Assessment of Cone Survival in Response to CNTF, GDNF, and VEGF<sub>165b</sub> in a Novel Ex Vivo Model of End-Stage Retinitis Pigmentosa

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**PURPOSE.** To develop a robust ex vivo model for evaluating cone survival in end-stage retinitis pigmentosa (RP) and apply this to quantify the effects of putative neuroprotective compounds.

**METHODS.** Rhodopsin knockout mice were crossed with OPN1-GFP reporter mice so that GFP-positive cones could be identified against the background of a rod-specific degeneration. Retinal explants were harvested from 10-week-old mice and maintained in organotypic culture. Ciliary neurotrophic factor (CNTF), glial cell–derived neurotrophic factor (GDNF), or vascular endothelial growth factor 165b (VEGF<sub>165b</sub>) was administered daily to treatment groups at three doses (200 ng/mL, 100 ng/mL, or 50 ng/mL; n = 5 explants per group). Fluorescence microscopy was performed on days 1, 3, 5, 7, 9, and 12 to document the number of GFP-expressing cones.

**RESULTS.** Cone survival could be assessed reliably and reproducibly in this model, and cone degeneration was significantly greater in the absence of rods, in keeping with clinical observations of RP. Daily administration of 200 ng/mL CNTF led to significantly increased cone survival compared with sham-treated controls. The effect was dose dependent; 100 ng/mL CNTF reduced cone loss but to a lesser extent, and 200 ng/mL GDNF showed significant protection against cone loss at later time points (day 9–12) but was much less effective than CNTF at all doses. VEGF<sub>165b</sub> showed no neuroprotective effect in this model at any dose.

**CONCLUSIONS.** This model allows precise quantification of the neuroprotective effects of various compounds on cone survival and may therefore provide a robust method of screening neuroprotective compounds before application in vivo. (Invest Ophthalmol Vis Sci. 2011;52:7340–7346) DOI:10.1167/iovs.11-7996

In retinitis pigmentosa (RP), the loss of rod photoreceptors is invariably followed by the loss of cones, even when the degeneration is caused by genes that are expressed only in rods. Cone degeneration in such instances is believed to occur because of the loss of sustaining proteins or other factors that are released by rods. Identifying a means of sustaining cones in the absence of rods is a critically important strategy in developing treatments for RP because humans are highly dependent on cone-mediated visual tasks for the activities of daily living. Many patients with RP go to the physician after most of their rod photoreceptors have degenerated, presenting significant challenges for rod gene replacement. Consequently, potential treatment strategies must necessarily focus either on photoreceptor replacement or on attenuating the loss of cones at a stage when a reasonable number of cones remain.

Recently, the administration of neuroprotective factors, or growth factors, has been described for the preservation of retinal ganglion cells in experimental models of glaucoma and in human clinical trials for the treatment of geographic atrophy in wet age-related macular degeneration (AMD). In particular, ciliary neurotrophic factor (CNTF) has been widely studied. CNTF is a type I cytokine that binds a specific CNTF receptor alpha (CNTFRα) and two general receptors, LIFRβ and gp130, resulting in the activation of Jak/STAT and MAPK pathways. Activation of STAT3 and ras-MAPK by CNTF has been associated with neuroprotection of ganglion cells in response to a wide variety of stress stimuli, including ocular hypotension, autoimmune optic neuritis, and optic nerve crush and has even been shown to promote axonal regeneration. Consequently, growth factors may provide a viable treatment for the preservation of cone photoreceptors secondary to rod loss in RP.

The neuroprotective effects of growth factors are typically determined in vivo, where their solubility and low molecular weight allow for intravitreal administration and subsequent diffusion through the vitreous into the neural retina. Testing requires either direct injection of the protein or expression of the protein from a vector or a slow release device. Although the former approach is the simpler, the short half-life of many neuroprotective compounds necessitates repetitive protein injections, with potential associated complications, including endophthalmitis. The latter approach allows for administration of a single dose but requires the production of either a viral vector or an encapsulated slow-release device, both of which are time consuming and often too expensive to be applied for a high-throughput screening approach. Although rod neuroprotection has previously been examined in vitro using dissociated photoreceptor cultures, disruption of the retinal morphology makes such methods unsuitable for studying the neuroprotection of cones secondary to advanced rod degeneration.

Secondary cone loss as seen in RP patients has also been observed in mouse strains with rod degeneration, such as the rhodopsin knockout mouse (Rho<sup>−/−</sup>). Cell loss in mice with retinal degeneration can be examined through the use of a fluorescent reporter by which intrinsic fluorescence acts as a
surrogate marker of cell viability. Herein, we used the OPN1LW-EGFP reporter mouse, which has fluorescent cone photoreceptors because of the expression of enhanced green fluorescent protein (EGFP) under the control of the human OPN1 long-wave promoter (OPN1LW), crossed to a rhodopsin knockout mouse, allowing the study of cone photoreceptor survival against a background of progressive rod degeneration.

In the present study, we adapted a previously described organotypic retinal culture system to maintain retinal explants from rhodopsin knockout OPN1LW-EGFP animals. In this novel ex vivo model of RP, cone neuroprotection is assessed longitudinally through repetitive imaging of retinal explants, with the survival of intrinsically fluorescent cones quantified. This model allows dose-controlled administration of neuroprotective factors and robust assessment of their effect on cone survival without recourse to in vivo administration. Herein, we validate our model by evaluating the neuroprotective effects on cone survival of two growth factors, CNTF and glial cell–derived neurotrophic factor (GDNF). Additionally, we explore the neuroprotective properties of the vascular endothelial growth factor 165b-isofrom (VEGF165b). This VEGF isoform has proved to be effective in the treatment of animal models of choroidal neovascularization and may prevent retinal and endothelial cell death.

METHODS

Mice

All animal experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and UK Home Office guidelines. Rhodopsin knockout mice (C57B/6.129 Rho+/−H11001, referred to herein as Rho+/−) have been described previously and were a kind gift obtained under material transfer agreement from Jane Farrar (Trinity College, Dublin, Ireland). OPN1LW-EGFP mice (C57BL/6/TgOPN1LW-EGFP85933Hue, referred to herein as B6TgOPN1LW-EGFP+/-) have been described previously and were obtained under MTA from the Mutant Mouse Regional Resource Centre, National Institutes of Health (000043-MU Opn1.gfp). Founders of this colony were obtained with the kind help of Rachel Pearson (University College London Institute of Ophthalmology, London, UK). Mice that express EGFP in cone photoreceptors and have a primary rod-specific degeneration (Rho+/−TgOPN1LW-EGFP+/−) were created through crossing of B6TgOPN1LW-EGFP+/- mice (homozygous for the OPN1LW-EGFP transgene insertion) with Rho+/− (homozygous for targeted rhodopsin knockout), followed by backcrossing of F1 progeny (Rho+/−TgOPN1LW-EGFP+/−) to the parental Rho+/− line. Mice were genotyped at weaning by PCR. Breeding pairs were established to maintain the EGFP transgene in a heterozygous state.

Retinal Explant Culture

Eyes were enucleated from B6TgOPN1LW-EGFP+/− and Rho+/−TgOPN1LW-EGFP+/− mice at postnatal day 70 and were transferred to room temperature 0.01% phosphate-buffered saline (PBS), after which the anterior segment and lens were removed. After isolation from the retinal pigment epithelium (RPE), the whole retina was transferred to complete culture media consisting of Neurobasal A, L-glutamine (0.08 mM), penicillin (100 U/mL), streptomycin (100 U/mL), B27 supplement (2%), and N2 supplement (1%, all Invitrogen Ltd., Paisley, UK). Using an operating microscope and Vannas scissors, retinas were divided into several 1- to 2-mm² pieces without bias toward orientation. Using a cutoff 5-ml pipette, to eliminate crush injury associated with forceps use, retinal pieces were transferred photoreceptor side down into individual 24-well plates containing an organotypic culture insert (cat. no. 353095; BD Falcon, Bedford, MA) and 700 μL complete media. Explants from Rho+/−TgOPN1LW-EGFP+/− mice were randomly assigned to treatment groups (n = 5 per group) and cultured at 54°C in a 5% CO2 environment.

RESULTS

Retinal Explants Can Be Maintained Long Term in Organotypic Culture and Individual Cone Photoreceptors Can Be Visualized

An ex vivo organotypic culture system was developed to explore the neuroprotection of cone photoreceptors after the administration of soluble growth factors. As a model of RP, retinal explants were harvested from Rho+/−TgOPN1LW-EGFP+/− mice, which express EGFP in cone photoreceptors and undergo primary rod degeneration leading to progressive thinning of the outer nuclear layer and the secondary loss of cones (Figs. 1A, 1B). However, evaluation of cone neuroprotection in such organotypic culture requires, first, that retinal explants be maintained over time so that treatment effects might be observed and, second, that the ability to identify individual fluorescent cone photoreceptors is maintained throughout, thus enabling quantification at repeated time points.

We initially used explants from B6TgOPN1LW-EGFP+/- mice to confirm previous reports that retinal explants could be maintained short term (<7 days) in serum-free organotypic culture. A comparison of media constituents revealed that both B27 and N2 supplements were necessary for the short-term survival of explants, with significantly reduced cone numbers observed by day 6 in the absence of one or both supplements (P < 0.001, two-way ANOVA) (Fig. 2A). Using optimized media in our organotypic culture system, it was possible to maintain explants from B6TgOPN1LW-EGFP+/- mice for more than 4 weeks (Fig. 2B). We observed a gradual decline in cone photoreceptor numbers, with significant reduction by day 9 (P < 0.05). Overall, the reduction in photoreceptor numbers was highly significant compared with day 1 (P < 0.001, one-way ANOVA). However, despite a reduction of ~50% in cone numbers by day
The observed explant survival was considerably longer than that of many other published reports.

Significantly, it was possible to identify individual cone photoreceptors and to follow their survival longitudinally in our culture system. The organotypic culture system provides good oxygenation of layered tissues, and the culture inserts used here permitted good visualization of intrinsically fluorescent cells through the membrane (Figs. 1C-E). We were able to identify distinct groups of cones over 12 days in explants from B6.Tg(OPN1LW-EGFP)11001/11002 mice (Fig. 2C), suggesting that the organotypic culture of retinas with intrinsic fluorescence allows for the long-term analysis of photoreceptor survival and provides an ideal ex vivo system with which to evaluate the neuroprotective effects of various soluble compounds.

CNTF Sustains Cone Photoreceptors in a Dose-Dependent Manner in Organotypic Culture

Having confirmed that retinal explants can be maintained long term in organotypic culture, we examined the neuroprotective effect of CNTF, which has been described previously to protect photoreceptors and ganglion cells in vivo.

Explants taken from B6.Tg(OPN1LW-EGFP)11001/11002 animals served as positive controls and showed the survival of cones on a wild-type background without rod photoreceptor degeneration. Cone photoreceptor survival was significantly lower in PBS-treated Rho−/− Tg(OPN1LW-EGFP)11001/11002 explants at all time points compared with positive controls (P < 0.001, two-way ANOVA) (Fig. 3A), confirming that the presence of rod photoreceptors is of great importance for the survival of cone photoreceptors. Daily administration of 200 ng/mL CNTF to Rho−/− Tg(OPN1LW-EGFP)11001/11002 explants led to significantly higher levels of cone survival from day 5 onward compared with the PBS-treated group (P > 0.001; two-way ANOVA treatment and age as factors), though cone survival was still significantly reduced compared with B6.Tg(OPN1LW-EGFP)11001/11002 explants at these time points (P < 0.001, two-way ANOVA). In addition to photoreceptor preservation, a reduction in autofluorescence was observed compared with PBS-treated explants (Fig. 3A). In our organotypic culture system, an increase in autofluorescence usually preceded tissue death and rapid cone loss and served as an indicator of the tissues’ general health. Administration of 100 ng/mL CNTF also led to the increased survival of cone photoreceptors compared with the PBS-treated group, with significant preservation apparent by day 5 (P < 0.01). Preservation was maintained and was highly significant by day 12 (P < 0.001) compared with negative controls (two-way ANOVA); 50 ng/mL CNTF did not have a significant protective effect over PBS treatment (Fig. 3A).

These results indicate, first, that CNTF has a dose-dependent, neuroprotective effect on cones, supporting the potential use of CNTF for the treatment of secondary cone loss in RP and, second, that the effects of neuroprotective compounds can be reliably and quantitatively assessed using our ex vivo model.

GDNF Is Protective of Cone Photoreceptors Only at High Doses

After the successful attenuation of cone degeneration by CNTF, alternative neuroprotective factors were evaluated, in particular GDNF, which has been described previously as having greater neuroprotective effects on photoreceptors than CNTF. Recombinant CNTF and GDNF proteins have similar molecular masses (22.9 kDa and 23.7 kDa, respectively); consequently, the doses administered here equate to comparable molar concentrations of...
protein. This allows us to draw direct comparisons regarding the neuroprotective effects of these two growth factors.

Administration of 200 ng/mL GDNF resulted in observable neuroprotective effects only at late time points (day 9, *P* < 0.01; day 12, *P* < 0.001) compared with the PBS-treated group (two-way ANOVA) (Fig. 3B). Treatment with 100 ng/mL GDNF showed a small but significant preservation of cone photoreceptors compared with PBS treatment at day 12 (*P* < 0.05, two-way ANOVA), whereas 50 ng/mL appeared to have no impact on cone survival. As with CNTF treatment, all groups had significantly reduced cone photoreceptor numbers compared with B6TgOPN1LW-EGFP+/−-positive controls (*P* < 0.001, two-way ANOVA) (Fig. 3B). However, autofluorescence was noticeably reduced in the high-dose GDNF treatment group compared with PBS-treated controls, indicating that GDNF administration does have a positive impact on explant health (Fig. 3B).

These results indicate that GDNF has a limited, dose-dependent, neuroprotective effect, but, even when administered at high dose (200 ng/mL), GDNF is not as effective as CNTF at attenuating secondary cone degeneration.

**VEGF<sub>165b</sub> Isoform Does Not Exhibit a Neuroprotective Effect Ex Vivo**

VEGF<sub>165b</sub>, a C-terminal splice variant of VEGF that has a molecular mass of 19.2 kDa, has previously been demonstrated to reduce neovascularization in animal models of oxygen-induced retinopathy<sup>24</sup> and to have protective effects on retinal epithelial and endothelial cells in culture.<sup>18,25</sup> Our results, however, indicate that VEGF<sub>165b</sub> does not provide neuroprotection to cone photoreceptors because there was no attenuation of cone loss at any dose or time point compared with PBS treatment (*P* > 0.05, two-way ANOVA) (Fig. 3C). Additionally, the level of autofluorescence was not noticeably reduced by the administration of VEGF<sub>165b</sub> (Fig. 3C). These results do not support the use of VEGF<sub>165b</sub> as a neuroprotective factor for the treatment of rod-cone dystrophy.

**DISCUSSION**

Herein, we evaluated the neuroprotective effects of three growth factors on the attenuation of cone loss secondary to rod
degeneration. A novel organotypic culture model was developed wherein cone survival was assessed ex vivo through the repetitive imaging of retinal explants with intrinsically fluorescent cone photoreceptors. Our primary finding was that CNTF has potent neuroprotective properties and is a potential compound to be used in the prevention of central vision loss in RP.

The model described is a robust tool for the high-throughput screening of neuroprotective compound and provides an

**Figure 3.** Survival of Rho<sup>−/−</sup> TgOPNL1LW-EGFP<sup>+/−</sup> explants in response to treatment at three different doses (200 ng/mL, 100 ng/mL, and 50 ng/mL) with three growth factors CNTF (A), GDNF (B), and VEGF<sub>165b</sub> (C). Note the increase in autofluorescence in untreated controls, indicating poor explant health. ns, not significant. *P < 0.05; **P < 0.01; ***P < 0.001; n = 5; two-way ANOVA treatment and age as factors.
alternative to costly in vivo assays, where repetitive ocular administration has inherent welfare concerns. It may have wider applications than the study of photoreceptor survival in RP and could be adapted to the study of other cell types for which fluorescent reporter lines exist. For example, the study of ganglion cell survival in response to oxidative stress may be conducted using retinal explants from the OPN4-GFP transgenic mouse in which GFP expression is restricted to retinal ganglion cells under the control of a cell-specific promoter. Our results show that CNTF administration leads to a robust dose-dependent protection of cone photoreceptors. To a lesser extent, GDNF also demonstrated neuroprotective properties, though these were only observed at high doses when administered in our ex vivo model. Interestingly, this finding is contradictory to those of a previous study that described GDNF as having the greater neuroprotective effect. However, unlike the in vivo expression of protein whereby levels of protein expression can be extremely variable, dosing in the model system we describe is precisely controlled. Consequently, the effects observed here may more faithfully reflect the absolute neuroprotective properties of these two compounds.

The neuroprotective effects of CNTF are thought to be mediated through Müller cell activation, resulting in STAT3 and ras-MAPK signaling after interaction with CNTFRA. However, in the vertebrate retina, CNTFRA expression is restricted to inner retinal neurons, particularly ganglion cells, and to rod outer segments, with no evidence of CNTFRA expression in photoreceptor cell bodies. Müller cells are also thought not to express CNTFRA and not to be directly affected by CNTF administration. However, in neurons, CNTFRA is anchored to the membrane by a unique glycosylphosphatidylinositol (GPI) linkage that, under certain physiological conditions, is degraded, allowing CNTFRA to be released from the membrane and solubilized. In the retina, this release occurs primarily from amacrine and horizontal cells. Soluble CNTFRA is able to bind free CNTF and to signal through the LIFR/ gp130 heterodimer, resulting in Müller cell activation mediated by Jak/STAT3 induction. Soluble CNTFRA does not appear to interact with photoreceptors directly, with no STAT3 or ras-MAPK activation having been reported previously. In contrast, Müller cells do express receptors for GDNF, namely tyrosine kinase Ret and growth factor receptor α-1, and downstream signaling after receptor binding likely occurs through a Jak/STAT or MAPK/ERK pathway similar to that of CNTF.

Activated or reactive astrocytes have been shown to play an essential role in neuronal survival in response to injury, with their ablation resulting in rapid neuronal death after injury in the adult CNS. One primary mechanism of activated glial cell-mediated neuroprotection is thought to involve the reduction of glutamate-induced cytotoxicity. CNTF has previously been shown to promote glial uptake of extracellular glutamate through the upregulation of glutamate transporters. However, GDNF is thought not to reduce levels of extracellular glutamate, as shown in the null mouse model of RP, perhaps contributing to the greater neuroprotective effects of CNTF in our ex vivo culture model. Müller activation is also noted to alter the expression of various neurotrophic factors, including CNTF, GDNF, basic fibroblast growth factor (bFGF), and nerve growth factor (NGF), which will additionally contribute toward neuroregeneration. Because of the presence of Müller cells in our retinal explants and the presence of glutamate in the culture media, the neuroprotective mechanisms occurring in this ex vivo culture system are likely to be similar to those in vivo as described. However, cone survival in this organ culture system will invariably be affected by additional stresses not normally associated with RP, particularly the trauma of the tissue dissection. Such stresses likely contribute to the slow degeneration of cones found in positive control explants.

VEGF antagonists have become the gold standard treatment for wet AMD, in which they limit angiogenesis. However, endogenous VEGF is required for proper visual function and Müller cell survival and is a critical neurotrophic agent in response to ischemic injury. Consequently, long-term inhibition of VEGF may negatively affect neuronal survival. Recently, C-terminus splice variants (b-isomers) have been described to be antiangiogenic and potentially neuroprotective. However, we observed no neuroprotective effects in our ex vivo model, contraindicating the use of VEGF165b for the treatment of cone loss in RP. Reassuringly, however, no acceleration of cone loss was seen at the doses tested, which is encouraging in the context of potential clinical applications for VEGF165b, for inhibiting angiogenesis in AMD, where foveal cones may be stressed.

In conclusion we have established a novel ex vivo model of RP whereby the neuroprotective effects of various compounds can be assessed longitudinally through the quantification of intrinsically fluorescent cone photoreceptors. Using this model, we demonstrated that CNTF and, to a lesser extent, GDNF were effective at attenuating the secondary loss of cones. This ex vivo model is widely applicable and could be adapted for studying the survival of any retinal cell providing a fluorescent reporter line exists.

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