Topical Pergolide Enhance Corneal Nerve Regrowth Following Induced Corneal Abrasion

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Purpose. Neurotrophic keratopathy is a degenerative disease that may be improved by nerve growth factor (NGF). Our aim was to investigate the use of pergolide, a dopamine (D1 and D2) receptor agonist known to increase the synthesis and release of NGF for regeneration of damaged corneal nerve fibers.

Methods. Pergolide function was evaluated by measuring axon length and NGF levels by enzyme-linked immunosorbent assay in cultured chicken dorsal root ganglion (DRG) cells with serial doses of pergolide (10, 25, 50, 150, and 300 µg/ml) and with different concentrations of a D1 antagonist. Pergolide function was further evaluated by cornea nerve fiber density and wound healing in a cornea scratch mouse model.

Results. Pergolide increased DRG axon length significantly at a dose between 50 and 300 µg/ml. Different concentrations of D1 antagonist (12, 24, 48, and 96 µg/ml) inhibited DRG axon length growth with pergolide (300 µg/ml). Pergolide (50 µg/ml) upregulated NGF expression in DRG cells at both 24 hours and 48 hours. Pergolide improved cornea nerve fiber density at both 1 week and 2 weeks. Pergolide also improved cornea wound healing.

Conclusions. We demonstrated that pergolide can act to promote an increase in NGF which promotes corneal nerve regeneration and would therefore improve corneal sensation and visual acuity in eyes with peripheral neurotrophic keratopathy.

Keywords: mouse cornea, DRG, NGF, pergolide

Neurotrophic keratopathy (NK) is a degenerative disease of the cornea that results in decreased or absent sensation to the cornea, corneal thinning, poor healing after corneal injury, persistent epithelial defects, corneal melting or perforation, and loss of vision. It can be caused by herpes zoster or simplex keratitis, chemical burns, physical injury, corneal surgery, long-term contact lens use, or injury to the trigeminal nerve.[1] NK has an estimated prevalence of 5/10,000 (0.05%).[2]

Corneal sensory nerves are essential for the maintenance of anatomical integrity, mitosis, and function of the corneal epithelium.[1] Corneal sensory nerve damage leads to altered levels of neuromodulators, resulting in morphologic changes such as decreased thickness of the corneal epithelium, loss of microvilli, abnormal basal lamina production, and eventually persistent epithelial defects. Neuromodulators that may play a role in the development of NK include nerve growth factor (NGF), substance P, neuropeptide Y, ciliary neurotrophic factor, and acetylcholine.[2–4] When these substances are reduced, there is a predisposition to developing neurotrophic keratopathy.[5]

The prognosis of NK depends on the etiology, degree of corneal hypoesthesia, and presence of other ocular diseases. Currently, most available treatments for NK promote corneal epithelial healing to prevent the progression of corneal damage. Cenergermin (a recombinant form of human nerve growth factor)[6] and corneal neurotization (surgical transposition of facial nerve branches),[7] therapeutic approaches to NK geared toward the improvement of corneal sensation or visual acuity, are quite expensive, pose surgical risks, and/or are difficult for patients. The ideal therapy for NK would improve corneal trigeminal innervation to restore the trophic supply of the corneal nerves and to stimulate corneal healing.[8–11] Promising therapeutic approaches include thymosin beta-4, which is under investigation,[12,13] and nicergoline, which may help patients with refractory neurotrophic keratopathy.[6]

Among the clinical trials for NK, nerve growth factor has shown the most promise. Two large open-label studies using murine-derived NGF demonstrated a high healing rate associated with improved corneal sensitivity, tear function, and visual acuity.[14,15] In the earlier study performed on

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43 patients (45 eyes), patients were administered murine NGF for 2 weeks after the ulcers had healed. All 45 eyes showed complete resolution of the persistent epithelial defect and some improvement in corneal sensation and visual acuity.[14] Another study with a nearly identical NGF regimen showed complete ulcer resolution in all 14 eyes, with improved corneal sensation in 13 eyes, and 2 eyes returning to normal.[15] Although NGF therapy shows promise, it is very expensive and NGF is difficult to store at room temperature.

Cabergoline and pergolide are ergot-derived dopamine receptor agonists that promote NGF expression. Cabergoline favors the D2 receptor (with very weak D1 activity) and is used to treat Parkinson's disease.[16] Pergolide is reported to favor D1 and D2 receptors equally and a murine study demonstrated a rapid elevation in NGF of 21-fold at 4 to 6 hours, and 84-fold at 24 hours.[16] We therefore hypothesized that pergolide and cabergoline, when administered as topical drops in a corneal epithelial scratch model in mouse, would elevate NGF and improve corneal nerve fiber regeneration.

Methods

Drug Preparation

Pergolide mesylate, cabergoline, cholesterol, and α-tocopherol were purchased from Sigma-Aldrich (St. Louis, MO) and propylene glycol from MP Biomedicals (Santa Ana, CA). 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (PC) was purchased from Cayman Chemical Company (Ann Arbor, MI). Phosphate buffered saline (PBS) (1×) pH 7.4 was obtained from Gibco by Life Technologies (Grand Island, NY). Syringe filter units were purchased from Sartosius Stedim (Goettingen, Germany) and isopore membrane filters from Millipore (Burlington, MA).

Pergolide was prepared with two formulations. The in vitro experiments in dorsal root ganglion (DRG) utilized a Marinosolv® (Marinomed Biotech; Wien, Austria) formulation, and pergolide containing liposomal microparticles was developed for the in vivo studies. This was done to overcome difficulties faced with turbidity, particle size, and drug concentration limitations in the liposomes. For efficacy comparison, the Marinosolv formulation was tested in vivo in addition to its use in vitro and was found to be similar.

Preparation of Drug-Loaded Marinosolv. Marinosolv is a proprietary solvent that enables the aqueous formulations of poorly soluble compounds.[17] The process can be described briefly as follows. The pergolide was dissolved in an organic solvent, propylene glycol, followed by the addition of water containing the buffer (pH 5.2), saponin, and dexpanthenol, resulting in spontaneous micelles that were a stable and clear solution that could be used as eye drops. The micelle size was ~2 to 5 nm with a slightly negative charge of –5 to –7 mV zeta potential and showed a globular shape.

Preparation of Drug-Loaded Liposomal Microparticles.

1. *Formulation:* The non-aqueous solution of pergolide was prepared by dissolution of pergolide in chloroform (3 mg/10 ml) along with PC (80 mg), cholesterol (10 mg), and vitamin E (18 µl) followed by sonication and vortexing to form a uniform solution. Thereafter, the organic solvent was removed under nitrogen and the resulting film was hydrated with 10 ml PBS (pH 7.4) to produce pergolide-loaded liposomes (0.3 mg/ml). The liposomes were sonicated and extruded through 0.4-µm isopore membrane filters to obtain even-sized microparticles. Vesicles were allowed to mature overnight under refrigeration, and final liposomal formulations were sterilized by filtration through 0.4-µm isopore membrane filters. Cabergoline-loaded liposomes were prepared in an identical fashion.
2. *Chromatographic conditions for pergolide analysis:* Chromatographic separation was performed on a Zorbax Eclipse XDB 4.6 × 250 mm 5µ with a column temperature of 40°C and injection volume of 20 µl; the flow rate of the mobile phase was set at 1.5 ml/min with detection by fluorescence spectrophotometer at λ_{ex} = 280 nm and λ_{em} = 345 nm. Mobile phase A contained 20-mM sodium 1-octanesulfonate and 1 ml glacial acetic acid in water; mobile phase B contained acetonitrile and water (1:1). Mobile phases A and B were used in a ratio of 50:50 for analysis. The calibration curve was generated for pergolide mesylate at concentrations ranging from 0.050 to 0.288 M, and the regression equation was calculated. There was an excellent correlation between peak area and drug concentration within the linear range of 0.050 to 0.288 M: $y = 5681.3x - 1.5184$ ($r = 0.9999$, n = 5), where y is the peak area and x is the concentration.
3. *Determination of the encapsulation efficiency of liposomes loaded with pergolide:* The encapsulation efficiency of pergolide liposomes was determined upon separation by centrifugation (4000 ×*g*, 4°C, 20 minutes) from the dispersing medium containing non-encapsulated pergolide, in triplicate, by the developed high-performance liquid chromatography method. The determination of the percentage of encapsulation efficiency (EE) in liposomes, was calculated by following formula:

$$EE\ (\%) = \frac{\text{Total amount of drug added} - \text{Unencapsulated amount of drug}}{\text{Total amount of drug added}}$$

The entire amount of pergolide in liposomes was measured by dissolving supernatant in methanol and further re-diluting with mobile phase (mobile phase A + B). The average drug entrapping efficiency of three batches of pergolide was determined to be 81.2%, which suggested that pergolide was efficiently entrapped inside the liposome during initial formulation.
4. *Determination of particle size:* Measurements of liposome particle size were carried out by Particle Sizing Systems (Entegris; Santa Barbara, CA). For analysis, formulations were diluted 1/20 (v/v) in an aqueous medium. All determinations were performed in triplicate at room temperature (25°C). The average particle diameter was less than 50 nm (particle diameter range was 43.9 nm to 55.4 nm).

In Vitro Characterization of DRG Neurite Outgrowth

Similar sized DRGs, which are easily isolated collections of central nervous system sensory neurons, were used and each experiment was repeated three times. Ten microliters

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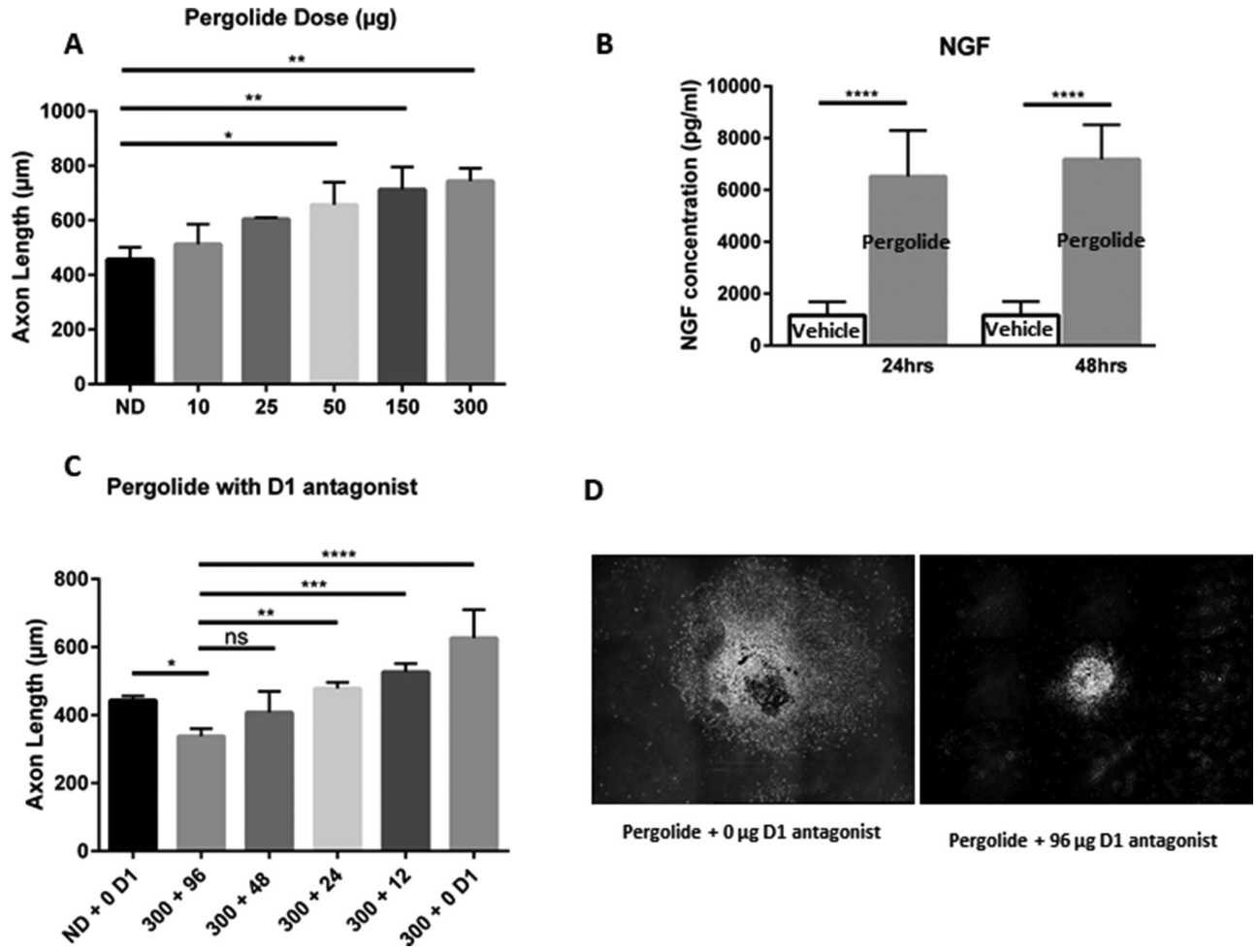

Figure 1. Pergolide improves DRG axon length, which is inhibited in the presence of dopamine D1 antagonist. **(A)** Pergolide improves DRG cell dendritic length. Different concentrations of pergolide (µg/ml) were incubated with chicken DRG cells and measured for DRG dendritic lengths after 72 hours (n = 4). **(B)** Pergolide upregulated NGF expression in DRG cells. The NGF enzyme-linked immunosorbent assay was measured at 24 hours and 48 hours after 50 µg/ml of pergolide incubation (n = 6). **(C)** DRG cells were incubated with 300 µg/ml of pergolide and different concentrations of D1 antagonist (0–96 µM) and imaged for axon length. **(D)** Representative images of DRG cells treated with 300 µg/ml pergolide and 0 µM or 96 µM of D1 antagonist. One-way analysis of variance; multiple comparisons were computed with Prism 6 software. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

of pergolide (loaded in Marinisolv) at increasing concentrations (10, 25, 50, 150, and 300 µg/ml) was added to the cell culture media. For control, DRG explants were incubated in cell culture medium matrix (as described below) with no additional drugs or growth factors added. Unless otherwise specified, all reagents for cell culture were purchased from Fisher Scientific (Hampton, NH).

Fertilized chicken eggs (Merrill Poultry Farm; Paul, ID) were incubated at temperatures between 37.2°C and 38.9 °C and at 100% relative humidity for 9 days. DRGs were dissected from the embryos under a stereomicroscope as described previously.[18] In brief, the embryo was dissected, and spine was exposed. The DRGs from the spine were gently separated and isolated for culturing in laminin-coated plates. Dulbecco's Modified Eagle Medium (Nutrient Mixture F-12) supplemented with 10% fetal bovine serum and 1% antimycotic/antibiotic solution was added to each well containing a single DRG and the specified therapeutic combination. The DRGs were cultured in a humid atmosphere at 37°C and 5% CO_2 for 72 hours. Thereafter, the DRGs were fixed in methanol and imaged at 4× magnification on a widefield microscope (Nikon Spinning Disk, Tokyo, Japan) with a phase contrast lens and a digital camera to capture images. Neurite extension was measured using the image processing software ImageJ 1.52c (National Institutes of Health; Bethesda, MD).[18,19] Average neurite length (lave) was calculated as $lave = (A_{tot}/\pi)^{1/2} - (A_{DRG}/\pi)^{1/2}$. The lave of all 4 DRGs in an experimental group were averaged for the reported results.

DRG Neurite Outgrowth and Assessment of D1 Antagonism

DRG cells were cultured with different concentrations of pergolide (10, 25, 50, 150, and 300 µg/ml). Based on the results of this experiment, 300 µg/ml was chosen as the concentration of pergolide for subsequent experiments. Next, DRG cells were incubated with 300 µg/ml pergolide for 24 and 48 hours. The NGF secreted by the cells was measured using NGF enzyme-linked immunosorbent assay (R&D Systems; Minneapolis, MN). Both of these experiments used DRG cells treated with vehicle only (no pergolide) as baseline control.

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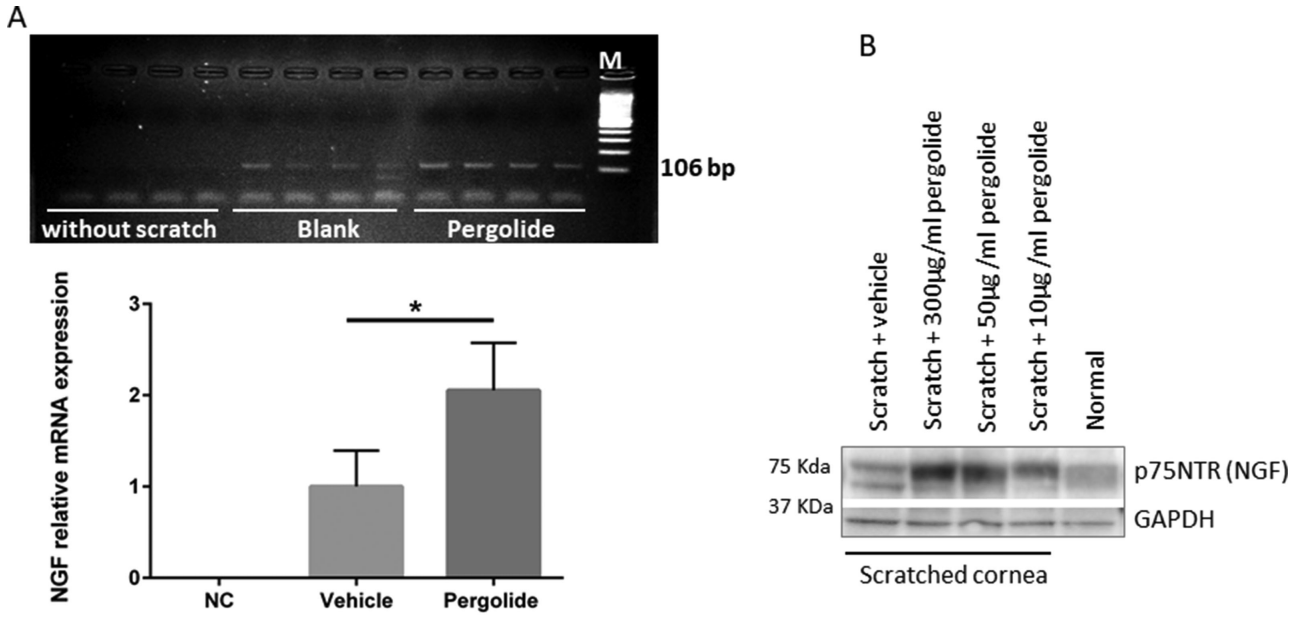

Figure 2. NGF is increased by pergolide. **(A)** Semiquantitative RT-PCR analysis of RNA extracted from cornea 24 hours after epithelial debridement. NGF gene expression in the corneas of the pergolide-treated group was 2.05-fold compared with the mean of the control group ($^{*}P < 0.05$; n = 4; analysis of variance repeated measures). **(B)** Western blots showing increased corneal NGF protein expression upon treatment with 10, 50, and 300 µg of pergolide compared with the control group.

It has been shown previously that pergolide can act via the D1 receptor.[16] To confirm the same in neural cells, DRGs were isolated as described previously and cultured along with 300 µg/ml of pergolide, followed by the addition of the D1 antagonist R(+)-SCH-23390 hydrochloride in different dosages (12, 24, 48, and 96 µM) and imaging for axon growth in the DRGs. The DRGs were cultivated for 72 hours. The drug was given in a single dose at the start of cultivation. DRG cells treated with no pergolide and no D1 antagonist served as a control.

In Vivo Effects of Drug Treatment on Injured Corneas

Animals. Male Balb/c mice 6 to 8 weeks old were purchased from the Jackson Laboratory (Bar Harbor, ME). All experiments were performed in accordance with the regulations of the Association for Research in Vision and Ophthalmology and were approved by the Institutional Animal Care and Use Committee of the University of Utah.

Corneal Epithelial Scratch Model. The mouse model of corneal wound injury has been described.[20] Briefly, three drops of 0.5% proparacaine hydrochloride ophthalmic solution (Bausch + Lomb; Rochester, NY) were applied, followed by IP injection of ketamine (90 mg/kg)/xylazine (10 mg/kg). After anesthesia, a trephine was used to introduce a 3-mm-diameter wound marker in the cornea of the right eye. Epithelium was removed with forceps. After wounding, erythromycin ophthalmic ointment (Perrigo; Minneapolis, MN) was used to prevent infection. The wound area was photographed and measured every 12 hours. The area of the epithelial defect was measured using ImageJ. The unhealed corneal epithelial defect was visualized by 1% fluorescein sodium staining and calculated as the percentage of the original defect. For treatment, the administration of pergolide (3 times/day) or control vehicle eye drops began the day the wound was made.

Preliminary Experiments with Pergolide and Cabergoline. Pergolide is reported to be a dopamine receptor D1 agonist and D2 agonist.[16] A separate group was treated with cabergoline, a dopamine receptor D2 agonist.[16] Groups of mice were subjected to corneal scratch injury and subsequently treated with blank liposomes (containing no drug), pergolide, or cabergoline-loaded liposomes in the form of eye drops (0.3mg/ml) three times a day for a period of 1 week. Eyes not subjected to any injury or treatment served as the normal control. Harvested corneas were subjected to immunostaining with class III β-tubulin antibody.

Immunofluorescence Staining. Corneal whole-mount staining was performed as previously described.[21] In brief, mouse eyes were collected a week after injury and treatment and fixed in acetone for 1 hour. The cornea was dissected around the scleral–limbal region. The cornea was blocked by PBS containing 0.1% Triton™ X-100 (Sigma-Aldrich) and 3% bovine serum albumin for 1 hour, and subsequently incubated in the same incubation buffer with nonconjugated class III β-tubulin polyclonal rabbit antibody (ab18207, 1:200) overnight at 4°C. Further, incubation with secondary antibody Alexa Fluor® 546 (Thermo Fisher Scientific; Waltham, MA) was for 1 hour at room temperature. The flat mounts were examined under an EVOS® fluorescence microscope (Life Technologies). The quantification of corneal innervation was calculated as the percentage of area positive for β-tubulin staining as previously described.[22]

RNA Extraction and PCR Analyses. Several neurotrophic factors associated with cornea nerve regeneration, including NGF, glial cell-derived neurotrophic factor, brain-derived neurotrophic factor, and vascular endothelial growth factor were determined by reverse transcription polymerase chain reaction (RT-PCR) (Supplementary data).

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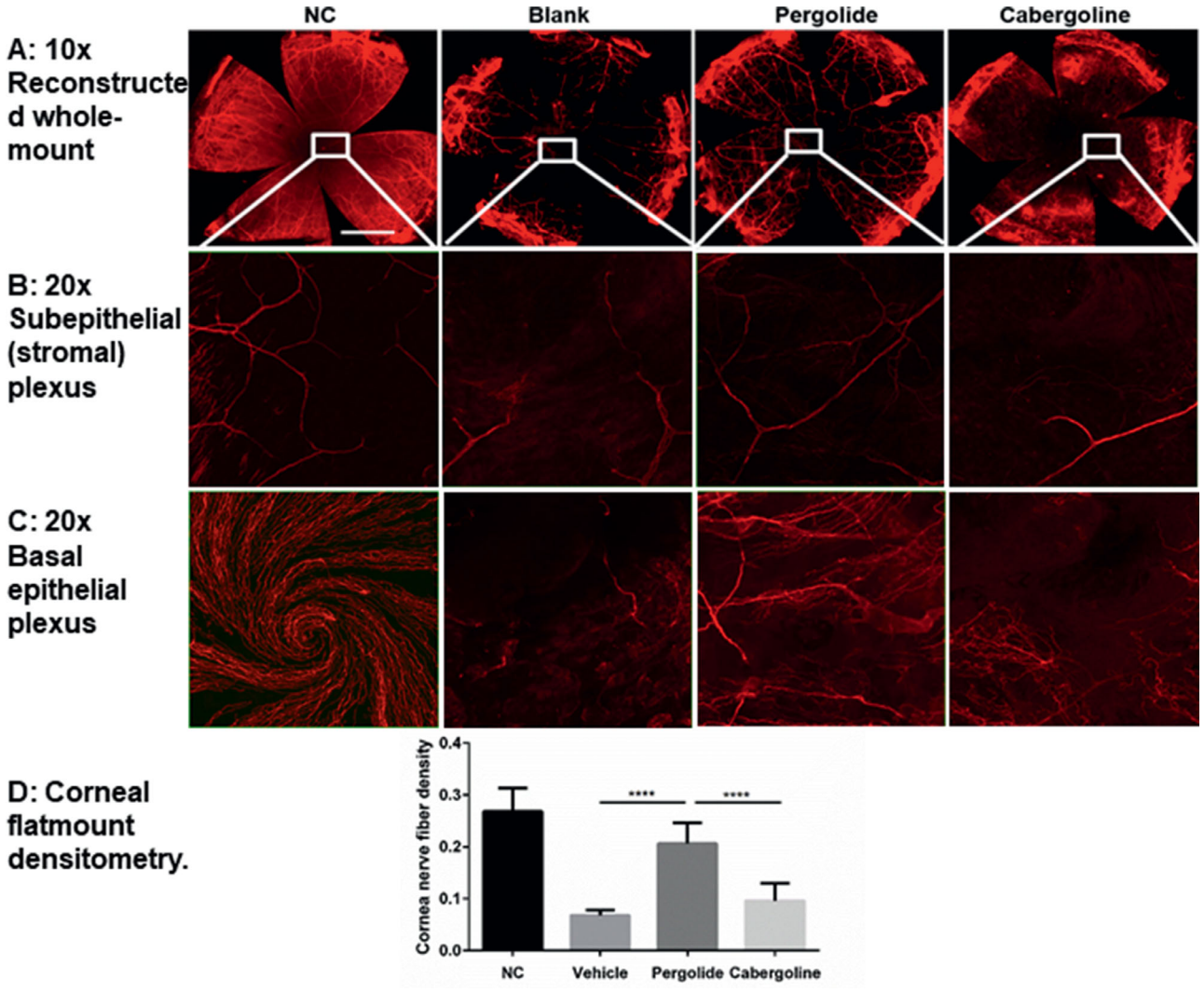

Figure 3. Pergolide, but not cabergoline, improves corneal nerve fiber regeneration. Balb/c mice (cornea scratch model) were treated with topical liposomes loaded with pergolide, cabergoline, and vehicle control (blank) eye drops 3 times per day for 1 week. Corneas were harvested and stained with β-tubulin antibody. Nerve density was closer to normal cornea with pergolide treatment. Cornea flatmount was calculated with ImageJ software; 10× scale: 1000 µm; 20× scale: 200 µm; n = 6. One-way analysis of variance; multiple comparisons were computed with Prism 6 software. $^{****}P < 0.0001$.

Total RNA was extracted from homogenates of cornea in each group (n = 3). Cornea was trephined with a 3.0-mm-diameter trephine 24 hours after debridement. The cornea was homogenized with the RNeasy® Mini Kit (Qiagen, Hilden, Germany). Single-strand cDNA was synthesized using a first-strand synthesis system for RT-PCR (Qiagen QuantiTect® Reverse Transcription Kit) and a random primer and was used as a template for PCR. PCR experiments were normalized to beta-actin gene expression. The PCR conditions were 5-minute hot start at 94°C, followed by 30 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 58°C, and extension for 1 minute at 72°C. Amplified products were separated by electrophoresis on a 1.0% agarose gel and visualized by ethidium bromide staining. To investigate the relative expression of NGF, band densities were measured with ImageJ software.

Western Blot. To determine NGF expression in corneas upon topical exposure to different concentrations of pergolide, proteins from normal and scratched corneas were blotted and probed with monoclonal primary antibody p75NTR, a low-affinity nerve growth factor receptor (Cell Signaling Technology; Danvers, MA) raised in rabbit at a 1:1000 dilution and 1:4000 dilution of goat anti-rabbit secondary antibody (Thermo Fisher Scientific). Glyceraldehyde 3-phosphate dehydrogenase (Abcam; Cambridge, MA) was used as a loading control. Bands were visualized by chemiluminescence at 75 kDa and 37 kDa for p75NTR and glyceraldehyde 3-phosphate dehydrogenase, respectively, by the Azure Biosystems cSeries imaging platform (Dublin, CA).

Statistics. Statistical analyses were performed using Prism 6 (GraphPad Software; San Diego CA). Data are presented as the mean ± standard deviation. Experiments were analyzed using data calculated by two-way *t*-test to determine overall differences, and a Tukey's multiple comparisons test was performed to determine statistically significant differences between treatment groups. Significance was accepted at a P value of <0.05. Experiments were repeated at least twice to ensure reproducibility.

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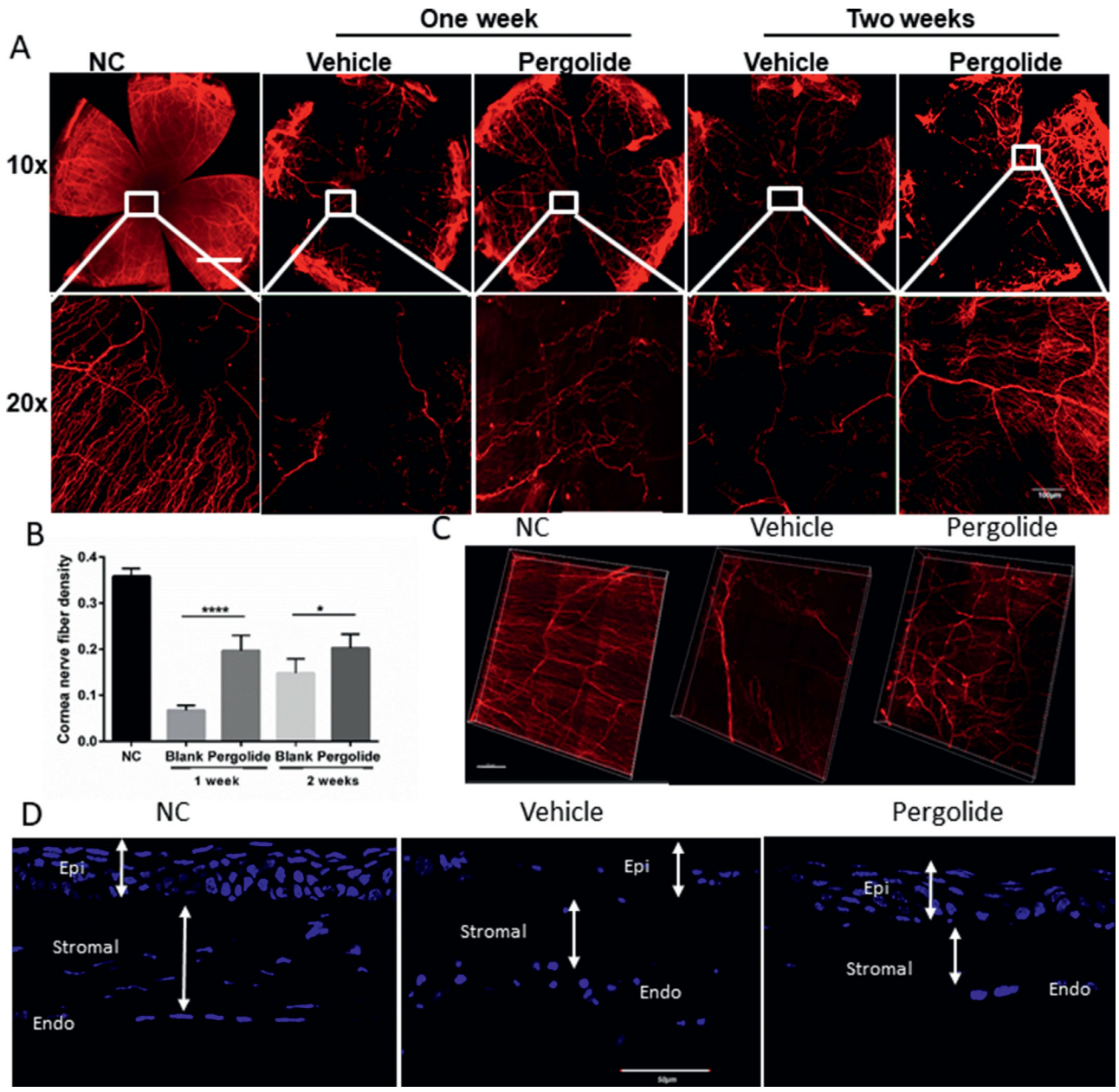

Figure 4. Pergolide hastens corneal reinnervation after scratch. **(A)** Balb/c mice that underwent cornea scratch were treated with topical pergolide loaded in Marinosolv and blank control (vehicle only, no pergolide) eye drops 3 times per day for 1 week or 2 weeks. Corneas were harvested and stained with β-tubulin antibody. **(B)** Cornea flatmount was calculated with ImageJ software and analyzed by Tukey's multiple comparison test. $^{***}P_{1\text{week}} = 0.00000032$; $^{*}P_{2\text{weeks}} = 0.0019$. **(C)** Representative three-dimensional images in mouse cornea epithelium layer demonstrate enhanced corneal nerve reinnervation (and density closer to uninjured normal cornea) after pergolide treatment for 1 week compared to vehicle treatment after corneal scratch injury. **(D)** Cornea stained with DAPI for visualization of epithelial layer and part of stroma, 24 hours after scratching by confocal microscopy (60×). Pergolide drops and vehicle were applied 3 times over 24 hours and showed improved epithelial density. NC, normal control

Results

Pergolide Induces DRG Neurite Growth

Chicken DRG axon length significantly increased at the higher dose treatments (50 to 300 µg/ml) (Fig. 1A). Moreover, NGF levels were elevated (Fig. 1B) in the pergolide-treated DRG cells after 24 hours and 48 hours. Further, to mechanistically confirm the role of D1 receptors, axon growth was measured in the presence of different concentrations of a D1 antagonist. DRG axon length extension was inhibited in a directly proportional dose-dependent fashion (Figs. 1C and 1D).

Pergolide Increases NGF mRNA and Protein Expression

We examined levels of several neurotrophic factors associated with cornea nerve regeneration, including NGF, glial cell-derived neurotrophic factor, brain-derived neurotrophic

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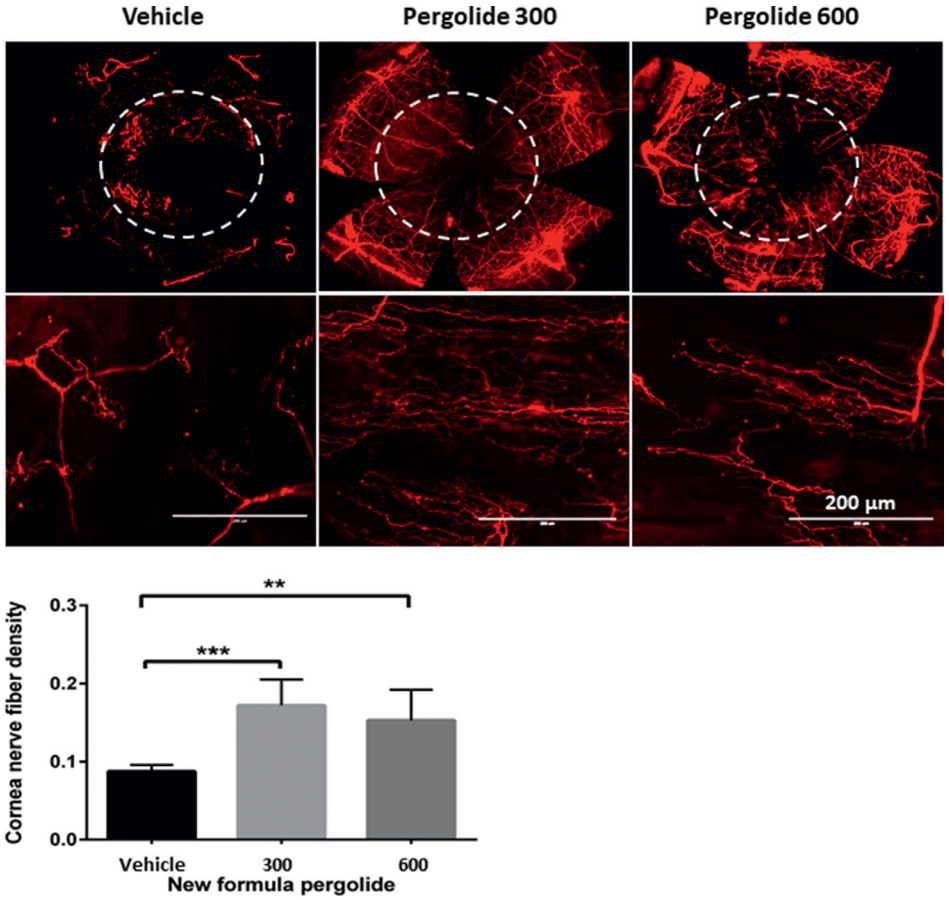

Figure 5. Dose–efficacy of pergolide in Marinosolv. The Balb/c cornea scratch model was created as before. Marinosolv formulation containing dissolved pergolide concentrations of 300 µg/ml or 600 µg/ml was applied 10 µl × 3 times per day for 1 week. Cornea nerve regeneration was evaluated by anti-β-tubulin III antibody (ab18207, 1:200 in 3% BSA) incubated with cornea flatmount. Only the scratch area was calculated. One-way analysis of variance was used to analyze statistical differences. $^{**}P < 0.01$; $^{***}P < 0.001$ (n = 6). Dash white circle: scratch area that was calculated.

factor, and vascular endothelial growth factor. RT-PCR demonstrated that only NGF was upregulated after the cornea was wounded. Further, upon treatment with liposomes loaded with pergolide, gene expression of NGF was significantly higher than in the vehicle control group (Fig. 2A). This was further confirmed by protein expression of NGF after treatment with different concentrations of pergolide as a clear aqueous solution with the Marinosolv formulation (10, 50, and 300 µg/ml) (Fig. 2B). Protein expression of NGF increased with pergolide treatment in a dose-dependent manner. Both liposomes and Marinosolv were effective as a vehicle for pergolide.

Pergolide, But Not Cabergoline, Improves Corneal Nerve Fiber Regeneration

Compared to blank control mice (vehicle only, no drug), only pergolide ($P < 0.0001$) but not cabergoline ($P > 0.05$) improved cornea nerve fiber regeneration (Fig. 3D). There was no difference between 1 and 2 weeks of treatment in mice (Fig. 4). Further, we tested 2 different treatment doses on mice (Fig. 5). Compared to blank control, 300-µg therapy induced superior recovery compared to the 600-µg dose. Representative three-dimensional images that clearly identify the corneal neuron axon regeneration are presented in Fig. 4C. Moreover, pergolide treatment hastened epithelial recovery and showed improved epithelial density in the scratched corneas (Fig. 4D).

Pergolide Improves Corneal Wound Healing

Corneal wound area decreased in a time-dependent fashion, with complete wound closure occurring after 2 days in mice without treatment. Corneal wound healing was faster in mice treated with pergolide (Fig. 6A) than in blank control mice (vehicle only, no drug). Quantification of the data confirmed that pergolide significantly improved corneal wound healing (Fig. 6B).

Discussion

Regeneration of corneal nerves and restoration of neural sensitivity is a cornerstone of therapies designed to target neurotrophic keratopathy; therefore, NGF has been of interest, as it restores corneal nerves and sensitivity and promote epithelial healing.[16,23] Corneal epithelium, keratocytes, and endothelium produce NGF in humans and mice.[23–26] NGF accelerates corneal epithelial proliferation, which aids

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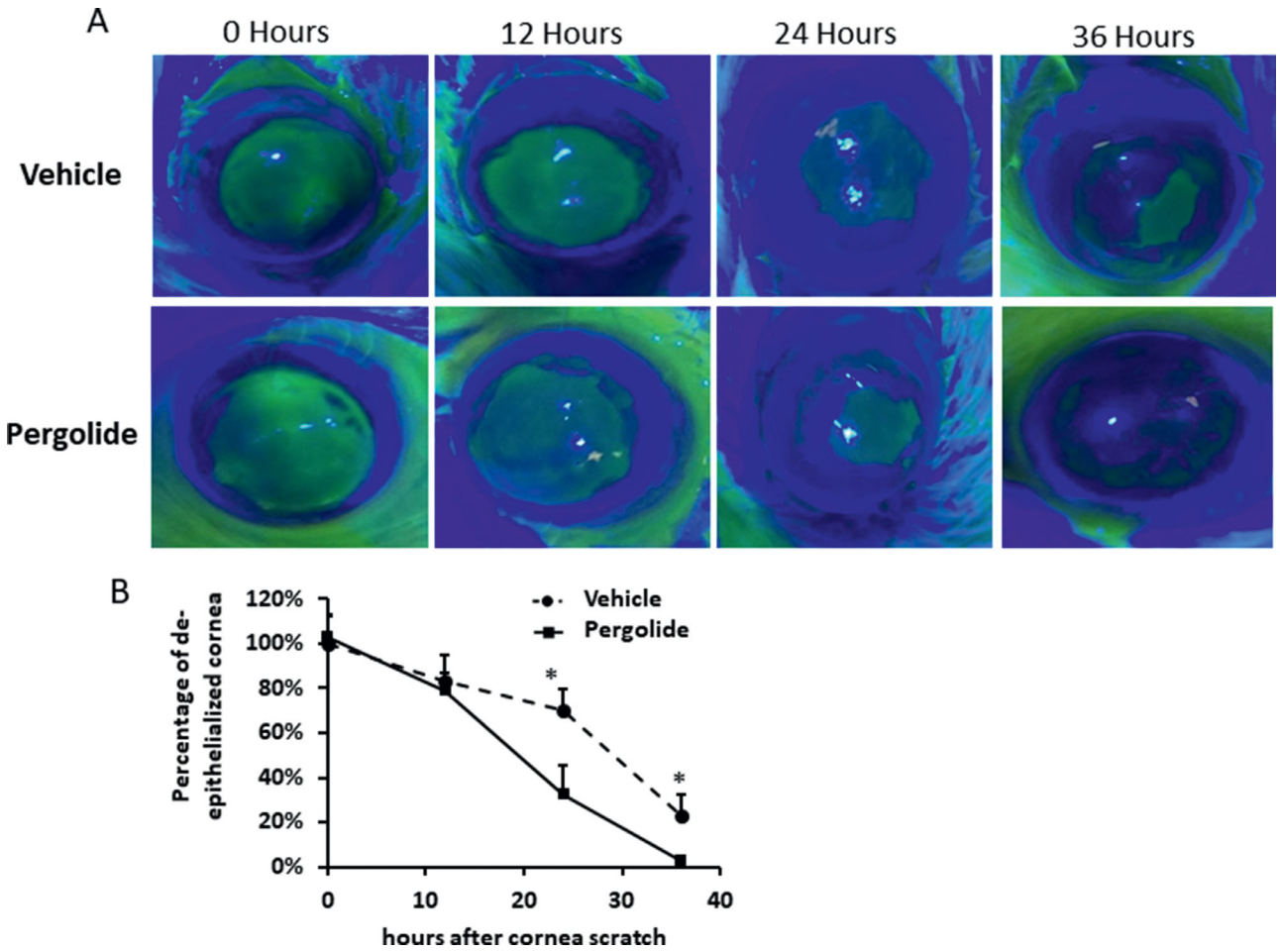

Figure 6. Pergolide improved cornea wound healing. **(A)** Representative macroscopic images of fluorescein-stained corneal wounds in BALB/c mice after treatment with or without pergolide eye drops for 2 days (3 times/day). **(B)** Wound area was measured every 12 hours after wounds were introduced (n = 6). Error bars indicate means ± standard deviation. Statistical analysis was performed with the unpaired Student's *t*-test for comparisons between two groups at the same time point.

healing and restoration of the injured epithelium. Moreover, NGF may play an important role in corneal nerve sensitivity by its release of several neuropeptides and its trophic effect on the peripheral nervous system.[27–29] Our experiments to test the efficacy of pergolide confirmed its ability to increase NGF and enhance nerve growth in vitro and in vivo.

Dorsal root ganglia and trigeminal nerve share many similar functions,[30] as both are somatic afferent fibers that release dopamine and other neurotransmitters. In addition, DRG neurons express multiple dopamine receptors, mainly D1R and D5R, but not D2R.[31] Therefore DRG neurons were deemed an appropriate model for the in vitro studies, which showed that the optimal therapeutic concentration of pergolide seemed to be 300 µg/ml (Figs. 1 and 5); therefore, this concentration was used for subsequent experiments. The 600-µg/ml concentration may not result in enhanced efficacy, possibly due to receptor saturation effects or pharmacodynamic issues.

Corneal wound healing in mouse cornea was significantly improved with pergolide treatment (Fig. 6). This corresponded with regeneration of corneal nerves in pergolide-treated mice as well as improved restoration of the epithelial cells (Figs. 3–5). In the cornea, nerve fiber morphology displayed obvious changes compared with normal cornea, as treated corneas showed less fiber density and more tortuosity (Figs. 1 and 2) and is similar to human cornea nerve morphology after LASIK.[32] Furthermore, NGF levels were distinctly upregulated with pergolide treatment, which correlated with the improved neural innervation in the mice (Figs. 1 and 2). This corroborates the findings of Ohta et al.,[16] who reported increased NGF levels in cultured astrocytes treated with pergolide, and Kawamoto et al.,[25] who reported accelerated wound healing with NGF in corneal ulcers in normal and healing-impaired diabetic mice.

The possible mechanism for the ameliorative effect exerted by pergolide on neural regeneration in corneal injury was examined (Fig. 3). Pergolide is a known dopamine receptor D1 and D2 agonist, and cabergoline is a D2 agonist/weak D1 agonist.[16] Although the observations for pergolide were supported by a previous study,[16] the results for cabergoline differed, with significantly lower innervation with cabergoline. This led us to speculate that the mechanism underlying neural regeneration with pergolide involves the dopamine receptor D1 but not D2. This hypothesis was corroborated by our experiment where inhibition of D1 blocked pergolide activity (Fig. 1C). Further, the corresponding increase in NGF levels suggests a connection between D1 and NGF that results in enhanced wound healing and innervation (Figs. 1–3). This is an exciting avenue for future studies and warrants deeper exploration

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to elucidate the pathway(s) involved. Future investigations should also evaluate the impact of pergolide on corneal maturation and intact epithelium (by immunohistology) and visual acuity. Further, the effect of pergolide in more complex models such as NK induced by herpes simplex virus (HSV) or diabetes would be of interest. Additionally, studies on the potential side effects and optimal dosing of pergolide as eye drops are warranted. However, it should be kept in mind that in the cornea most of the nerve fibers are sensory nerves originating from the trigeminal nerve,[5] whereas in HSV-1 keratitis there could be repeated damage to the sensory nerve, preventing nerve regeneration. Instead, sympathetic nerve ingrowth with associated inflammation may be seen in HSV-1 keratitis.[33]

Pergolide was used in two different formulations in this study. Because it is poorly soluble in aqueous solutions, we initially incorporated it into liposomes. However, this posed difficulties including turbidity, particle size, and limited concentration loading. These problems were overcome by using Marinosolv loaded with pergolide, which came with the added advantages of being a clear solution and suitable for intravitreal injections, as well. Marinosolv-based eye drops gave in vivo results equivalent to those of the initial liposome formulation (Figs. 3–5).

Pergolide was originally developed as a drug for Parkinson's disease; however, systemic administration of pergolide resulted in increased cardiac valvulopathy, which led to its withdrawal from US and Canadian markets. It continues to be available as a drug for human use in other countries, including the United Kingdom and Australia.[34] There remains a strong rationale for repurposing pergolide as a therapeutic for ocular neuronal conditions in which drug delivery can be confined to injured tissues, eliminating the possibility of off-target effects. The bioavailability of drugs administered to the surface of the eye is very low compared to systemic administration due to the anatomic isolation of the eye, small surface for absorption, corneal metabolism, binding proteins in tear fluid, blinking, small volume of eye drops, and blood–retina barriers.[35,36] This should permit localized effects of pergolide on the injured corneal tissues while avoiding systemic side effects. Further, Marinosolv allows the lipophilic drug to be loaded and dispensed as a clear solution and therefore can be explored in future studies. Topical aqueous eye drops are preferred over suspensions and emulsions, as the formulation is generally less complex, easy to administer, and more comfortable to use, resulting in better patient compliance.[36] Further, although both liposomes and Marinosolv were effective in delivering pergolide, because of its various advantages the latter may be the preferred choice in subsequent studies. In the future, we will also assess pergolide safety in the mouse heart and other small animals after long-term treatment with topical eye drops, prior to a clinical trial.

In conclusion, pergolide was effective in enhancing corneal neural regeneration and epithelial wound healing. Although the entire pathway is not understood, it is apparent that pergolide could exert its effects by upregulating NGF levels, making it a potential drug candidate and a novel therapy for neurotropic keratopathy. Additionally, Marinosolv was identified as a feasible aqueous drug carrier with distinct advantages for formulation of pergolide as eye drops. Pergolide loaded in Marinosolv could be a potentially efficacious therapeutic approach for the restoration of corneal sensation and visual acuity loss due to neurotrophic keratopathy.

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