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Article

Conjunctivally Applied BDNF Protects Photoreceptors from Light-Induced Damage

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Purpose: To test whether the topical eye treatment with BDNF prevents the effects of continuous light exposure (LE) in the albino rat retina.

Methods: Two groups of albino rats were used. The first group of rats received an intraocular injection of BDNF (2 μL, 1 μg/μL) before LE, while the second group was treated with one single drop of BDNF (10 μL, 12 μg/μL) dissolved in different types of solutions (physiological solution, the polysaccharide fraction of Tamarind gum, TSP, and sodium carboxy methyl cellulose), at the level of conjunctival fornix before LE. The level of BDNF in the retina and optic nerve was determined by enzyme-linked immunosorbent assay. We recorded the flash electroretinogram (fERG) in dark adapted rats 1 week after LE. At the end of the recording session, the retinas were removed and labeled so that the number of photoreceptors nuclear rows and thickness of the outer nuclear layer was analyzed.

Results: Intravitreal injection of BDNF before LE prevented fERG impairment. Different ophthalmic preparations were used for topical eye application; the TSP resulted the most suitable vehicle to increase BDNF level in the retina and optic nerve. Topical eye application with BDNF/TSP before LE partially preserved both fERG response and photoreceptors.

Conclusions: Topical eye treatment with BDNF represents a suitable, noninvasive tool to increase the retinal content of BDNF up to a level capable of exerting neuroprotection toward photoreceptors injured by prolonged LE.

Translational Relevance: A collyrium containing BDNF may serve as an effective, clinically translational treatment against retinal degeneration.

Introduction

Neurodegeneration of photoreceptors represents a group of inherited (Mendelian, most of the cases) or induced retinal diseases for which no treatment is available; the neurodegeneration process is similar to the programmed cell death occurring in different nuclei of the central nervous system with progressive apoptosis of photoreceptors. Molecules with neurotrophic actions including neurotrophic factors of NGF family (neurotrophins) were shown to be effective in slowing down cell death in various models of neurodegenerative diseases.[1–4] The biological action of neurotrophins is mediated by specific receptors present on the cell membrane. Various types of neurotrophin receptors have been characterized: a low-affinity receptor (p75) capable of binding NGF as well as other neurotrophins and a group of tyrosine kinase type receptors (Trks) specific for each neurotrophin. Trk receptors increase their autophosphorylation activity and trigger a cascade of intracellular events responsible for cell survival and differentiation, phenotype maintenance, and plasticity.[5] In the mammalian retina, BDNF and its TrkB receptor are highly expressed.[6–9] Genetic targeting of BDNF, or disruption of BDNF signaling, impairs

transmission of electrical signals from photoreceptors to second-order neurons[10] and results in alterations of the inner retina.[11] Remarkably, intraocular injection of BDNF was capable of preventing light induced apoptosis of photoreceptors in albino rats.[12,13]

So far BDNF, as well as other growth factors, has been typically administered to the internal ocular tissues by intravitreous or retrobulbar injection; these methods of treatment are associated with the risk of various complications such as the ocular bulb perforation and infections.[14] In the present work, we searched for an alternative strategy permitting an easy, safe, and repeatable treatment to increase the level of neurotrophic factors in the retina. To this aim, we exploited the possibility of administering BDNF using the external ophthalmic route, that is, in the form of collyrium; previous results by our group showed that topical eye application of BDNF was able to increase the BDNF retinal content in the mouse and rat retina.[15] We have selected one model of neurodegeneration to study retinal protection by topical eye application of BDNF. This model is based on light-induced damage (light-damage) to the albino rat retina caused by exposure to continuous bright light; this is an in vivo model to induce a fast and synchronized program of photoreceptor apoptosis.[2] Exposure to bright continuous light was previously used as a model system to assess quantitatively the photoreceptor survival-promoting activity of various neurotrophic agents, including BDNF.[12,13] In the present paper, human recombinant BDNF was intravitreally injected and topically applied at the level of conjunctival fornix in separate groups of albino rats. Different vehicles were used for topical eye application of BDNF; the retina and optic nerve levels of the BDNF protein were measured in tissue extracts taken at different times after treatment using high sensitive enzyme-linked immunosorbent assay (ELISA). The flash electroretinogram (fERG) and histology of outer retina were investigated in treated and untreated eyes of light exposed (LE) albino rats. Our results clearly indicated that topical eye application of BDNF in the form of collyrium was able to preserve the retinal response to flash stimuli and to prevent alteration of photoreceptors in light-damage of albino rat retinas.

Material and Methods

Ethics Statement

All experiments were conducted according to Ministry of Health (the regulatory authority for controlling the use of laboratory animals and ethics on animal experiments in Italy) guidelines (Legislative Decree n. 116/92) and in accordance with the European Community guidelines (European Directive 86/609/EEC). The experimental protocol (IACUC document) was approved by the Ministry of Health (n. 152/2013; IACUC is renewed every 3 years). The authors confirm their adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

BDNF Preparations

Thirty-four 16-week-old male Wistar albino rats (Charles River Italia, Lecco, Italy) were used. Before BDNF administration, rats were anesthetized with an intraperitoneal injection of Avertin at 2 mL/100 g body weight (1.25% [w/v] 2,2,2-tribromoethanol and 2.5% [w/v] 2-methyl-2-butanol; Aldrich, St. Louis, MO). Human recombinant BDNF (QED Bioscience, Inc., San Diego, CA) at concentrations comprised between 1 and 12 μg/μL was dissolved in a physiological solution (0.9% NaCl) and applied as a drop (10 μL) at the level of conjunctival fornix under dissecting microscope (Leica, Germany); the site of application was gently compressed for a few minutes. In a second group of rats, we used the polysaccharide fraction of Tamarind gum (TSP; 0.25%, Farmigea Holding Srl, Pisa, Italy) or sodium carboxy methyl cellulose (CMC; 0.2%, Farmigea Holding Srl) as vehicles, with the aim to produce thickened ophthalmic solutions facilitating prolonged BDNF delivery. In a parallel group of albino rats, BDNF was also supplied using single intravitreal injection (human recombinant BDNF at the concentration of 1 μg/μL in 2 μL of 0.9% NaCl). Topical eye treatment and intravitreal injection were performed 3 hours before prolonged exposure to light (light damage) with animals left to recover from anesthesia.

Light-Induced Damage

The protocol for light damage (photolesion) derives from that described by LaVail et al.[12] with minor modifications. Briefly, albino rats were reared on a 12-hour light/dark cycle, with the illumination level below 60 photopic lux, until the day of LE. At that time, the rats were housed separately in transparent Plexiglas cages and exposed to continuous light produced by four 36-W white fluorescent bulbs (Osram Sylvania, Munich, Germany). Light sources were suspended 30 cm above (two bulbs) and under (two bulbs) cages to produce a uniform

illumination of 1000 lux. Animals had free access to food and water. After continuous illumination for 48 hours (LE), animals were returned in a room with a 12-hour light/dark cycle for 5 days before recording and subsequent retinal histological analysis.

Flash Electroretinogram

Flash electroretinogram was performed according to previously published methods.[16,17] Briefly, the electrophysiological signals were recorded through gold plate electrodes inserted under the lower eyelids in contact with the cornea, previously anesthetized with oxybuprocaine (Novesine, Novartis Pharma, Switzerland). Corneal electrodes from each eye were referred to a needle electrode inserted subcutaneously at the level of the corresponding ear region. The different electrodes were connected to a two-channel amplifier (LACE, Pisa, Italy). Animals were dark adapted (360 minutes), anesthetized using Avertin as described and loosely mounted in a stereotaxic apparatus under dim red light with the body temperature maintained at 37.5°C and the heart rate monitored; recordings were preferably performed at noon to avoid amplitude oscillations linked to circadian rhythms. FERG was evoked by 10-ms single flashes of different luminances generated through a Ganzfeld stimulator (LACE) in scotopic conditions. The amplitude of the a- and b-wave for each eye was plotted as a function of increasing luminance (transfer curve); for each animal group the mean a- and b-wave amplitude ± standard error of the mean (SEM) was plotted.

Histological Analysis

After fERG recording, rats were sacrificed and their eyecups were harvested and fixed by immersion in a solution of 4% paraformaldehyde (PFA) in 0.01 phosphate buffer (PB, at pH 7.4) in the light phase during the diurnal cycle; a cut was made in the top of eye cup for successive orientation (the 12:00 meridian of eye was marked). Then, eye cups were washed in PB several times and cryoprotected in 30% sucrose in PB at 4°C overnight. Eye cups were then embedded in cryomatrix (Tissue Tek, Reichert-Jung, Nuloch, Germany) and frozen. Serial frozen sections (10 μm) were cut on a cryostat (Reichert-Jung) perpendicular to the 12:00 meridian, starting from the superior border of the cornea. We used two sampling methods. In a preliminary series of experiments, sampling was performed along the vertical meridian, at a distance of 750 μm, this resulting in 15 retinal sites of sampling according to Maccarone et al.[18]; we used a window covering an area of 250 × 250 μm. Since LE for 48 hours dramatically reduced the thickness of the outer nuclear layer (ONL) homogenously along the vertical meridian, we decided to use a second sampling method. We performed sampling at two sites along the vertical meridian taken at different distances from the optic nerve head: one site in the peripheral retina (3.5 mm from the optic nerve head) and one site in the central retina (1 mm from the optic nerve head). In this way, two peripheral and two central retinal regions (two regions for superior and two regions for inferior quadrants, this resulting in eight retinal sites; we used a window covering an area of 250 × 250 μm) were selected along the vertical meridian for each retina, and sampled together with other retinas within each experimental group, holding separate peripheral and central retinal sites. We decided to use this second method unless otherwise stated. Sections were collected onto gelatin-coated slides, air-dried, and stored at −20°C until further processing. Propidium Iodide (Molecular Probes; 1:1000 in phosphate-buffered saline; Thermo Fisher Scientific, Waltham, MA), a nuclear stain fluorescent in the red spectra, was used to compare the number of rows of photoreceptors or thickness of ONL. Images were taken by confocal microscope (Leica) and fluorescence microscope (Nikon, Japan). To quantify photoreceptors, the number of nuclei rows in the ONL and the ONL thickness were separately assessed for each retina.

Enzyme-Linked Immunosorbent Assay

Retinas and optic nerves were extracted in anesthetized rats and immediately homogenized in five volumes of 100 mM Tris buffer (pH 7.4) containing 500 mM NaCl, 2% TritonX-100, 1% NP40, 10% glycerol, and protease and phosphatase inhibitors: 1 mM PMSF, 10 g/mL aprotinin, 1 g/mL leupeptin (Sigma Aldrich, Milano, Italy). This homogenate was centrifuged at 10,000 rpm for 30 minutes, and the supernatant fraction was used for ELISA. We performed a sandwich ELISA using the BDNF Emax immunoassay system from Promega (Promega Corporation, Madison, WI) to measure retinal and optic nerve levels of BDNF. Briefly, 96-well plates were coated with anti-BDNF monoclonal antibody and incubated at 4°C for 18 hours. The plates were incubated in a blocking solution for 1 hour at room temperature, then samples were added. The samples and BDNF standards were incubated for

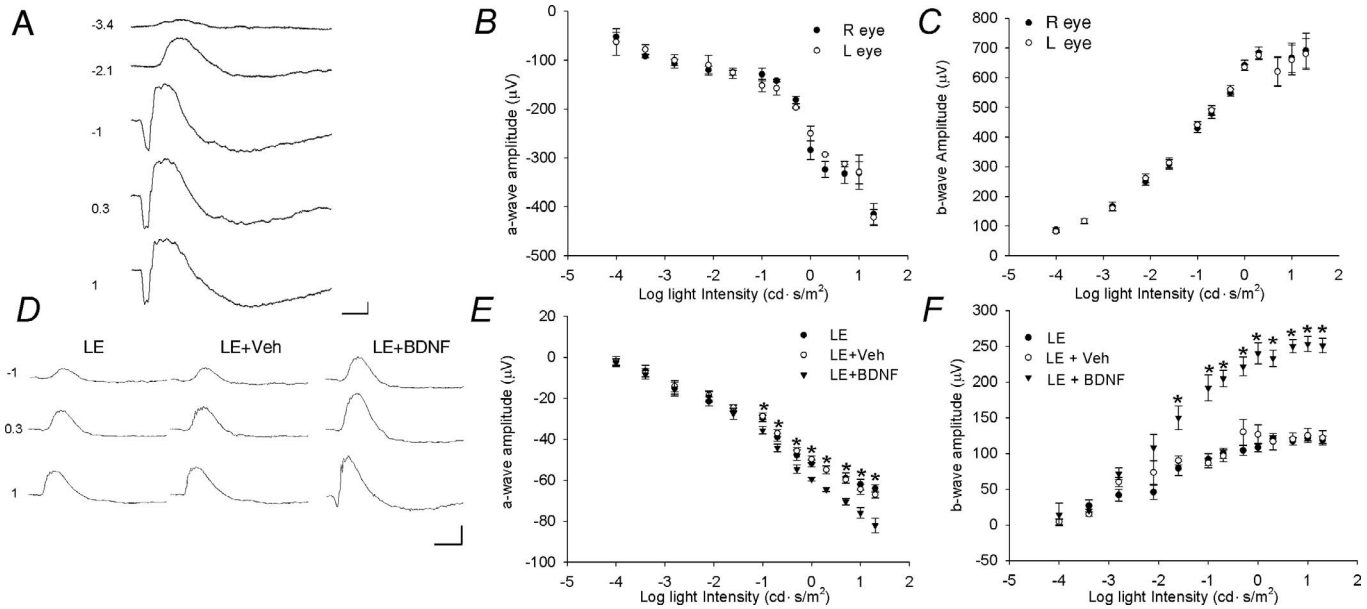

Figure 1. Flash ERG (fERG) in control and light-damaged rats. (A) Representative traces of dark-adapted fERG evoked at five different luminances (top-down traces, log units). Calibration bars: horizontal bar = 100 ms, vertical bar = 100 μV, top and bottom deflection indicates positive and negative voltage, respectively. (B, C) We reported the transfer curve for a-wave (B) and b-wave (C) amplitude (μV) recorded at different light intensities (log cd s/m^2) for stimulation of right (R) and left (L) eye ($n = 5$). (D) Representative traces recorded at three different luminances (top-down traces, log units) in LE rats, LE rats whose eye was intraocularly injected with vehicle (LE + Veh; NaCl 0.9%, 2 μL), and LE rats whose eye was intraocularly injected with BDNF (LE + BDNF; 2 μL of hBDNF at the concentration of 1 μg/μL). Calibration bars: horizontal bar = 100 ms, vertical bar = 100 μV, top and bottom deflection indicates positive and negative voltage, respectively. LE dramatically decreased the a- and b-wave amplitude at different luminances; BDNF injection partially prevented the fERG impairment. (E) The transfer curve for a-wave amplitude (μV) recorded at different light intensities (log cd s/m^2). (F) The transfer curve for b-wave amplitude (μV) recorded at different light intensities (log cd s/m^2). The results in E and F show that a- and b-wave amplitudes in the LE eyes injected with BDNF (LE + BDNF) were significantly higher compared to those measured in LE vehicle-treated eyes (LE + Veh); five eyes for each group were analyzed (one-way ANOVA, $*P < 0.05$).

2 hours, followed by washing with the appropriate buffer. Successively, the plates were incubated with anti-human BDNF polyclonal antibody at room temperature for 2 hours, washed, and then incubated with specific anti-IgG antibody conjugated to horseradish peroxidase for 1 hour at room temperature. The plates were finally incubated with chromogen substrate to produce color reaction. The reaction was stopped with 1M HCl, and absorbance was measured at 450 nm using a microplate reader (Model 550, Bio-Rad Laboratories, Milano, Italy).

Statistics

The statistical significance of differences between untreated, control vehicle treated, and BDNF-treated eyes was achieved using individual Student's *t*-test and one-way analysis of variance (ANOVA) for ELISA and histological analysis; for fERG analysis, we used two-way ANOVA (Sigma Stat) followed by Holm-Sidak all-pairwise multiple comparison procedure; $P < 0.05$ was considered significant.

Results

Previous reports[12,13] showed that single intraocular injection of BDNF at the concentration comprised between 1 and 10 μg/μL was able to protect retinal cells in different animal models of retinal degeneration,[2] including light-damage. We repeated these experiments in our experimental context. We used intravitreal injection of BDNF 3 hours before LE (1 μg/μL in NaCl; volume = 2 μL). After 3 hours from BDNF treatment, the albino rats were exposed to constant light (1000 Lux; LE) for 48 hours and, successively, left to recover in their cages; 7 days after light-damage (calculated from the first day of LE) rats were anesthetized and dark adapted for fERG recordings. Retinal responses to flashes of different intensities in normal untreated eyes are reported in Figure 1 (A) as single traces and Figure 1 (B, C) as the plot of a- and b-wave amplitudes at different light intensities (cd s/m^2). We found that a single intraoc-

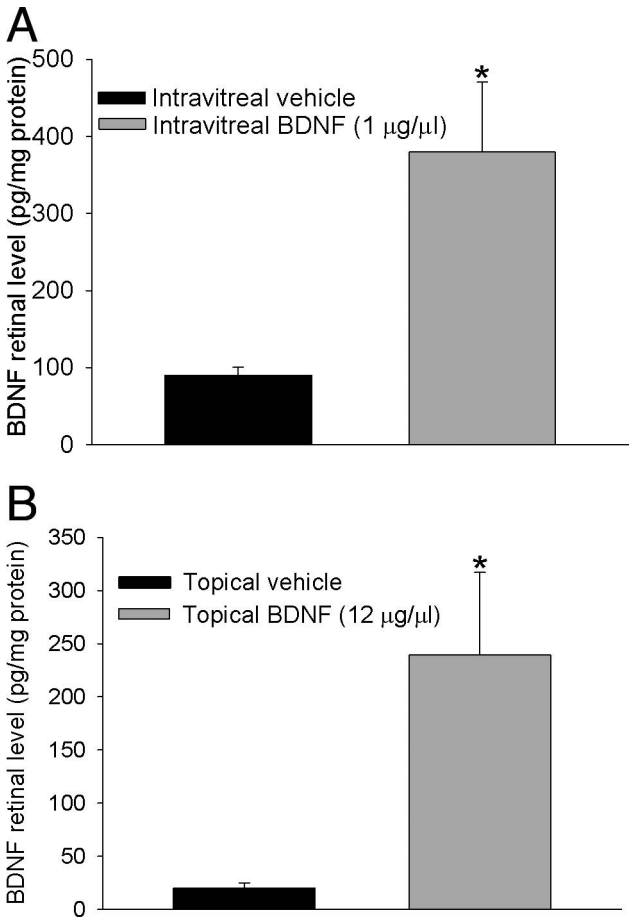

Figure 2. Retinal level of BDNF following intravitreal and topical eye treatment. (A) The histogram shows the rat retinal BDNF level (pg/mg protein, ELISA) in the BDNF injected eye (BDNF concentration = 1 μg/μL in physiological solution, volume 2 μL; $n = 5$ retinas) and vehicle injected eye (physiological solution, volume = 2 μL; $n = 5$ retinas). (B) The histogram reports the rat retinal level of BDNF following single topical eye treatment with BDNF (concentration = 12 μg/μL, volume = 10 μL, $n = 5$ retinas) respect to vehicle treated eye (physiological solution, volume = 10 μL). Retinal level of BDNF was determined by ELISA 6 hours after treatment; $^*P < 0.05$ (Student's *t*-test).

ular injection of BDNF before LE was able to prevent, at least partially, the fERG impairment (Figs. 1D, 1E, 1F); as clearly visible in Figure 1, the fERG was nearly abolished by LE with a- and b-wave amplitude dramatically decreased at all light intensities (Figs. 1D, 1E, 1F, LE + vehicle and LE). Previous data by our group showed that the retinal BDNF level was significantly increased compared to untreated eye, when BDNF was intraocularly injected at the same concentration of 1 μg/μL and measured 6 hours after by ELISA.[15] Thus, intraocular injection of BDNF was able to prevent impairment of fERG caused by LE.

The next step was to know whether BDNF topical application to the eye was capable of increasing the retinal content of BDNF. We used BDNF at the concentration of 12 μg/μL in 0.9% NaCl according to recent results obtained in the mouse eye.[15] In Figure 2, we compared BDNF retinal level in the rat using intravitreal injection and topical eye treatment. Retinal BDNF level was significantly increased respect to the contralateral eye when BDNF was intravitreally injected (Fig. 2A) and topically applied (Fig. 2B). In addition, we explored whether different BDNF ophthalmic-based preparations characterized by high viscosity should facilitate the administration and continuous delivery of BDNF to the eye as liquid drop. To increase the viscosity of the solution, we used sodium CMC or the polysaccharide fraction of TSP (ophthalmic preparation frequently used as artificial tear). TSP was previously utilized as vehicle to carry pharmacologically active molecules for topical treatment of the eye surface.[19–22]; TSP is able to facilitate the absorption of active molecules by increasing their retention time on ocular surface. BDNF (5 μL at the concentration of 24 μg/μL in physiological solution) was added to 0.4% CMC and 0.5 % TSP (5 μL) to have a final concentration of 12 μg/μL in one drop (10 μL) of 0.2% CMC and 0.25 % TSP, respectively. Results in Figure 3 (A) showed that single drop application of BDNF/TSP (6 hours after treatment, $n = 5$, mean = 478.6 pg/mg protein, SEM = 85.5) induced a significant increase of BDNF level in the retina compared to control vehicle treated eyes (6 hours after treatment, $n = 5$, mean = 46.6 pg/mg protein, SEM = 16.8), BDNF/NaCl ($n = 5$, mean = 239.98, SEM = 80.44), and BDNF/CMC ($n = 5$, mean = 26.91, SEM = 3.6) topical eye treatment. Interestingly, similar results were obtained when BDNF was measured in homogenates from the optic nerve of the treated eye (Fig. 3B). The pharmacokinetic study of BDNF/TSP topical eye application showed that BDNF level in the retina (Fig. 4A) and optic nerve (Fig. 4B) was significantly higher when measured 6 hours after treatment returning slowly toward basal level by 24 hours; importantly, 12 hours after treatment, the level of the protein in the retina was still elevated over basal value ($n = 4$, $^*P < 0.05$).The reported results suggest that TSP represents a suitable vehicle for topical eye BDNF treatment.

Having demonstrated that a single drop of BDNF/TSP to the eye is able to increase the rat retinal content of BDNF, similarly to our recent results in mouse retina,[15] we afforded the question of whether this BDNF delivery was sufficient to prevent the

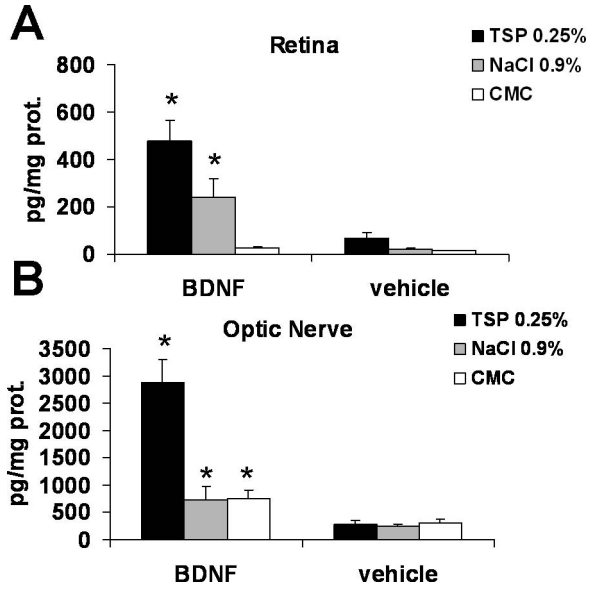

Figure 3. Topical eye application of BDNF increases BDNF level in the retina and optic nerve. We applied one single drop (10 μL) of different solutions (NaCl 0.9%, TSP 0.25%, CMC 0.2%) containing BDNF (12 μg/μL BDNF) at the level of conjunctival fornix in Wistar rats; the contralateral eye was treated with different solutions used for preparing BDNF. To increase the viscosity of solution, we used sodium carboxymethyl cellulose (CMC, 0.2%) and the polysaccharide fraction of TSP (0.25%). Our results show that single drop application of BDNF/TSP (12 μg/μL BDNF in 0.25% TSP) induced a significant increase of BDNF level in the retina (A) and optic nerve (B), compared to control vehicle treated eyes, BDNF/NaCl and BDNF/CMC topical eye treatment ($n = 5$ retinas in each group, $^*P < 0.05$). Retinal level of BDNF was determined by ELISA 6 hours after treatment; $^*P < 0.05$ (Student's *t*-test).

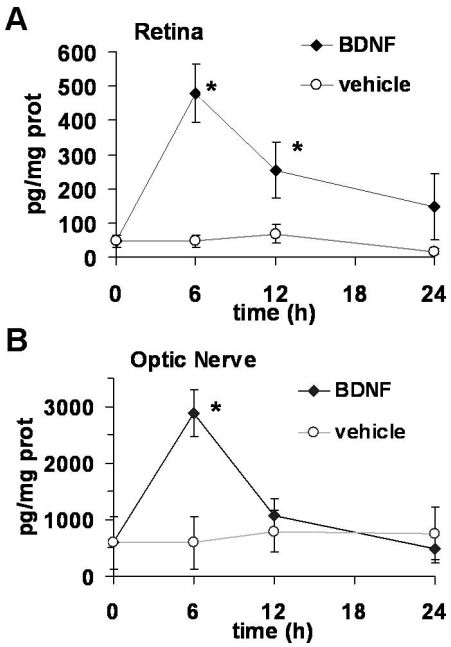

Figure 4. The pharmacokinetic study of BDNF/TSP topical application showed that BDNF level in the retina (A) and optic nerve (B) was significantly higher when measured 6 hours after treatment returning slowly towards basal level by 24 hours; importantly, 12 hours after treatment the level of the protein in the retina was still elevated over basal value ($n = 5$, $^*P < 0.05$).

impairment of retinal responses to flash and photoreceptor degeneration that follows a prolonged exposition to constant bright light in albino rats (light-damage). Seven days after LE (calculated from the first day of LE), rats were anesthetized and dark adapted for fERG recordings; as clearly visible in Figure 5 (A), the fERG was nearly abolished by LE with a- and b-wave amplitude dramatically decreased at different light intensities. We used one single drop containing BDNF/TSP (10 μL, 12 μg/μL of BDNF in 0.25% TSP) in the conjunctival fornix 3 hours before LE. At first, we controlled that BDNF/TSP treatment did not affect fERG in normal rats ($n = 3$). We found that one drop of BDNF/TSP did not impact on retinal responses to light as evaluated by recording the dark adapted fERG 12 and 24 hours after the treatment (data not shown). Remarkably, topical eye application of BDNF/TSP before LE was able to preserve fERG responses and to prevent dramatic a- and b-wave alteration in rats exposed to LE and recorded 7 days after (Fig. 5A, LE + BDNF). In Figure 5 (B, C), we reported the transfer curve for a- and b-wave amplitude at different light intensities showing that a- and b-wave amplitudes in the eyes treated with BDNF/TSP before LE were significantly higher compared to those measured in vehicle-treated eyes (LE + vehicle) and untreated eyes (LE), at least in the range of luminances comprised between 0.1 and 20 cd s/m^2. Thus, increasing the retinal content of BDNF by topical eye application was capable of preventing the disappearance of retinal responses to flash in the light-damage model.

Also, we investigated whether topical eye application of BDNF might prevent alteration of photoreceptors after LE. We conducted an histological analysis of retinas (Figs. 6A, 6B, 6C) using the fluorescent dye Propidium Iodide and confocal microscopy; at the end of the recording sessions, the retinas were processed and analyzed. We investigated the retinas from eyes treated with one single drop of BDNF/TSP (10 μL, 12 μg/μL of BDNF in 0.25% TSP; LE + BDNF), eyes treated with vehicle (TSP 0.25%; LE + vehicle), untreated eyes (LE), and unexposed eyes (CycL). In Figure 6 (A, B) it is visible that LE for 48 hours dramatically reduced the

photoreceptor rows and photoreceptor ONL thickness in both the central and peripheral retina; in the ONL, the normal 10 to 12 rows of photoreceptor nuclei present in the rat retina of eyes unexposed to light (rats reared on a 12-hour light/dark cycle, CycL) reduced to 3 to 4 rows both in the central and peripheral retina of eyes exposed to bright light in agreement to previous results[12,13]; accordingly, the thickness of ONL in LE rats was strongly reduced. The histograms in C showed a significantly higher number of rows of photoreceptor nuclei in the LE eyes treated with single drop of BDNF/TSP compared to vehicle-treated retina; this photoreceptor rescue was present both in the central and peripheral retina as also assessed by measuring the thickness of ONL (Fig. 6C).

Thus, topical eye treatment with BDNF in the form of collyrium represents a suitable, reliable, and noninvasive tool to increase the retinal content of BDNF up to a level capable of exerting neuroprotection toward photoreceptors injured by light.

Discussion

The main result obtained in the present paper indicates that BDNF following topical eye application can reach the retina inducing the increase of BDNF protein level both in the retina and optic nerve of albino rats in agreement to what was recently reported in the mouse.[15] Remarkably, topical eye treatment with BDNF mitigates the retinal damage that follows an exposure to continuous bright light in albino rats.

Light-damage is an in vivo model used to induce synchronized and fast photoreceptor cell death by apoptosis. Photoreceptor death by apoptosis is the final step of several retinal dystrophies including inherited diseases such as the family of *Retinitis pigmentosa* and age-related senile macular degeneration. Previous studies investigated the efficacy of neurotrophic factors to prevent photoreceptor degeneration; b- and a-FGF, GDNF, CNTF, and BDNF resulted suitable agents to prevent cell death in light damage.[2] In particular, b-FGF and GDNF are thought to exert a direct trophic action on photoreceptors that express b-FGF and GDNF receptors.[23] BDNF was also consistently reported to promote photoreceptor survival in light damage,[12,13] including cones,[24] although its specific receptors are largely absent in these retinal cells; indeed, low expression of TrkB has been described only in one cone subtype.[25] This supports the possibility that BDNF might induce

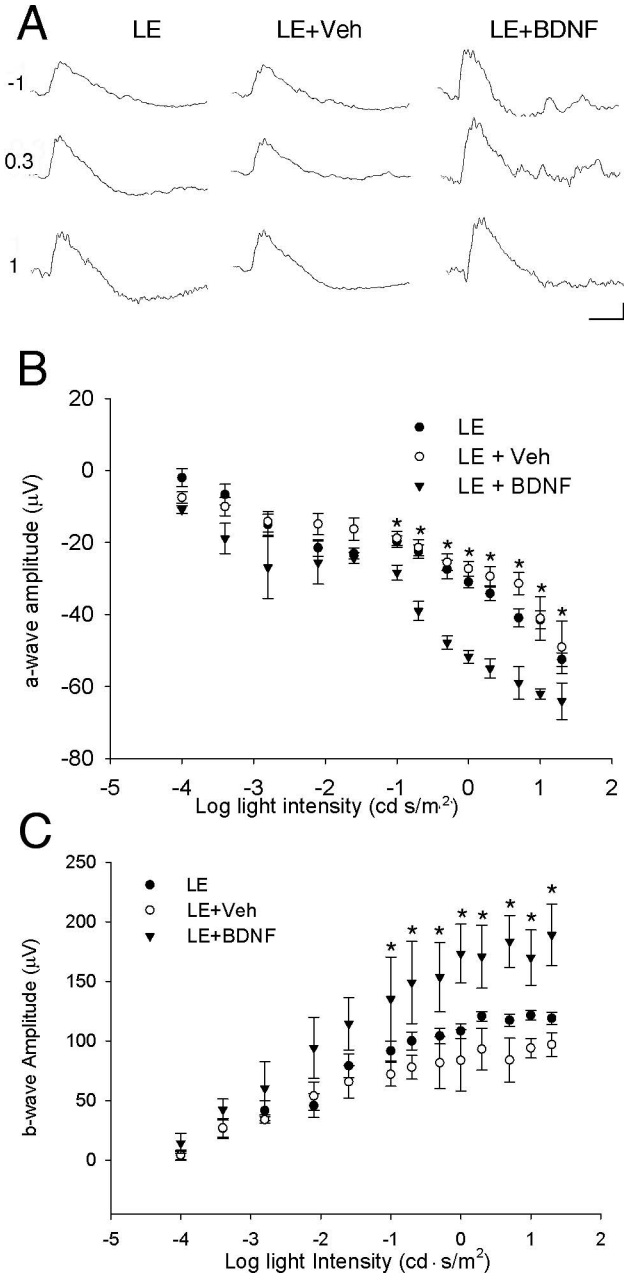

Figure 5. Topical eye application of BDNF prevents fERG impairment. (A) One week after LE, fERG was evoked by different luminances (top-down traces, log units) and recorded from the untreated eye (left column, LE), eye treated with vehicle (middle column, LE + vehicle, TSP 0.25%, 1 drop = 10 μL), eye treated with BDNF/TSP (right column, LE + BDNF; 12 μg/μL BDNF in 0.25% TSP, 1 drop = 10 μL). Topical application of BDNF/TSP before LE was able to partially preserve fERG responses recorded 1 week after light damage. Calibration: horizontal bar = 100 ms, vertical bar = 50 μV. (B, C) In panels B and C, we reported the transfer curve for a-wave and b-wave amplitude (μV), respectively, recorded at different light intensities (log cd s/m^2). The results show that a- and b-wave amplitudes in the LE eyes treated with BDNF/TSP (LE + BDNF) were significantly higher compared to those measured in LE vehicle-treated eyes (LE + Veh); four eyes for each group were analyzed (one-way ANOVA, *$P < 0.05$).

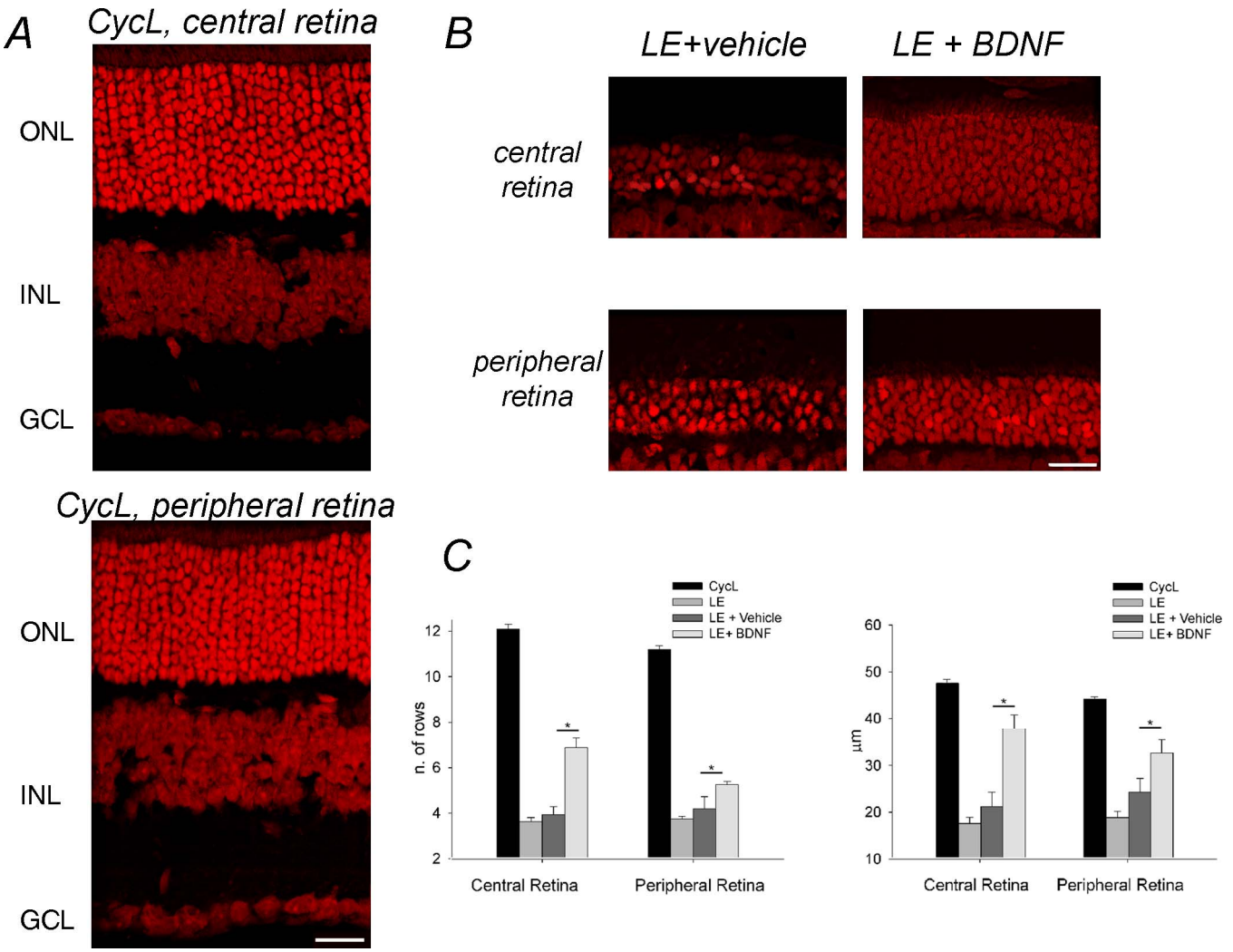

Figure 6. Using Propidium Iodide staining and confocal microscopy, we conducted an histological analysis of ONL from the central and peripheral retina of normal eyes (rats reared on a 12-hour light/dark cycle; Cycl), LE eyes treated with vehicle (LE + vehicle; TSP 0.25%) and LE eyes treated with one single drop of BDNF/TSP (LE + BDNF; 12 µg/µL in 0.25% TSP). (A) *Top* panel and *bottom* panel refer to the central and peripheral retina, respectively, of a rat reared on a12-hour light/dark cycle (Cycl). (B) One week after LE, the rows of photoreceptor nuclei appear strongly reduced (left column, LE + vehicle) both in the central (*top row*) and peripheral retina (*bottom row*). BDNF/TSP topical eye treatment was able to prevent the dramatic lost of photoreceptors in light-damage (right column, LE + BDNF). Horizontal calibration bar = 20 µm. Panel C reports histograms showing a significantly higher (*$P < 0.05$) number of photoreceptor rows (left histogram) and greater ONL thickness (right histogram) in the light-damaged central and peripheral retina of rats whose eye was treated with BDNF (LE + BDNF) respect to contralateral vehicle-treated eye (LE + vehicle), untreated eye (LE); CycL refers to retina of rats reared on a 12-hour light/dark cycle. Four retinas for each group were processed and analyzed.

signaling in other cells of the retina, thus acting indirectly on photoreceptors.[26] Indeed, Müller cells express TrkB and react to BDNF exposure by increasing the release of b-FGF and GDNF.[23] In agreement to this hypothesis, recent results on TrkB knockout mice showed that BDNF signaling in retinal glia has important roles in protection of retinal cells.[27]

Regardless of whether BDNF acts directly or indirectly on photoreceptors, this neurotrophin was widely applied to prevent photoreceptor apoptosis that follows an exposition to continuous bright light.[2] So far, BDNF as well as other neurotrophic factors, have been administered to the internal ocular tissues mainly by intravitreal or retrobulbar injection, two methods of treatment that involve the risk of various complications such as the ocular bulb perforation, infections, and lesions.[14] These unwanted effects prevented an extensive use of neurotrophic factors as survival promoting agents in retinal diseases.

Recently, we reported that repeated topical eye applications of BDNF were able to rescue retinal responses in a murine model of glaucoma.[15] Here, to facilitate a prolonged delivery of BDNF applied in the conjunctival fornix we used a TSP, a polysaccharide fraction of TSP, largely employed as artificial tear and vehicle to carry pharmacological active molecules.[19–22] We reported that the efficacy of TSP to facilitate the increase of BDNF retinal level is higher than that of physiological solution and CMC. Pharmacokinetic study of BDNF/TSP topical eye application showed that retinal BDNF level was higher than in control eye 6 hours after application remaining elevated for 12 hours and returning to basal value in 24 hours.

In the second part of the present paper, we afforded the fundamental question of whether topical eye treatment with BDNF was able to ameliorate the retinal alterations induced by prolonged exposition to continuous bright light in albino rats. We showed that BDNF topical application to the eye immediately before LE was able to conserve the retinal response to light and to promote survival of photoreceptors in LE albino rats, similarly to intraocular injection. A single intraocular injection of BDNF is probably more effective than a single topical eye BDNF application in preserving fERG amplitudes at different luminances, possibly for different dynamics in the release to vitreous humor, retina, and optic nerve.

Pharmacological compounds can reach the retina through two routes when topically applied to the eye: through the cornea and through the conjunctiva. Since BDNF, as well as other neurotrophic factors, is high molecular protein, it is very difficult that it can reach the retina by crossing the cornea. In a previous report, NGF was topically applied at the level of conjunctival fornix similarly to the method followed in the present paper. By measuring the NGF level in the conjunctiva and/or sclera, the authors suggested that NGF reached the posterior eye segment travelling through conjunctiva/sclera.[28] Trans-scleral delivery requires BDNF to permeate through several layers of ocular tissue (sclera, Bruch's membrane-choroid, retinal pigment epithelium) to reach the retina. We expect that novel trans-scleral drug delivery systems might ameliorate delivery of drugs to retina; for instance, we reported that TSP is more effective than physiological solution and CMC.

The alternative possibility is that BDNF might be delivered to the internal ocular tissues after absorption in choroidal tissue and plasma transport. In our experimental conditions, the contralateral eyes treated with vehicle did not show increased BDNF retinal levels respect to basal values and, more importantly, the retinas of vehicle treated eye resulted unprotected from light damage, thus contrary to what was expected in the case that BDNF was adequately adsorbed and transported to several tissues including the contralateral untreated eye.

The present results suggest that topical application to the eye of BDNF and, possibly, additional survival promoting agents, might represent a new therapeutic strategy to protect retinal cells from dysfunction/degeneration. In particular, we reported that the topical eye treatment with BDNF prevents impairment of flash ERG and loss of photoreceptors in light damage. It remains to be proved whether protracted topical eye treatments with BDNF will be feasible for the human retina that needs to be maintained in a functional state for a longer time to prevent cell degeneration in different retinal diseases.

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