Neurotrophic Factors Minimize the Retinal Toxicity of Verteporfin Photodynamic Therapy

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PURPOSE. A prior study showed that brain-derived neurotrophic factor (BDNF) rescues photoreceptors from collateral retinal damage caused by photodynamic therapy (PDT). This study was conducted to determine whether cilary neurotrophic factor (CNTF), a combination of BDNF and CNTF, or pigment epithelial cell-derived growth factor (PEDF) might protect photoreceptors and retinal function more effectively than BDNF. Also investigated was whether protection would be observed after a second round of PDT with adjunctive BDNF treatment.

METHODS. Normal rats received intravitreal injections of BDNF, CNTF, a combination of BDNF and CNTF, or PEDF in one eye and PBS in the other 2 days before PDT. Retinal function and photoreceptor survival were assessed with multifocal ERG (mfERG) and histology 1 week after PDT. Another group of rats received two courses of PDT 3 months apart, with injection of BDNF 2 days before each treatment.

RESULTS. All factors significantly increased photoreceptor survival. The combination of BDNF and CNTF rescued more photoreceptors than either factor alone. Only BDNF improved retinal function 1 week after PDT, with CNTF and the combination of BDNF and CNTF reducing mfERG responses. BDNF injection before a second round of PDT improved mfERG responses and retinal structure.

CONCLUSIONS. BDNF is the most effective single factor among those tested for neuroprotection and improvement of retinal function after PDT, although a combination of BDNF and CNTF rescues more photoreceptors. Adjunctive treatment with BDNF also protects retinal structure and function through two rounds of PDT, suggesting its potential value for patients who require multiple treatments. (Invest Ophthalmol Vis Sci. 2007; 48:430–437) DOI:10.1167/iovs.06-0690

Photodynamic therapy (PDT) has been used widely for the treatment of neovascular age-related macular degeneration (AMD), the leading cause of blindness among elderly patients in the United States and Europe.¹–³ PDT uses verteporfin, a photosensitizing dye that releases oxygen radicals when activated by a low-energy laser, to close the pathologic blood vessels of choroidal neovascularization (CNV). However, PDT is not absolutely selective for CNV and damages the overlying retina in animal models.¹–⁹ Retinal toxicity may explain the common visual disturbances and occasional severe vision loss experienced by patients after PDT.¹⁰–¹⁵ Multifocal (mf) ERG studies of patients have revealed depression of retinal function after PDT, which gradually recovers.¹⁴,¹⁵ For those patients who require repeated treatment at 3-month intervals,¹⁶ cumulative toxicity could contribute to disappointing visual outcomes.¹¹ In animal models, collateral damage to normal retina is cumulative with repeated treatment.¹²,¹³

In a previous study, we asked whether the neuroprotective agent, brain-derived neurotrophic factor (BDNF), could reduce the retinal toxicity of PDT in a rat model.¹⁷ We found that pretreatment with BDNF significantly increased the number of photoreceptors surviving 1 week after PDT. Using mfERG as an accepted surrogate measure of local retinal function, we found that BDNF also improved function in the PDT-treated area. Although promising, these initial findings raised further questions that must be answered before adjunctive neuroprotective treatment can be considered for clinical use. Is neuroprotection effective in the long term? Can BDNF protect the retina through multiple courses of PDT?

We also wondered whether other neuroprotective factors, or combinations of factors, might be more effective than BDNF. Ciliary neurotrophic factor (CNTF) protects photoreceptors in several animal models of inherited retinal degeneration¹⁷–²² and in the setting of constant light toxicity.¹⁸,²⁸ These findings have prompted a clinical trial of CNTF in patients with retinitis pigmentosa.²⁹ A potential drawback of CNTF therapy is depression of retinal function measured by ERG,²³,²⁴,²⁶ but some data indicate that this effect is transient²⁵ (Luthert PJ, personal communication, 2001; Wen R et al. IOVS 2004;45:ARVO E-Abstract 785; McGill TJ et al. IOVS 2006;47: ARVO E-Abstract 4815). Pigment epithelial cell-derived growth factor (PEDF) is a potent anti-angiogenic factor,³⁰–³¹ that also protects photoreceptors from genetic and environmental insults³²–³⁶ and has been studied as a potential treatment for CNV in animal models³⁴,³⁷–³⁹ and in a clinical trial.⁴⁰ Its combination of neuroprotective and antiangiogenic activities suggests that PEDF could be a particularly valuable adjunct to PDT³⁶ (Young TA et al. IOVS 2004;45:ARVO E-Abstract 2231).

In this study, a combination of BDNF and CNTF protected more photoreceptors from the retinal toxicity of PDT than did BDNF alone, whereas CNTF alone and PEDF were less effective than BDNF. Only BDNF improved retinal function, as measured by mfERG. We observed significant recovery of retinal function.

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in PBS-injected eyes during a 3-month period after PDT, suggesting that retinal function can improve even after severe damage to photoreceptors. When administered before two successive rounds of PDT, BDNF protected retinal function and structure.

METHODS

Animals, Injections, and Photodynamic Therapy
All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the University of California San Francisco Committee on Animal Research. Brown-Norway rats 4 to 6 months of age were maintained in a 12:12-hour light–dark cycle at in-cage illuminance of less than 150 lux. Anesthesia, pupillary dilation, and intravitreal injections were performed as described previously. Doses of neurotrophic factors injected were 4 μg BDNF (2 mg/mL in PBS; gift of Regeneron Pharmaceuticals, Tarrytown, NY), 4 μg CNTF (2 mg/mL in PBS; R&D Systems, Minneapolis, MN), a combination of 4 μg BDNF and 4 μg CNTF (each at a final concentration of 1 mg/mL in PBS), or 10 μg PEDF (5 mg/mL in water; BioProducts MD, Middletown, MD). All factors were injected in a volume of 2 μL, except for the combination of BDNF and CNTF, which was injected in a volume of 4 μL. Each animal received an injection of one of these factors (or the combination) in one eye and an equivalent volume of PBS in the other. Animals were randomized with respect to which eye received the neurotrophic factor(s). Eyes were checked after injection for vitreous hemorrhage, cataract, or other complications. Animals with complications were not treated or analyzed further. All subsequent experiments were performed and evaluated by experimenters who were masked to the factor-injected eye.

PDT was performed as described, 2 days after intraocular injections. A dose of 6 mg/m² verteporfin and a laser fluence of 10 J/cm² were used for all animals. The right eye was treated beginning 3 minutes after verteporfin injection, and the left eye at 4 minutes. Some animals received a second round of BDNF and PBS injections 3 months after the first, followed 2 days later by PDT. In these experiments, each eye received the same injection (BDNF or PBS) before both rounds of PDT.

Electroretinography
Multifocal ERGs were recorded and analyzed as described, except as follows. The 16 traces with the lowest root mean square (RMS) values in each array were averaged. Our 3.0-mm PDT laser spot corresponded to 16 to 18 hexagons in the 61-hexagon stimulus array. In rare cases in which the 16 traces with the lowest RMS values included a trace that was not contiguous with the PDT-treated area, this trace was excluded and the trace with the next lowest RMS value was used instead. The trace representing the optic nerve head (positioned in the center of the third row from the bottom in all recordings) and those inferior or immediately adjacent to it were excluded from analysis.

Histology
Tissue processing and localization of PDT-treated areas in histologic sections were performed as described. To ensure that the vertical meridian of the PDT-treated area (its greatest diameter) was analyzed, sections including both the optic nerve head and a diode laser burn (placed immediately before perfusion to mark the superior border) were used to quantify photoreceptor survival. The entire PDT-treated area was photographed in histologic sections. The length of this area was measured, and all photoreceptor nuclei within it were counted. Surviving photoreceptors were quantified as the number of nuclei per 100-μm length of the treated area. We used this method because the disruptions of retinal architecture observed 3 months after PDT, or after a second round of PDT (Fig. 6), made it difficult to measure reproducibly the outer nuclear layer (ONL) area or to count rows of photoreceptor nuclei, as was done in our previous study. We reanalyzed our histologic sections from the earlier study according to the method used in the current one, to enable comparison with new data. All methods of analysis yielded essentially identical results for these sections (data not shown). Rosettes were identified as circular formations of photoreceptor nuclei completely enclosing remnants of inner and outer segments (examples in Fig. 6). In some cases, combined inner and outer segment length was determined in rosettes or in scalloped regions of ONL (e.g., Fig. 3C), by measuring the length from the outer limiting membrane to the point where the outer segments met either the RPE, a macrophage that was often present, or a mass of debris membranes containing many macrophages. Measurements were made at the 3, 6, and 9 o'clock positions in each of five rosettes or scalloped regions in a given retina. For all experiments, interocular comparisons between factor- and PBS-injected eyes used Student’s paired, two-tailed t-test.

RESULTS

Effects of Different Neurotrophic Factors on Retinal Function and Photoreceptor Survival after PDT
mfERG responses from untreated rats were largely uniform except for a depression at the optic nerve head (Fig. 1A). As we demonstrated previously, PDT caused a localized depression in mfERG responses 1 week after treatment, corresponding to the treated area (Fig. 1B). Adjunctive treatment with BDNF increased responses in the PDT-treated area at this time point (Figs. 1E, 2). We asked whether pretreatment with other neurotrophic factors could also ameliorate the focal depression of mfERG responses observed 1 week after PDT. Despite its established neuroprotective activity, CNTF depressed mfERG responses throughout the entire area studied 1 week after PDT (Figs. 1C, 1D, 2). CNTF, but not the other factors tested, significantly increased the latency of mfERG responses (Fig. 2A; mean ± SEM: PBS, 48.2 ± 1.7 ms; CNTF, 55.1 ± 1.1 ms; P < 0.04; n = 6). These findings are consistent with the known effects of CNTF on the full-field ERG. A combination of BDNF and CNTF also depressed mfERG responses, whereas PEDF had little effect (Fig. 2).

All factors tested significantly increased the number of photoreceptors surviving 1 week after PDT (Figs. 3, 4). The normal rat retina contained 10 to 11 rows of photoreceptor nuclei in the ONL (Fig. 3A). PDT reduced the ONL to three to four irregularly arranged rows of photoreceptors, with disruption or loss of most inner and outer segments and RPE pigment clumping and attenuation (Fig. 3B), consistent with earlier findings. All histologic changes were confined to the area treated with PDT. Pretreatment with BDNF increased the number of surviving photoreceptors by 21% (Fig. 4). CNTF (14% increase) and PEDF (13% increase) were somewhat less effective; whereas the combination of BDNF and CNTF increased photoreceptor survival by 28% (Fig. 4). The remaining inner and outer segments were significantly longer in factor-injected (23.6 ± 0.5 μm) than in PBS-injected eyes (15.1 ± 1.2 μm; P < 0.03; n = 3 rats; see Figs. 3B, 3C).

Rescue by BDNF after Repeated PDT Treatment
Because BDNF was most effective in rescuing both photoreceptor function and structure, we asked whether the protective effect of BDNF would extend to 3 months after a single course of PDT and through a second round of PDT preceded by BDNF injection, 3 months after the first. This is the recommended interval for patients who require more than one PDT treatment.
Both PBS-injected and BDNF-injected eyes had increased mfERG responses 3 months after a single round of PDT, compared with 1 week after PDT (Figs. 5A, 5B). At 1 week, responses were significantly higher in the BDNF-injected eyes than in the PBS-injected eyes (Fig. 5A). After 3 months, however, mfERG responses in the PBS-injected eyes had significantly recovered compared with 1 week (\( P < 0.001 \), unpaired \( t \)-test), so that no significant difference remained between PBS- and BDNF-injected eyes (Fig. 5B). Some recovery of mfERG responses was observed over the same period in PDT-treated animals that received no intraocular injection (data not shown). However, their degree of recovery (11%) was less than that observed in PBS-injected eyes (34%), suggesting that intraocular injection of PBS contributed to recovery of retinal func-

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**Figure 1.** PDT caused focal depression of mfERG responses. (A) Pseudocolor plot of averaged mfERG responses from six untreated eyes. A physiologic depression corresponding to the optic nerve head appears inferiorly. (B) PDT after PBS injection depresses mfERG responses in the PDT-treated area, which appears centrally in this plot. (C) CNTF injection before PDT (in the fellow eye of the animal shown in B) depresses mfERG responses throughout the retinal region studied. Responses are further depressed in the PDT-treated area, also present centrally. (D) Overlay of trace arrays used to generate (B) and (C). Black traces: the PBS-injected eye; red: the CNTF-injected eye. CNTF reduced responses relative to PBS throughout the region studied. Responses in each eye were lower in the center of the array, where PDT was applied. (E) Trace arrays from a PBS-injected eye (black) and the BDNF-injected fellow eye (red) show functional rescue in the BDNF-injected eye in most responses in the PDT-treated area (located more superiorly than in B–D) and no change in most responses outside this area. (D, E) Traces are shown on a slightly different scale from that used in (A–C), since pseudocolor plots use the RMS value of each trace rather than the absolute amplitude.

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933236/)

**Figure 2.** Pretreatment with BDNF improved retinal function 1 week after PDT, whereas CNTF impaired retinal function. (A) Sums of 16 mfERG responses from PDT-treated areas of representative animals injected with different factors. Heavy lines: factor-injected eyes; dashed lines: PBS-injected fellow eyes; thin lines: sum of 16 responses averaged from the untreated eyes shown in Figure 1A, corresponding to the positions in the trace array occupied by a typical PDT-treated area. (B) Effects of different factors on mfERG responses in PDT-treated areas of individual animals 1 week after PDT, expressed as the percentage difference in factor-injected relative to PBS-injected (control) fellow eyes. (C) Averages of data shown in (B). BDNF increased responses by 37.4% ± 12.7% (\( P < 0.005; n = 17 \)). CNTF caused a 30.1% ± 8.3% decrease in mfERG responses (\( P < 0.05; n = 6 \)), whereas a combination of BDNF and CNTF reduced mfERG responses by 15.5% ± 7.0% (\( P < 0.05; n = 12 \)). PEDF caused a small, nonsignificant decrease (\( P > 0.6; n = 9 \)).
tion after PDT. The observed increase in mfERG responses with time was not simply a function of increasing age, because serial mfERG studies of untreated animals revealed no change between 3 and 9 months of age (data not shown).

The functional effect of the second round of BDNF injection followed by PDT was similar to the first. One week after the second PDT treatment, mfERG responses were again significantly greater in BDNF-injected eyes than in PBS-injected eyes (Fig. 5C). Therefore, BDNF preserved retinal function through two rounds of PDT.

A beneficial effect of BDNF on retinal structure was also apparent. One week after PDT, the surviving photoreceptors in PBS-injected eyes were irregularly arranged, lending a scalloped appearance to the ONL (Fig. 3B). Three months after PDT, severe ONL disruption was observed in PBS-injected eyes. The ONL appeared split in some places, with the innermost photoreceptors arranged in large rosettes (Fig. 6A). Fewer such abnormal structures were seen in BDNF-injected eyes (Fig. 6B), although the difference did not reach significance when quantified (Fig. 6E). Significantly fewer rosettes were seen in BDNF-injected eyes than in PBS-injected eyes 1 week after a second course of factor injection and PDT (Figs. 6C, 6D, 6F). Inner and outer segments also appeared longer in BDNF-injected eyes at this time point (Figs. 6C, 6D). BDNF significantly increased the number of surviving photoreceptors 1 week after PDT (Fig. 4), but not at 3 months after the first treatment or 1 week after the second (data not shown). Therefore, after the second PDT treatment, the improved retinal function observed in BDNF-injected eyes correlated with improved retinal architecture rather than an increase in the number of photoreceptors.

**DISCUSSION**

We found in our previous study that adjunctive treatment with BDNF increases the number of surviving photoreceptors and the magnitude of local retinal responses to light 1 week after verteporfin PDT. In the present study, we asked whether other neurotrophic factors might be superior to BDNF. Although both CNTF and PEDF significantly increased the num-

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**Figure 3.** Trophic factors rescued photoreceptors 1 week after PDT. (A) Untreated retina with 10 to 11 rows of photoreceptor nuclei in the outer nuclear layer (ONL). IS, photoreceptor inner segments; OS, outer segments; RPE, retinal pigment epithelium; arrowheads, outer limiting membrane. (B) In an eye treated with PDT preceded by PBS injection, photoreceptor loss resulted in a thinner, scalloped ONL. Few inner and outer segments remained (asterisks), extending beyond the outer limiting membrane (arrowheads). The photoreceptor inner segments (IS) extended beyond the outer limiting membrane (arrowheads). The outer segments (OS) were mostly disorganized, but some of these structures were more clearly delineated (arrows). Scale bar, (A–C) 20 μm; (D) 10 μm.

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933236/)

**Figure 4.** Counts of surviving photoreceptors in PDT-treated areas one week after treatment, expressed as the percentage increase in factor-injected eyes relative to PBS-injected (control) fellow eyes. Significant rescue was observed for all factors tested. BDNF treatment increased photoreceptor survival by 20.9% ± 2.9% (P < 10^{-6}; n = 17; data reanalyzed from Ref. 9), whereas CNTF produced a 13.9% ± 3.6% increase (P < 0.02; n = 4). The rescue effect of a combination of BDNF and CNTF was greater than either of their individual effects (28.1% ± 8.5% increase; P < 0.04; n = 5), although not significantly different from either of the single factors. PEDF increased the number of surviving photoreceptors by 12.8% ± 3.8% (P < 0.02; n = 8).
BDNF was again apparent (PBS, 78.7 ± 0.5; BDNF, 113.9 ± 8.8 nV; *P* < 0.005; *n* = 17). After the second PDT treatment, responses in PBS-injected eyes recovered significantly compared with 1 week (*P* < 0.001, unpaired *t*-test), and the difference between BDNF-injected and PBS-injected eyes was no longer significant (PBS, 113.9 ± 5.0 nV; BDNF, 120.1 ± 7.5 nV; *P* > 0.5; *n* = 15). One week after a second injection of PBS or BDNF, followed by a second PDT treatment, significant functional rescue by BDNF was again apparent (PBS, 78.7 ± 6.2 nV; BDNF, 104.5 ± 5.9 nV; *P* < 0.05; *n* = 8).

The rescue effect of the combination of BDNF and CNTF was somewhat greater than their individual effects, perhaps reflecting the fact that they act through distinct signal transduction pathways. Previous investigations have also found that combinations may be more effective than individual neurotrophic factors. The combination depressed mfERG responses, although to a lesser degree than did CNTF alone. Further experiments are needed to determine whether the combination of BDNF and CNTF would produce photoreceptor survival superior to BDNF alone in the long run and whether retinal function would therefore be better after allowing time for recovery from the transient suppressive effect of CNTF (Luthert PJ, personal communication, 2001; Wen R et al. *IOVS* 2004;45:ARVO E-Abstract 785; McGill TJ et al. *IOVS* 2006;47:ARVO E-Abstract 4815). If so, the clinical utility of combination treatment would depend on the tradeoff between short-term suppression of mfERG responses and improved long-term photoreceptor survival.

BDNF significantly improved retinal function 1 week after a single PDT treatment, but no difference between BDNF-injected and PBS-injected eyes was observed 3 months later. mfERG responses increased in both sets of eyes over this period, but the change in PBS-injected eyes was quantitatively greater than in BDNF-injected eyes, so that they were not significantly worse than BDNF-injected eyes after 3 months. Depression and gradual recovery of mfERG responses have also been documented in patients treated with PDT. Therefore, we cannot exclude the possibility that CNTF used in conjunction with PDT might result in improved visual acuity for patients.

PEDF had no appreciable effect on mfERG responses at the dose tested compared to PBS injection. This may have resulted from its relatively modest degree of photoreceptor rescue. However, it remains possible that the antiangiogenic activity of PEDF could be valuable in the treatment of CNV. Our experiments did not address this question, but they do suggest that if PEDF were used for its antiangiogenic activity, it would also have a moderate neuroprotective effect.
served some rescue in PBS-injected eyes relative to noninjected eyes, demonstrated by recovery of retinal function assessed with mfERG but not by retinal histology, that was significant only at the 3-month time point.

The functional recovery with time that we observed in PBS-injected eyes may have resulted from at least two different mechanisms, which are not mutually exclusive. First, the depression of mfERG responses by PDT may reflect not only the death of some photoreceptors, but also sublethal injury to surviving cells. For example, inner and outer segments were more severely disrupted in PBS-injected eyes than in factor-injected eyes 1 week after PDT (Figs. 3B, 3C). They lengthened over the following 3 months (Figs. 6A, 6B), but were again shorter in PBS-injected than in BDNF-injected eyes after a second PDT treatment (Figs. 6C, 6D). These changes in inner and outer segment length may account for the changes in mfERG responses over time by altering photon capture or other aspects of photoreceptor physiology. This model suggests that BDNF-injected eyes maintain relatively robust mfERG responses throughout the period studied (Fig. 5) because their inner and outer segments are largely protected from damage due to PDT.

Second and more speculatively, the injured retina may undergo a process of synaptic remodeling. Death of photoreceptors after PDT would deprive inner retinal neurons of some photoreceptor inputs. These neurons may form new connections with the surviving photoreceptors, possibly strengthening inner retinal responses to light over time in PBS-injected eyes despite the decreased number of photoreceptors. Synaptic remodeling does occur in retinas with stressed or dying photoreceptors, although there is as yet no evidence that the newly formed synapses can mediate normal visual function. Whatever the mechanism of functional recovery in our model, it is likely to have relevance to understanding the effects of PDT on the human retina.

It is possible that continuous delivery of BDNF, rather than the single bolus injection used in our studies, would further improve retinal function in BDNF-treated eyes 3 months after PDT. Neurotrophic factors are degraded within several days after single injections and are generally insufficient for long-term protection, prompting investigators to work toward continuous delivery methods. These include encapsulated cell technology and viral delivery of genes encoding neurotrophic factors.

BDNF rescued retinal function after a second course of PDT, suggesting that the benefits of neuroprotection could be sustained in patients requiring multiple treatments. Protection of function after the second treatment was associated with improved retinal architecture, rather than with an increase in the number of surviving photoreceptors. Rosette formation is a fairly nonspecific finding and has been reported in retinitis pigmentosa, other inherited retinal disorders, and exposure to teratogens. The disorganization that we observed in PBS-injected eyes progressed with time: only scalloping was evident 1 week after PDT, but full-fledged rosettes appeared by 3 months (Figs. 3B, 6A). BDNF presumably reduces this abnormal reorganization of the ONL by ameliorating the initial injury from PDT. The structural damage in PBS-injected eyes, and protection by BDNF, were more marked after a second PDT treatment. Our data suggest that BDNF would have long-term benefits for retinal organization, particularly in patients requiring more than one treatment.

Our findings point to at least two potential benefits of adjunctive neuroprotective treatment for patients receiving PDT. First, visual disturbances and depression of mfERG responses with gradual recovery have been documented after PDT in patients. We observed similar depression and recovery in mfERG responses over time in PBS-injected eyes, whereas BDNF largely prevented these changes, maintaining relatively robust mfERG responses. These findings suggest that BDNF may also prevent visual disturbances in patients after PDT. Protection from acute severe vision loss could confer a major benefit on a subset of patients. Second, animal studies have shown that retinal damage is cumulative with successive PDT treatments. Our data add to this body of evidence and show that BDNF preserves retinal structure through two rounds of PDT, a benefit that would be expected to persist in the long term. Structural preservation may be particularly important in the presence of CNV, which is not a feature of our model, but which represents a preexisting disruption of retinal structure in patients with neovascular AMD. As mentioned earlier, it is possible that our study did not capture the full potential benefit of BDNF, because we delivered only a single bolus dose of the factor. Emerging continuous delivery approaches may be needed to maximize the long-term therapeutic value of BDNF.

Although the treatment of neovascular AMD is evolving with the introduction of therapies targeting vascular endothelial growth factor (VEGF), PDT may continue to play an important role. An ongoing clinical trial has compared a combination of PDT and ranibizumab, an anti-VEGF antibody fragment, with ranibizumab monotherapy. The combination treatment requires fewer intraocular injections of ranibizumab to achieve an equivalent clinical benefit (Schmidt-Erfurth UM et al. IOVS 2006;47:ARVO E-Abstract 2960). By preserving retinal structure and function after PDT, adjunctive neuroprotective therapy may contribute to optimal visual outcomes in patients with AMD.

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