An Agonistic TrkB mAb Causes Sustained TrkB Activation, Delays RGC Death, and Protects the Retinal Structure in Optic Nerve Axotomy and in Glaucoma

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Purpose. Brain-derived neurotrophic factor (BDNF) receptors TrkB and $p75^{NTR}$ are expressed in the retina. However, exogenous BDNF does not provide retinal ganglion cells (RGCs) with long-lasting neuroprotection in vivo during optic nerve axotomy or in glaucoma rat models of neurodegeneration. The authors set out to answer the hypothesis that a selective TrkB agonist might afford more efficient neuroprotection.

Methods. Animal models of acute neurodegeneration (complete optic nerve axotomy) and chronic neurodegeneration (ocular hypertension, glaucoma) were used. After intravitreal delivery of test agents or controls, surviving RGCs were quantified. Transient or sustained activation of TrkB receptors in vivo was quantified by Western blot analysis retinal samples for TrkB-phosphotyrosine. Time-dependent changes to the neuronal retinal layers were quantified longitudinally by Fourier domain–optical coherence tomography.

Results. The authors show that a selective TrkB agonist caused long-lived TrkB activation and significantly delayed RGC death in these models of acute and chronic retinal injury in vivo. Importantly, using noninvasive retinal imaging, they also show that a selective TrkB agonist caused preservation of the retinal structure in both animal models, with maintenance of the layers comprising neurons and neuronal fibers.

Conclusions. In animal models of acute and chronic neurodegeneration, a TrkB agonist affords long-lasting neuroprotection by causing sustained TrkB activation. The use of structural end points could have prognostic value to evaluate neuroprotection. This work contributes to the understanding of neurotrophic mechanisms underlying RGC death in glaucoma and optic nerve axotomy. (*Invest Ophthalmol Vis Sci.* 2010;51:4722–4731) DOI:10.1167/iovs.09-5032

Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family. The receptors for BDNF include TrkB and $p75^{NTR}$. In adult neurons these two receptors are usually expressed at varying ratios. BDNF binding to the TrkB receptor mediates prosurvival signals, whereas binding to $p75^{NTR}$ generally mediates pro-death signals, depending on the biological context.[1] However, the retinal ganglion cells (RGCs) are phenotypically unlike most other neurons because normal RGCs express TrkB but do not express $p75^{NTR}$.[2] In fact, in normal retina, the $p75^{NTR}$ receptor is expressed preferentially on glial cells.[3] The levels of TrkB and $p75^{NTR}$ can be upregulated in disease states of the retina, such as optic nerve (ON) axotomy[3] and glaucoma.[4]

Experimental ON axotomy is a model of acute injury in which the optic nerve is completely severed, causing the RGCs to die and their cell bodies to degenerate rapidly.[3] The episcleral vein cautery (EVC) experimental glaucoma model is a chronic and progressive neuropathy associated with high intraocular pressure (IOP). The experimental glaucoma model causes slow and progressive RGC death.[4–7]

The selective cellular distribution of TrkB and $p75^{NTR}$ receptors on developmentally and functionally different cell types in the retina prompted the use of BDNF to study neurotrophic mechanisms in experimental animal models of neurodegeneration. Administration of exogenous BDNF can delay RGC death during ON axotomy[8–10] or glaucoma.[2,11–13]

However, the pharmacologic use of BDNF affords short-lived neuroprotection. Moreover, efficacy with BDNF requires administration at high doses or high frequency. These results are paradoxical. First, it is unclear why high doses of exogenous BDNF administration are required when the upregulation of endogenous BDNF in disease states[4] makes this growth factor already present at relatively high levels. Second, neurotrophins activate Trk receptors with sustained kinetics,[14] making it unclear why high frequency of exogenous BDNF administration is required.

The relative failure of BDNF may be attributed to poor activation of retinal TrkB in vivo,[15] to poor pharmacokinetics, or to transient Trk activation in the retina caused by BDNF. Sustained Trk activation leads to long-lived physiological effects in neurons,[16,17] whereas transient activation leads to incomplete or limited physiological effects.[18] Additionally, high levels of BDNF could cause $p75^{NTR}$ activation in glia with the release of neurotoxic factors such as pro-neurotrophins or TNF-α.[19,20]

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To address these issues, we report the evaluation of an agonist that selectively targets TrkB while excluding binding to $p75^{NTR}$. We use a pair of anti–TrkB mAbs that bind selectively to TrkB but not to $p75^{NTR}$. One anti–TrkB mAb is an agonist, and the other anti–TrkB mAb is a functionally inert control. We quantified long-lasting TrkB activation and long-lasting neuroprotection of RGCs in rat models of ON axotomy and glaucoma. A noninvasive imaging system, Fourier-Domain optical coherence tomography (FD-OCT),[21] showed that neuroprotection afforded quantitative preservation of the retinal structure.

Materials and Methods

Production and Characterization of mAbs

Mouse mAbs were produced through standard hybridoma techniques, as we described previously for other agonistic anti–Trk receptor mAbs.[22,23] Splenocytes from immunized female Balb/c mice (8 weeks old) were fused to SP2/0 myelomas, and hybridomas were screened by differential binding in an enzyme-linked immunosorbent assay (ELISA) using the original TrkB immunogen (data not shown). Several mAbs were identified in ELISA, and mAbs 1D7, B3, and 21G3 were selected for further study. All mAbs were purified by affinity column chromatography to >95% (Sigma, Montreal, Quebec, Canada).

Cell Lines

We used HEK293 stably transfected with rat TrkB constructs. For specificity controls, we used wild-type HEK293 cells or HEK293 stably transfected with rat $p75^{NTR}$, human TrkC, human TrkA, or rat TrkA.[24] We also used the neuronal cell line SY5Y stably transfected with human TrkB cDNA (SY5Y-TrkB). For specificity, wild-type SY5Y cells were used.[25]

Antibody Binding Flow Cytometry Assays

Characterization of mAb 1D7, B3, and 21G3 binding to the cell surface was made by flow cytometry (FACScan; Becton Dickinson, San Jose, CA) assays using live cells, as described.[22] Cells (2.5×10^5) in 0.1 mL binding buffer (Hanks' balanced salt solution), 0.1% bovine serum albumin, and 0.1% NaN_3 were incubated with primary mAbs (7 nM) for 30 minutes at 4°C and washed in binding buffer, followed by FITC-conjugated goat anti–mouse secondary antibody for 30 minutes at 4°C. As negative controls, no primary (background fluorescence) or irrelevant mouse IgG primary (Sigma) was used. Cells were acquired and analyzed on a fluorescence-activated cell scanner (FACScan; Becton Dickinson) using an analysis program (Cell Quest; Becton Dickinson). Flow cytometry reveals specific binding to native cell surface receptors. mAbs 1D7 and 21G3 bind cell surface TrkB and were thus used in biological assays, but mAb B3 does not bind to native cell surface receptors.

Functional Assays for TrkB Agonist Activity

The biological properties of the ligands were measured by quantification of TrkB tyrosine phosphorylation by Western blot analysis after treatment of cell lines with ligands and by effects on MTT-based survival/proliferation assays.

The tyrosine phosphorylation of TrkB was studied after treatment of cells in culture with TrkB ligands mAb 1D7, mAb 21G3, control mouse IgG, or control BDNF (each at 10 nM) for 12 minutes at 37°C. Then cells were solubilized, and protein concentrations were determined (Detergent Compatible Protein Assay; Bio-Rad, Hercules, CA). Western blot analysis was performed as described[26] with antiphosphotyrosine mAb 4G10 or with anti–phospho-TrkB serum (a kind gift of Moses Chao, Weill Medical College of Cornell University, New York, NY) and the enhanced chemiluminescence system (PerkinElmer Life Sciences, Waltham, MA). Tyrosine phosphorylation of TrkB provides a direct measure of TrkB activation.[27] Anti–actin antibody or anti–βIII tubulin antibody (Sigma) was used to standardized protein loading.

For survival assays, SY5Y-wt or SY5Y-TrkB cells (10,000 cells/well) in serum-free media (PFHM-II; Gibco, Toronto, Canada; supplemented with 0.2% BSA) were added to 96-well plates (Falcon, Lincoln Park, NJ) with or without mAb 1D7, mAb 21G3, mouse IgG as negative control, BDNF as positive control (each at 10 nM), or serum (final 10% FBS) as normal culture conditions. Cell survival was quantified spectrophotometrically at 595 nm with MTT (Sigma) 48 to 72 hours after plating, as described.[24]

Animals and Anesthesia

All animal procedures were conducted in accordance with the guidelines set out by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with Institutional Animal Care and Use Committee (IACUC) protocols. Wistar female rats (weight range, 250–300 g; Charles River Laboratories, Wilmington, DE) were housed in a 12-hour light/12-hour dark cycle with food and water ad libitum. Deep anesthesia (ketamine, xylazine, acepromazine injected intraperitoneally; 50/5/1 mg/kg, as per IACUC recommendations) was used for ECV (glaucoma), ON axotomy, Fluorogold labeling, intraocular injection procedures, FD-OCT measurements, and euthanatization. Light anesthesia (a gas mixture of oxygen/2% isoflurane, at a rate of 2.5 L/min, in accordance with IACUC recommendations) was used for measuring IOP.

Rat Retinal Degeneration Models

Optic Nerve Transection Model. The procedure was performed as described.[3,7] In brief, a 1.5- to 2.0-cm skin incision was made along the edge of the right orbit bone; lachrymal glands and orbital fats were excised, and extraocular muscles were separated to expose the optic nerve. An 18-gauge needle was used to lacerate the sheath longitudinally so as not to disturb the ophthalmic artery; the ON parenchyma was then extricated, lifted by a homemade hook, and completely transected 0.5 to 1.0 mm posterior to the eyeball with micro-tweezers. At 7 days and 14 days after axotomy, respectively, ~50% and ~90% of RGCs die.[3]

Glaucoma Model. The EVC model of glaucoma[7] is validated in comparative studies.[28] Radial incisions were made in conjunctiva, and three of the episcleral veins (two dorsal episcleral veins located near the superior rectus muscle and one temporal episcleral vein located near the lateral rectus muscle) were cauterized with a 30′ cautery tip. The contralateral control eyes underwent sham surgery without cauterization to isolate the three veins.

IOP was measured immediately after the EVC surgery and every week until the end point of each experiment. The mean normal IOP of rats under light anesthesia was measured with an applanation tonometer (Tono-Pen XL; Reichert, East Morris Plains, NJ) immediately after the EVC surgery and every week until the end point of each experiment. Normal IOP was 10 to 14 mm Hg. One week after cauterization, IOP was elevated to ~23 mm Hg in ~90% of the rats and remained at that level throughout the duration of the experiment. High IOP is chronic and causes progressive disease; after 6 weeks of disease, ~70% RGCs remain alive.[5,7]

IOP Measurements. The mean normal IOP of rats under light anesthesia was 10 to 14 mm Hg. In the glaucoma model, the IOP was elevated ~1.7-fold throughout the duration of the experiment. We reported stable and chronic high IOP in this model.[5,7] IOP was measured with an applanation tonometer (Tono-Pen XL; Reichert)[29,30] immediately after the EVC surgery and every week until the end point of each experiment. In our experiments, 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. We reported IOP data previously in many similar studies.[5,7] Approximately 90% of the cauterized eyes experienced sustained elevations of IOP. We discarded the animals (and censored all the data) in the rare cases in which the retinal vasculature showed signs of ischemia, 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 and in the rare cases in which the animals had cataracts.

Intraocular Injections and Drug Treatment

A 30-gauge needle was used for intraocular injections. The needle was injected at a 45° angle 2 mm behind the corneoscleral limbus until the entire bevel of the needle was inserted into the vitreous body without damaging the lens. The procedure was finished in 2 minutes. After the injection, the needle was left in place for another minute to allow dispersion of the compound into the vitreous. The experimental eyes were injected with test agent or control vehicle, and the contralateral eyes served as normal uninjected controls. Our previous publications showed that normal contralateral eyes are not different from each other when they remain uninjected or receive control PBS injections.[5,7]

Drug treatments were performed with the experimenters blinded to the treatment code. For the glaucoma model, the intraocular injections were made at days 14 and 21 after cauterization; the end point was at day 42 of high IOP. Thus, in this paradigm, before treatment, there is preexisting damage for 14 days (causing ~8% RGC death[5,7]). For the axotomy model, the intraocular injections were performed within 5 minutes of injury, and the end point was at day 14 after ON transection. Recombinant BDNF and anti–TrkB mAbs 1D7 and 21G3 were prepared in PBS. Intraocular injections in retinas used for therapeutic assays (end points at 14 days in ON axotomy and 42 days in glaucoma) delivered 3 μL with 3 μg compound. Intraocular injections in retinas used for biochemical assays (end points at 6 or 18 hours) delivered 3 μL with 3 μg compound.

Fluorogold Retrograde Labeling

RGCs were retrogradely labeled with a 4% Fluorogold solution (Fluorochrome, Englewood, CO) applied bilaterally to the SC.[3,5,7] Briefly, rats were mounted onto a stereotactic apparatus (Kopf Instruments, Tujunga, CA), holes were drilled at a position 1.3 mm lateral to the sagittal suture and 2.5 mm anterior to the lambda suture on each side, and Fluorogold (3 μL) was injected into the SC at the depth of 6.0 mm below the skull. Then the holes were filled with sterile sponge (Gelfoam; Pfizer, New York, NY) soaked in 4% Fluorogold. In the glaucoma model, retrograde labeling was performed at day 35 after ocular hypertension (7 days before the experimental end point), whereas in the axotomy model, retrograde labeling was carried out 7 days before optic nerve transection (21 days before the experimental end point). These times afford excellent labeling efficacy and are practical and compatible with experimental procedures, including the long-lived glaucoma model (6 weeks).

In Vivo TrkB Phosphorylation

Rats ($n = 4$ per group) received 1 μg of the indicated treatment. In axotomy drugs were injected within 5 minutes after ON transection. In glaucoma, drugs were injected after 14 days of high IOP. Six or 18 hours after drug treatment retinas were dissected and lysed in 80 μL SDS-PAGE sample buffer containing 2% SDS. After SDS-PAGE and transfer, membranes were subjected to Western blot analysis with rabbit antisera to TrkB-pTyr (a gift of Moses Chao), followed by goat anti–rabbit secondary antibodies conjugated to horseradish peroxidase (Sigma) at a 1:10,000 dilution. Loading was controlled with antibodies to β-tubulin (Sigma). Detection was by enhanced chemiluminescence. For digital quantification, membranes were scanned and analyzed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

Statistical Analysis

Statistical software packages (Prism 5 [GraphPad Software Inc., San Diego, CA] and SPSS 13.0 [SPSS Inc., Chicago, IL]) were used. After quantification of Western blot data, the mean ± SD is shown. For IOP data, FD-OCT data, and RGC counting data, the mean ± SEM is shown. For IOP and FD-OCT results, the data were analyzed by repeated measures ANOVA software (SPSS 13.0; SPSS Inc.) followed by post hoc tests (Tukey HSD) for comparisons among the three groups. One-sample *t*-test analysis was used for data obtained from in vivo TrkB phosphorylation. Statistical significance was declared if $P \leq 0.05$.

RGC Survival Quantification and Statistical Analysis

Quantification of surviving RGCs was performed as reported previously.[3,5,7] At day 14 of ON axotomy or at day 42 of glaucoma, both eyes were enucleated; the anterior parts were cut out, and the remaining part was fixed in 4% paraformaldehyde for 30 minutes. Then the retinas were flat-mounted on a glass slide and dissected by four radial cuts to facilitate flattening of the retinas into a Maltese cross shape, with the vitreous side up. Pictures for each freshly flat-mounted retina were taken using a fluorescence microscope (Carl Zeiss Meditec, Jena, Germany), with 12 pictures/retina at 20× magnification. For each quadrant there were 3 pictures at a distance of 1 mm, 2 mm, and 3 mm radially from the optic nerve (indicated as areas 1, 2, and 3). Microglia and macrophages that incorporated Fluorogold after phagocytosis of dying RGCs were excluded based on their morphology, as previously reported.[3,5,7]

In all cases, manual RGC counting was performed by two independent persons. One person was the experimental performer, blinded to the drug treatment code, and the second person was unrelated to the experiment and was blinded to the entire protocol. Standardization of the percentage of RGC survival in each rat was calculated as the ratio of the experimental eye (right eye, OD) compared with the control eye (left eye, OS) ($RGC_{experimental}/RGC_{contralateral}$). Mean ± SEM was calculated for all percentages of RGC survival data, for each experimental group (untreated, PBS, BDNF, 1D7, 21G3). Data analysis was performed (Prism 5; GraphPad Software Inc.). Comparison between the RGC survival rates was made using one-way ANOVA with Dunnett's multiple comparison test (comparison of treatment groups against the controls) and Bonferroni test (comparison of experimental groups). $P \leq 0.05$ was considered statistically significant.

Fourier Domain–Optical Coherence Tomography

A noninvasive prototype spectrometer-based FD-OCT system was used to acquire the retinal images. FD-OCT is a noninvasive method that allows time-kinetic studies in the same animal, with axial resolution in tissue nominally better than 4 μm and repeatability of the measurements from B-scans better than 1 μm. Data acquisition was performed using custom software written in C++ for rapid frame grabbing, processing, and display of two-dimensional images. Manual segmentations were used to measure the thicknesses of the rat retinas in glaucoma and axotomy experiments. After anesthesia, the rats were placed on a homemade rack, and the head was oriented to an angle at which the eye was properly aligned to the optical beam. The pupils were dilated with a topical solution (atropine sulfate 1%; Alcon, Fort Worth, TX). Refraction of light at the cornea was cancelled by placing over the eye a flat coverslip coated with a generic artificial tear gel. Alignment of the optical system to the rat retina required a few minutes and was followed by rapid acquisition of data (~5 s/vol). During retinal scanning, three volumes were acquired in different sectors of the retina containing the ON head and retinal blood vessels as landmarks. This is sufficient to comprise most of the retina. The volumes can be rendered in 3D or visualized en face as a fundus image. After processing, six B-scans were randomly selected from each volume. The retinal thickness measurements were performed with ImageJ software using the saved data. In each B-scan, the thickness of the nerve fiber layer (NFL)–ganglion cell layer (GCL)–inner plexiform layer (IPL), hereafter referred to as NGI, was measured at four adjacent points at a distance nominally 1.5 mm from the ON head.

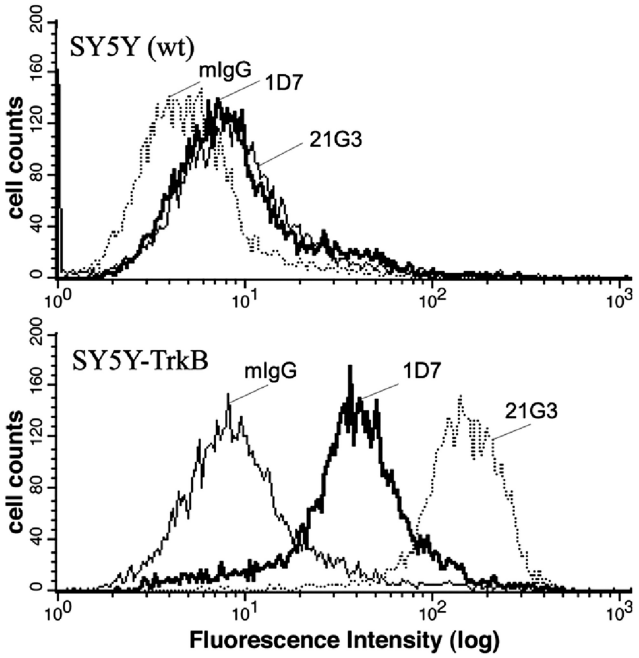

FIGURE 1. mAbs 1D7 and 21G3 bind to cell surface human TrkB. Flow cytometry binding profile of mAbs 1D7 and 21G3 binding to wild-type SY5Y cells or human TrkB-transfected SY5Y cells. Mouse IgG is the negative control. mAb B3 does not bind to native cell surface TrkB.

RESULTS

Generation and Characterization of Anti–TrkB mAbs 1D7 and 21G3

Initial screening of hybridomas was made by ELISA binding studies (data not shown). Clones mAb 1D7, mAb 21G3, and mAb B3 were selected for flow cytometry binding assays using live cells.

Clones mAb 1D7 and mAb 21G3 bind to SY5Y cells stably transfected to express human TrkB (SY5Y-TrkB) but do not bind to wild-type SY5Y cells above background control mouse immunoglobulin (Fig. 1). On U represented on a logarithmic scale, the fluorescence mean can be measured from bell-shaped histograms. The mean fluorescence of SY5Y-TrkB cells exposed to background mouse immunoglobulin was 8 ± 3 U; when exposed to mAb 1D7 or mAb 21G3, the mean fluorescence increased to 44 ± 7 U and 153 ± 14 U, respectively. In controls, the mean of wild-type SY5Y cells exposed to background mouse immunoglobulin was 4 ± 2 U, and when exposed to mAb 1D7 or mAb 21G3 the mean was 9 ± 3 and 8 ± 3 U, respectively.

In further tests, mAb 1D7 and mAb 21G3 also bind to HEK293 cells transfected with rat TrkB but do not bind to HEK293 cells transfected with rat $p75^{NTR}$, human TrkC, human TrkA, or rat TrkA receptors (Table 1). Because the cells are intact, the flow cytometry data indicate that mAb 1D7 and mAb 21G3 bind to native TrkB ectodomain expressed on the cell surface. The data also indicate that mAb 1D7 and mAb 21G3 can bind selectively to TrkB and that they can bind both human TrkB and rat TrkB. In contrast, mAb B3 does not bind to any of the cells in flow cytometry, indicating that it does not recognize native cell surface receptor.

Further work aimed at characterizing the binding site of these mAbs on the TrkB ectodomain. mAb 1D7 and mAb B3 recognize denatured TrkB on Western blot analysis when samples are resolved in nonreduced SDS-PAGE. In contrast, mAb 21G3 does not recognize denatured TrkB under any condition and cannot be studied using Western blot analysis.

To identify the TrkB domain in which mAb 1D7 and mAb B3 bind, we studied HEK293 cells expressing transfected chimeric receptors. In these transfectants, a domain of rat TrkA was spliced in to replace the corresponding domain in rat TrkB[24,31] (Fig. 2a). mAb 1D7 does not bind chimeras 2.1 and 2.2, indicting that it recognizes the D2-D3 domain(s) of rat TrkB. mAb B3 does not bind chimeras 2.2 and 3.1, indicating that it recognizes the D4 domain of rat TrkB (Fig. 2b).

The expressed chimeric receptors are functional, as shown in phosphotyrosine Western blot analysis after treatment of live cells with 2 nM BDNF (Fig. 2c). The 3.1 chimera was activated without ligand, as previously reported.[24,31] Chimeras 2.1 and 2.2, not recognized by mAb 1D7, were activated by BDNF. These data indicate that the receptor sites for BDNF binding and activation do not overlap with the receptor sites for mAb 1D7 binding. This notion is further supported by our observations that prebinding of BDNF to cell surface TrkB did not inhibit mAb 1D7 binding to TrkB.

Anti–TrkB mAbs 1D7 Is an Agonist

Functional assays were performed with mAb 1D7 and mAb 21G3 because they recognize native cell surface receptors. These functional studies were not carried out with mAb B3 because it does not recognize native TrkB on the cell surface and, therefore, cannot be studied functionally.

Treatment of SY5Y-TrkB cells with mAb 1D7 (10 nM) or BDNF (10 nM) resulted in bands consistent with TrkB tyrosine

TABLE 1. Summary of Flow Cytometry Data

Cells	Mean Channel Fluorescence			
	Mouse Immunoglobulin	1D7	21G3	B3
SY5Y	4 ± 2	9 ± 3	8 ± 3	9 ± 3
SY5Y-TrkB (human)	8 ± 3	**44 ± 7**	**153 ± 14**	11 ± 4
HEK293	10 ± 3	14 ± 4	15 ± 4	15 ± 4
HEK293-TrkB (rat)	9 ± 3	**93 ± 11**	**186 ± 15**	13 ± 4
HEK293-TrkA (rat)	6 ± 3	9 ± 3	12 ± 4	11 ± 4
HEK293-TrkA (human)	4 ± 2	7 ± 3	9 ± 3	10 ± 3
HEK293-TrkC (human)	8 ± 3	9 ± 3	5 ± 2	11 ± 4
HEK293-$p75^{NTR}$ (rat)	8 ± 3	8 ± 3	11 ± 4	10 ± 3

Flow cytometry binding profile of the three anti–TrkB mAbs 1D7, 21G3, and B3 (or control mouse immunoglobulin) binding to the indicated cell lines. Specificity controls are the untransfected SY5Y parental cells or untransfected HEK293 parental cells. Data shown are the mean channel fluorescence from bell-shaped histograms as shown in Figure 1, with 5000 cells analyzed (average ± SEM from three or more independent assays). Numbers in bold indicate increases over background and over control cells.

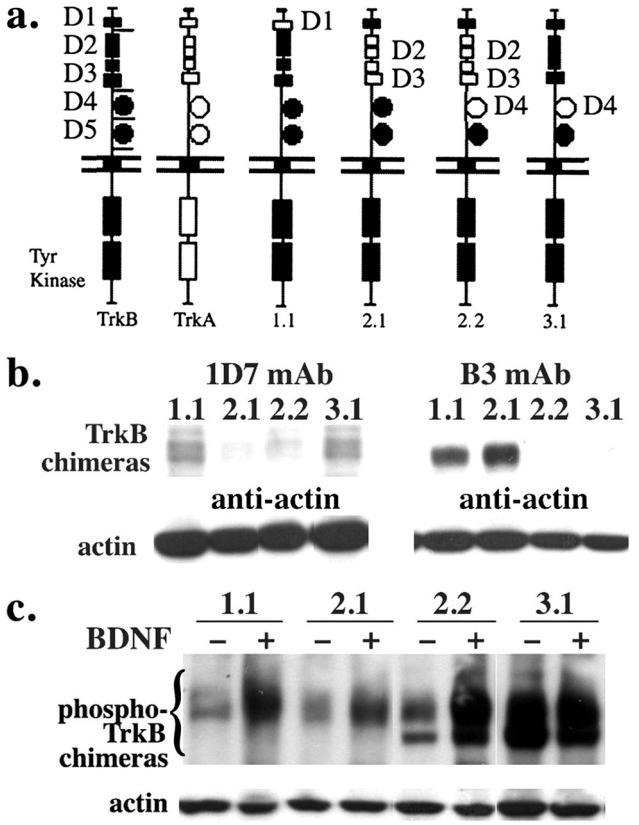

FIGURE 2. mAb 1D7 binds to the D2–D3 domain of rat TrkB. (**a**) Scheme of the chimeric receptors. TrkB (*black*) domains were replaced with TrkA (*white*) domains. These chimeras were expressed in HEK293 cells.[24,31] This research was originally published in *The Journal of Biological Chemistry*. Zaccaro MC, Ivanisevic L, Perez P, Meakin SO, Saragovi HU. p75 coreceptors regulate ligand-dependent and ligand-independent Trk receptor activation, in part by altering Trk docking subdomains. *J Biol Chem*. 2001;276:31023–31029. © The American Society for Biochemistry and Molecular Biology. (**b**) Western blots of mAb 1D7 and B3 binding to samples prepared from cells expressing the chimeric receptors. Actin is shown as the loading control. mAb 21G3 does not bind in Western blot analysis. (**c**) Cells express functional chimeric receptors, as previously reported.[24,31] Anti–phosphotyrosine Western blot of samples prepared from cells expressing the chimeric receptors after cells were treated or not treated for 15 minutes with 2 nM BDNF, as indicated.

phosphorylation (Fig. 3a). The heterogeneity of TrkB-pTyr bands in these cells has been reported.[24,32] Treatment with mAb 21G3 (10 nM) or with control mouse immunoglobulin did not induce TrkB tyrosine phosphorylation. mAb 1D7 is a TrkB ligand that activates this receptor in a manner similar to BDNF. Treatment of control wild-type SY5Y cells with the reagents, including mAb 1D7 and BDNF, did not result in the detection of phospho-TrkB as expected because they do not express TrkB (Fig. 3a).

We then measured agonism in survival assays using wild-type SY5Y cells or SY5Y-TrkB cells. These cells die by apoptosis when placed in serum-free conditions, but they can be protected from death by supplementation with trophic support (Fig. 3b). mAb 1D7 and BDNF support SY5Y-TrkB cell survival in a dose-dependent manner. In contrast, mAb 21G3 and mouse immunoglobulin do not support SY5Y-TrkB cell survival. In specificity controls, neither the mAbs nor BDNF can support the survival of wild-type SY5Y cells. As a further positive survival control, supplementing serum-free cultures with 10% serum supports the survival of SY5Y-TrkB and wild-type SY5Y cultures (Fig. 3b).

Together the data indicate that mAb 1D7 and mAb 21G3 bind to native TrkB on the cell membrane. Although mAb 1D7 is agonistic to TrkB, mAb 21G3 is biologically inert. This pair of TrkB ligands (one agonistic and one inert) was tested in vivo and were compared with BDNF, which is an agonist of TrkB and $p75^{NTR}$.

Anti–TrkB mAb 1D7 Supports Long-lasting RGC Survival in Glaucoma and ON Axotomy

We compared in vivo the neuroprotective effect of the mAbs as TrkB ligands compared with BDNF and PBS controls. Equal protein concentrations of test agents were injected intravitreally (3 μg/3 μL) because little is known about pharmacokinetics, bioavailability, or biodistribution within the retina. However, because mAbs have greater molecular weight, they likely result in a lower retinal molarity than BDNF. The concentration of BDNF was selected from doses reported to be efficacious.[2,8–13] We quantified whether treatments promote RGC survival in vivo.

In a rat model of ON axotomy, a single intravitreal injection of the test agents or PBS control was performed immediately after axotomy. RGCs were quantified at the 14-day end point. Representative micrographs of retinas show the labeled RGCs (Fig. 4a). From these pictures we quantified the percentage of surviving RGCs after ON axotomy (Fig. 4c) compared with the contralateral naive eye. In the untreated axotomy group, 11.3% ± 1.5% of the RGCs remained alive. Injection of PBS had no effect, and 12.2% ± 1.1% of the RGCs remained alive. BDNF (14.6% ± 1.3% RGC survival) and mAb 21G3 (15.7% ± 1.4% RGC survival) did not prevent RGC death compared with the PBS control group. However, mAb 1D7 afforded statistically

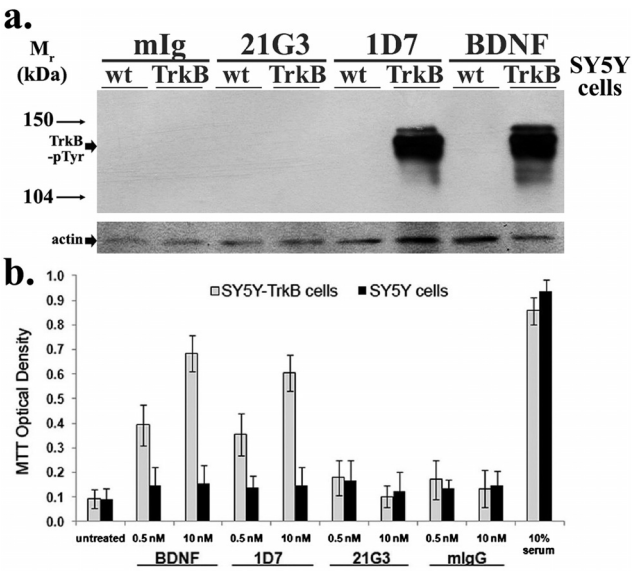

FIGURE 3. mAb 1D7 is a TrkB agonist. (**a**) Representative anti–phosphotyrosine Western blot of whole cell lysates from cells treated with 2 nM BDNF, 10 nM mAb 1D7, 10 nM mAb 21G3, or 10 nM mIgG. SY5Y-TrkB (TrkB) and control wild-type SY5Y (wt) cells were studied. A heterogeneous protein containing phosphotyrosine, consistent with the relative gel mobility (M_r) of TrkB, is recognized only for SY5Y-TrkB cells activated by BDNF or mAb 1D7. M_r is indicated, based on molecular markers. (**b**) Cell survival was studied by MTT assay. SY5Y or SY5Y-TrkB cells were cultured in serum-free media to induce cell death. Indicated concentrations of BDNF, mAb 1D7, or 21G3 were added and further incubated for 3 days. Fetal bovine serum (10%) was used as positive control (set to 100% survival). Average optical density ± SEM; $n = 4$.

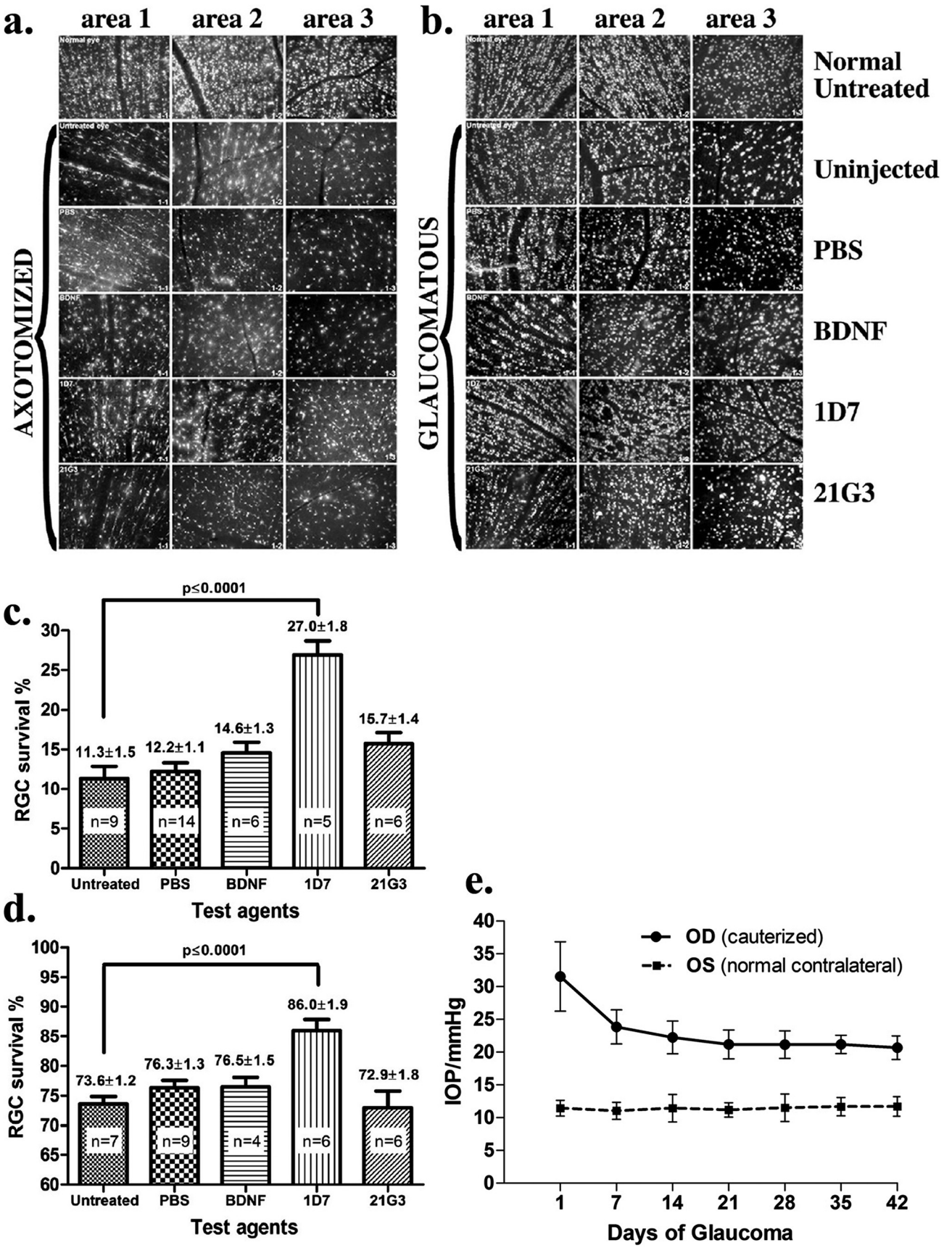

FIGURE 4. Quantification of surviving RGCs in axotomy and glaucomatous retina. (**a**, **b**) Representative pictures of Fluorogold-labeled RGCs after the indicated disease (14 days of axotomy, 42 days of glaucoma) and treatment. Areas denote standardized distances from optic nerve. (**c**, **d**) Summary of RGC survival in ON axotomy and in glaucoma. For each treatment RGC survival was quantified compared with normal contralateral control from the indicated number of eyes ± SEM. (**e**) Averages of the IOPs measured weekly in rats used for glaucoma experiments; $n = 18 \pm$ SD. OD, cauterized right eye; OS, naive left eye.

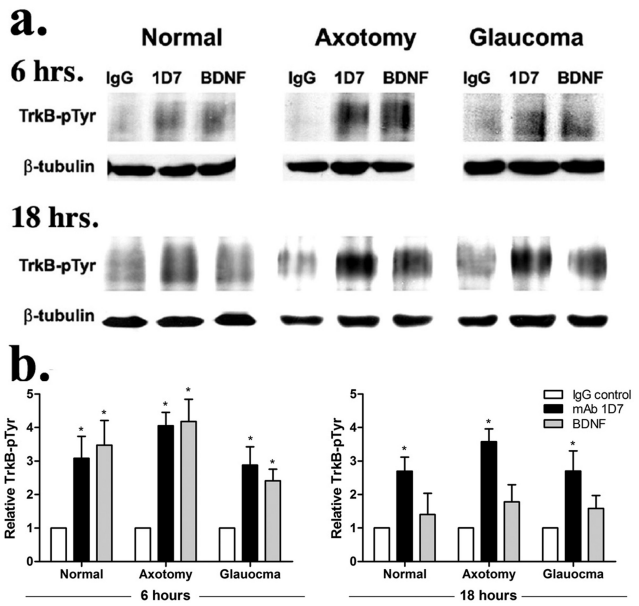

FIGURE 5. Quantification of surviving RGCs in axotomy and glaucomatous retina. (**a**) Representative phospho-TrkB Western blots of protein extracts from retinas (normal, day 1 axotomy, day 14 glaucoma) collected 6 hours or 18 hours after the indicated treatments. (**b**) Quantification of the phospho-TrkB compared with β-tubulin. Control IgG-treated samples are standardized as 1. Average ± SEM; $n = 4$ independent retinas each group. $^*P \leq 0.05$. Statistical analyses was performed with one-sample *t*-test analysis.

significant neuroprotection, and 27.0% ± 1.8% RGCs remained alive ($P \leq 0.0001$ vs. PBS control group; Fig. 4c).

In a rat model of glaucoma, two intravitreal injections of the test agents or PBS control were performed at day 14 and day 21 of glaucoma, and RGCs were quantified at the day 42 end point of glaucoma (i.e., 21 days after the last treatment). Representative micrographs of retinas show the labeled RGCs (Fig. 4b). From these pictures we quantified the percentage of surviving RGCs after glaucoma (Fig. 4d) compared with the contralateral naive eye. The IOP measured in the rats used for the glaucoma studies show sustained IOP elevation in the cauterized eyes (Fig. 4e), as previously shown.[4,5] It is noteworthy that in this paradigm there was preexisting RGC loss of approximately 8% to 12%[4,5] before drug treatment was applied. Moreover, the retina endured constant stress because the measured IOP remained high throughout the experiment.

In the untreated glaucoma group, 73.6% ± 1.2% of the RGCs remained alive. Injection of PBS had no effect, and 76.3% ± 1.3% of the RGCs remained alive. BDNF (76.5% ± 1.5% RGC survival) and mAb 21G3 (72.9% ± 1.8% RGC survival) did not prevent RGC death compared with the PBS control group. However, mAb 1D7 afforded statistically significant neuroprotection, and 86.0% ± 1.9% labeled RGCs remained alive ($P \leq 0.0001$ vs. PBS control group; Fig. 4d).

Long-lived RGC Survival Correlates with Long-lasting TrkB Activation

Functional activation of TrkB in vivo was tested by measuring receptor phosphorylation. Normal, axotomized, or glaucomatous eyes were injected intravitreally with BDNF, mAb 1D7, or mIgG control. Retinal protein extracts were analyzed either 6 hours or 18 hours later for TrkB tyrosine phosphorylation using a specific anti–phospho-TrkB rabbit antisera (Fig. 5a).

At the 6-hour point, treatment with BDNF and mAb 1D7 significantly increased TrkB tyrosine phosphorylation compared with mIgG control. However, at the 18-hour point only mAb 1D7 significantly increased TrkB tyrosine phosphorylation compared with mIgG control (Fig. 5a; data quantified in Fig. 5b). Comparable data were obtained by using a generic anti–phosphotyrosine mAb 4G10 (data not shown). Thus, although both BDNF and mAb 1D7 induce pTyr-TrkB, only mAb 1D7 can induce sustained pTyr-TrkB and can protect RGCs from death in retinal diseases.

A Selective TrkB Agonist Maintains Retinal Structures in Glaucoma and ON Axotomy

We measured structural changes using FD-OCT, a noninvasive method that allows time-kinetic studies of retinas degenerating after axotomy or glaucoma (Fig. 6). In the normal rodent retina, the RGCs form a single layer (the GCL), bracketed by the NFL and the IPL. With FD-OCT it is possible to quantitatively measure the combination of the NGI layers. The NGI is relevant because it is where the RGC soma and the projecting RGC fibers are located.

The experimental protocol is described in Figure 6a. The combined thickness of NGI in normal retinas was 71 ± 0.6 μm. Fourteen days after axotomy, the NGI thickness was 54.7 ± 1.2 μm, whereas in the mAb 1D7-treated group it was 60.8 ± 0.3 μm (significant versus axotomy, $P \leq 0.001$; representative data are shown in Fig. 6b and are summarized in Fig. 6c). Forty-two days after glaucoma, the NGI thickness was 51.2 ± 2.6 μm, whereas in the mAb 1D7-treated group it was 60.4 ± 0.1 μm (significant versus untreated glaucoma, $P \leq 0.02$; Fig. 6d).

Thus, mAb 1D7 treatment significantly protected the structure of the retinal neuronal layers in both acute (ON axotomy) and chronic (glaucoma) conditions. However, mAb 1D7 treatment does not protect the NGI fully. At the experimental end points (day 14 for ON axotomy, day 42 for glaucoma), the NGI of mAb 1D7-treated rats was significantly thinner than in normal retinas ($P \leq 0.0001$). This is not surprising given that these eyes endured continuous stress and had a low treatment frequency and dose.

DISCUSSION

This work reports on the characterization of novel anti–TrkB mAbs and their application in vivo to the neurodegenerating retina. The mAbs will be important research tools and perhaps clinical tools because they bind to both human TrkB and rat TrkB.

mAb 21G3 is biologically inert, whereas mAb 1D7 is a functional agonist. The potency of mAb 1D7 in ex vivo assays is equivalent to BDNF on a molar basis. However, mAb 1D7 binds to TrkB at a site distinct from BDNF. Because mAb 1D7 recognizes denatured TrkB on Western blot analysis, it is likely that it recognizes a linear or a stable epitope within the D2-D3 domains of the receptor. The domain and the epitope recognized by mAb 1D7 can be defined as an agonistic hot spot.[24,33,34]

Not all mAbs that are directed to the TrkB ectodomain are functionally active. This could be a consequence of each mAb having different binding sites. However, one property that all mAbs share is multivalency; hence, they have the potential to induce receptor dimerization. Any putative TrkB dimerization functionally inert mAb 21G3might induce is nonfunctional in terms of receptor pTyr and in terms of neuronal survival ex vivo and in vivo.

Other anti–TrkB agonistic mAbs have been reported,[35] one of which was recently shown to protect axotomized RGCs.[36] However, our study is the first to account for structural end points in axotomized retina to elucidate the in vivo biochemical differences between a mAb TrkB agonist and BDNF and to

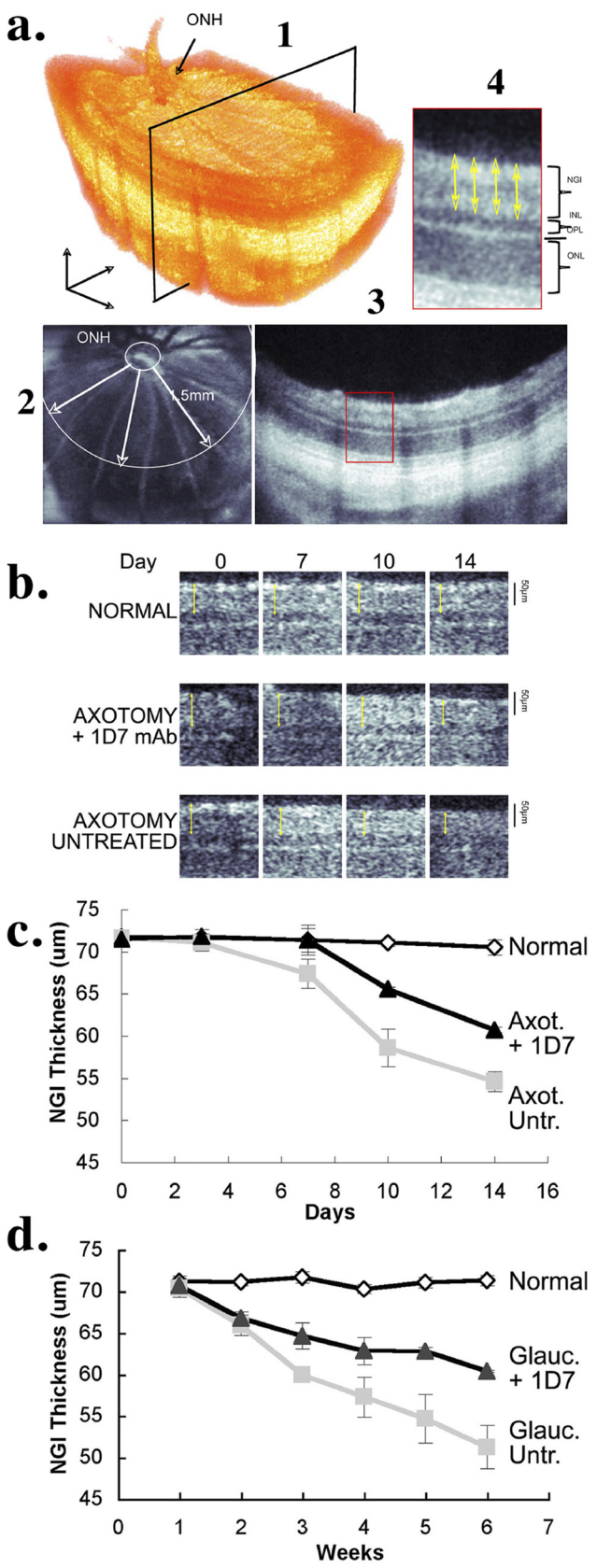

Figure 6. Quantification of retinal structures in vivo. (**a1**) 3D reconstruction of a rat retina with 600 B-scans taken every 4 mm in the *x*- and *y*-axes (2.4 mm). A single isolated B-scan (*rectangle*) shows the *z*-axis, with the inner segment at the top. (**a2**) Fundus (top view) of the image in (**a1**). Three volumes were randomly selected in different sectors of the retina at a distance of ~1.5 mm from the optic nerve head. (**a3**) From each volume six B-scans were randomly selected. (**a4**) In each B-scan, four measurements of NGI thickness were completed (*yellow arrows*). Seventy-two measurements per eye were thus performed and averaged. Each group consisted of three rats (axotomy or glaucoma ± mAb 1D7 treatment). The normal contralateral eye of each animal served as the control. (**b**) Representative sections of B-scan images for normal, axotomy ± mAb 1D7. The images are unmodified and are not enhanced. Note the progressive loss of NGI thickness over time, particularly in the untreated axotomy sample. (**c**) Axotomy, time-dependent (days) changes to average NGI thickness ± SD; $n = 3$ eyes. 1D7 treatment is significant compared with untreated control at day 7 ($P < 0.05$), day 10 ($P < 0.005$), day 14 ($P < 0.001$) by repeated-measures ANOVA. (**d**) Glaucoma, time-dependent (weeks) changes to average NGI thickness ± SD; $n = 3$ eyes. 1D7 treatment is significant compared with untreated control at week 3 ($P < 0.02$), week 4 ($P < 0.05$), week 5 ($P < 0.05$), and week 6 ($P < 0.02$) by repeated-measures ANOVA.

evaluate a nonactivating anti–TrkB mAb in vivo. Testing an inert TrkB ligand such as mAb 21G3 in vivo is an important variable that controls for possible ligand-induced receptor dimerization. It also controls for nonspecific effects because in vivo cell-bound antibodies can activate immune pathways such as opsonization of complement fixation.

Our study is the first to evaluate a selective TrkB agonist in glaucoma. Finding novel approaches to prevent neurodegeneration is important, especially in glaucoma, which is a frequent condition worldwide affecting 2% of people older than 40. Vision loss is clinically evident when >30% of RGCs have died.[37]

High IOP is a risk factor for glaucoma. However, because high IOP is indolent, glaucoma is often not diagnosed, and patients have some degree of nerve damage before therapy is initiated. All current pharmacologic therapy aims to normalize IOP, and the drugs are effective. Yet the disease remains chronic, with continuing RGC death and thinning of the NFL (which is routinely assessed using OCT in humans). The most effective IOP-lowering drugs only delay vision loss in patients: 53% progress to blindness after 6 years and 70% after 8 years.[38] In addition, depending on genetic background, 18% to 35% of patients have normal tension glaucoma[39] and have no therapeutic recourse.

Preventing neurodegeneration is attractive as a cotherapy for glaucoma and for an increasing number of conditions for which it is desirable to increase TrkB signaling selectively. Those other conditions include regulation of food intake, Rett syndrome, Huntington's disease, and depression.[40]

Why Did mAb 1D7 Succeed but BDNF Fail in Our Experimental Paradigm?

Although both BDNF and mAb 1D7 induce pTyr-TrkB, only mAb 1D7 can protect RGCs from death in retinal disease. The pharmacologic efficacy of mAb 1D7 may be attributed to its causing a long-lived TrkB activation, resulting in long-lived physiological effects. Trk receptors are activated with very long kinetics compared with other receptor tyrosine kinases.[14] Sustained Trk activation leads to long-lived physiological effects in neurons[16,17] even after the agonists and the activated receptors have been cleared.

In contrast, the failure of BDNF may be attributed to its causing short-lived TrkB activation because it is known that transient Trk activation leads to incomplete physiological effects.[18] There are two possible reasons for short-lived TrkB activation by exogenous BDNF. First, BDNF may have poor pharmacokinetics, and neurotrophins are known to have short half-lives in vivo.[41] Second, it has been suggested that BDNF

may have poor bioavailability,[42] and the presence of BDNF inhibitory factors in the retina has been proposed.[15]

It is possible that the dose and frequency used for BDNF might have been limiting in our experimental conditions. However, if this were a concern, they would have been even more limiting for the mAbs because they were administered at an eightfold lower molar dose. This was done deliberately. Our analyses of the literature showed that therapeutic BDNF efficacy is reported only when BDNF is applied using methods that result in high local doses.[2,8–13] In addition, others have shown that BDNF can protect RGCs only under certain forms of retinal injury (e.g., distal axotomy but not proximal axotomy)[43] and that in the postnatal retina TrkB activity is not required unless there is some form of stress.[44]

Location of Ligand-Dependent TrkB Activation

In retina, there are two sources of endogenous BDNF. One source is local production of BDNF in retina. The BDNF that is produced locally in retina acts primarily at the RGC soma. Experimental drugs such as exogenous BDNF and mAb 1D7 applied intravitreally also act primarily at the RGC soma. In retinal degeneration the local production of endogenous neurotrophins (including BDNF) are significantly upregulated.[4] For this reason, it seemed counterintuitive to us that injecting even more BDNF in the locale of the retina would be effective.

Another source of BDNF is a target-derived supply. BDNF produced in cortex binds receptors at the RGC terminals, and the complex is retrogradely transported to the RGC cell body in vesicles. These complexes continue to signal in the vesicles.[14,45,46] Retrogradely transported neurotrophic signals are intrinsically different from neurotrophic signals activated at the cell body.[47–49] This particular set of retrogradely transported signals is compromised in axotomy and glaucoma because retrograde transport of neurotrophins and vesicles is impaired.[2,13] Thus, it is conceivable that long-lived and efficacious BDNF signals may require retrograde transport, whereas signals activated by mAb 1D7 do not. Indeed, a recent study in humans[50] indicates a correlation between reduced TrkB activation and disease progression in glaucoma because of a mutation in the *NT-4* gene (which encodes for a TrkB-activating ligand).

Another important difference is that BDNF binds and activates $p75^{NTR}$, but the mAb 1D7 does not. Activation of $p75^{NTR}$ may be undesirable given that $p75^{NTR}$ is upregulated in disease states. BDNF activation of $p75^{NTR}$ may cause glial release of neurotoxic pro-neurotrophins or TNF-α[19,20] and could compromise any benefits of TrkB activation.

Correlations between RGC Survival and Sparing of Structural Damage

Although FD-OCT is routinely used in humans, only relatively simple models of retinal degeneration have been imaged with FD-OCT in rodents.[51–53] Recently, FD-OCT was validated against histology[21] but quantitative studies are not well documented.

During glaucoma progression, the quantitative changes of the thickness of the NGI (structural data obtained with FD-OCT) appear to be similar to the quantitative loss in RGC counts (data obtained by counting Fluorogold-labeled RGCs). At 42 days of glaucoma there is a close correlation between loss of structural integrity (~30% loss of thickness) and cellular loss (~30% RGC death). Neuroprotection with mAb 1D7 results in the survival of approximately half the RGCs that would have died and the maintenance of approximately half of the NGI thickness that would have been lost. Thus, the use of FD-OCT to measure preservation of the retinal structure may be a predictive end point for neuroprotective efficacy in glaucoma.

In contrast, in optic nerve axotomy there is no parallel between RGC counts and retinal structure. After 7 and 14 days of axotomy, the rates of cell death were ~50% and ~90%, respectively,[3,7] but only 6% and 23% loss of thickness, respectively. The delay in loss of structure may be attributed to the acute death causing hypertrophic axonal swelling and thickening of the IPL[9] or to the fact that optic nerve axotomy is an extraretinal injury with a different pathologic course than glaucoma.[43]

The present report contributes to neurotrophic mechanisms underlying RGC death in glaucoma and ON axotomy and validates TrkB-selective agonists for neuroprotection and for maintenance of the retinal structure.

Acknowledgments

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