Neuroprotectin D1 Restores Corneal Nerve Integrity and Function After Damage From Experimental Surgery

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Submitted: March 21, 2013

Accepted: May 16, 2013

Citation: Cortina MS, He J, Russ T, Bazan NG, Bazan HEP. Neuroprotectin D1 restores corneal nerve integrity and function after damage from experimental surgery. *Invest Ophthalmol Vis Sci.* 2013;54:4109–4116. DOI:10.1167/iovs.13-12075

PURPOSE. To investigate if topical treatment of neuroprotectin D1 (NPD1) increases regeneration of functional nerves after lamellar keratectomy.

METHODS. An 8-mm stromal dissection was performed in the left eye of each rabbit. The rabbits were treated with NPD1, pigment epithelial-derived factor (PEDF) in combination with docosahexaenoic acid (DHA) or vehicle for 6 weeks, and corneas were obtained at 8 weeks. After fixation, corneal wholemounts were stained with mouse monoclonal anti- β III-tubulin antibody and double stained with chicken anti-calcitonin gene-related peptide (CGRP) antibody. Corneal sensitivity and tear secretion were measured using the Cochet-Bonnet esthesiometer and the Schirmer's test, respectively. Additional rabbits were treated with NPD1, PEDF+DHA, or vehicle, and corneal sections were stained with a rat monoclonal anti-neutrophil antibody. Cultures of trigeminal ganglia from 5-day-old mice were treated with NPD1, PEDF+DHA, lipoxin A4 (LXA4), 12- or 15-hydroxyeicosatetraenoic acid (12[S] or 15[S]-HETE), and nerve growth factor (NGF) as positive control.

RESULTS. NPD1 increased subepithelial corneal nerve area three times compared with vehicle-treated rabbits. The effect was similar to PEDF+DHA-treated animals. There was recovery of CGRP-positive neurons and an increase in corneal sensitivity and tear secretion in NPD1-treated animals. NPD1 decreased neutrophil infiltration after 2 and 4 days of treatment. In the in vitro cultures, NPD1 and PEDF+DHA induced a 3-fold increase in neurite outgrowth compared with cultures without supplementation. Treatments with LXA4, 12(S)-, and 15(S)-HETE did not stimulate neurite outgrowth.

CONCLUSIONS. NPD1 has anti-inflammatory and nerve regenerative properties. This study demonstrates that NPD1 may offer an effective treatment for neurotrophic corneas.

Keywords: neuroregeneration, corneal sensitivity, pigment epithelial-derived factor, neuroprotectin D1, dry eye

As one of the most richly innervated tissues in the body,^{1–3} the cornea relies in part on its sensory and autonomic nerve fibers to maintain homeostasis and promote wound healing after injury. Damage to corneal nerves can result in decreased sensitivity, dry eye symptoms, and neurotrophic keratitis. Dry eye resulting in corneal nerve damage has been observed after laser vision correction with laser in situ keratomileusis (LASIK) and photorefractive keratectomy (PRK), longstanding contact lens use, and diabetes, among others causes.^{4–7} More severe forms of neurotrophic keratitis are observed in patients with herpes viral infections and penetrating keratoplasty.^{8–10}

Nerve terminals regenerate after injury; however, this can be a slow and often incomplete process. For example, patients who undergo penetrating keratoplasty with complete transection of corneal nerves may continue to have very poor innervation even 30 years after transplantation.¹⁰ The speed at which corneal nerves regenerate correlates with the depth of the injury.¹¹ However, even after more superficial injuries like LASIK and PRK, innervation of the cornea is slow,⁷ and some patients continue having dry eye symptoms for several years.⁵

In a neurotrophic cornea, epithelial breakdown may occur, which can be difficult to resolve due to the impaired wound healing. Neuropeptides synthesized by corneal nerves have been used in animal models^{12–14} of corneal injury and in patients who failed to respond to conventional therapies.¹⁵ This highlights the important role corneal nerves play in this process. However, we still lack an effective treatment that can stimulate regeneration of damaged corneal nerve fibers and thus provide a more definitive solution to the problem.

Docosahexaenoic acid (DHA), an omega-3 essential fatty acid, has been shown to induce synaptogenesis and dendrite formation in the brain.¹⁶ This fatty acid is the precursor of neuroprotectin D1 (NPD1), a lipid mediator derived from the selective oxygenation of DHA by 15-lipoxygenase-1 (15-LOX-1) that has potent anti-inflammatory and neuroprotective actions.^{17,18} Pigment epithelial-derived factor (PEDF) is a broad acting neurotrophic and neuroprotective factor involved in angiogenesis, neuronal cell survival, and cell differentiation.¹⁹ Studies have shown that PEDF strongly stimulates the synthesis of NPD1 from its precursor DHA.²⁰ In addition, we have shown that corneas treated with PEDF and DHA exhibit increased NPD1 synthesis.²¹

Treatment with PEDF in association with DHA after lamellar keratectomy increases regeneration of corneal nerves.²¹ We also have shown that corneal sensation returns to normal levels in treated animals 8 weeks after surgery.²² However, the mechanism by which PEDF and DHA exert their effect on corneal nerve regeneration is not known. We hypothesize that this effect is due to the synthesis of NPD1 from DHA,²¹ and that NPD1 is the lipid mediator involved in modulating this response. The purpose of this study is to define if topical NPD1 treatment increases regeneration of functional corneal nerves after surgery.

We also compare the effect of different 12/15-lipoxygenase mediators derived from the omega-6 fatty acid, arachidonic acid (AA), with PEDF+DHA and NPD1 on axonal growth in neurons in culture.

METHODS

Animals

Male New Zealand albino rabbits weighing 2 to 3 kg were used. Pregnant Swiss Webster mice were obtained from Charles River Laboratories (Wilmington, MA). Five-day-old pups were used to obtain trigeminal ganglia for cell cultures. Animals were treated according to the guidelines for the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and protocols were approved by the Institutional Animal Care and Use Committee at the Louisiana State University Health Sciences Center, New Orleans.

Surgery and Treatments

Each rabbit received intramuscular xylazine (10 mg/kg) and ketamine hydrochloride (50 mg/kg) anesthesia. Tetracaine eye drops were used as topical anesthesia. An 8-mm stromal dissection through a 3-mm incision was performed in the left eye of each rabbit. No sutures were used. Moxifloxacin ophthalmic eye drops were instilled before and after the surgical procedure.

Treatment was started immediately after surgery. Nine rabbits received topical application of NPD1 drops (100 ng) three times a day for 6 weeks (a gift from Resolvix Pharmaceuticals, Bedford, MA). Three rabbits received PEDF (50 ng; BioProducts, Middletown, MD) with >95% purity tested by SDS-PAGE plus DHA (10 µg; Cayman Chemical, Ann Arbor, MI) complexed to 25% human albumin by means of a 72-hour collagen shield (Oasis, Glendora, CA) soaked in the drug; previous studies have shown that this was an optimal way to deliver the compounds.²⁰⁻²³ Collagen shields were changed twice a week for 6 weeks. The control group (three rabbits) received vehicle drops (0.02% ethanol in PBS). Rabbits were sacrificed at 8 weeks with an overdose of sodium pentobarbital via ear vein injection.

To study neutrophil infiltration, 12 additional rabbits receive similar surgery and were treated with NPD1, PEDF+DHA, or vehicle for 2 and 4 days, respectively.

Corneal Sensitivity

Corneal sensitivity measurements of the central cornea inside the surgical area were performed weekly in each eye using the Cochet-Bonnet esthesiometer (Luneau Ophtalmologie, Chartres Cedex, France). The technique has been previously described and validated.¹¹ Briefly, the length of the monofilament was varied from 6.0 to 0.5 cm in 0.5-cm fractions until the corneal touch threshold was found. At each monofilament length, the cornea was touched 10 times. A positive response

was considered if the animal blinked >50% of the times stimulated. If no blink response could be elicited at a monofilament length of 0.5 cm, corneal sensitivity was recorded as 0. The same examiner performed all the measurements.

Measurement of Tear Production

Schirmer's test (Zone-Quick; Menicon America, Inc., San Mateo, CA) was used to assess tear production after surgery according to the manufacturer's instructions. The test was performed without anesthesia. Measurements were obtained weekly in the injured as well as the uninjured eye for comparison in every group. The same examiner blinded to the different treatment groups performed all the measurements.

Immunocytochemistry

To assess corneal innervation, whole corneas were excised and fixed in 2% fresh paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours at room temperature or overnight at 4°C. After three thorough washings in 0.1 M PBS for 5 minutes each, the whole cornea was incubated with mouse monoclonal anti-β III-tubulin antibody (1:1000; Covance Antibody Services, Inc., Berkeley, CA) as previously described.² For double staining, chicken anti-calcitonin gene-related peptide (CGRP; 1:1500; Biotechnology, Inc., Temecula, CA) was added first in 1% goat normal serum plus 0.15% Triton X-100 in 0.1 M PBS for 72 hours and fixed with fresh paraformaldehyde for 30 minutes, then washed and double stained with anti-βIII-tubulin antibody.²² After washing with 0.1 M PBS five times for 5 minutes each, the corneas were incubated with FITC-conjugated secondary antibodies (1:1500, Alexa Fluor goat anti-chicken IgG or Alexa Fluor 594 donkey antimouse IgG; Molecular Probes, Eugene, OR) for 2 hours at room temperature. Finally, the injured area of the cornea was viewed and photographed with a fluorescence microscope (Eclipse TE200; Nikon, Tokyo, Japan) equipped with a digital camera (DXM 1200; Nikon) using commercial imaging software (MetaVue; Molecular Devices, Sunnyvale, CA). The βIII-tubulin-positive tissue nerve area at the subepithelial level was calculated and compared with the total area with an image analysis program (Image pro Plus 4.5; Cybernetics, Inc., Silver Spring, MD). CGRP-positive nerves were calculated and compared with the β-III-tubulin positive nerves. Ten different images of different areas within the injured zone were analyzed per rabbit and then averaged.

To quantify neutrophils, corneas on day 2 and 4 after surgery were incised along the limbus and fixed in 4% paraformaldehyde at room temperature for 2 hours, cut in half and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA). Serial cryostat sections (6 µm) were cut, air dried, and stored at -20°C until use. For immunofluorescence staining, the sections were washed in PBS, blocked with 10% goat serum, 0.1% Triton-X100 in PBS for 30 minutes at room temperature, and then incubated overnight at 4°C with a rat monoclonal neutrophil antibody (1:500; Abcam, Cambridge, MA). Afterwards, the sections were incubated with Alexa Fluor 488 goat antirat Ig (H+L) antibody (Molecular Probes) for 1 hour at room temperature. 4',6'-diamino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO) staining was performed to localize the nuclei. The sections were examined with a fluorescent microscope under ×200 magnification. Positive cells were counted in a blind fashion from four randomly selected fields per cornea and averaged.

FIGURE 1. NPD1 increases β III tubulin and CGRP-positive nerves after lamellar keratectomy. **(A)** Immunocytochemistry of subbasal nerve plexus of rabbit corneal wholemounts stained with anti- β III tubulin after 6 weeks of treatment and 8 weeks of surgery. **(B)** Quantification of β III tubulin subepithelial nerves in NPD1- (nine rabbits) and PEDF+DHA (three rabbits)-treated groups. *Significant difference with respect to vehicle-treated animals (three rabbits). **(C)** Quantification of CGRP-positive nerves against the total nerve area for each group. There were six rabbits in the NPD1 group and three rabbits in the other groups. **(D)** Immunofluorescence analysis of rabbit corneal wholemounts with anti-CGRP (red) and anti- β III-tubulin (green) antibodies 8 weeks after lamellar keratectomy. The overlay images show CGRP-positive nerves in orange for vehicle-, NPD1-, and PEDF+DHA-treated corneas. *Significant difference with respect to vehicle-treated animals.

Trigeminal Ganglia Cell Cultures

Five-day-old pups were euthanized by an injection of pentobarbital. Then trigeminal ganglia were harvested rapidly in Dulbecco's modified Eagle's medium (DMEM) F-12 media plus 10% fetal bovine serum (FBS) and digested with 0.125% collagenase II (Sigma-Aldrich) for 15 minutes at 37°C, followed by 0.25% trypsin for an additional 15 minutes at 37°C. Dissociated cells were resuspended in DMEM-F12 media containing gentamicin, pen-strep, glutamine, and 10% FBS supplemented with 6 μ g/mL of 5-fluoro-2'-deoxyuridine (5-FDU) and 14 μ g/mL uridine, both from Sigma-Aldrich, as antimetabolites and preplated on uncoated polystyrene plates for

30 to 60 minutes at 37°C to remove contaminant cells. Cells from the supernatant (containing the neurons) were counted and plated in four-well glass chambers coated with Poly-D-Lysine/laminin for 24 hours. The culture media was then changed to DMEM-F12 media plus 0.5% horse serum supplemented with antibiotics and antimetabolites and then treated with 50 nM DHA + 50 ng/mL PEDF; 50 nM NPD1; 50 ng/mL of nerve growth factor (NGF) as positive control; lipoxin A4 (LXA4); 12-hydroxyeicosatetraenoic acid (12[S]HETE) or 15(S)HETE (all 50 nM; Cayman Chemical). Pictures were taken 24 hours after treatments with a phase contrast microscope and neurite cell lengths were measured in a blind fashion using CellSens Software (Olympus, Tokyo, Japan).

Data Analysis

All data are expressed as mean $\pm$ SD. Statistical comparison between NPD1 or PEDF+DHA and vehicle groups was performed by Student's *t*-test. Comparison between several groups was done by one-way ANOVA. *P* values <0.05 were considered significant.

RESULTS

Effects of NPD1 on Corneal Nerve Area After Lamellar Keratectomy

Immunohistochemistry of corneal wholemounts stained with β III tubulin showed an increase in the subepithelial corneal nerve area at 8 weeks after surgery in the group receiving treatment with NPD1 (24.91%) compared with the vehicle-treated group (8.32%; Figs. 1A, 1B). As reported before, the group treated with PEDF+DHA also stimulates innervation (22.53%). These differences were statistically significant and represent almost a 3-fold increase in nerve area. There was no statistically significant difference in the amount of staining observed between NPD1- and PEDF+DHA-treated groups.

One of the main neuropeptides produced by corneal sensory nerves is CGRP.¹ Immunohistochemistry of corneal wholemounts stained for β III tubulin and CGRP showed that 44% of fibers of the total nerve area staining with β III tubulin are positive for CGRP in rabbits without injury (Figs. 1C, 1D). Eight weeks after injury, the percentage of corneal nerve fibers positive for CGRP decreased to 22% in the vehicle-treated group. Corneas of NPD1-treated animals had an increase in the CGRP-positive neurons compared with the vehicle-treated group (39%, $P = 0.005$). As previously shown, the PEDF+DHA-treated group recovered CGRP-positive nerve fibers to control values²² with 41% of CGRP positive nerve fibers ($P = 0.001$).

Corneal Sensitivity and Tear Secretion After NPD1 Treatment

Corneal sensitivity measured with a Cochet-Bonnet esthesiometer was increased in NPD1-treated animals compared with vehicle-treated animals starting 4 weeks after surgery (Fig. 2A). The NPD1 group almost reached control (no injury) values at 7 weeks after surgery. Although a tendency toward higher sensitivity values was observed with NPD1, no statistically significant difference was found between NPD1 and PEDF+DHA-treated groups (Fig. 2B).

Basal tear secretion as measured by Schirmer's test showed an increase in tear production in NPD1-treated animals (Fig. 2C). By week 2, the values were similar to nonoperated corneas (control). Similar results were observed in animals treated with PEDF+DHA. A statistically significant difference in Schirmer's test measurements was observed at 3 and 4 weeks after injury between vehicle and NPD1 or PEDF+DHA treatments. Values between the groups became closer toward the end of the 5-week follow-up period. No significant difference was observed between NPD1 and PEDF+DHA-treated groups.

Effect of NPD1 on Neutrophil Infiltration

To study if NPD1 has an anti-inflammatory action on corneas after injury, corneas were processed 2 and 4 days after surgery by immunohistochemistry with an anti-neutrophil antibody. Two days after surgery, there was an active infiltration of polymorphonuclear neutrophils (PMNs; Fig. 3) in the injured area that decreased by day 4. Corneas treated with NPD1 presented a 60% ($P = 0.0001$) reduction in neutrophil-positive

FIGURE 2. Effect of NPD1 on corneal sensitivity and tear secretion after surgery. (A) Central corneal sensitivity was measured with the Cochet-Bonnet esthesiometer once a week starting 1 week after surgery and treatment. *Significant difference between NPD1 group and vehicle ($P < 0.05$). (B) No differences were found between NPD1- and PEDF+DHA-treated group. (C) Tear secretion was measured with Schirmer's test. The test was performed without anesthesia. Measurements were obtained weekly in the injured as well as uninjured eye for comparison in every group. *Significant differences at 3 and 4 weeks with respect to vehicle. There were six rabbits in the NPD1 group and three rabbits each in the other groups.

cell staining and a 50% ($P = 0.0008$) decrease with PEDF+DHA. There was a further decrease in PMN infiltration at 4 days after treatment with NPD1 (70%, $P = 0.0004$). Interestingly, at day 4, there was also a significant decrease of PMNs in NPD1-treated corneas compared with PEDF+DHA ($P = 0.0002$).

FIGURE 3. Immunostaining of PMNs in corneas 2 and 4 days after surgery. Corneas were stained with anti-neutrophil antibody and counted in a blind fashion. Four sections/corneas were counted in randomly selected fields. *Significant decrease compared with vehicle-treated corneas at 2 days after surgery. **Significant decrease compared with vehicle-treated corneas at 4 days after surgery. ***Significant decrease compared with PEDF+DHA-treated corneas at 4 days after surgery.

Trigeminal Ganglia Neurite Outgrowth Induced by NPD1 or PEDF+DHA

To determine if the action on nerve regeneration was selective for the 15-lipoxygenase mediator NPD1, experiments were conducted using primary cultures of trigeminal ganglion neurons from newborn mice. Treatment of the cells for 24 hours with NPD1 or with PEDF+DHA produced a 3-fold increase ($P = 0.0005$) in neurite outgrowth compared with cultures without supplementation (Fig. 4). Nerve growth factor used as a positive control showed a similar increase. Treatment with the 12/15-LOX derivatives from arachidonic acid, 12(S) HETE, 15(S) HETE, and LXA4 did not stimulate neurite outgrowth in the conditions of these experiments.

DISCUSSION

Alterations in corneal innervation may result in impaired corneal sensation, severe dry eye, and neurotrophic keratitis. These alterations frequently occur after refractive surgery, corneal transplant, herpetic infection, chemical burns, contact lens wear, Sjogren's syndrome, advanced aging, and diabetes mellitus.^{4,6,24,25} It is well established that corneal nerves and epithelial cells have a close relationship, in which corneal nerves promote epithelial cell proliferation and differentiation and that corneal sensitivity correlates with the state of the ocular surface.^{26,27} In addition, a recent study suggests a critical role of nerves in maintaining corneal epithelial stem cells.²⁸

After corneal nerve damage, there is an impaired ability of the corneal epithelium to respond to injury. This is due in part

FIGURE 4. Comparison of NPD1 and PEDF+DHA with different lipid mediators on neurite outgrowth. Neurons were obtained from trigeminal ganglia of 5-day-old Swiss Webster mice. Cells were stimulated with PEDF (50 ng/mL) plus DHA (50 nM), NPD1, LXA4, 15(S)HETE or 12(S)HETE (all 50 nM). Controls without lipids and positive control with NGF (50 ng/mL) were added. After 24 hours, cells were fixed and pictures taken with a phase contrast microscope. (A) Neurite length was quantified in a blind fashion. The values correspond to the average of five fields from four different wells/condition. The experiment was repeated twice with similar results. (B) Phase contrast pictures of isolated neurons incubated for 24 hours with NPD1, PEDF+DHA, or NGF.

to reduced trophic support provided by the nerves that may lead to spontaneous and persistent epithelial defects, corneal ulcers, melting, and perforation.²⁹ Although there are treatments for alleviating dry eye symptoms, there are no efficacious treatments to compensate for the loss of innervation and epithelial breakdowns. This is a challenging issue to resolve in neurotrophic cornea.

Our previous studies have shown that treatment with PEDF+DHA can stimulate corneal nerve regeneration after a lamellar keratectomy that transects the stromal nerves.²¹ The treatment induces synthesis of NPD1, a docosanoid with antiapoptotic and neuroprotective actions in retinal pigment epithelial cells and models of brain damage.^{21,30} We have also shown that recovery of corneal sensitivity is correlated with the regeneration of nerve fibers after surgery.²² We now demonstrate that application of NPD1 after lamellar keratectomy produces nerve regeneration similar to PEDF+DHA treatment. NPD1 action is selective since other 12/15-LOX derivatives, such as 12(S) and 15(S) HETEs, and LXA4, could not stimulate neurite growth of trigeminal neurons in culture.

Corneal nerves contain neuropeptides such as CGRP and substance P (SP) that have neurotrophic influences over corneal epithelial cells.¹ Our previous studies have shown that SP is strongly expressed in epithelial cells after injury and that treatment with PEDF+DHA does not affect its expression or localization.²² Therefore, in this study we only investigated how CGRP-positive neurons were recovered after injury. In the rabbit cornea, 40% of their total nerve fibers were positive for CGRP, and we found that NPD1-treated animals recover the same proportion of CGRP-positive neurons 8 weeks after

injury. A similar response is obtained with PEDF+DHA treatment suggesting that NPD1 may be a mediator of this effect.

A large proportion of the sensory fibers present in the cornea respond to mechanical stimuli.³¹ In the present study, we evaluated the mechanical component of corneal sensitivity and demonstrated a correlation between subbasal nerve regeneration induced by NPD1 and PEDF+DHA treatments and corneal sensation, indicating functionality of regenerated corneal fibers. Similarly, increased tear secretion with NPD1 and its precursors, PEDF and DHA, suggest that restoration of the tear reflex arc by functional neurons occurs sooner after treatment conditions and that treatment may have a protective effect on the ocular surface from dry eye-induced damage after surgery.

Previous studies have shown an inverse relationship between supplementation with omega-3 polyunsaturated fatty acids and dry eye syndrome.^{32,33} Recently, a potent anti-inflammatory effect of omega-3 α -linolenic acid (ALA) has been reported in human corneal epithelial cells in culture.³⁴ Increasing the amount of omega-3 ALA increases its product DHA, which, under cell stress, could induce the synthesis of NPD1. In fact, in cornea organ culture experiments, we recently showed that injury to corneal epithelium increases the release of PEDF that, in the presence of DHA, synthesizes NPD1.^{35,36}

The exact mechanism by which NPD1 promotes corneal nerve regeneration is still not understood. It is believed that communication between corneal nerve fibers and epithelial cells is bidirectional and that corneal epithelial cells release

soluble factors that promote neurite extension and survival.^{37,38} NPD1 promotes wound healing in the cornea and is synthesized by corneal epithelial cells.^{35,39} In agreement with these findings, we have shown that combined treatment PEDF and DHA accelerates wound healing in rabbit corneas.²² NPD1 has been shown to be anti-inflammatory, displaying inflammatory resolving activities, and also induces cell survival. For example, NPD1 promotes neuronal cell survival in models of Alzheimer disease and modulates proinflammatory responses, resulting in retinal pigment epithelial cell survival during oxidative stress.^{30,40} It has also shown effectiveness in the treatment of ischemic brain injury in stroke animal models.⁴¹ Recently, NPD1 was shown to regulate TRPV1/TNF- α -mediated spinal synaptic plasticity with possible analgesic effects in inflammatory pain.⁴²

We speculate that the effect of NPD1 on corneal nerves may result in part from a modulation of the inflammatory response induced by injury, leading to corneal epithelial cell survival. Corneal injury induces an influx of inflammatory cells that migrate to the cornea from the limbal vessels. An early inflammatory response seems to be important for efficient corneal wound healing and nerve regeneration,⁴³ but this response needs to be resolved in order to restore tissue homeostasis. Our present study shows that treatment with NPD1 and PEDF+DHA significantly decreases the influx of PMNs after surgery. To support the idea that chronic inflammation may be deleterious to nerve regeneration processes, a study in patients with bacterial and fungal corneal infections has shown a correlation between an increase in the inflammatory immune response and the decrease in subbasal corneal nerves.⁴⁴

In summary, the data presented in this study support the hypothesis that NPD1, the mediator synthesized by PEDF+DHA, has anti-inflammatory and nerve regenerative properties. NPD1 may offer interesting options for therapeutic intervention in the treatment of disorders involving corneal nerve damage.

Acknowledgments

Supported by the National Eye Institute of the National Institutes of Health under award number R01EY019465. TR was the recipient of a research supplement award for Grant R01EY019465 to promote diversity in health-related fields. The authors alone are responsible for the content and writing of the paper.

Disclosure: **M.S. Cortina**, None; **J. He**, None; **T. Russ**, None; **N.G. Bazan**, None; **H.E.P. Bazan**, None

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