Neurotrophic Effect of a Novel TrkB Agonist on Retinal Ganglion Cells

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PURPOSE. Retinal ganglion cells (RGCs) die in glaucoma and virtually all optic neuropathies. Recently, novel tropomyosin-related kinase B (TrkB) monoclonal antibodies have been shown to activate TrkB receptors and exert neuroprotective and neurotrophic effects. In the present study, the authors examined the ability of one of them, 29D7, to elicit RGC survival and neurite growth both in culture and in vivo.

METHODS. RGCs from postnatal day (P)3 to P4 Sprague-Dawley rats were isolated by sequential immunopanning using a monoclonal antibody to Thy1. RGCs were cultured in serum-free defined medium in 96-well plates. RGC viability was assessed after 1 to 3 days by MTT assay. Activation of TrkB and downstream signaling molecules was confirmed by Western blot analysis. Intravitreal injections of 29D7 were performed after optic nerve axotomy, and subsequent RGC survival was quantified using β-III tubulin immunostaining. Regeneration was assessed using retrograde fluorogold tracing in an optic nerve-peripheral nerve graft model.

RESULTS. Similar to brain-derived neurotrophic factor (BDNF), the 29D7 antibody strongly promoted RGC survival and neurite growth in vitro compared with medium alone or control IgG. Forskolin, which weakly supported RGC survival on its own, potentiated the effect of 29D7. Intravitreal injection of 29D7 enhanced RGC survival but not regeneration in vivo 2 weeks after optic nerve injury.

CONCLUSIONS. Together, these findings demonstrate the potential for antibody-mediated TrkB agonism as a potential therapeutic approach to enhance RGC survival after optic nerve injury. Further studies are needed to elucidate the mechanistic differences between this TrkB agonist and BDNF.

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Materials and Methods

Animals

All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the University of Miami Institutional Animal Care and Use Committee. For in vitro experiments, litters of Sprague-Dawley (SD; Harlan Laboratories, Inc., Allen Park, MI) were used at postnatal day (P) 3. For in vivo experiments, adult female SD rats, each weighing 200 to 250 g, were used. All surgical procedures on rats were performed under general anesthesia using an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (7.5 mg/kg). Rats also received subcutaneous injection of buprenorphine (0.05 mg/kg; Bedford Laboratories) as postoperative analgesic. Eye ointment containing erythromycin was applied to protect the cornea.

Immunopanning of RGCs

RGCs from P3 to P4 SD rats were purified through sequential immunopanning to 99.5% purity, as previously described.15,16 RGCs were cultured on poly-D-lysine (PDL; 70 kDa, 10 μg/mL; Sigma, St. Louis,
MO) and laminin (1 μg/mL; Invitrogen, Carlsbad, CA) in neurobasal (NB) serum-free defined medium as described, containing insulin (5 μg/mL), sodium pyruvate (1 mM), l-glutamine (1 mM), triiodothyronine (T3; 40 ng/mL; Sigma), N-acetyl cysteine (NAC; 5 μg/mL; Sigma), and B27.16

Survival Assays

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) survival assays were performed as described.16 Briefly, MTT was dissolved in PBS at 5 mg/mL. This stock solution was added to the culture well (1:9) and incubated at 37°C for 1 hour. Viable cells with active mitochondria cleaved the tetrazolium ring into a visible dark blue formazan reaction product. The viable and dead cells in each well were counted by bright-field microscopy. All values are given as the mean ± SEM of at least three wells and at least three separate experiments. Results of representative experiments are shown.

Quantitation of Neurite Growth

RGCs were plated at 2000 cells/well in a 96-well plate in 150 μL serum-free medium in the presence of factors as described. After 1 to 3 days, the fluorogenic substrate calcine-AM (Sigma) was added to a final concentration of 1 mg/mL. After 1 hour at 37°C, fluorescent viable cells were visualized with a fluorescence microscope (Zeiss, Thornwood, NY). Three pictures were captured in similar positions around the center in each well; approximately 200 cells were counted in each well, and images were gathered with a charge-coupled device camera and analyzed with imaging processing, enhancement, and analysis software (Image-Pro 3DS 5.1; Media Cybernetics, Silver Spring, MD).

Western Blot Analysis

Approximately 3 × 10^5 purified RGCs were incubated in NB/0.02% BSA (NB control) or NB containing control IgG (10 μg/mL), 29D7 antibody (10 μg/mL), or BDNF (50 ng/mL) at 37°C for 2 hours. Cells were then collected, snap frozen, and stored at −80°C. Before Western blot analysis, cells were lysed in NP-40 buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40) containing protease inhibitor (Complete Mini; Roche) and phosphatase inhibitor cocktail (catalog no. 78428; Pierce, Rockford, IL). Protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules, CA). Samples (30 μg) were separated by SDS-PAGE in 10% Tris-glycine precast gels (NuSeP, Sydney, Australia) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). The membrane was incubated for 1 hour in blocking solution containing 3% BSA and 0.1% Tween-20, pH 7.6. This was followed by overnight incubation at 4°C in the blocking buffer containing a rabbit anti-phospho-Trk (1:500; catalog no. 9141; Cell Signaling, Danvers, MA), rabbit anti-phospho-Akt (1:1000; catalog no. 34-8400; Zymed Laboratories, South San Francisco, CA), or rabbit anti-phospho-ERK1/2 antibody (1:1000; no. E7028; Sigma). Subsequently, the labeled proteins were visualized by incubation with a horseradish peroxidase (HRP)-conjugated anti-goat or rabbit IgG (1:2500; Santa Cruz Biotechnology, Santa Cruz, CA) followed by development with a chemiluminescence substrate for HRP (Pierce). To determine the amounts of total TrkB, MAPK, or Akt present in each lane, the PVDF membranes were stripped of the antibodies in stripping buffer (Bio-Rad) for 5 minutes and incubated with a mouse anti-TrkB (1:500; catalog no. 610101; Becton Dickinson, Franklin Lakes, NJ), goat anti-ERK1 (1:1000; catalog no. sc-93-G; Santa Cruz Biotechnology), or rabbit anti-Akt antibody (1:1000; catalog no. 9272; Cell Signaling) and were visualized as described.

Intraorbital Optic Nerve Axotomy and Intravitreal Injection

The left optic nerve (ON) was exposed intraorbitally, and its dura was opened. ON was transected approximately 1.5 mm behind the globe. For animals receiving a peripheral nerve (PN) graft, a segment of PN graft was then sutured (10-0 suture; Ethicon, Somerville, NJ) onto the proximal stump of the cut ON, and the distal end was placed over the skull and secured with 6-0 suture to connective tissue. Care was taken to avoid damaging the blood supply to the retina. Intravitreal injections were performed just posterior to the pars plana with a pulled glass pipette connected to a 50-μL Hamilton syringe. Care was taken not to damage the lens. Rats with any significant postoperative complications (e.g., retinal ischemia, cataract) were excluded from further analysis. Animals were allocated to different experimental groups. One control group received no intravitreal injections; the other groups received injection of 5 μL PBS, control IgG (12 μg/μL), 29D7 antibody (16 μg/μL), or BDNF (5 μg/5 μL) at 3 days or injections at 3 and 10 days after ON axotomy.

Retrograde Labeling of Regenerating RGCs

The number of RGCs growing axons into the PN graft was determined 4 weeks after surgery. To retrogradely label regenerating RGCs, rats were anesthetized as described, the graft lying on the skull was exposed, and 0.2 μL of 4% fluorogold (FG; Fluorochrome, Denver, CO) was slowly injected into the distal end of the graft. Three days later, rats were deeply anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Retinas and PN grafts were removed and postfixed for 1 or 2 hours, respectively. After a wash, retinas were flat-mounted with the RGC layer uppermost. To determine the total number of FG-labeled RGCs, a grid was placed over each retina, and the number of FG-labeled RGCs was counted at each grid intersection. The sample points were equally distributed across the whole retina. Each sample field measured 0.235 mm × 0.235 mm, and 60 to 80 fields were sampled per retina.

Immunohistochemical Staining and Counting of Viable RGCs

Methods used to quantify RGCs after injury have limitations: retrograde label could be phagocytosed by microglia and other cells, producing inaccurate counting;17 counting axons in ON sections reflects axon count rather than cell survival; and staining RGCs with antibodies misses RGCs that downregulate certain markers before cell death.18-20 Several cell markers have been suggested to be RGC specific in the retina, such as Thy1, Brn3a,21 NeuN,20 and γ-synuclein.22 With these limitations in mind, we used βIII tubulin as a marker for surviving RGCs because it is an efficient and reliable method for selective
labeling of viable RGCs in retinal whole-mounts after ON injury. 23–27 To immunostain for βIII tubulin, retinas were blocked in 10% normal goat serum and 0.2% Triton X-100 for 1 hour, then incubated in the same medium with βIII tubulin antibody (1:500) overnight at 4°C. After further washes, retinas were incubated with Alexa 594-conjugated goat anti–mouse secondary antibody (1:400; Invitrogen) overnight at 4°C. The same sampling procedures described for counting FG+ (regenerating) RGCs were used to quantify the number of βIII tubulin-positive cells.

**Statistical Analysis**

Statistical analysis was performed using statistical software (GraphPad InStat version 3.06 for Windows; GraphPad Software, San Diego, CA). RGC numbers from different groups were analyzed by one-way analysis of variance (ANOVA). Dunnett’s post hoc test was used to compare mean values of experimental groups against the same control group; Bonferroni’s posttest was used to compare mean values for all groups.

**RESULTS**

**Effect of 29D7 on RGC Survival In Vitro**

To examine the survival effect of the TrkB agonist antibody 29D7, we cultured highly purified P3 RGCs on PDL and laminin in defined, serum-free medium with or without growth factors. 16 29D7 significantly increased RGC survival compared with control IgG at 1 day in culture. The adenylate cyclase activator forskolin, which showed a modest effect on RGC survival on its own, demonstrated an additive effect with either BDNF or 29D7, together significantly promoting RGC survival at 1 day to 3 days in culture (Fig. 1). Interestingly, 29D7 had a similar or better effect on survival than BDNF (Fig. 1). To further test whether the effect of 29D7 is dose dependent, we treated RGCs with varying concentrations of 29D7 (from 160 ng/mL to 0.5 ng/mL) in the presence of the cAMP analogue cyclopentylthio (CPT)-cAMP. We observed a dose-dependent rescue of RGCs as shown in Figure 2. Without CPT-cAMP, the effect of 29D7 reached plateau levels at approximately 10 ng/mL (Fig. 2A). In the presence of CPT-cAMP, the effect of 29D7 reached plateau levels at 0.1 ng/mL (approximately 10⁻⁹ M) and did not show any decreased ability to enhance RGC survival at concentrations even 100 times this level (Fig. 2B). Interestingly, the effect of BDNF in the presence of CPT-cAMP decreased after 10 ng/mL (approximately 10⁻⁹ M) (Fig. 2B). Thus, 29D7 strongly enhanced RGC survival in culture in a dose-dependent manner, and this effect was enhanced by cAMP elevation, consistent with prior observations on the ability of cAMP to enhance the RGC response to BDNF for survival and axon growth. 16, 28

**Effect of 29D7 on RGC Neurite Growth In Vitro**

Given that 29D7 can promote RGC survival in vitro and that BDNF supports both survival and axon growth of RGCs in...
we next evaluated the effect of 29D7 on RGC neurite growth. Primary RGC cultures were treated with control IgG, 29D7, or BDNF. After 1 to 3 days, RGCs were labeled with the vital dye calcein. All neurites longer than one cell body were counted and measured; 29D7 or BDNF alone significantly enhanced the percentage of surviving RGCs that extended neurites as well as the average neurite length per cell (Fig. 3). Combined with forskolin, the effect of either 29D7 or BDNF was modestly increased (Fig. 3). Therefore, 29D7 promoted neurite growth of cultured RGCs in a fashion similar to that of BDNF, and this effect was enhanced by pharmacologic elevation of cAMP levels.

Activation of PI3K/Akt and MAPK/ERK Pathways

29D7 can activate proximal and secondary signaling molecules downstream of TrkB receptors in cell lines. We next asked whether 29D7 similarly activates these pathways in purified, primary RGCs treated with NB (control) medium, control IgG (10 μg/mL), 29D7 (10 μg/mL), or BDNF (50 ng/mL). Note that 29D7 was previously shown to have no ability to activate TrkA or TrkC as, of course, is also true of BDNF. Western blot analysis revealed a significant increase in the activation of TrkB, PI3K/Akt, and MAPK/ERK pathways in response to 29D7 (Fig. 4). 29D7 and BDNF significantly increased TrkB phosphorylation (Fig. 4A) and ERK1/2 phosphorylation approximately twofold (Fig. 4B). More dramatically, 29D7 increased PI3K/Akt phosphorylation approximately fourfold over control levels, and BDNF increased PI3K/Akt phosphorylation more than five times that of control levels (Fig. 4C). Thus, at concentrations that elicit peak levels of RGC survival, 29D7 activates signaling pathways downstream of TrkB at levels similar to those of BDNF.
**Distribution of 29D7 Antibody after Intravitreal Injection**

In anticipation of testing the ability of 29D7 to enhance RGC survival and regeneration in vivo, we asked whether 29D7 localized to the retina after intravitreal injection. To determine the localization of 29D7 antibody after intravitreal injection, RGCs were retrogradely labeled from the superior colliculus with 4% FG 1 week before eye injection. We injected 3 μL mouse control IgG (10 μg), 29D7 antibody (10 μg), or BDNF (5 μg) into the eye. Rats were euthanatized after 1 or 2 days, their retinas were cryosectioned, and immunostaining against the injected murine antibodies was performed. One day after injection, strong fluorescence immunostaining for mouse IgG was found around the cytoplasm in the ganglion cell layer in animals that received 29D7 injection, whereas in animals that received control IgG injections, immunostaining against the injected murine antibodies was performed. One day after injection, strong fluorescence immunostaining for mouse IgG was found around the cytoplasm in the ganglion cell layer in animals that received 29D7 injection, whereas in animals that received control IgG injections, immunostaining against the injected murine antibodies was performed. One day after injection, strong fluorescence immunostaining for mouse IgG was found around the cytoplasm in the ganglion cell layer in animals that received 29D7 injection, whereas in animals that received control IgG injections, immunostaining against the injected murine antibodies was performed. One day after injection, strong fluorescence immunostaining for mouse IgG was found around the cytoplasm in the ganglion cell layer in animals that received 29D7 injection, whereas in animals that received control IgG injections, immunostaining against the injected murine antibodies was performed. One day after injection, strong fluorescence immunostaining for mouse IgG was found around the cytoplasm in the ganglion cell layer in animals that received 29D7 injection, whereas in animals that received control IgG injections, immunostaining against the injected murine antibodies was performed. One day after injection, strong fluorescence immunostaining for mouse IgG was found around the cytoplasm in the ganglion cell layer in animals that received 29D7 injection, whereas in animals that received control IgG injections, immunostaining against the injected murine antibodies was performed.

**Effect of 29D7 on RGC Survival In Vivo**

To assess the effect of 29D7 on RGC survival in vivo, we first tested a model of complete optic nerve transection. 29D7 (48 μg/3 μL), control IgG (36 μg/3 μL), or BDNF (5 μg/3 μL) was applied at 3 days after surgery. Animals were euthanatized 14 days after axotomy. The density of surviving RGCs was assessed in retinal whole-mounts using βIII tubulin staining. The number of surviving RGCs in animals with a single 29D7 injection was slightly increased but was not statistically significantly different from that in retinas that received an injection of control IgG, whereas BDNF significantly rescued RGCs after injury (Fig. 6A). We next tested whether injecting 29D7 or BDNF twice, at 3 and 10 days after injury, significantly enhanced RGC survival. We found that 29D7 significantly increased RGC survival compared with controls but to a lesser degree than in BDNF-treated retinas (Fig. 6B).

Next, we examined the effect of 29D7 on RGC axon regeneration in a peripheral nerve autograft model. Animals received three intravitreal injections—on days 3, 10, and 17—after optic nerve axotomy and peripheral autograft. At 4 weeks, there was no significant difference between control IgG (27.8 ± 7/mm²; n = 6) and 29D7 groups (31.4 ± 8/mm²; n = 4). Therefore, 29D7 promoted RGC survival after complete optic nerve transection but did not promote axon regeneration in the presence of a peripheral nerve graft.

**DISCUSSION**

RGCs and other adult CNS neurons die after axon injury, but it is not known why. Neurotrophic factors have been widely studied in this context, and several lines of research suggest that TrkB agonists are a promising approach. This study provides additional evidence for the potential therapeutic utility of TrkB agonists in peripheral nerve injury models, as well as in the retina. The results suggest that 29D7, a TrkB agonist, may promote RGC survival after complete optic nerve transection but may not promote axon regeneration in the presence of a peripheral nerve graft.

**Figure 5.** Retinal penetration and activity of 29D7 after intravitreal injection. Immunofluorescence images of retinal cryosections. 29D7 or mouse control IgG (10 μg in 3 μL) was injected into the vitreous of adult rats. At 1 and 2 days later, rats were euthanatized, and immunostaining against mouse IgG was performed. A peak in immunofluorescence was seen at 48 hours for both the control antibody and 29D7. *Insets*: colocalization of FG and immunostaining. Scale bar, 50 μm (125 μm in insets).
BDNF binds to receptors, including TrkB and the low-affinity receptor p75NTR. The TrkB receptor is expressed by RGCs, but its expression decreases after optic nerve axotomy, and p75NTR is expressed in developing RGCs and may play a role in the developmental cell death of RGCs. In the adult retina it is expressed in Müller cell processes but may also be expressed in adult RGCs in vivo. Its expression may be elevated under stress, and inhibition of p75NTR is neuroprotective to RGCs in vivo, though not necessarily in a cell-autonomous manner. We saw effects of 29D7 both in early postnatal RGCs in vitro and in adult RGCs in vivo. It is possible that as adult RGCs decrease their trophic responsiveness after axon injury, delivery of 29D7 could be more effective with cAMP analogs.

Although BDNF has demonstrated potential for the treatment of a number of neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease, it did not show efficacy in ALS or in a pilot study of peripheral neuropathy, when tested in humans. This lack of effect in human trials may be the result of BDNF binding to p75NTR. Indeed, the role of p75NTR is more likely to contribute to cell death for a mini-neurotrophin that activates Trk receptors in the absence of p75NTR binding showed a better effect on neurite growth in the presence of a myelin inhibitor from cerebellar neurons than did BDNF. Inhibition of p75NTR in Müller glia could potentiate the neuroprotective effect of NGF.

Thus, there is motivation to study more highly selective receptor targeting approaches (e.g., to take advantage of an antibody’s specific stimulation of Trk receptors without activating any potentially detrimental signaling from p75NTR). Here we examined one such agonist antibody, 29D7, for its ability to stimulate the survival of RGCs in vitro and in vivo. We show that this antibody can stimulate RGC survival in vitro and in vivo, consistent with prior work demonstrating neuroprotective and neurotrophic effects on cultured neurons in vitro. At very low cell densities, forskolin alone has been shown to have very small or no effect on RGC axon growth. In culture, stimulation with 29D7 led to the activation of downstream signaling pathways, including ERK1/2 and PI3K/AKT, two pathways we have previously shown play a necessary role in RGC survival and axon growth in response to BDNF. Unlike a previous report that found a transient enhancement in BDNF-induced MAP kinase phosphorylation only at 0.5 and 1 hour but not at 2 hours, we observed a strong effect at 2 hours. This could be explained by the younger postnatal RGC ages used here (P3). Thus, 29D7 shares many of the neuroprotective properties of BDNF.

Interestingly, however, we found a number of differences between stimulation with BDNF and 29D7. First, the dose-response curves between BDNF and 29D7 differed in that BDNF reached a higher peak effect on survival, but then its efficacy declined at higher concentrations. At very short time periods, trkB activation is directly proportional to BDNF concentration, but ERK activation generally peaks at much lower concentrations. The highest concentrations of BDNF may demonstrate a decrease in efficacy for survival, possibly because of failure to dimerize TrkB receptors or because of activation of lower affinity death receptors such as p75NTR. In contrast, 29D7 did not show a similar decline in efficacy at higher concentrations, possibly because 29D7 is unable to bind to p75NTR or because 29D7 may not depend on dimerization to induce TrkB activation. Specific trkA agonism is also neuroprotective for RGCs, consistent with these data.

Second, 29D7 consistently demonstrated a weaker ability to elicit survival, axon growth, and phosphorylation of TrkB and downstream signaling molecules. In particular, the effect of...
29D7 in vivo depended heavily on the dosage and injection times, suggesting different mechanisms of metabolism or different effects on TrkB or p75NTR activation in vivo. The molecular weight of 29D7 is much higher than that of BDNF (150 kDa vs. 13 kDa). This may limit the ability of 29D7 to diffuse through the inner limiting membrane and access RGCs. This was unlikely, however, because we observed strong immunostaining against mouse IgG in the ganglion cell layer 1 to 2 days after intravitreal injection of 29D7 antibody (Fig. 5), and other antibodies can penetrate the retinal tissues.60 One of the receptors for immunoglobulin Fc domains, the neonatal Fc receptor (FcRN), modulates IgG transport across the placenta.61 In the eye, FcRN is expressed at the blood-retinal barrier and possibly functions in transporting IgG from the retina to the systemic circulation62 and may contribute to the rapid antibody clearance seen here and by others.63 Additionally, after ON injury, activated microglia may express Fc receptors as well, such that exogenous 29D7 may become engulfed by microglia.64 Both these factors may contribute to the weaker effect seen with 29D7 treatment compared with BDNF. Testing this hypothesis with Fab fragment or single-chain antibody would address these possibilities. The weaker effect of 29D7 could also be the result of differences in endosome signaling or transport after binding TrkB, consistent with our finding differences in ERK1/2 and PI3K phosphorylation. We did not, however, perform a dose-response study to look at the supra-plateau concentrations of BDNF compared with 29D7; we hypothesize, based on their dose-response curves, that BDNF efficacy may fall off, whereas 29D7 efficacy may be retained. This could serve a potential clinical advantage if, for example, high concentrations of 29D7 were to be delivered to enhance pharmacokinetics (e.g., drug persistence) without sacrificing pharmacodynamics (e.g., initial drug efficacy).

Taken together, these findings demonstrate the potential for antibody-mediated TrkB agonism as a potential therapeutic approach for RGCs. In addition, these reagents may allow further dissection of neurotrophic signaling pathways, with the ultimate goal of enhancing neuronal survival and axon growth after optic nerve injury or in degenerative diseases such as glaucoma and ischemic optic neuropathy.

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References


