

In Glaucoma the Upregulated Truncated TrkC.T1 Receptor Isoform in Glia Causes Increased TNF- α Production, Leading to Retinal Ganglion Cell Death

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PURPOSE. Glaucoma is a distinct neuropathy characterized by the chronic and progressive death of retinal ganglion cells (RGCs). The etiology of RGC death remains unknown. Risk factors for glaucomatous RGC death are elevated intraocular pressure and glial production of tumor necrosis factor- α (TNF- α). Previously, the authors showed that glaucoma causes a rapid upregulation of a neurotrophin receptor truncated isoform lacking the kinase domain, TrkC.T1, in retina. Here they examined the biological role of TrkC.T1 during glaucoma progression.

METHODS. Rat and mouse models of chronic ocular hypertension were used. Immunofluorescence Western blot analysis and in situ mRNA hybridization were used to identify cells upregulating TrkC.T1. A genetic model of engineered mice lacking TrkC.T1 (TrkC.T1^{-/-}) was used to validate a role for this receptor in glaucoma. Pharmacologic studies were conducted to evaluate intravitreal delivery of agonists or antagonists of TrkC.T1, compared with controls, during glaucoma. Surviving RGCs were quantified by retrograde-labeling techniques. Production of neurotoxic TNF- α and α 2 macroglobulin were quantified.

RESULTS. TrkC.T1 was upregulated in retinal glia, with a pattern similar to that of TNF- α . TrkC.T1^{-/-} mice had normal retinas.

However, during experimental glaucoma, TrkC.T1^{-/-} mice had lower rates of RGC death and produced less TNF- α than wild-type littermates. In rats with glaucoma, the pharmacologic use of TrkC antagonists delayed RGC death and reduced the production of retinal TNF- α .

CONCLUSIONS. TrkC.T1 is implicated in glaucomatous RGC death through the control of glial TNF- α production. Overall, the data point to a paracrine mechanism whereby elevated intraocular pressure upregulated glial TrkC.T1 expression in glia; TrkC.T1 controlled glial TNF- α production, and TNF- α caused RGC death. (*Invest Ophthalmol Vis Sci.* 2010;51:6639–6651) DOI:10.1167/iovs.10-5431

Neurotrophin (NT)-3, one of the members of the neurotrophin family, regulates multiple events in the development and maturation of the peripheral nervous system (PNS) and the central nervous system (CNS). TrkC, the main receptor for NT-3 is expressed in the PNS, CNS, and other tissues.¹ Full-length TrkC (TrkC.FL) is a approximately 150-kDa type 1 receptor tyrosine kinase protein that relays trophic signals.

By alternative splicing, the trkC locus can generate truncated receptor isoforms such as TrkC.T1, which lacks the kinase domain and has a unique short intracellular domain. Overexpression of TrkC.T1 causes defects in the nervous system.² Neurodegeneration can ensue because TrkC.T1 acts as a dominant-negative receptor of TrkC.FL or because TrkC.T1 sequesters NT-3.^{3,4} These mechanisms are indirect and do not require TrkC.T1 to signal.

However, we recently showed that truncated Trk receptors can signal in a ligand-dependent manner, leading to the activation of Rac1 GTPase, the ruffling of the plasma membrane, and the formation of cellular protrusions.^{5–7} To further study the biological function of TrkC.T1 in vivo, we took advantage of the observation that the TrkC.T1 isoform is significantly upregulated during the early phase of glaucoma. TrkC.T1 upregulation was selective for glaucoma; it was not seen in optic nerve axotomy.⁸

We sought to determine whether TrkC.T1 was relevant to neurodegeneration in glaucoma. Glaucoma is a group of optic nerve neuropathies characterized by the chronic and progressive death of retinal ganglion cells (RGCs). Elevated intraocular pressure (IOP) is a major risk factor.⁹ Although the etiology of RGC death in glaucoma is multifactorial, a key contributor is the production by retinal glia of factors that are neurotoxic to RGCs. Two known neurotoxic factors are tumor necrosis factor- α (TNF- α)^{10–13} and α 2-macroglobulin (α 2m).¹⁴

These factors are secreted by the retinal glia in normal eyes and in glaucomatous eyes. However, the mechanism by which the retinal glia can finely regulate baseline secretion versus upregulated secretion of proteins that cause progressive RGC

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death in a chronic condition such as glaucoma is unknown.¹⁵ Therefore, we explored the mechanisms that regulate the production of neurotoxic factors that cause RGC death in glaucoma.

Here we provide genetic, anatomic, and pharmacologic evidence correlating the glaucoma-induced expression of TrkC.T1 and the production of TNF- α in activated retinal glia or Müller cells leading to RGC death over time. Together, these data suggest a paracrine mechanism whereby high IOP causes early upregulation of TrkC.T1, which in turn regulates TNF- α production, causing glaucomatous RGC death. This work provides new evidence on the relevance of truncated neurotrophin receptors in disease and potentially validates TrkC.T1 as a target for glaucoma therapy.

MATERIALS AND METHODS

All animal procedures were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and adhered to the protocols approved by the McGill University Animal Welfare Committee.

Animals

Mice (male and female C57BL/6) and Wistar rats (female, 250 to 300 g; Charles River Laboratories, Wilmington, MA) were kept in a 12-hour light/12-hour dark cycle and were provided food and water ad libitum. All animal manipulations were performed between 9 am and 12 am.

Targeting Vectors, Electroporation, and Selection

For targeting of the TrkC.T1-specific exons, a replacement-type targeting vector was constructed by microhomologous recombination in yeast^{16,17} using a 129/SV mouse genomic fragment. Electroporation

and selection were performed using the CJ7 embryonic stem (ES) cell line, as described elsewhere.¹⁸ DNA derived from G418/FIAU-resistant ES clones was screened using a diagnostic *Scal* restriction enzyme digestion using 5' and 3' probes external to the targeting vector sequence (not shown). Recombinant clones containing the predicted rearranged band were obtained at a frequency of 1/300. After germline transmission of the targeted ES cell clone, diagnostic *Bgl*II restriction enzyme digestion using the internal probe indicated in Figure 1 was used for screening because it allowed us to distinguish between the wild-type and all the targeted alleles, including those generated by Cre recombination. A targeted ES cell clone injected into C57Bl/6 blastocysts generated chimeras that transmitted the mutated allele to the progeny.¹⁹

TrkC.T1 Mice Genotypic Screening

After confirmation of germline transmission by Southern blot analysis, we devised a PCR-based method for subsequent screening of the mutant animals. PCR conditions were as follows: TrkC.T1 primer 1, 5'-GAC ATA GAG CCT TCC TGA CCC-3'; primer 2, 5'-CCA TCA CCA TAA ATT CTG CCC-3'; primer 3, 5'-CCC TTC AGG AAC CCT GTA CCT A-3'. PCR reaction conditions were as follows: primers 1, 2, 3 (100 ng/ μ L), each 0.25 μ L, 6 μ L H₂O, and 7 μ L 2 \times master mix (SYBR Green; Promega, Madison, WI) and 0.25 μ L diluted tail DNA. PCR conditions were performed as follows: 35 cycles at 96°C for 2 minutes, 94°C for 30 minutes, 58°C for 30 minutes; 35 cycles at 72°C for 40 minutes, 72°C for 7 minutes.

Anesthesia

Deep anesthesia was used for rat and mice undergoing cauterization (glaucoma), fluorogold labeling, intraocular injection procedures, and euthanization (ketamine, xylazine, and acepromazine injected intraperitoneally: 50/5/1 mg/kg, in accordance with IACUC recommendations). For measuring IOP, light anesthesia was used (mixture of oxy-

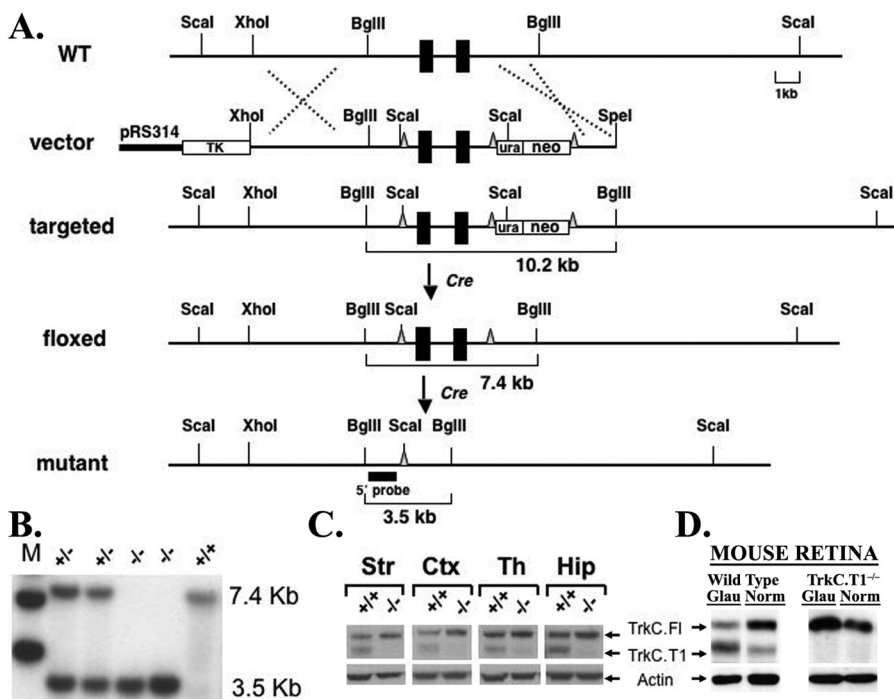


FIGURE 1. Deletion of TrkC.T1 in mouse and expression of TrkC isoforms. (A) Schematic representation of the strategy used to target the exons encoding the TrkC.T1-specific isoform. A targeting vector for conditional removal of the two exons of interest (approximately 2.5 kb) was generated using 6 kb of the upstream DNA sequence and 1.5 kb of the downstream DNA sequence. A fusion URA neo cassette was used for the positive selection, and a HSV-TK, thymidine kinase cassette was used for the negative selection. The 5' probe was used to detect an endogenous *Bgl*II wild-type (WT) band of 7.4 kb that was of similar size in the floxed allele and the LoxP sites. After Cre-induced recombination to excise the TrkC.T1-specific exons, the *Bgl*II band was reduced to 3.5 kb. WT, wild-type allele; TK, HSV-TK, thymidine kinase cassette. *Triangles*: LoxP sites; the restriction endonucleases used for DNA digestion *Scal*, *Xho*I, and *Bgl*II are indicated. (B) Southern blot analysis of *Bgl*II-digested tail DNA derived from a litter obtained from the intercross of two mutant heterozygous mice. Note

the 7.4-kb band derived from the wild-type allele and the 3.5-kb mutant band. (C) Western blot analysis of striatum, cortex, thalamus, and hippocampus dissected from mutant (-/-) or wild-type (+/+) and hybridized with an antiserum directed against the juxtamembrane domain of the trkC receptor (serum 656). Blots were hybridized with an anti-actin-specific antibody to control for loading. (D) Western blot analysis of whole retinas dissected from mutant or wild-type mice (normal eyes or day 14 glaucomatous eyes), with an antiserum directed against the juxtamembrane domain of the trkC receptor (serum 656). An anti-actin-specific antibody was used to control for loading. None of the specific bands of the expected MW were seen when the primary serum was replaced with control normal rabbit serum (data not shown).

gen and 2% isoflurane at a rate of 2.5 L/min, in accordance with IACUC recommendations).

Glaucoma Model

The episcleral vein cauterization (EVC) model of rat and mice glaucoma^{20,21} has been validated in comparative studies.^{22,23} We have described this model.^{8,14,24} Radial incisions were made in the conjunctiva, and three of the episcleral veins were cauterized with a 30 cautery tip. Contralateral control eyes underwent sham surgery to isolate the three veins but without cauterization. Approximately 90% of the cauterized eyes experienced sustained elevations of IOP, irrespective of TrkC.T1 genotype. We euthanized the animals (and censored the data) in the rare instances in which the retinal vasculature showed signs of ischemia and when an average IOP of <1.4-fold (too low) or >2.8-fold (too high) in the cauterized eyes occurred at any point during the study.

Intraocular Pressure

IOP was measured with an applanation tonometer (Tono-Pen XL; Reichert, Depew, NY)^{25,26} immediately after the EVC surgery and every week until the end point of each experiment. Four consecutive readings were obtained from each eye with a coefficient of variation <5%, and the average number was taken as the IOP for the day according to criteria in the manufacturer's manual. In this animal model of glaucoma, we achieved significantly elevated IOP of approximately 1.6-fold (mice) and approximately 1.7-fold (rat) over normal in more than 90% of the animals. The increase in IOP is chronic. In mice, normal IOP under light anesthesia was 8 to 12 mm Hg. In the EVC glaucoma model, approximately 1.6-fold elevated IOP was maintained for 5 weeks. The mean normal IOP of rats under light anesthesia was 10 to 14 mm Hg, and significantly elevated IOP was maintained for as long as 4 months. To avoid excessive manipulation of the animals and the eyes, day-to-day and diurnal-nocturnal fluctuations of IOP were not recorded.

Intravitreal Injections

A 30-gauge needle was used in intravitreal injections, as we reported previously.^{14,24} The entire procedure was finished in 2 minutes. After injection, the needle was left in place for another minute to allow dispersion of the compound into the vitreous. Our previous publications^{8,14,24} showed that normal contralateral eyes were not different from each other when they remained uninjected or received control PBS injections. Experimental eyes were injected with test agents or control vehicle, and normal contralateral eyes served as naive controls.

Drug Regimen

All intravitreal injections delivered 2 μ L, which contained 2 μ g of the agents. Drug treatments were performed with the experimenters masked to the treatment code. In all cases the dilution medium vehicle was PBS, and it was used as control.

Intravitreal injections in rat eyes with glaucoma were made at days 14 and 21 of glaucoma; the end point for measuring RGC survival was at day 42 (e.g., 21 days after the last injection). Thus, in this paradigm, there was preexisting damage for 14 days before treatment, which we have reported at approximately 8% RGC death.^{8,14,24}

When drugs were injected in normal eyes, fluorogold-labeled RGCs were counted after 14 or 21 days. These time points corresponded to the days of drug exposure in the glaucoma paradigm (in glaucoma, eyes were treated at days 14 and 21 of glaucoma, with the end point at day 42 of glaucoma (21 days after treatment).

Drugs

As the TrkC-selective agonist, we used anti-TrkC mAb 2B7, an anti-TrkC ectodomain antibody with agonistic properties for TrkC.²⁷ In biochemical and biological assays, mAb 2B7 induced the same trophic signals as

NT-3 but did not bind to the p75^{NTR} coreceptor, whereas NT-3 did bind to p75^{NTR}.

As TrkC-selective antagonists, we used the small molecule peptidomimetics KB1413 and KB1368, related to the compounds reported.^{28,29} These antagonists block ligand-dependent TrkC activity and the ligand-independent baseline activity of TrkC that is overexpressed in cell lines.

Fluorogold Retrograde Labeling

RGCs were retrogradely labeled with a 4% fluorogold solution (Fluorochrome, Englewood, CO) applied bilaterally to the superior colliculus, with minor modifications from our previous reports.^{14,24} In rats, retrograde labeling was performed at day 35 after ocular hypertension (e.g., 7 days before the experimental end point). In mice, retrograde labeling was performed at day 28 after ocular hypertension (7 days before end point). These times afford excellent labeling efficacy and are practical and compatible with experimental procedures, including the long-lived glaucoma model. RGCs were labeled throughout the whole retina, both in glaucomatous eyes and in normal eyes. The RGC numbers reported for rats and mice are consistent with those reported by other groups.^{30,31}

Preparation of Retinas

At the end point of glaucoma, both eyes were enucleated and fixed in 4% paraformaldehyde, and retinas were flat-mounted in the shape of a Maltese cross, vitreous side up, on a glass slide. Pictures were taken with a fluorescence microscope (Carl Zeiss Meditec, Jena, Germany), with 12 pictures per retina at 20 \times magnification, each area encompassing 0.219 mm². For each quadrant there were three pictures for rats (at a radial distance of 1 mm, 2 mm, and 3 mm from the optic nerve) and two pictures for mice (at a radial distance of 1 mm and 2 mm from the optic nerve). Microglia and macrophages, which incorporated fluorogold after the phagocytosis of dying RGCs, were excluded according to their morphology, as previously reported.³²

Quantification of RGC Survival

Quantification of fluorogold-labeled RGCs was performed as reported previously.^{14,24,31} In all cases, manual RGC counting was performed by two independent persons. One was the experimental performer, masked to the drug treatment code, and the other was unrelated to the experiment and was masked to the entire protocol. Separately, automated quantitative counting was conducted with image analysis software (Metamorph; Molecular Devices, Sunnyvale, CA) using the module Count Nuclei to identify cells as unique objects.³³ Selected parameters for retinal ganglion cells were 8 to 15 μ M for the width range. Manual counts and automated counts were generally in accordance, and deviations among them were smaller than 5% per image. Fluorogold retrograde labeling measures RGC retrograde transport. Given that transport deficits precede glaucomatous RGC death,^{26,34} quantification of labeled RGCs is a valid outcome measure of both events. For simplicity, however, we used the terms *RGC survival* and *RGC death*.

Statistical Analysis

Standardization of the percentage of RGC survival in each rat was calculated as the ratio of the experimental versus contralateral normal control (RGC_{experimental}/RGC_{contralateral}; OD/OS). The percentage of RGC survival for each experimental group (untreated, PBS, 2B7, KB1314, KB1368) was averaged \pm SEM (n = number of eyes indicated in each graph and legend). Data analysis was performed using statistical software (Prism 5; GraphPad Software Inc., San Diego, CA). Comparison between the RGC survival rates was made using one-way ANOVA with Dunnett's multiple comparison test; $P \leq 0.05$ was considered statistically significant.

Western Blot Analysis

For Western blot analyses of retinal TrkC and TrkC.T1, tissues were collected from brain (striatum, hippocampus, cortex, cerebellum) and from both retinas (left eye normal, right eye glaucomatous). Results were standardized to loading control and were averaged \pm SEM ($n = 4$ animals per group). Each sample was immediately placed on ice in protein extraction buffer^{14,35} with protease inhibitor cocktail (Sigma). After homogenization and measurement of protein concentration (Bio-Rad, Hercules, CA), 10 μ g each sample was loaded onto an SDS-PAGE gel. After Western transfer, membranes were immunoblotted with rabbit polyclonal antibody 656, which recognizes all TrkC isoforms⁷ at a 1:2000 dilution. Goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Sigma) were used at a 1:8000 dilution. Loading was controlled with antibodies to β -actin (Sigma, St. Louis, MO). For digital quantification, membranes were scanned and analyzed with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

Immunohistochemistry. The eyes of mice or rats were enucleated and immersed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight, then transferred to 30% sucrose at 4°C for 2 to 3 days until the eyes sank to the bottom of the tube. Rat eyes were embedded in paraffin, and sections (5 μ m) were prepared in the histopathology service. Sections were deparaffinized in xylene solution (3 \times , 5 minutes) and then washed with PBS (3 \times , 5 minutes) before immunostaining, as described. Mouse eyes were embedded in OCT (Tissue-Tek; Sakura Finetek, Torrance, CA), and frozen radial cryosections (14 μ m) were placed onto gelatin-coated slides and blocked with 3% BSA in PBS with 1% Triton 100 for 30 minutes at room temperature. Sections were exposed to the primary antibody: 2B7 mouse mAb (0.5 μ g/mL) or 656 antiserum (rabbit 1:1000 dilution). Secondary antibodies were rhodamine conjugated (Jackson Immunochemical Laboratories, Avondale, PA) anti-mouse or anti-rabbit (1:500 dilution) for 1 hour at room temperature. After washing, the sections were coverslipped with medium (Immu-Mount; Shandon, Pittsburgh, PA) and studied by fluorescence microscopy (Zeiss). All immunostaining was performed simultaneously on all sections from normal IOP and high IOP.

In Situ mRNA Hybridization. The digoxigenin (DIG) PCR probe synthesis kit (Roche, Basel, Switzerland) was used with DIG-dUTP to generate DNA labeled with DIG. Labeled TrkC or TNF- α probes were purified. The TrkC probes bind to both TrkC.FL and TrkC.T1 isoforms. The DIG label increased the M_r of the products by 100 to 200 U, indicating efficient labeling. Retinal cryosections (10 μ m) or paraffin-embedded sections (5 μ m) were prepared from normal or day 14 glaucomatous retinas. Sections on coverslips were air dried (5 minutes), and 80 ng DIG-labeled probes in 20 μ L and coverslips were placed over each cryosection. Then the slides were placed on a heat plate at 95°C for 5 minutes to denature DNA; this was followed by cooling the slides for 5 minutes on ice. Slides were further incubated at 42°C overnight in a humidified chamber. After the coverslips were removed, the slides were washed twice for 5 minutes with 2 \times SSC at 20°C and once for 10 minutes with 0.1 \times SSC at 42°C. Negative controls used the same probes without DIG label. Detection was performed with a DIG nucleic acid detection kit (Roche) in accordance with the manufacturer's instructions using nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate. To show the morphology, retinal sections were stained for 10 to 30 minutes in neutral red (5 mg/mL in water, pH 6.8; Roche), followed by washing in PBS and mounting with coverslips. The slides were examined under a microscope, and images were recorded.

TNF- α and α 2-Macroglobulin. For Western blots of retinal TNF- α and α 2m, both retinas (one glaucomatous, one normal) of each animal were studied in independent gels, standardized to loading control. The ratio of the glaucomatous eye versus the normal contralateral eye was calculated, and results were averaged \pm SEM ($n = 4$ animals per group). Sample processing was as described. After SDS-

PAGE and Western transfer, membranes were immunoblotted with rabbit polyclonal antisera against TNF- α (PeproTech, Rocky Hill, NJ) or α 2m (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:3000 dilution. Goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Sigma) were used at a 1:10,000 dilution. Loading was controlled with antibodies to β -actin (Sigma). For digital quantification, membranes were scanned and analyzed using ImageJ software.

Wild-type versus TrkC.T1^{-/-} Mice. The right eyes of wild-type mice ($n = 4$) or TrkC.T1^{-/-} mice ($n = 4$) were cauterized to induce glaucoma for 14 days. The naive left eyes served as internal controls. The mice received no drug treatment, and the comparison for TNF- α and α 2m expression was made between the wild-type group and the TrkC.T1^{-/-} group.

Rat Glaucoma Treated Pharmacologically. The right eyes of rats were cauterized to induce glaucoma. The naive left eyes were normal controls. After 14 days of glaucoma, each right eye received a single intravitreal injection of test agent or control (PBS, TrkC agonist, or antagonist), and each normal left eye received a single intravitreal injection of PBS control. Comparisons were made across treatment groups. Quantification of TNF- α or α 2m expression after treatment was made at different time points (each time point with three or four eyes). Each set of retinas was harvested at day 15 (1 day after intraocular injection), day 17 (3 days after intraocular injection), or day 19 (5 days after intraocular injection).

Animal Groups

In all cases, the right eye of each animal was the experimental eye, and the left eye was the control naive (no increased pressure) and untreated (no injection) or injected with PBS vehicle. IOP of the experimental eyes was increased to induce glaucoma, as indicated. Experimental eyes were uninjected or were injected with PBS vehicle (untreated group) or were injected with the indicated agents (TrkC agonist group, TrkC antagonist group). For the end point of counting RGCs, glaucoma was extended for 5 weeks (mice) or 6 weeks (rats). For the end point of quantifying TNF- α or α 2m expression, glaucoma was extended for 2 weeks (histochemistry or in situ hybridization) or for 2 weeks with or without 3 to 5 days of posttreatment with TrkC antagonists.

RESULTS

Generation of TrkC.T1-Deficient Mice

To investigate the functional significance of TrkC.T1 upregulation after the elevation of IOP, we generated a mouse mutant with a targeted deletion of this receptor isoform.

TrkC.T1 is generated by the alternative splicing use of exons 13b and 14b.³⁶ In the TrkC locus these exons are located between the transmembrane-encoding exons and the kinase domain-encoding exons. Based on a comparison of the murine and human genomic sequences, we introduced the LoxP sites in genomic areas upstream and downstream of exons 13b and 14b that are not conserved between the two species (data not shown). This was done to minimize the risk of disrupting potential regulatory regions that may be required for proper splicing or expression of the *trkC* receptor isoforms (Fig. 1A). After targeting of the TrkC.T1-encoding exons in ES cells and introducing the mutation into the mouse germline, crosses with a line ubiquitously expressing the Cre recombinase generated a complete null mutant line of the TrkC.T1 isoform, as demonstrated by RT-PCR studies of their mRNA (Fig. 1B).

Different brain areas dissected from mutant TrkC.T1 and control mice were subjected to Western blot analysis to verify targeted deletion of the TrkC.T1 isoform and to evaluate expression of the full-length TrkC (TrkC.FL) receptor isoform (Fig. 1C). Expression of TrkC.T1 protein was completely abolished in the knockout mice, whereas TrkC.FL protein showed

an approximately 1.6-fold increase. Thus, in the brains of knockout mice, there appears to be partial compensation by replacement of the nonexpressed truncated isoform for the full-length isoform.

In normal wild-type retinas TrkC.T1 protein levels were very low (Fig. 1D). After 14 days of glaucoma, TrkC.T1 protein levels in retinas of wild-type mice increased 1.8 ± 0.3 -fold compared with retinas without glaucoma ($P \leq 0.05$, considered significant when the data were standardized to actin control). In contrast, the TrkC.FL protein levels showed no significant changes in glaucoma when the data were standardized to actin control (Fig. 1D). These data confirm previous reports that in wild-type rodents it is TrkC.T1 protein that is selectively upregulated in glaucoma.⁸

In TrkC.T1^{-/-} retinas, the TrkC.T1 protein was not detected regardless of the presence of glaucoma. However, in glaucomatous TrkC.T1^{-/-} retinas, TrkC.FL expression was elevated 1.3 ± 0.1 -fold compared with normal TrkC.T1^{-/-} retinas (nonsignificant).

Thus, after 14 days of glaucoma in wild-type mice, significant elevations of TrkC.T1 protein levels but relatively normal TrkC.FL protein levels were observed; in TrkC.T1^{-/-} mice, TrkC.T1 was not observed but TrkC.FL protein levels were relatively normal.

IOP and RGC Numbers Are Indistinguishable in Wild-type and Knockout Mice

The normal IOP of naive eyes (left eye [OS]) was the same irrespective of TrkC.T1 genotype. The cauterized eyes (right eye [OD]) in wild-type ($n = 16$), TrkC.T1[±] heterozygous ($n = 24$), or TrkC.T1^{-/-} homozygous ($n = 20$) mice experienced comparable elevations of IOP over the 5-week term (Fig. 2A). Thus, the absence of TrkC.T1 did not prevent IOP elevation in this glaucoma model.

To investigate whether the lack of TrkC.T1 could affect RGC development, we quantified RGC numbers in knockout (KO) and wild-type mice with normal IOP. Retinas showed no differences in RGC numbers irrespective of TrkC.T1 genotype (Fig. 2B). Even further, similar RGC numbers were quantified for KO and wild-type mice at ages 3, 6, and 12 months (Fig. 2C). Thus, the RGCs of TrkC.T1 KO mice appeared to go through normal developmental proliferation, pruning, differentiation, maturation, and aging.

TrkC(T1)^{-/-} Mice Are Resistant to Glaucomatous RGC Death

We investigated whether the deletion of TrkC.T1 would have an impact on RGCs during glaucoma. Labeled RGCs were counted after 5 weeks of elevated IOP and were compared against those in the contralateral naive eyes.

The wild-type group ($n = 16$) had approximately 64% RGCs labeled (or 36% loss), the heterozygous TrkC.T1[±] group ($n = 23$) had approximately 70% RGCs labeled (or 30% loss), and the homozygous TrkC.T1^{-/-} group ($n = 17$) had approximately 80% RGCs labeled (or 20% loss). The TrkC.T1^{-/-} group exhibited a significant difference from wild-type ($P \leq 0.001$) and from TrkC.T1[±] ($P \leq 0.05$; Fig. 3A).

Therefore, the TrkC.T1^{-/-} mice exhibited relative resistance to glaucomatous RGC death. The difference in RGC loss between wild-type mice with glaucoma (36%) and KO mice with glaucoma (20%) corresponded to approximately half the total RGC damage. Note that this resistance was observed in spite of constant stress because of high IOP over the 5-week period.

The data above were also analyzed by segregating the data according to age. Resistance to glaucomatous RGC death in TrkC.T1^{-/-} mice was independent of age. Young, adult, and aged TrkC.T1^{-/-} mice (3 months, 6 months, and

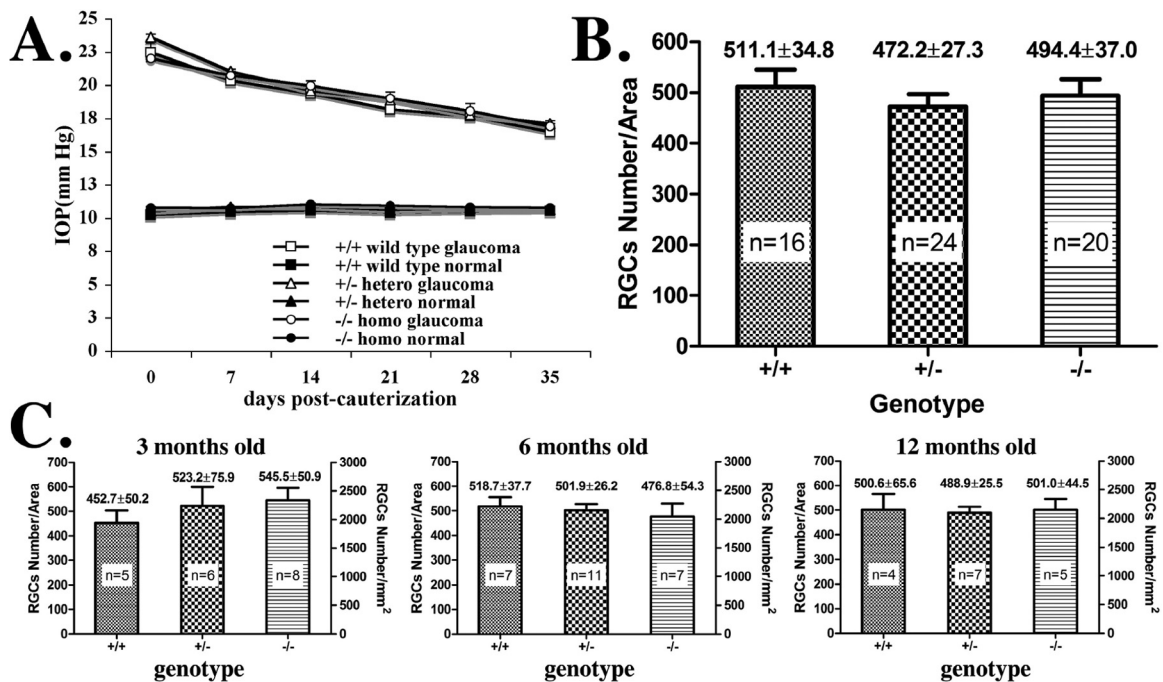


FIGURE 2. Characterization of the retinas of TrkC.T1 KO mice. (A) Sustained increase in IOP. Right eyes (*open symbols*) were cauterized to increase IOP. Left eyes (*filled symbols*) were normal controls. Differences in IOP between the right and left eyes were significantly different at all the times shown ($P < 0.05$). Sustained high IOP was the same in wild-type, heterozygous TrkC.T1 KO, or homozygous TrkC.T1 KO mice. (B) In normal retinas, the number of RGCs was the same regardless of genotype. RGC numbers shown are the average per area counted in 12 areas (0.219 mm^2) and are represented per square millimeter. (C) In normal retinas the number of RGCs was the same regardless of age.

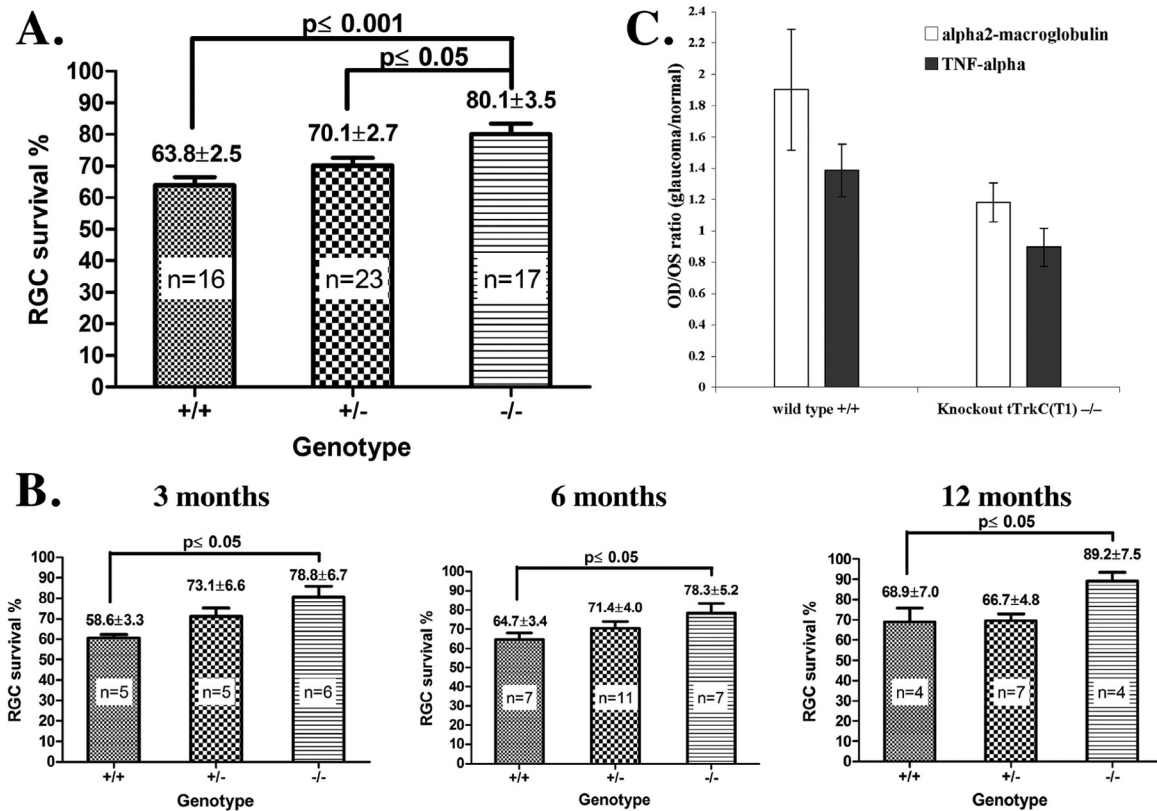


FIGURE 3. During experimental glaucoma, homozygous TrkC.T1 KO mice lost RGCs at a lower rate and expressed less neurotoxic factors. Live RGCs were counted in normal retinas (OS, standardized to 100%) or glaucomatous (OD) retinas after 35 days of constant ocular hypertension. All animals in a group were averaged \pm SEM (n = as indicated). **(A)** Data were pooled by genotype regardless of age. **(B)** Data were broken down by each genotype according to age (3, 6, and 12 months). **(C)** In glaucoma there was elevation of TNF- α and α 2M expression in wild-type mice but not in TrkC.T1^{-/-} mice. The right eyes (OD) of wild-type (n = 4 mice) or TrkC.T1^{-/-} (n = 4 mice) mice were cauterized to induce glaucoma for 14 days. Contralateral eyes (OS) served as internal controls. Expression of TNF- α and α 2M for each eye was adjusted to β -actin levels, and the OD/OS ratio was calculated. Data for each group were averaged \pm SEM (n = 4).

12 months) showed no statistically significant differences in their resistance to glaucomatous RGC death (Fig. 3B).

Multivariate analysis of total IOP over the time period and mouse age indicated that the only explanatory variable was TrkC.T1 genotype. However, the aged group, regardless of genotype, also showed a trend toward more resistance to glaucomatous RGC death than did the young group.

TrkC.T1 Regulates Expression of α 2m and TNF- α in Glaucomatous Retinas

These data suggest that the expression of TrkC.T1 is deleterious for RGCs. It is known that in glaucomatous eyes, TNF- α ^{13,37} and α 2m¹⁴ mediate RGC death. Hence, we studied whether the expression of TrkC.T1 is relevant to the production of these neurotoxic factors.

We quantified α 2m and TNF- α protein in retinal samples of homozygous TrkC.T1^{-/-} mice or control wild-type mice aged 3 to 5 months. Densitometric quantification and analyses of the Western blot data compared protein expression in day 14 glaucomatous eyes with those of contralateral normal eyes (n = 5 mice/group \pm SEM; standardized versus β -actin loading control; Fig. 3C).

In TrkC.T1^{-/-} mice, glaucoma does not significantly elevate α 2m (1.2 ± 0.13 glaucoma/normal eye ratio) or TNF- α (0.9 ± 0.12 glaucoma/normal eye ratio). In contrast, in wild-type mice with glaucoma, α 2m expression was significantly elevated (1.9 ± 0.38 glaucoma/normal ratio) (also reported

elsewhere¹⁴) as was TNF- α expression (1.4 ± 0.17 glaucoma/normal ratio; also reported elsewhere^{13,37}).

These data suggest that the expression of TrkC.T1, which is induced in wild-type mice with experimental glaucoma, may be associated with the efficient upregulation of the neurotoxic factors TNF- α and α 2m. If this is true, the relative resistance of TrkC.T1^{-/-} mice to glaucomatous RGC death may be associated with their inability to upregulate the expression of neurotoxic factors.

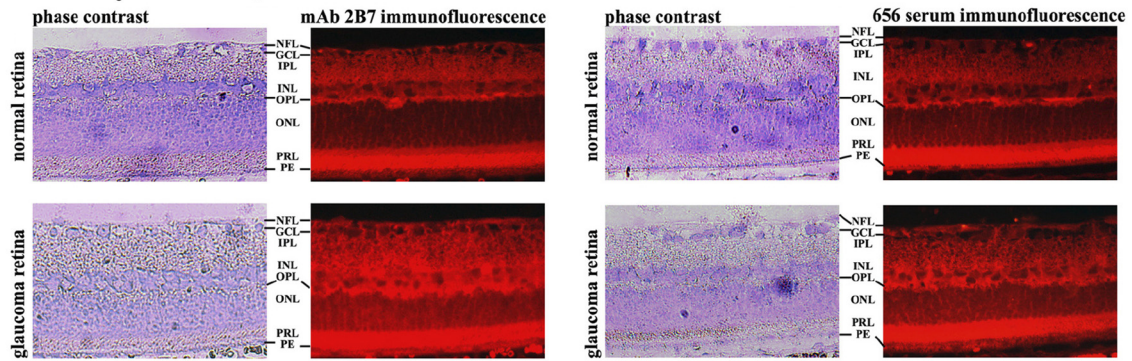
TrkC.T1 Is Upregulated in Glia/Müller Cells

In glaucoma TrkC.T1 is the main receptor isoform to be upregulated⁸ (Fig. 1D). Hence, we studied which cell populations in the retina upregulate TrkC.T1. Immunohistochemistry with two different anti-TrkC antibodies was performed using antibodies that bind to the ectodomain and detect all the TrkC isoforms, mAb 2B7,²⁷ and rabbit serum 656.⁷

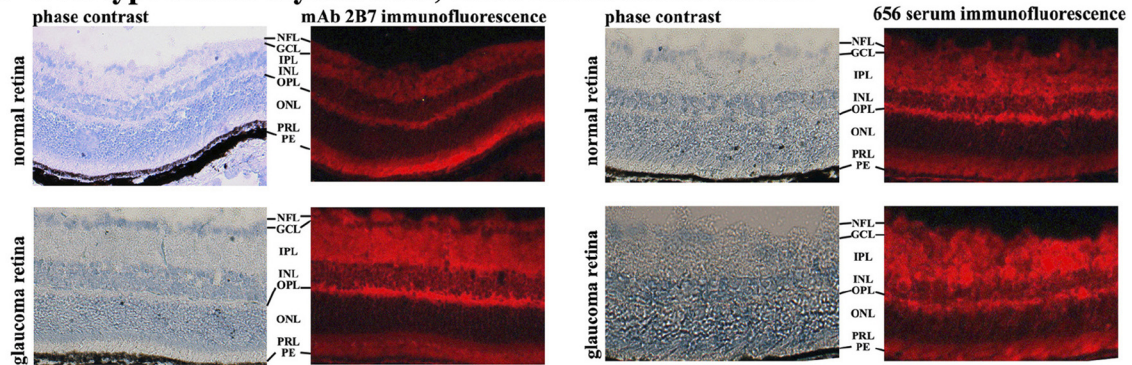
Day 14 retinas of rats and of wild-type mice with or without glaucoma (Figs. 4A, 4B) were immunostained. Higher magnification is shown in Figure 4C. In experimental glaucoma there was a robust increase of TrkC immunoreactivity at the inner nuclear layer (INL) and the inner plexiform layer (IPL) and some increase at the nerve fiber layer (NFL) compared with normal retinas.

There was also strong TrkC immunoreactivity at the photo-receptor layer (PRL), the pigmented epithelium (PE) layer, and some staining at the outer plexiform layer (OPL), but the

A. Rat cryosections, TrkC immunofluorescence



B. Wild type mouse cryosections, TrkC immunofluorescence



C.

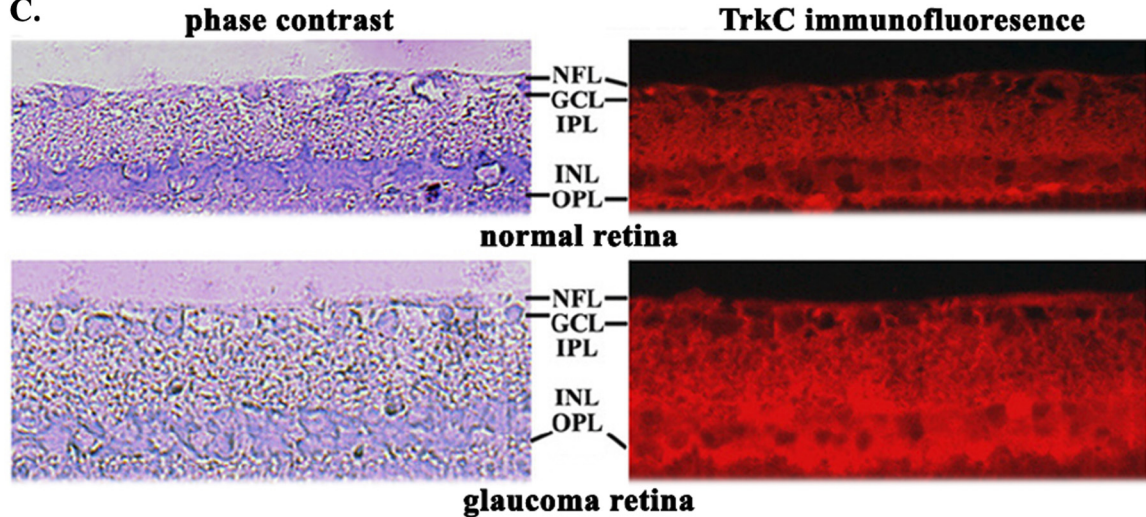


FIGURE 4. Immunolocalization of TrkC in normal and glaucomatous retinas. Right eyes (OD) of rats or mice were cauterized to induce glaucoma for 14 days. Left eyes (OS) were the normal controls. ONL, outer nuclear layer. (A) Rat retinal cryosections (15 μ m) were immunostained with anti-TrkC mAb 2B7 or anti-TrkC rabbit serum 656, followed by rhodamine-conjugated secondary antibody. Corresponding phase-contrast images are shown. No specific immunoreactivity was seen when the primary reagent was replaced with normal mouse IgG (control for mAb 2B7) or normal rabbit serum (control for serum 656) (data not shown). Magnification, 20 \times . (B) Same as in (A), but cryosections from wild-type mice were immunostained. Magnification, 20 \times . (C) 100 \times magnification of images shown in (A).

immunoreactivity in these layers did not change after glaucoma.

Thus, immunoreactivity data indicate that TrkC upregulation during experimental glaucoma takes place largely at the INL (where the cell bodies of glia and Müller cells reside), at the IPL (which contains the Müller cell processes), and at the NFL. Because the NFL contains Müller cell endfeet and astrocytes, it is possible that the immunoreactivity was caused by the TrkC produced in these cells.

Note that the ganglion cell layer (GCL) containing the RGC somata did not exhibit strong TrkC immunoreactivity, either in normal or in glaucoma sections. Specifically, in immunofluorescence, the RGC somata appear as black spheres but are visible in the corresponding phase-contrast images.

In negative controls, there was no significant immunoreactivity when irrelevant primary antibodies (mouse IgG or normal rabbit serum) were used (data not shown). It is also gratifying that the two different anti-TrkC antibodies gave sim-

ilar results, both in mouse retinas and in rat retinas with or without glaucoma.

TrkC.T1 Colocalizes with TNF- α in Glia/Müller Cells

Next, we studied retinal sections from wild-type mice with or without glaucoma and TrkC.T1^{-/-} mice with or without glaucoma by in situ mRNA hybridization using probes for TrkC and for TNF- α . Eight retinas (two wild-type normal IOP, two wild-type day 14 glaucoma, two TrkC.T1 KO normal IOP, two TrkC.T1 KO day 14 glaucoma) were processed. Four sections from each retina were probed for TrkC and for TNF- α . Representative data are shown in Figure 5A. Larger magnifications are shown for sections of wild-type mice studied with TrkC and TNF- α probes (Fig. 5B).

In wild-type mice with glaucoma, there was a robust increase of TrkC mRNA at the IPL and INL, whereas in normal retinas these layers did not express detectable TrkC mRNA. The OPL, PRL, and PE layers had TrkC mRNA, but there were no major differences in these layers between the normal IOP and day 14 glaucoma sections.

Parallel studies with sections from TrkC.T1^{-/-} mice revealed no significant upregulation of TrkC mRNA expression in glaucoma. These data confirm previous reports⁸ that in glaucoma most upregulated TrkC is of the TrkC.T1 isoform.

Overall, the in situ TrkC mRNA hybridization data are consistent with TrkC immunohistochemistry results, with the ex-

ception that in the NFL TrkC protein was present but TrkC mRNA signals were low. This may mean that Müller cells can transport TrkC protein to their endfeet at the NFL. Thus, it appears that TrkC mRNA was upregulated selectively in glia and Müller cells and perhaps in astrocytes.

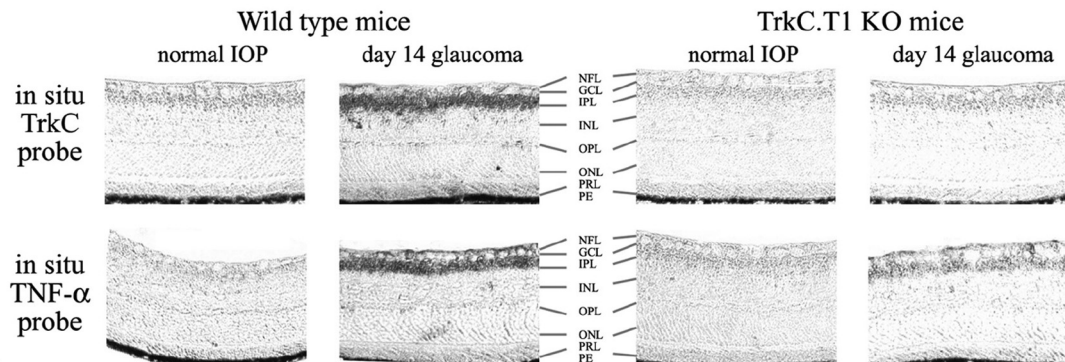
The data also show that in wild-type mice, glia, astrocytes, and Müller cells express TNF- α messaging and that they upregulate TNF- α messaging during glaucoma. These data are consistent with the cells reported to express TNF- α ³⁸ and with the location of TNF- α in human glaucomatous eyes.¹¹ TNF- α was predominantly localized in the inner retinal layers. The most intensely stained layer in the glaucomatous retina was at the NFL and in areas around the GCL and IPL. The morphology of the stained cells suggests that astrocytes (which are located in this layer) may express TNF- α . The TNF- α signals were notably increased in the INL, where the cell bodies of the Müller cells are located.³⁹

Overall, the data demonstrated that in glaucoma there was a temporal and anatomic overlap between the upregulation of TrkC (likely TrkC.T1) and of TNF- α . Upregulation of these proteins took place before significant RGC death (at day 14 of experimental glaucoma, when only approximately 8% of RGCs died).

Glial TrkC.T1 Regulates Production of TNF- α

Next, we tested the hypothesis that TrkC.T1 may be directly implicated in the glial production of TNF- α , leading to subse-

A. WT and TrkC.T1 KO mouse sections, TNF- α and TrkC.T1 in situ hybridization



B. 100X magnification of WT mouse day 14 glaucoma sections

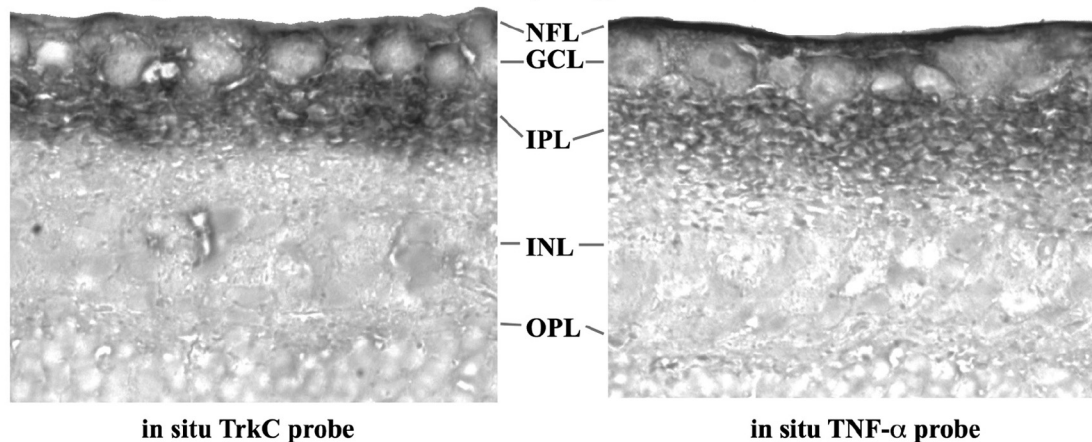


FIGURE 5. Localization of TrkC and TNF- α in normal and glaucomatous retinas. (A) In situ mRNA hybridization with TrkC probes and TNF- α probes in wild-type and TrkC.T1 KO mice with day 14 glaucomatous retinas or normal retinas. Paraffin sections (5 μ m) were studied, and images were taken at 20 \times . (B) In situ mRNA hybridization with TrkC probes and TNF- α probes in wild-type mice after 14 days of glaucoma. Images were taken at 100 \times .

quent RGC death. Because the activity of TrkC.T1 is ligand dependent,⁷ we tested the effects of the pharmacologic antagonists of TrkC we described previously.^{28,29} The TrkC antagonists bound to the TrkC ectodomain and selectively inhibited TrkC-mediated biochemical and biological signals but not TrkA or TrkB signals.

The right eyes of rats with day 14 glaucoma either were untreated or were treated with TrkC antagonists. The corresponding left eyes were untreated normal IOP controls. Three days (e.g., glaucoma day 17) or 5 days (e.g., glaucoma day 19) after treatment with TrkC antagonists, the levels of TNF- α and α 2m were quantified by Western blot analysis. Representative pictures (Fig. 6A) and densitometric quantification (Figs. 6B, 6C) are shown.

As reported, untreated day 17 glaucoma ($n = 4$ analyzed vs. normal contralateral eyes) showed elevated levels of TNF- α (2.7 ± 0.4 -fold) and α 2m (3.3 ± 0.2 -fold) ($P \leq 0.05$, with data standardized to loading control). Three days after treatment of day 14 glaucomatous eyes with the TrkC antagonist KB1413, there was a significant reduction in TNF- α ($P \leq 0.05$ vs. untreated day 17 glaucoma; not significantly different from normal retinas). This decrease brought TNF- α to nearly normal levels (Figs. 6B, 6C). Although less dramatic, treatment of glaucomatous eyes with the TrkC antagonist KB1368 also significantly reduced the expression of TNF- α .

Reduced TNF- α production in retina was sustained and detectable 5 days after treatment. This is remarkable given that the eyes experienced constant stress because of continuous elevated IOP. These data suggest that TrkC.T1 may be selectively involved in the regulation of TNF- α because α 2m expression was reduced only marginally and transiently by the TrkC antagonist KB1413 and was not affected at any time by KB1368.

Together, this evidence indicates that pharmacologic antagonism of TrkC.T1 has a potent and sustained effect at selectively reducing the expression of the neurotoxic factor TNF- α , even in the presence of constant high IOP insult.

TrkC.T1 Regulation of TNF- α Expression Is Deleterious to RGCs

The findings that TrkC antagonists (likely antagonists of TrkC.T1) reduce TNF- α levels in wild-type rats with in glaucoma prompted us to investigate whether TrkC antagonists could afford long-term RGC survival in glaucoma (Fig. 7).

The IOP of experimental eyes was elevated and was sustained throughout the experiment^{8,14,24} (IOP data not shown). The experimental end point was day 42 of glaucoma.

Glaucomatous eyes were treated at days 14 and 21 of glaucoma with TrkC antagonists KB1413 and KB1368 or with vehicle PBS. Labeled RGCs were counted at day 42 of glaucoma and compared with normal untreated contralateral eyes. Without treatment, approximately 73% of the RGCs remained labeled after 42 days of glaucoma ($P \leq 0.0001$ vs. normal contralateral eyes). Intravitreal injection of the TrkC antagonist KB1413 significantly protected RGCs to approximately 88% survival ($P \leq 0.001$ vs. control untreated or PBS-treated glaucoma). Treatment with the TrkC antagonist KB1368 afforded marginal protection, and approximately 81% RGCs remained labeled (not significantly different from the control untreated glaucoma).

Treatment with vehicle PBS did not alter RGC loss (approximately 75% of the RGCs remained labeled). Previously, we showed that control treatments with irrelevant, nonbinding peptidomimetics of similar structure did not alter RGC death in glaucoma. Moreover, we previously showed that treatment with a structurally related peptidomimetic that acted as a TrkA agonist was neuroprotective in this glau-

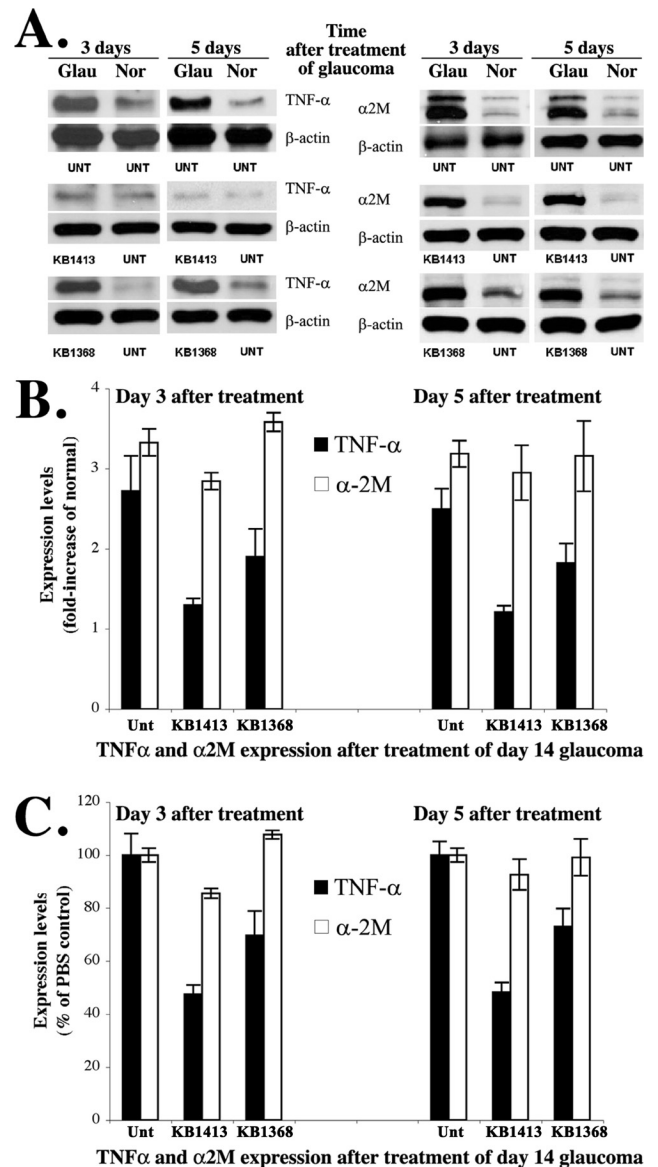


FIGURE 6. TrkC antagonists block the expression of TNF- α during experimental glaucoma. Right eyes of wild-type rats were cauterized to induce glaucoma (Glau). After 14 days, the glaucomatous eye received a single intraocular injection of the test agents or control (PBS, TrkC antagonists). In each rat the contralateral eye served as the normal untreated control (Nor). Retinas ($n = 4$ per group) were processed for Western blot analysis 3 days or 5 days after intraocular injection. (A) Representative Western blots of samples processed at the indicated days. (B) Fold-increase of TNF- α and α 2M expression. The densitometric signal for each eye was adjusted to β -actin, and the ratio of right eye/left eye was calculated. Data for each group were averaged \pm SEM ($n = 4$). The comparison shows that TrkC antagonists cause a significant reduction of the fold-increase in TNF- α levels to levels near that of the normal contralateral control eye. (C) Data are presented by standardizing the expression of TNF- α and α 2M to the PBS control glaucoma group as 100%.

coma paradigm.²⁴ Together, these controls indicated that the pharmacologic treatment was responsible for the neuroprotection of RGCs in glaucoma.

To exclude the possible confounding effects of antagonism of TrkC.FL, assays tested the antagonists in normal eyes. Normal eyes expressed TrkC.FL but had very low TrkC.T1 levels. In these controls, intravitreal injections of the TrkC antagonists KB1413 and KB1368 did not change

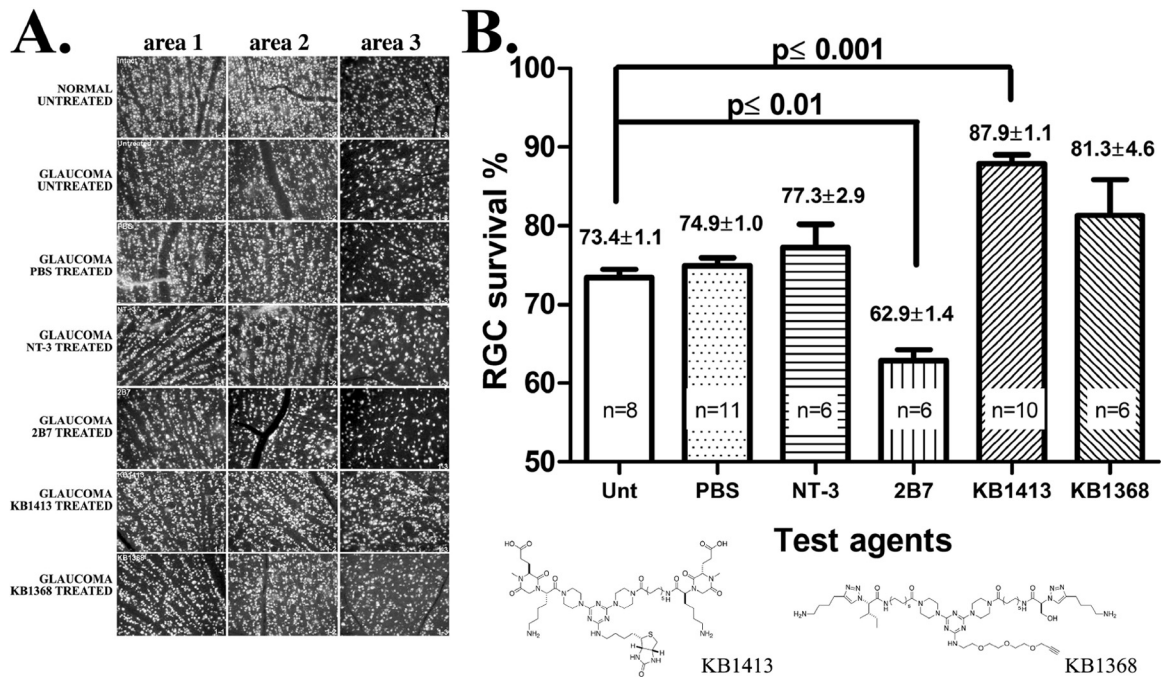


FIGURE 7. Pharmacologic modulation of RGC death by targeting TrkC in glaucoma. Right eyes of rats were cauterized to induce glaucoma. Left eyes were normal controls. At days 14 and 21 of glaucoma, each right eye received a single intraocular injection of test agents or control (PBS, TrkC agonists, or antagonists), and the contralateral eye was untreated. At day 42 of glaucoma, live RGCs were counted. (A) Representative pictures of fluorogold-labeled RGCs. Areas 1, 2, and 3 represent concentric distances from the optic nerve head, each measuring 0.219 mm². (B) RGC survival in day 42 glaucoma. RGC counts in each rat were standardized to the normal eye (100%). Data for a group were the average ± SEM of the indicated number of rats.

the numbers of RGCs counted at days 14 and 21 after injection ($n = 3$ eyes per compound). These time points corresponded to the days of drug exposure in the glaucoma paradigm (in glaucoma, eyes were treated at days 14 and 21 of glaucoma, with the end point day 42 of glaucoma, which was 21 days after treatment). These controls suggest that the biological effects of TrkC antagonists were due primarily to blocking TrkC.T1. These data also suggest that TrkC.FL expressed in normal retinas does not have a major role in RGC health and maintenance.

Together, this evidence indicated that pharmacologic antagonism of TrkC.T1 has a potent and sustained effect at selectively reducing the expression of the neurotoxic factor TNF- α , even in the presence of constant high IOP insult, with a consequent reduction of RGC death seen during glaucomatous stress.

Is TrkC.T1 Regulation of TNF- α Dependent on a TrkC Agonist?

Given that the antagonism of TrkC (likely antagonism of TrkC.T1) afforded significant protection to RGCs in glaucoma and reduced TNF- α , we investigated whether treatment with a TrkC agonist would have the opposite effect.

As a selective TrkC agonist, we used agonistic mAb 2B7, directed to the TrkC ectodomain, which induces NT-3-like biochemical and biological signals by selective activation of TrkC but without binding to the p75^{NTR} receptor.²⁷

Treatment with mAb 2B7 at days 14 and 21 of glaucoma accelerated the loss of RGCs counted at the day 42 end point. Labeled RGCs decreased to approximately 63% (significant vs. untreated glaucoma or PBS-treated glaucoma; $P \leq 0.01$). In contrast to mAb 2B7, control injection of exogenous NT-3 did not alter RGC loss in glaucoma, and approximately 77% RGCs remained labeled ($P \leq 0.0005$ vs. normal contralateral eyes).

This value was not different from that of the untreated glaucoma or the PBS-treated glaucoma group.

Accelerated RGC loss in glaucoma caused by mAb 2B7 was consistent with its ability to upregulate TNF- α levels. Compared with untreated glaucomatous eyes, treatment with mAb 2B7 significantly increased TNF- α levels measured at 24 hours after injection (i.e., day 15 of glaucoma). mAb 2B7 caused an increase of approximately 40% (1.37 ± 0.11 mAb 2B7/PBS TNF- α ratio; $n = 3$). This represented a substantial increase, considering that the eyes already had up-regulated TNF- α levels because of the stress of glaucoma. However, the increase in TNF- α caused by mAb 2B7 was transient. Measurements at 3 days (i.e., glaucoma day 17) or 5 days (i.e., glaucoma day 19) after injection of mAb 2B7 showed that TNF- α levels were the same as in untreated glaucomatous eyes.

Hence, mAb 2B7 seemed to create an early “spike” of TNF- α levels that exacerbated RGC death. Given that mAb 2B7 was injected twice intravitreally (at days 14 and 21 of glaucoma) in the experiments counting surviving RGCs and that counting was done at the day 42 glaucoma end point, these spikes seemed sufficient to accelerate RGC death and were consistent with reports in which injection of TNF- α caused RGC death measured 3 weeks later.^{13,37}

Together our data indicate that ocular hypertension induced the upregulation of TrkC.T1 in glia, which was deleterious to RGCs through a mechanism that involved increased production of TNF- α by glia. It is likely that the activity of TrkC.T1 in glia was ligand dependent. A TrkC agonist can accelerate the production of TNF- α neurotoxicity, resulting in faster RGC death during glaucoma. TrkC antagonists can attenuate the production of TNF- α neurotoxicity, resulting in RGC neuroprotection during glaucoma. Given that glia also express the p75^{NTR}, a receptor that has been implicated in TNF- α neurotoxicity, in future work we

will explore whether there is a functional relationship between TrkC.T1 and p75^{NTR} in glia.

DISCUSSION

In this study, we used rat and mouse ocular hypertension models to study the function of truncated TrkC.T1 in pathologic conditions. Data were obtained using TrkC.T1 knockout mice and pharmacologic agonists and antagonists of TrkC. The results implicated TrkC.T1 in the regulation of neurotoxic factors by retinal glia during experimental glaucoma. The data point to a paracrine or a non-cell autologous mechanism of RGC death.

Retinas of the TrkC.T1 knockout mice had RGC numbers similar to those of wild-type mice. Even the age variable proved to be insignificant, suggesting that TrkC.T1 is not relevant to the normal development and maturation of RGCs. Moreover, TrkC.T1 knockout mice had normal ocular pressure; experimentally we could increase IOP in a manner similar to that in wild-type mice, suggesting that TrkC.T1 is not relevant to regulating IOP.

Despite the similarities with normal wild-type retinas, the glia of TrkC.T1 knockout mice did not elevate the production of TNF- α or α 2m during experimental glaucoma. In addition, the RGCs of TrkC.T1 knockout mice were resistant to glaucomatous death. These data suggest that TrkC.T1 may play a deleterious role for neurons during stress but not in the normal state. In homozygous TrkC.T1^{-/-} mice, glaucomatous RGC death was reduced but not fully prevented. This is not surprising given that RGC death in glaucoma is likely multifactorial.^{40,41} Thus, TrkC.T1^{-/-} mice may be useful for exploring additional factors relevant to RGC death.

We also found that TrkC antagonists protect RGCs from death by selectively inhibiting the upregulation of TNF- α during experimental glaucoma. TNF- α is a neurotoxic factor produced by glia.¹⁰⁻¹³ The reduction of TNF- α expression was sustained for at least 5 days after treatment with TrkC antagonists. This is remarkable because in the experimental paradigm retinal damage preexisted for 14 days and the eyes experienced constant stress due to continuous elevated IOP.

Interestingly, the effect of TrkC antagonists seems to be selective at reducing TNF- α because expression of another neurotoxic factor, α 2m, was unaffected or was reduced only marginally and transiently. Ineffective suppression of α 2m by TrkC antagonists was not surprising because upregulation of α 2m mRNA and protein has been shown to be long-lived in glaucoma and persists even when high IOP is normalized.¹⁴ The cell population in which most of the upregulated production of α 2m takes place is the same cell population in which most of the upregulated production of TNF- α takes place, the activated microglia. The inability to normalize α 2m with TrkC antagonists may explain continuous RGC death even when TNF- α levels are normalized. Again, this is consistent with the view that RGC death in glaucoma is multifactorial.

Lastly, a TrkC agonist accelerated TNF- α production and RGC death. The TrkC agonist-dependent effect was consistent with evidence that TrkC.T1 acts in a ligand-dependent manner.⁷ We postulate that the 2B7 agonist preferentially activates TrkC.T1 in glia because it is the major receptor isoform upregulated in experimental glaucoma.⁸

Accelerated RGC death in glaucoma by mAb 2B7 is not caused by generalized toxicity based on four observations. First, the injection of mAb 2B7 in eyes with normal pressure ($n = 3$) did not result in RGC loss (97% RGCs alive compared with uninjected contralateral eyes) at the end point of 28 days after injection. Second, systemic injection of mAb 2B7 does not cause the degeneration of TrkC-expressing motor

neurons (HUS, manuscript in preparation). Third, control injections with PBS or with nonbinding mouse IgG did not accelerate RGC death (data not shown). Fourth, intravitreal injection of an agonistic mAb directed to TrkB is neuroprotective,³⁵ indicating that mAb binding to a target expressed in the retina does not lead to RGC death.

Evidence in this report indicates that pharmacologic antagonism or genetic manipulation of TrkC.T1 has a potent and sustained effect at selectively reducing the expression of the neurotoxic factor TNF- α in glaucoma, even in the presence of constant high IOP insult. This effect results in a partial rescue of RGCs from death. The evidence is thus consistent with a paracrine or nonautologous mechanism of RGC death. High IOP causes the overexpression of TrkC.T1 in glia early during experimental glaucoma, which in turn regulates TNF- α production in glia and which, in turn, causes RGC death.

The implication of TrkC.T1 in glaucoma suggests that TrkC.T1 may be a therapeutic target. The therapeutic use of TrkC antagonists may be difficult from a regulatory; unfortunately, the enzymes that process TrkC mRNA into the different isoforms are unspecified so they cannot be targeted at this time.

Based on correlative data, it is likely that NF- κ B may be implicated in TrkC.T1 actions leading to TNF- α . A recent study showed that NMDA toxicity activates NF- κ B in glia, causing TNF- α upregulation.³⁰ NMDA toxicity and glaucoma may share the pathway of NF- κ B activation because excitotoxic damage (hyperactive NMDA receptors, elevated glutamate, Ca²⁺, and nitric oxide) account for some RGC death in glaucoma.⁴²⁻⁴⁵ However, it is unlikely that NMDA toxicity shares with glaucoma the TrkC.T1 upregulation reported in the present article. Moreover, TrkC.T1 upregulation was not detected in another ocular injury model, optic nerve axotomy.⁸

In both NMDA toxicity and glaucoma, TNF- α upregulation seemed to account for approximately 50% of the RGC death because the inhibition of TNF- α activity was protective of approximately 50% of the RGCs that otherwise would have died. However, NMDA toxicity is a very different model of retinal damage than glaucoma. The former is rapid and acute, with significant RGC death taking place within 3 hours, whereas the latter is slow, progressive, and chronic, with significant RGC death measurable after weeks.

High IOP is a risk factor for glaucoma. Besides IOP-lowering drugs, there are two proposed approaches for the management of glaucoma and other retinal neurodegenerative conditions. One approach aims at direct neuroprotection by the activation of neurotrophic pathways mediated by the neurotrophic receptors TrkA, TrkB, CNTF, and GDNF.⁴⁶ A second approach aims to reduce neurotoxic injury by inhibiting the action of TNF- α , α 2M, NMDA-R, β -amyloid, p75^{NTR}, and calcineurin.^{14,47-49} From a mechanistic and a translational research point of view, it would be important to explore whether "reducing neurotoxicity" and "increasing neuroprotection" can impact each other in a synergistic or an additive manner. These questions are currently under study.

Depending on genetic backgrounds, 18% to 35% of patients with glaucoma have normal tension glaucoma.⁵⁰ It is attractive to speculate that these patients may have a genetic predisposition to increased TrkC.T1 levels either in retinal glia or in the optic nerve head astrocytes and that it can progress to pathologic levels of TNF- α . This hypothesis is also under study.

Acknowledgments

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