles, to ensure the same orientation in all eyes. Eyecups were embedded in Epon/Araldite following published procedures (39). Semithin (1 μm) sections were cut with a glass knife, stained

with toluidine blue solution, and coverslipped using Permount medium. Cells in the RGC layer were counted in these sections, using a 60× objective on a Nikon microscope. To be counted, a cell had to contain the nucleus and nucleolus. As each eye was cut in exactly the same orientation, accurate cell numbers, counting cells from the entire retinal circumference (ventral to dorsal), can be obtained. For statistical analysis, data were expressed as an average $\pm$ standard deviation, and a two-tailed *t* test was used to determine significance levels.

Electron microscopy (EM)

Tissues were collected as described for semithin sections. Optic nerves were postfixed in reduced osmium, stained with 2% uranyl acetate, dehydrated, and flat-embedded in Epon. Thin sections were cut, poststained with lead citrate and uranyl acid, and examined and photographed with an electron microscope. Electron micrographs were taken at 2500× magnification systematically in an S shape, covering the entire optic nerve in 12 images. These images were digitized and analyzed using Adobe PhotoShop and NIH Image J software. All axon profiles in each digitized image were outlined and filled, using Adobe PhotoShop, producing 1 image for myelinated and 1 for unmyelinated axons. These binary images were exported into Image J to determine numbers of myelinated and unmyelinated axons as well as their axon surface area. Optic nerve areas were determined using digitized light-microscope images to allow for the calculation of the absolute number of RGC axon profiles in the optic nerve. The mean and standard deviation of profiles were calculated. We counted the total number of axons per image taken rather than using stereology, since total cell counting provides a more accurate count than even the most rigorous randomized sampling technique.

RESULTS

Expression Profiles of TrkB and BDNF in the Mouse Retinocollicular System

If target-derived BDNF, signaling through TrkB, plays a role in early postnatal RGC survival, TrkB must be expressed in early differentiating RGCs while BDNF is expressed in the SC at the time of target innervation (P2). To examine the distribution of BDNF, we used the BDNF^{lacZ} mice. In the presence of the substrate X-gal, all the cells that would normally express BDNF are stained blue. At P2, BDNF was found to be expressed in the SC (Fig. 1A). At P2, the layers of the mouse SC are poorly differentiated. The dorsal part of the SC will differentiate into two layers, the stratum opticum and stratum griseum superficiale, which receive visual inputs from retinal ganglion cells, whereas the ventral part of the SC will differentiate into layers that are reciprocally connected with brainstem areas and receive inputs from other neocortical areas (17). BDNF expression was found to be moderate in the upper part of the SC and more abundant in the lower half. Interestingly, RGCs appear also to produce their own BDNF (Fig. 1B), and there is additional expression in secretory cells overlying the ciliary body (Fig. 1B). The pattern of BDNF expression in the retina appears to be almost identical from P0 to adulthood ((5); B. Rohrer personal observation).

Retinas from BDNF^{lacZ} heterozygous mice were sectioned and immunostained for both β -galactosidase (Fig. 2A), as a monitor of endogenous BDNF, and TrkB (Fig. 2B), using an

antibody to the extracellular domain of TrkB. At all ages examined (P0 to adult), RGCs expressed both BDNF and its receptor. Figure 2 depicts an example of a P10 retina in which both β -galactosidase and TrkB are shown to be colocalized in the same RGCs. While anti-TrkB recognizes all isoforms of TrkB, data by Suzuki *et al.* (44) have shown that all RGCs express the truncated isoform of TrkB while approximately 70% express the kinase-containing isoform.

TrkB^{-/-} RGCs Project Appropriately to the Superior Colliculus

To determine whether the RGCs of the *trkB*^{-/-} animals project appropriately to and initiate branching in the retinorecipient layers of the dorsal SC, we DiI-labeled RGC projections at various ages. In mouse, axons arrive at the rostral SC at E15, project diffusely by P0, and initiate axonal arborization and synaptogenesis by P6 (18). We DiI-labeled RGC projections at two time points: birth and P6.

Small amounts of DiI were injected into the RGC layer of anesthetized mouse pups. Four hours was found to be sufficient time for anterograde transport to occur at that age *in vivo*. Postnatal day 0 RGC projections were labeled with a small DiI injection close to the optic nerve head. As expected, we found that RGC axons from both wild-type and *trkB*^{-/-} animals have entered the SC by that time (Fig. 3A), and no obvious differences between the two genotypes were observed. The slightly stronger staining in the *trkB*^{-/-} SC was due to a larger area of retina covered by the label. Postnatal day 6 projections were identified by DiI injections into the nasal or temporal retina to identify posterior and anterior projections, respectively. Here we show an example of P6 *trkB*^{-/-} nasal retinal ganglion cell axons projecting to the posterior SC (Fig. 3C) and initiating branching (arrows in Fig. 3D). This pattern was not detectably different from projection patterns seen in WT littermates (data not shown). We did not investigate possible minor differences in branching pattern between the two genotypes, but the overall projection pattern were very similar.

Cell Survival in the RGC Layer in *trkB*^{-/-} Mice

Cells in the RGC layer were examined in radial semithin plastic sections stained with toluidine blue (Fig. 4A). As all of the sections were taken from the central retina, and numbers of cells in the RGC layer are very constant between animals, a loss of RGCs should be readily detectable. Numbers of cells in the retinal ganglion cell layer were counted in wild-type ($n = 11$), heterozygous ($n = 5$), and homozygous ($n = 6$) littermates between P10 and P16. As the period of cell death is over by P6 (52) and cells are not generated in the RGC layer after E18, we pooled the numbers for each genotype. Interestingly, no significant difference in cell numbers could be detected between the three groups. As shown in Fig. 5A, all three had about 330 retinal ganglion cells per circumference (WT, 339.5 ± 6.3 ; Het, 325.6 ± 20.9 ; KO, 323.5 ± 13.2 ; ns). In addition, no increase in pyknotic profiles in the RGC layers of any of the mutant retinas was detected (data not shown).

RGCs make up only about 40% of all the cells in the RGC layer. The other 60% are displaced amacrine cells (26). To examine the possibility of cellular compensation in the RGC layer, we examined P16 *trkB*^{-/-} retina with antibodies specific for RGCs (Fig. 4B) and displaced amacrine cells (Figs. 4C and 4D). Brn3A is a transcription factor that labels the

majority of RGC nuclei (25); while in the RGC layer, calbindin and parvalbumin label predominantly displaced amacrine cells and a few RGCs (23). With each of the antibodies, we could not detect a significant reduction in number of stained cells in the RGC layer of the *trkB*^{-/-} retinas, consistent with our cell counts.

Number of RGC Axons Is Not Reduced by Loss of TrkB

Although cell counts and immunohistochemical analyses did not reveal any apparent differences in numbers of immunopositive cells in the RGC layer, structural changes could have gone unnoticed. We therefore examined the optic nerves by electron microscopy from P14 WT and *trkB*^{-/-} animals (three animals per genotype). Numbers of myelinated and unmyelinated axons were compared and axon areas measured (Fig. 5B).

Counts of axons in the optic nerve (Fig. 5C) confirmed that there is no significant difference in the total number of retinal ganglion cells between the wild-type and the *trkB*^{-/-} animals ($66,100 \pm 11,400$ versus $70,800 \pm 16,800$, ns). These numbers are comparable with data collected by Williams *et al.* (48), who reported numbers between $50,800 \pm 1100$ and $75,800 \pm 2200$, depending on the mouse strain analyzed. There is, however, a difference in the number and percentage of myelinated axons ($18,252 \pm 1876$ versus 5598 ± 1750 , $P < 0.001$). Compared to wild-type, the *trkB*^{-/-} optic nerve contains approximately 70% fewer myelinated axons. The average area of an individual myelinated axon was not affected by the knockout (0.54 ± 0.061 versus $0.50 \pm 0.072 \mu\text{m}^2$, ns), while the average area of an unmyelinated axon was slightly reduced (0.16 ± 0.010 versus $0.11 \pm 0.003 \mu\text{m}^2$, $P < 0.003$, Fig. 5D). Similar results were obtained by Cellierino *et al.* (8), in the BDNF knockout mouse where they reported a 50% reduction in number of myelinated RGC and CNS axons without an effect on myelination of peripheral nerves. These authors, therefore, argued that the malnutrition or reduced body weight could not explain the hypomyelination. We have not examined myelination in CNS versus PNS neurons in the *trkB*^{-/-} mouse, but expect that it is similar to that found in the BDNF^{-/-} mutant.

TrkB Activation Appears to Slow Adult RGC Degeneration

Results presented above demonstrate that TrkB expression does not regulate the survival of immature RGCs at the time of target innervation. However, the life span of *trkB*^{-/-} animals is limited to a few weeks. To determine whether TrkB modulates the later loss of RGCs observed in aging adult animals, we used a second line of *trkB* transgenic animals in which a *trkB* cDNA followed by an SV40 poly(A) sequence was inserted into the first coding exon immediately following the ATG. Animals homozygous for this allele do not express truncated isoforms of TrkB and express only about 25% of the normal amount of the kinase-containing isoform of TrkB. The pattern of TrkB expression appears identical to that of TrkB in wild-type animals, which is not surprising since only a few hundred bases of exon DNA were deleted from the genome during construction of this allele (49).

The rodent retina is physiologically mature by P50 (21), and adult RGCs appear to decline by approximately 30% by 27 months of age (47). At 3 months of age, numbers of RGC in the wild-type retina were not significantly lower than at P14 (compare Fig. 5A to Fig. 6A). In animals expressing low amounts of *trkB*, the number of cells in the RGC layer was

reduced by 20% at 3 months of age (Fig. 6A). The RGC layer of wild-type animals contained 323.3 ± 16.2 cells per circumference ($n = 6$), in comparison to 267.1 ± 24.1 cells per circumference in the RGC layer of $\text{trkB}^{\text{fbz/fbz}}$ ($n = 6$; $P < 0.001$). Observations consistent with these counts were obtained by electron microscope analysis of sections of P90 optic nerves (Fig. 6B). To confirm a 20% reduction in absolute numbers using electron microscopy is virtually impossible due to the nature of statistical sampling, unless very large numbers of samples are counted. Thus, we confirmed only the overall trend that the number of axons is reduced by approximately 16% ($78,495 \pm 15,212$ versus $66,069 \pm 12,582$; $P < 0.3$, Fig. 6C) with the greatest effect on the number of myelinated axons ($73,329 \pm 15,582$ versus $61,525 \pm 9822$, $P < 0.3$) in comparison to unmyelinated axons (5167 ± 2089 versus 4545 ± 3080 , $P < 0.8$). The average area of myelinated (0.57 ± 0.081 versus $0.51 \pm 0.089 \mu\text{m}^2$, ns) and unmyelinated axons (0.26 ± 0.028 versus $0.28 \pm 0.023 \mu\text{m}^2$, ns) was not affected by the reduction in TrkB expression (Fig. 6D). Even though this dataset does not reach statistical significance, these EM results appear consistent with the results obtained by light microscopy.

DISCUSSION

Although RGCs express a variety of growth factor receptors, experiments have suggested that their postnatal survival is regulated by a ligand of TrkB, BDNF, NT-4, or possibly NT-3 (15, 29, 31, 32). Using X-gal staining and immunohistochemistry, we demonstrated that TrkB is expressed in RGCs and BDNF is expressed in the target areas in the SC as well as in the RGCs. Expression in RGCs and SC potentially allows for both paracrine and autocrine mechanisms of TrkB activation. RGCs of $\text{trkB}^{-/-}$ animals were found to project appropriately to the SC. Contrary to our expectations, increased apoptosis of RGCs was not observed during the target-dependent phase of RGC survival. The absence of TrkB, however, did result in a significant reduction in the extent of axonal myelination. Since these animals are small, and malnutrition has been reported to affect myelination in early postnatal animals (1), it is not certain that this effect is direct. A reduction of 75% in TrkB kinase in the hypomorphic animals, on the other hand, leads to a small loss of approximately 20% of RGCs at 3 months of age without affecting myelination, suggesting that TrkB-mediated signaling may help prevent degeneration of RGCs in the mature retina.

RGCs Do Not Require TrkB Activation for Survival During Target Innervation

It has been shown that the number of RGCs in the $\text{BDNF}^{-/-}$ mouse is not reduced (8). Although these results suggest that TrkB activation is not required for RGC survival, two other ligands for TrkB, NT-3 and NT-4, are also present in the mouse visual system. NT-3 appears to be present in some cells in the RGC layer (5) and NT-4 has been demonstrated in the mouse retina by reverse transcriptase-PCR (B. Rohrer, unpublished observation). Interestingly, in mice that lack all isoforms of the receptor TrkB, RGCs project to the SC and branch in retinorecipient layers (Fig. 3), and their survival is unaffected at least until P16 (Figs. 4 and 5), the oldest time point studied due to the limited survival-time of the $\text{trkB}^{-/-}$ animals. These findings are interesting with respect to pathfinding and axonal branching, as well as RGC survival and function. TrkB activation was thought to promote both axonal branching (9, 11) and survival (29). We did not investigate other aspects of RGC physiology.

This has been done in the BDNF^{-/-} mice, and it is expected that results in trkB^{-/-} animals will be similar (40). The experiments presented here are consistent with the results obtained in the BDNF^{-/-} mouse, suggesting that immature RGCs do not require TrkB activation by BDNF or its other ligands for their survival. They do not, however, eliminate the possibility that TrkB and BDNF promote RGC survival, but are not essential because of other compensatory survival mechanisms.

TrkB and Age-Related Retinal Ganglion Cell Loss

Age-related structural changes in the rodent retina include a gradual loss of RGCs (7, 38, 47), which is consistent with changes described in the human optic nerve (16). One possible cause for the gradual cell loss is oxidative stress. As the retina requires large amounts of oxygen and is exposed to visible light, it produces reactive oxygen intermediates such as free radicals, hydrogen peroxide, and singlet oxygen as the by-products of oxygen metabolism. The young retina appears to create its own protective environment in response to light by increasing retinal levels of antioxidants and through expression of bFGF and CNTF (28). Additional neuroprotective mechanisms include the retrograde transport of BDNF and NGF (45).

Unfortunately, stress response genes and antioxidants appear to be downregulated in the aging retina and brain (4, 6, 10). In addition, less BDNF and TrkB are present in the aging compared to the young brain (12, 33). Consequently, oxidative stress could cause cellular damage and possibly trigger diseases such as age-related RGC degeneration. Our observation on the elevated loss of RGC in an animal expressing low levels of TrkB receptor (trkB^{fbz/fbz}) suggest that TrkB activation may help maintain cellular antioxidant defenses in the mature retina. Alternatively, the developmental cell loss of RGCs that occur during target innervation may never stop, but rather progress at a slow rate.

Potential Mechanisms of TrkB Action

Which BDNF-responsive neurotrophin receptors are expressed by the RGCs? Here we have demonstrated that RGCs in the mouse retina express TrkB at the time of target innervation (Fig. 2B). Although we were not able to distinguish between the expression patterns of the kinase-containing and the truncated isoforms of TrkB, because the antibody used in this study was raised against the extracellular domain of TrkB, recent data using mRNA probes specific for either the truncated isoforms or the full-length form of trkB have demonstrated that all RGCs express the truncated form of trkB, and approximately 70% of all RGCs also express the full-length form of trkB (44). A similar-sized subset also expresses p75NTR, although no attempt was made to study colocalization between p75NTR and the two TrkB isoforms. Thus all three receptor isoforms are present in RGCs to mediate potentially the neurotrophin effect.

A comparison of different neurotrophin receptor knockout mice could possibly explain the survival effects we have seen in our experiments. During early postnatal development, RGCs in the trkB^{-/-} animals that lack both TrkB.T1/T2 and TrkB kinase do not die at a higher rate than their wild-type counterparts (Figs. 4 and 5), but long-term maintenance of adult RGCs appears to be impaired (Fig. 6). On the other hand, Pollock and co-workers (34) have

reported that in a different *trkB* mutant mouse, in which only the kinase exon has been deleted (27), a sevenfold increase in RGC pyknosis was observed at P6. Unfortunately, no data on RGC numbers are available on the *TrkB.T1*-overexpressing mouse (41). Finally, postnatal survival of RGCs from *p75NTR*^{-/-} animals has not been investigated. The comparison of these sets of results allows for a couple of interpretations: (1) Activation of full-length *TrkB* promotes long-term survival of RGCs and is thus responsible for maintaining the adult neuronal phenotype or (2) an imbalance of full-length *TrkB* and *TrkB.T1/T2* in favor of the truncated isoform may be detrimental during the target-dependent phase of RGC development. *TrkB.T1/T2* activation by excess neurotrophin in the absence of full-length *TrkB* may activate a cell death pathway in a way similar to that in human retinoblastoma tumor cells, in which a favorable tumor prognosis (i.e., the activation of the cell death pathway) depends on increased levels of *TrkB.T1* (2). Alternatively, sequestration of BDNF, NT-4, or NT-3 by *TrkB.T1/T2* may inhibit an alternate, neurotrophin-dependent survival pathway. Taken together, RGCs may survive the lack of full-length *TrkB* stimulation if truncated isoforms of *TrkB* are not present, by relying on other endogenous survival factors such as CNTF (14, 46) and basic fibroblast growth factor (53) or neurotrophins acting through *p75NTR* (51).

CONCLUSION

Studies of naturally occurring cell death have attracted much attention as molecules involved in these early processes could also play a role in degenerative processes or aging. We were interested in investigating further the roles in RGC survival of BDNF, NT-4, and NT-3 acting through their receptor *TrkB*. Although both BDNF and its receptor are expressed during both the period of naturally occurring cell death and beyond, we were unable to establish a survival role for neurotrophin signaling through *TrkB*, but found possible evidence for their involvement in early aging. As RGCs express other receptors for growth factors that can be provided by both target areas and the retina itself, we will further investigate the role of survival factors in additional mouse mutants. It will be interesting to study RGC development in double mutants or conditional mutants to further understand the dependence of RGCs on survival factors. This is particularly important in light of the potential use of growth factors as therapeutic agents for macular degeneration.

Acknowledgments

We thank Baoji Xu for the two *TrkB* lines. This study was supported in part by NIH Research Grants EY11349, EY01919, EY02162, and NS P01-16033; the Howard Hughes Medical Institute; the Foundation Fighting Blindness; and That Man May See, Inc. L.F.R. is an Investigator and B.R. was a Research Associate of the Howard Hughes Medical Institute. M.M.L. is a Research to Prevent Blindness Senior Scientist Investigator. K.R.J. was supported by grants from the Muscular Dystrophy Association and the American Cancer Society.

References

1. Almeida ML, Guedes RC, Hokoç JN, Martinez AM. 2000; Malnutrition effects on the optic nerve of adult and developing rat. *Soc Neurosci Abstr.* 26:2116.
2. Aoyama M, Asai K, Shishikura T, Kawamoto T, Miyachi T, Yokoi T, Togari H, MY, Kato T, Nakagawara A. 2000; There are different expressions of BDNF and its variants between human neuroblastomas with favorable prognoses and with unfavorable prognoses. *Soc Neurosci Abstr.* 26:2120.

3. Baxter GT, Radeke MJ, Kuo RC, Makrides V, Hinkle B, Hoang R, Medina-Selby A, Coit D, Valenzuela P, Feinstein SC. 1997; Signal transduction mediated by the truncated trkB receptor isoforms, trkB.T1 and trkB.T2. *J Neurosci.* 17:2683–2690. [PubMed: 9092589]
4. Beatty S, Koh H, Phil M, Henson D, Boulton M. 2000; The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol.* 45:115–134. [PubMed: 11033038]
5. Bennett JL, Zeiler SR, Jones KR. 1999; Patterned expression of BDNF and NT-3 in the retina and anterior segment of the developing mammalian eye. *Invest Ophthalmol Visual Sci.* 40:2996–3005. [PubMed: 10549663]
6. Bernstein SL, Liu AM, Hansen BC, Somiari RI. 2000; Heat shock cognate-70 gene expression declines during normal aging of the primate retina. *Invest Ophthalmol Visual Sci.* 41:2857–2862. [PubMed: 10967038]
7. Cano J, Machado A, Reinoso-Suarez F. 1986; Morphological changes in the retina of aging rats. *Arch Gerontol Geriatr.* 5:41–50. [PubMed: 3718070]
8. Cellerino A, Carroll P, Thoenen H, Barde YA. 1997; Reduced size of retinal ganglion cell axons and hypomyelination in mice lacking brain-derived neurotrophic factor. *Mol Cell Neurosci.* 9:397–408. [PubMed: 9361277]
9. Choi O, O'Leary DDM. 2000; Potential role for BDNF and TrkB in developing topographic retinotectal projections. *Soc Neurosci Abstr.* 26:291.
10. Christen Y. 2000; Oxidative stress and Alzheimer disease. *Am J Clin Nutr.* 71:621S–629S. [PubMed: 10681270]
11. Cohen-Cory S, Fraser SE. 1995; Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. *Nature.* 378:192–196. [PubMed: 7477323]
12. Croll SD, Ip NY, Lindsay RM. 1998; Decreased expression of BDNF and trkB as a function of age and cognitive performance. *Brain Res.* 812:200–208. [PubMed: 9813325]
13. Cui Q, Harvey AR. 1995; At least two mechanisms are involved in the death of retinal ganglion cells following target ablation in neonatal rats. *J Neurosci.* 15:8143–8155. [PubMed: 8613749]
14. Cui Q, Lu Q, So KF, Yip HK. 1999; CNTF, not other trophic factors, promotes axonal regeneration of axotomized retinal ganglion cells in adult hamsters. *Invest Ophthalmol Visual Sci.* 40:760–766. [PubMed: 10067981]
15. Di Polo A, Aigner LJ, Dunn RJ, Bray GM, Aguayo AJ. 1998; Prolonged delivery of brain-derived neurotrophic factor by adenovirus-infected Müller cells temporarily rescues injured retinal ganglion cells. *Proc Natl Acad Sci USA.* 95:3978–3983. [PubMed: 9520478]
16. Dolman CL, McCormick AQ, Drance SM. 1980; Aging of the optic nerve. *Arch Ophthalmol.* 98:2053–2058. [PubMed: 7436843]
17. Edwards MA V, Caviness SJ, Schneider GE. 1986; Development of cell and fiber lamination in the mouse superior colliculus. *J Comp Neurol.* 248:395–409. [PubMed: 3722463]
18. Edwards MA, Schneider GE, Caviness VSJ. 1986; Development of the crossed retinocollicular projection in the mouse. *J Comp Neurol.* 248:410–421. [PubMed: 3722464]
19. Eide FF, Vining ER, Eide BL, Zang K, Wang XY, Reichardt LF. 1996; Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. *J Neurosci.* 16:3123–3129. [PubMed: 8627351]
20. Frade JM, Barde YA. 1999; Genetic evidence for cell death mediated by nerve growth factor and the neurotrophin receptor p75 in the developing mouse retina and spinal cord. *Development.* 126:683–690. [PubMed: 9895316]
21. Fulton AB, Hansen RM, Findl O. 1995; The development of the rod photoresponse from dark-adapted rats. *Invest Ophthalmol Visual Sci.* 36:1038–1045. [PubMed: 7730013]
22. Hamano K, Kiyama H, Emson PC, Manabe R, Nakauchi M, Tohyama M. 1990; Localization of two calcium binding proteins, calbindin (28 kD) and parvalbumin (12 kD), in the vertebrate retina. *J Comp Neurol.* 302:417–424. [PubMed: 2289978]
23. Haverkamp S, Waessle H. 2000; Immunocytochemical analysis of the mouse retina. *J Comp Neurol.* 424:1–23. [PubMed: 10888735]

24. Huang EJ, Wilkinson GA, Farinas I, Backus C, Zang K, Wong SL, Reichardt LF. 1999; Expression of Trk receptors in the developing mouse trigeminal ganglion: In vivo evidence for NT-3 activation of TrkA and TrkB in addition to TrkC. *Development*. 126:2191–2203. [PubMed: 10207144]
25. Huang EJ, Zang K, Schmidt A, Saulys A, Xiang M, Reichardt LF. 1999; POU domain factor Brn-3a controls the differentiation and survival of trigeminal neurons by regulating Trk receptor expression. *Development*. 126:2869–2882. [PubMed: 10357931]
26. Jeon CJ, Strettoi E, Masland RH. 1998; The major cell populations of the mouse retina. *J Neurosci*. 18:8936–8946. [PubMed: 9786999]
27. Klein R, Smeyne RJ, Wurst W, Long LK, Auerbach BA, Joyner AL, Barbacid M. 1993; Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. *Cell*. 75:113–122. [PubMed: 8402890]
28. Liu C, Peng M, Laties AM, Wen R. 1998; Preconditioning with bright light evokes a protective response against light damage in the rat retina. *J Neurosci*. 18:1337–1344. [PubMed: 9454843]
29. Ma YT, Hsieh T, Forbes ME, Johnson JE, Frost DO. 1998; BDNF injected into the superior colliculus reduces developmental retinal ganglion cell death. *J Neurosci*. 18:2097–2107. [PubMed: 9482796]
30. Mansour-Robaey S, Clarke DB, Wang YC, Bray GM, Aguayo AJ. 1994; Effects of ocular injury and administration of brain-derived neurotrophic factor on survival and regrowth of axotomized retinal ganglion cells. *Proc Natl Acad Sci USA*. 91:1632–1636. [PubMed: 8127857]
31. Mey J, Thanos S. 1993; Intravitreal injections of neurotrophic factors support the survival of axotomized retinal ganglion cells in adult rats in vivo. *Brain Res*. 602:304–317. [PubMed: 8448673]
32. Meyer-Franke A, Kaplan MR, Pfrieger FW, Barres BA. 1995; Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. *Neuron*. 15:805–819. [PubMed: 7576630]
33. Muto Y, Hayashi T, Higashi Y, Endo T, Yamamoto T, Sato K. 1999; Age-related decrease in brain-derived neurotrophic factor gene expression in the brain of the zitter rat with genetic spongiform encephalopathy. *Neurosci Lett*. 271:69–72. [PubMed: 10477104]
34. Pollock GS, Boyd KA, Kerkel KA, Lyles J, Frost DO. 2000; Roles of trkB ligands in developmental retinal ganglion cell death. *Soc Neurosci Abstr*. 30:847.
35. Pollock GS, Vernon E, Forbes ME, Yan Q, Ma YT, Hsieh T, Robichon R, Frost DO, Johnson JE. 2001; Effects of early visual experience and diurnal rhythms on BDNF mRNA and protein levels in the visual system, hippocampus, and cerebellum. *J Neurosci*. 21:3923–3931. [PubMed: 11356880]
36. Quigley HA, McKinnon SJ, Zack DJ, Pease ME, Kerrigan-Baumrind LA, Kerrigan DF, Mitchell RS. 2000; Retrograde axonal transport of BDNF in retinal ganglion cells is blocked by acute IOP elevation in rats. *Invest Ophthalmol Visual Sci*. 41:3460–3466. [PubMed: 11006239]
37. Reichardt, LF, Farinas, I. *Neurotrophic Factors and Their Receptors: Roles in Neuronal Development and Function*. Oxford Univ. Press; New York: 1997.
38. Ricci A, Bronzetti E, Amenta F. 1988; Effect of aging on the nerve fibre population of rat optic nerve. *Gerontology*. 34:231–235. [PubMed: 3220258]
39. Rohrer B, Korenbrot JI, LaVail MM, Reichardt LF, Xu B. 1999; Role of neurotrophin receptor TrkB in the maturation of rod photoreceptors and establishment of synaptic transmission to the inner retina. *J Neurosci*. 19:8919–8930. [PubMed: 10516311]
40. Rothe T, Baehring R, Carroll TH, Grantyn R. 1996; Repetitive firing deficits in brain-derived neurotrophic factor knockout mice. *Soc Neurosci Abstr*. 22:2009.
41. Saarelainen T, Pussinen R, Koponen E, Alhonen L, Wong G, Sirvio J, Castren E. 2000; Transgenic mice overexpressing truncated trkB neurotrophin receptors in neurons have impaired long-term spatial memory but normal hippocampal LTP. *Synapse*. 38:102–104. [PubMed: 10941145]
42. Segal RA, Greenberg ME. 1996; Intracellular signaling pathways activated by neurotrophic factors. *Annu Rev Neurosci*. 19:463–489. [PubMed: 8833451]
43. Simon DK, O'Leary DD. 1991; Relationship of retinotopic ordering of axons in the optic pathway to the formation of visual maps in central targets. *J Comp Neurol*. 307:393–404. [PubMed: 1856329]

44. Suzuki A, Nomura S, Morii E, Fukuda Y, Osaka J. 1998; Localization of mRNAs for trkB isoforms and p75 in rat retinal ganglion cells. *J Neurosci Res.* 54:27–37. [PubMed: 9778147]
45. von Bartheld CS. 1998; Neurotrophins in the developing and regenerating visual system. *Histol Histopathol.* 13:437–459. [PubMed: 9589902]
46. Weise J, Isenmann S, Klocker N, Kugler S, Hirsch S, Gravel C, Bahr M. 2000; Adenovirus-mediated expression of ciliary neurotrophic factor (CNTF) rescues axotomized rat retinal ganglion cells but does not support axonal regeneration *in vivo*. *Neurobiol Dis.* 7:212–223. [PubMed: 10860786]
47. Weisse I, Loosen H, Peil H. 1990; Age-related retinal changes—Comparison between albino and pigmented rats. *Lens Eye Toxic Res.* 7:717–739. [PubMed: 2100190]
48. Williams RW, Strom RC, Goldowitz D. 1998; Natural variation in neuron number in mice is linked to a major quantitative trait locus on Chr 11. *J Neurosci.* 18:138–146. [PubMed: 9412494]
49. Xu B, Gottschalk W, Chow A, Wilson RI, Schnell E, Zang K, Wang D, Nicoll RA, Lu B, Reichardt LF. 2000; The role of brain-derived neurotrophic factor receptors in the mature hippocampus: Modulation of long-term potentiation through a presynaptic mechanism involving trkB. *J Neurosci.* 20:6888–6897. [PubMed: 10995833]
50. Xu B, Zang K, Ruff NL, Zhang YA, McConnell SK, Stryker MP, Reichardt LF. 2000; Cortical degeneration in the absence of neurotrophin signaling: Dendritic retraction and neuronal loss after removal of the receptor TrkB. *Neuron.* 26:233–245. [PubMed: 10798407]
51. Yamashita T, Tucker KL, Barde YA. 1999; Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. *Neuron.* 24:585–593. [PubMed: 10595511]
52. Young RW. 1984; Cell death during differentiation of the retina in the mouse. *J Comp Neurol.* 229:362–373. [PubMed: 6501608]
53. Zhang C, Takahashi K, Lam TT, Tso MO. 1994; Effects of basic fibroblast growth factor in retinal ischemia. *Invest Ophthalmol Visual Sci.* 35:3163–3168. [PubMed: 8045711]

FIG. 1.

BDNF localization using heterozygous $BDNF^{lacZ}$ mice. X-gal staining was used to identify BDNF localization in superior colliculus (SC) and retina. (A) At P2, BDNF is expressed in the upper half of the SC that will differentiate into retinorecipient layers, as well as in deeper, corticorecipient layers. (B) BDNF is also expressed in retinal ganglion cells and secretory cells overlying the ciliary body. Abbreviations: CB, ciliary body; D, dorsal; RGC, retinal ganglion cells; V, ventral. Scale bars, 100 μ m.

FIG. 2.

BDNF and TrkB colocalization in RGCs. Paraffin sections of the retina of a P10 mouse were stained, using antibodies against (A) lacZ and (B) TrkB. The antibody against lacZ labels retinal ganglion cells, whereas the TrkB antibody labels retinal ganglion cells and blood vessels (arrow), with the receptor being localized predominantly to the plasma membrane of RGCs. The digital images were superimposed (C), demonstrating the coexpression in the same cells. Please note that the labeling of the basement membrane (arrowhead) is due to nonspecific labeling of the Texas red secondary antibody. Scale bar, 20 μm .

FIG. 3.

Retinocollicular projections in wild-type and $\text{trkB}^{-/-}$ mice. Retinocollicular projections were identified using anterograde transport of DiI *in vivo*. (A and B) The ingrowth of the RGC axons at P0 in (A) the wild-type and that in (B) the $\text{trkB}^{-/-}$ animals are comparable. Please note that the difference in numbers of axons labeled in the $\text{trkB}^{-/-}$ animal is due to the slightly larger bolus of DiI being delivered to the retina. In addition, by P6 (C and D) $\text{trkB}^{-/-}$ nasal RGC axons were found to project to the posterior SC where they initiate branching (D, arrows), just like their wild-type counterparts (data not shown). D is an enlarged area of the posterior SC identified by arrowheads in C. Scale bars, (A, C) 100 μm , (B) 200 μm , (D) 50 μm .

FIG. 4.

Cell survival in the RGC layer is not affected by the loss of TrkB. (A) One-micrometer plastic retina sections were counterstained with toluidine blue and quantitatively analyzed for numbers of cells in the RGC layer. Cell profiles appear to be similar in number between the two mouse genotypes at P16 (wild type, left column; knockout, right column). For absolute numbers please refer to Fig. 5A and the text. To distinguish between RGCs and displaced amacrine cells, 7- μ m paraffin sections were stained with (B) the nuclear marker Brn3A and with cytoplasmic markers (C) calbindin and (D) parvalbumin. No significant loss of cellular staining in the retinal ganglion cell layer was apparent. Please note that calbindin and parvalbumin also label amacrine cells in the INL in a mirror image fashion, which was not investigated in this report, but has been published elsewhere (39). Abbreviations: BV, blood vessel; IPL, inner plexiform layer; RL, retinal ganglion cell layer. Scale bar, 20 μ m.

FIG. 5.

RGC survival is not affected by the loss of *trkB*. (A) Cell profiles were counted in the RGC layer of P10–16 wild-type ($n = 11$) and *trkB*^{-/-} ($n = 6$) mice in 1- μm plastic sections stained with toluidine blue. Each radial section contained approximately 330 cells, regardless of the genotype. (B) Analysis of EM sections ($\times 2500$) demonstrated that the *trkB*^{-/-} optic nerve at P14 has fewer myelinated axons (right) than its wild-type littermate (left). (C) The numbers of RGC axons are not significantly different between the two genotypes by P14 (3 mice each); but the number of myelinated axons (light gray) is significantly reduced in the *trkB*^{-/-} optic nerve. (D) Whereas the average area of the myelinated axons (light gray) is not significantly affected in the *trkB*^{-/-} optic nerve, the average area of the unmyelinated axons (dark gray) appears to be significantly reduced. For statistics, refer to Results in the text.

FIG. 6.

Age-related RGC degeneration in *trkB^{fbz/fbz}* young adult mice. (A) Numbers of cells in the RGC layer (counted as in Fig. 5A) were significantly reduced by 20% in the 3-month-old hypomorphic animal. (B) EM sections ($\times 2500$) did not reveal any obvious morphological difference between the two genotypes (left, wild type; right, *trkB^{fbz/fbz}*). (C) By P90, the majority of axons in the optic nerve of both genotypes are myelinated, although as in the cell count, the mutant animals have approximately 16% fewer RGC axons. (D) The areas of both myelinated (light gray) and unmyelinated (dark gray) axons are indistinguishable between the two genotypes. For statistics, refer to Results in the text.