BASIC SCIENCE

Exogenous modulation of intrinsic optic nerve neuroprotective activity

**Sinisa D. Grozdanic · Tatjana Lazic · Markus H. Kuehn · Matthew M. Harper ·
Randy H. Kardon · Young H. Kwon · Erin B. Lavik · Donald S. Sakaguchi**

Abstract

Background To characterize the molecular and functional status of the rat retina and optic nerve after acute elevation of intraocular pressure (IOP).

Methods Retinal ischemia was induced in rats by increasing the IOP (110 mmHg/60 minutes). Microarray analysis, quantitative RT-PCR (qRT-PCR) and immunohistochemistry were used to characterize retinal tissue. PLGA microspheres containing neurotrophic factors (BDNF, GDNF, or CNTF) or empty microspheres were injected into the vitreous of operated animals 1 day after elevation of IOP. Pupil light reflex (PLR) parameters and electroretinograms (ERG) were monitored at multiple time points during the 60-day postoperative recovery period.

Results Molecular analysis showed a significant intrinsic up-regulation of CNTF at 10 and 25 days after induction of the acute ocular hypertension ($p=0.0067$). Molecular tissue analysis of GDNF and its receptors (GDNFR1, GDNFR2), and BDNF and its receptor (trkB) showed no change in expression. Animals that received CNTF microspheres had no significant functional recovery compared to animals which received blank microspheres ($p>0.05$). Animals that received GDNF or BDNF microspheres showed significant PLR recovery ($p<0.05$ and $p<0.001$ respectively) compared to non-treated animals.

Conclusions Continuous release of neurotrophic growth factors (NGFs) significantly protects optic nerve function in the experimental model of retinal ischemia observed by PLR analysis.

Keywords Neuroprotection · Retina · BDNF · GDNF · CNTF

Sinisa D. Grozdanic and Donald S. Sakaguchi equally contributed to this manuscript.

S. D. Grozdanic (✉) · T. Lazic · M. M. Harper
Department of Veterinary Clinical Sciences,
College of Veterinary Medicine, Iowa State University,
Ames, IA 50011, USA
e-mail: sgrozdan@iastate.edu

M. M. Harper · D. S. Sakaguchi
Department of Genetics, Development and Cell Biology and the
Neuroscience Program, Iowa State University,
Ames, IA 50011, USA

M. H. Kuehn · R. H. Kardon · Y. H. Kwon
Department of Ophthalmology and Visual Sciences,
University of Iowa Hospitals and Clinics,
Iowa City, IA 52242, USA

S. D. Grozdanic · T. Lazic · M. H. Kuehn · M. M. Harper ·
R. H. Kardon · D. S. Sakaguchi
United States Veterans Affairs Center for the Prevention and
Treatment of Visual Loss, VA Medical Center,
Iowa City, IA 52242, USA

E. B. Lavik
Department of Biomedical Engineering,
Case Western Reserve University,
10900 Euclid Avenue,
Cleveland 44106, OH, USA

Introduction

Ischemic insults to the retina and optic nerve often lead to blindness due to serious perturbation of neuronal and glial retinal elements. Currently, glaucoma and ischemic disorders of the eye, including diabetes, ischemic optic neuropathy and retinal vascular occlusions are among the most prevalent causes of blindness worldwide.

Numerous studies have demonstrated that the rat model of acute elevation of intraocular pressure is a useful model for studying not only retinal ganglion cell damage, but also pan-retinal changes caused by retinal ischemic disorders [1, 2].

The presence of ischemic damage to all retinal neuronal cell types provides a unique advantage of testing possible neuroprotective molecules and their biological effect on different sub-populations of retinal neurons. By this approach, screening time for the best candidate molecules can be dramatically shortened, and successful therapeutic molecules can be further validated in specific animal models in which pathological changes affect defined neuronal populations.

Ischemic retinal and optic nerve diseases (glaucoma and diabetic retinopathy being the most frequent ones) are characterized by the development of progressive visual field defects and retinal neuronal death [3, 4]. Unfortunately, in many patients, despite adequate control of the IOP or glucose levels, the loss of vision continues.

Current medical and surgical treatments can achieve control of the IOP in glaucomatous patients, or provide increased vascularization and/or oxygenation in patients with different retinal/optic nerve ischemic diseases. However, there are still no adequate treatments that provide protection of remaining healthy neurons and/or recovers function in retinal neurons already compromised by the disease process. This necessitates development of alternative therapeutic strategies that could act directly on the retinal neurons to promote their survival, even in situations where the causative mechanism of damage (e.g. elevated IOP and ischemia due to completely or partially obstructed retinal blood vessels, diabetes) is still not well-controlled. It has been previously demonstrated that exogenous application of neurotrophic factors can promote survival in glaucoma [5–9] and in eyes with different forms of retinal degeneration [10–14]. We previously demonstrated that rodent retinas exposed to acute and chronic elevation of IOP are capable of significant intrinsic, but temporary, recovery of the retina and optic nerve function [15–17], suggestive of intrinsic ocular mechanisms capable of preserving retinal function and structure.

We had two separate goals for this study. The first goal was to characterize expression profile of genes, which may be responsible for the observed temporary functional recovery in rodent eyes exposed to acute elevation of intraocular pressure, with a particular focus on neurotrophic growth factor expression. The second goal was to evaluate whether exogenous application of neurotrophic factors can modulate (increase or decrease) functional and morphological recovery in ischemic retinas.

Materials and methods

Induction of retinal ischemia by elevated intraocular pressure (IOP)

All animal studies were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research, principles of laboratory animal care" (NIH publication No. 85-23, revised 1985), the OPRR Public Health Service Policy on the Humane Care and Use of Laboratory Animals (revised 1986), and the U.S. Animal Welfare Act, and procedures were approved by the Iowa State University Committee on Animal Care. Procedure to generate an ischemia–reperfusion insult in rats was performed as previously described [17]. Briefly, adult Brown Norway rats ($n=63$) were initially anesthetized with 4% halothane, 30% nitrous oxide (NO) and 70% oxygen (0_2). Anesthesia was maintained with 2.5% halothane, 30% NO and 70% O_2, and body temperature was maintained using a heating pad. The pupils were dilated with topical 2.5% phenylephrine hydrochloride and 1% tropicamide. After topical instillation of 0.5% propracaine hydrochloride, the anterior chamber was cannulated with a 25-gauge needle connected to a reservoir containing 0.9% NaCl. The IOP in experimental eyes was controlled by the height of the reservoir to maintain a pressure of 110 mmHg for 60 minutes. Retinal ischemia was confirmed by the blanching of the iris and retinal circulation. At the end of the period of elevated IOP, the needle was removed, and reperfusion of the retinal vasculature was confirmed by ophthalmoscopic examination. To prevent potential infection, antibiotic ointment (neomycin + polymyxin B + bacitracin; Bausch & Lomb Pharmaceuticals Inc; Tampa, FL, USA) was applied topically after the procedure. After surgery, pain was controlled by acetaminophen (100 mg/kg) + codeine (75 mg/kg) in the drinking water for 3 days.

Molecular profiling

Groups of five rats were sacrificed at 10, 25 and 42 days after acute elevation of IOP. Retinal tissue was collected for microarray and qRT-PCR analysis and immunohistochemical analysis.

Immunohistochemical analysis

For antibody staining, retinal tissue sections were washed in phosphate buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM $Na_2 HPO_4$, 1.47 mM KH_2PO_4) and incubated in blocking solution [5% goat serum; 0.4% bovine serum albumin, BSA (Sigma, St. Louis, MO, USA), and 0.2% Triton X-100 (Fisher Scientific)] in PBS. A specific primary antibody directed against CNTF alpha (Antibodies Inc., Davis, CA, USA) was used at a dilution of 1:250 and diluted in blocking solution, and preparations incubated overnight at 4°C in a humid chamber. On the following day, the preparations were rinsed with PBS and incubated with Cy3 conjugated donkey-anti-goat secondary antibody (Jackson ImmunoResearch, West Grove, PA,

USA) diluted at 1:1,000 in blocking solution for 90 minutes at room temperature. Sections were washed in PBS and cover-slipped with Gelmount Mounting media (Fisher). Negative controls were run in parallel during all processing, and included the omission of the primary antibody and/or secondary antibody, and no non-specific labeling was observed under these conditions.

Microarray and quantitative RT-PCR analyses

Total retinal RNA was extracted from retinas of rat eyes using Qiagen RNeasy (Qiagen, Valencia, CA, USA) columns and treated with DNAse. The quantity and quality of total RNA was determined (Bioanalyzer; Agilent, Santa Clara, CA, USA). Two micrograms of total RNA from each individual retina in each experimental group was pooled for microarray studies, which were performed using array chips (Rat Genome 230 2.0; Affymetrix, Santa Clara, CA, USA). All hybridizations were performed at the University of Iowa Microarray Core Facility. Quantitative RT-PCR analysis was performed for by using 200 ng retinal RNA from each individual animal, which was reverse transcribed in a random primed reaction. PCR primers were designed to span at least one intron to avoid the generation of PCR signal from potentially remaining genomic DNA (Table 1).

Five micrograms of the resulting cDNA was used as template for each amplification reaction, and all samples were analyzed in triplicate. Samples underwent 35 cycles of amplification in an ABI type 7700 machine using SYBR Green as the reporter dye (QuantiTect, Qiagen). Amplification controls included wells containing genomic DNA only and those containing no target (water controls). Transcript levels were determined based upon standard curves for each primer pair. Melt curve analyses were carried out following each amplification reaction to ascertain the absence of unspecific amplification products or primer dimers. Expression values obtained were normalized to transcript levels for ubiquitin C (UBC) in each sample.

Microsphere fabrication

GDNF microsphere fabrication

Glial cell lined-derived neurotrophic factor (GDNF) microspheres were fabricated using a modified version of the spontaneous emulsification technique as previously described [8, 18]. Briefly, 200 mg of poly(lactic-co-glycolic acid) (PLGA) 502H (50:50, Mn~10 kDa, Boehringer Ingelheim, GmbH, Ingelheim, Germany) was dissolved in 5 ml of a 1:4 (vol:vol) dicholoromethane (Sigma-Aldrich, St. Louis, MO, USA) to trifluoroethanol solution (Sigma-Aldrich). Ten micrograms of GDNF (R&D Systems, Minneapolis, MN, USA), 700 µg bovine serum albumin (BSA, Sigma-Aldrich), and 11.125 µg of AOT (Aerosol-OT, Sodium bis-2-ethylhexyl-sulfosuccinate, Sigma-Aldrich) were dissolved in 300 µl phosphate-buffered saline (PBS). The solutions were vortexed. The subsequent emulsion was poured into 200 ml of a 5% (w/v) poly(vinyl alcohol) (PVA) solution (Polysciences, Inc., Warrington, PA, USA), which was stirred to allow for solvent transfer and hardening of the microspheres. Following this step, the microspheres were collected by centrifugation and washed three times in deionized water to remove the residual PVA solution from the spheres. The microspheres were then quick- frozen in liquid nitrogen, lyophilized overnight and stored at−20°C in a dessicator until use. Blank microspheres were made at the same time using the same conditions except that no growth factor was added.

BDNF microsphere fabrication

Brain-derived neurotrophic factor (BDNF) microspheres were made in a manner identical to the GDNF microspheres, except that a blend of 50% PLGA 502H and 50% PLGA-b-plys-g-PEG was substituted for the PLGA 502H. PLGA-b-plys-g-PEG was fabricated by coupling PLGA 502H with poly-L-lysine (Sigma, Mn~1000 Da, deprotected) following [19]. Methyl-terminated poly(ethylene glycol) (PEG) (Polysciences, Warren, PA, USA: Mn~2000 Da)

Table 1 Sequence of primers used for real-time PCR reactions

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
GDNF	AAGTTATGGGATGTCGTGGC	GTCAGGATAATCTTCGGGCA
GFRA1	AAGTCTCTGTACAACTGCCGCT	AAGAGCATCCCGTAGCTGTGCTT
GFRA2	AATGAGCTGTGTGCGGCTGAAT	ATCCAGGCAGTGGTTGCTTT
BDNF	ATCCACTGAGCAAAGCCGAACT	AGCCTTCATGCAACCGAAGT
TrkB	CAAGCTGACGAGTTTGTCCA	CCTGGAGAGTCTTGAGCCAC
CNTF	TTGTGTCCTGGGACAGTTGA	AGTCGCTCTGCCTCAGTCAT
CNTFR	ACACCACGGCTATCACCTTC	ATTCGAGAGCTCCACATGCT
UBC	ATCTTTGCAGGCAAGCAGCTGGAA	TCTTGCCTGTCAGGGTCTTCACAA

was activated with carbonyldiimidazole (CDI) following [20] and [21]. The activated PEG was added to the PLGA-b-plys and precipitated in ether.

CNTF microsphere fabrication

Ciliary neurotrophic factor (CNTF) microspheres were prepared in the same manner as the GDNF microspheres, except that 20 µg of CNTF was added, and 98 µg of AOT as recently described [22].

Characterization of microspheres

Particle sizing Microspheres were characterized using a Beckman Coulter Multisizer 3 apparatus with a 100 µm diameter aperture. The mean diameters reported here are volume weighted diameters.

Scanning electron microscopy (SEM) Microspheres were sputter coated with gold for 30 s at 25 mA. SEM micrographs were taken on a Hitachi S-530 SEM operating at 4 kV.

Release study Three sets each of growth-factor-containing and blank microspheres were prepared by adding 1 ml of PBS to 10 mg of microspheres. The mixtures were then incubated at 37°C on a Labquake rotating shaker. At specific time points (2, 8 h, 1, 3, 5 days, etc. until no pellet could be identified following centrifugation), the mixture was centrifuged, and the supernatant was removed. Fresh PBS was added, and the microspheres were returned to the shaker. The supernatant for each of the sets of microspheres was frozen and stored at−80°C for subsequent analysis using ELISA kits for specific growth factors (R&D Systems, Duo Set) (Fig. 1).

Injection of polymer microspheres

Twenty four hours postoperatively, rats were anesthetized; the conjunctival and ocular surface was cleansed with 1:40 betadine solution and rinsed with a sterile saline solution.

Microspheres were injected into the vitreous using pulled and beveled glass needles with an outer diameter of approximately 100 µm at the tip. A 2% suspension of microspheres was prepared in PBS (for each growth factor separately) and vortexed briefly before each injection to ensure a uniform dispersion of spheres in the injection mass. Using a 25 µl Hamilton Syringe (Hamilton Co.; Reno, NV, USA) connected via PVC tubing to the injection needle, 3 µl of 2% suspension of the microspheres was injected into the vitreous.

Fig. 1 Release kinetics of PLGA microspheres (**a**, **b**, **c**)—steady release of neurotrophic growth factors was detected for at least 60 days (**a**, BDNF), 80 days (**b**, GDNF) and 50 days (**c**, CNTF). Scanning electron microscopy of the microspheres (**d**). Scale bar in D=50 µm
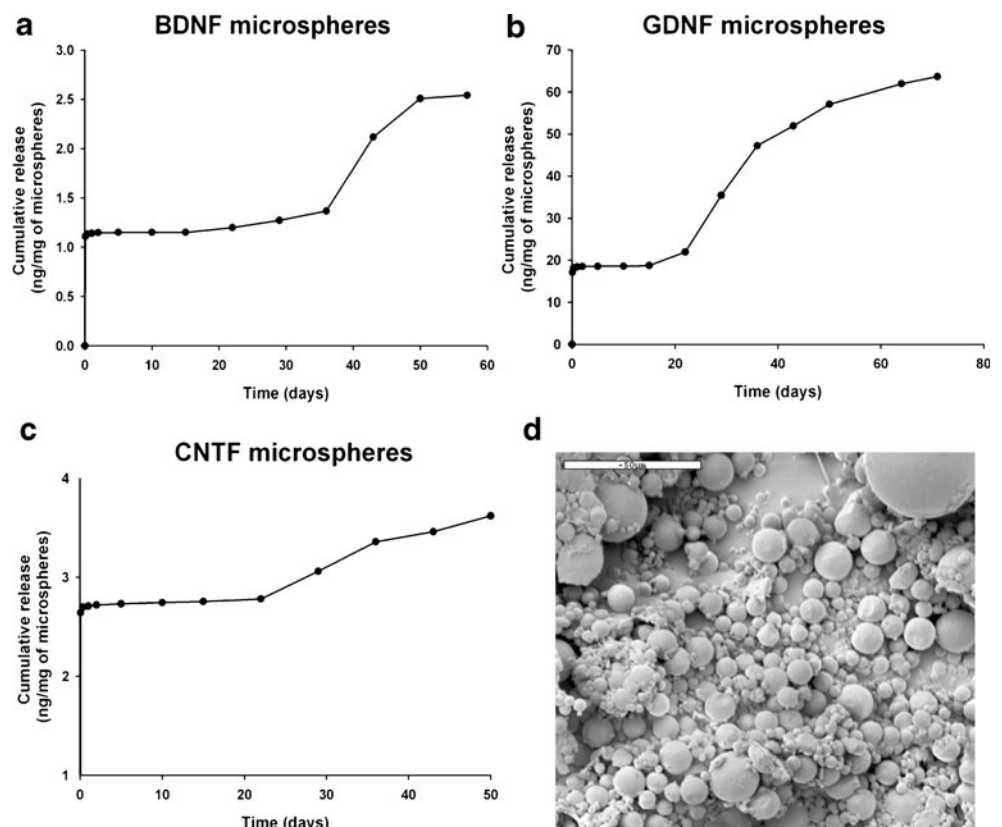
Three different groups of animals received growth-factor-engineered microspheres containing: BDNF ($n=10$), CNTF ($n=9$) or GDNF ($n=7$). A fourth, control group received blank PLGA microspheres ($n=9$), which were not impregnated with growth factors, while a fifth group of rats ($n=13$) did not receive any injections.

Computerized pupillometry

The pupil light reflex was evaluated with a custom-made computerized pupillometer (University of Iowa, Iowa City, IA, USA) preoperatively and on days 10, 20, 30, 40, 50 and 60 postoperatively as previously reported [23]. Animals whose tissue was collected for molecular analysis were recorded at 1, 10, 25 and 42 days postoperatively. Briefly, animals were anesthetized initially with 4% halothane, 30% NO and 70% O_2. A light plane of anesthesia was maintained with 1% halothane, 30% NO and 70% O_2 to avoid suppression of the pupil light reflex response that occurs with the use of higher doses of anesthetic. The computerized pupillometer was attached to two infrared-sensitive CCTV video cameras for simultaneous visual monitoring of both pupils. However, a single channel computerized pupillometer was used to record the movement of the pupil from the control (non-operated) eye, while the stimulus light was alternated between the control and operated eye. The stimuli were delivered through a mask equipped with four green (light-stimulus-delivering) and three infrared (iris-illuminating) diodes per eye. Custom made software routines (Winnana Software, University of Iowa) were used to analyze the recorded tracings of the pupil movements in response to light stimuli and to objectively determine the timing and amplitude of the pupil reflex responses. Since the opposite, non-operated eye was used as a control at the same testing time, any defect which was caused by ischemia–reperfusion injury was monitored longitudinally over time.

Electroretinography

To quantitate post-ischemic recovery after acute elevation of the IOP, electroretinography was performed at 62 days post-operatively as previously reported [23]. Briefly, animals were dark-adapted for at least 6 h, anesthetized as described previously and the pupils dilated with 1% tropicamide and 2.5% phenylephrine. The animals were placed in a specially designed dome whose interior was completely covered with aluminum foil to obtain a Ganzfeld effect. Body temperature was maintained using a microwave-heated thermal pad (R.G. Barry Corporation, Pickerington, OH, USA). A light stimulus was delivered through the ceiling of the dome using a PS-22 stimulator (Grass-Telefactor, West Warwick, RI, USA). Two DTL fiber electrodes (Chieldex Trading GmbH, Palmyra, NY, USA), were used to simultaneously obtain signals from both eyes. The reference electrode was positioned in the ear, while the ground electrode was on the back (subcutaneous). A Neuropack-MEB 7102 Evoked Potential Measuring System (Nihon-Kohden America, Foothill Ranch, CA, USA) was used to deliver a triggered output to the flash stimulator and collect signals from both eyes. A flash ERG routine was delivered at a 0.2 Hz frequency (ten averaged signals per recording session, sensitivity 100 µV/division, low-cut frequency 0.5 Hz, high-cut frequency 10 kHz, analysis time 500 ms). Oscillatory potentials were recorded by delivering light stimuli at a 0.2 Hz frequency (ten averaged signals per recording session, sensitivity 100 µV/division, low-cut frequency 50 Hz, high-cut frequency 500 Hz, analysis time 100 ms). Isolated cone responses were recorded from previously light adapted eyes by delivering stimuli at 20 Hz (50 averaged signals per recording session, sensitivity 50 µV/division, low-cut frequency 0.5 Hz, high-cut frequency 10 kHz, analysis time 500 ms).

Histological examination

We previously demonstrated that the rat model of acute ocular hypertension injury is characterized by severe damage of all retinal layers (17). As such, we decided to perform morphometric analysis by measuring the thickness of different retinal layers in sections obtained at the optic nerve head level, as described in our previous studies [15, 17]. Briefly, 65 days postoperatively, rats were deeply anesthetized with a high dose of phenobarbitol (100 mg/kg) and perfused intracardially with ice-cold heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Eye globes containing optic nerves were postfixed, paraffin embedded, and 7 µm-thick sections of the retina (sections through the optic nerve) were collected onto poly-l-lysine-coated glass slides, and stained with hematoxylin and eosin. Tissue sections were examined with a Nikon Microphot FXA photomicroscope (Nikon Corporation, New York, NY, USA). Images were captured using a Kodak Megaplus Camera (Model 1.4; Kodak Corp., San Diego, CA, USA) connected to a MegaGrabber Framegrabber in a Macintosh 8100/80 AV computer (Apple Computer, Cupertino, CA, USA) using NIH Image 1.58 VDM software (W.Rasband, NIH, Bethesda, MD, USA). Sections of the retina at the level of the optic nerve head were prepared, and each section was divided into 12 optic fields (six fields of the central retina and six fields of the peripheral retina). Within each field, we measured the thickness of the nerve fiber layer and retinal ganglion cell layers (NFL + RGC), inner nuclear (INL) and outer nuclear layer (ONL) by measuring four values per layer and calculating averaged values per section. Measurements of retinal layer thickness were

Table 2 Genes down-regulated more than threefold in retinas exposed to acute elevation of IOP. Gene pattern is suggestive of the continuous loss of genetic activity associated with essential retinal neuronal functional and structural properties in early and late time points

Gene title/sequence description	Reference sequence accession no.	Relative change 10 days	Relative change 25 days
Nfl, neurofilament, light polypeptide	BF394545	6.1	4.9
Nefh, neurofilament, heavy polypeptide	AF031879	4.3	3.8
Opn1mw, opsin 1 (cone pigments), medium-wave-sensitive (color blindness, deutan), green opsin	NM_053548	5.1	3.6
Arr3, arrestin 3, retinal (X-arrestin)	AI112106	3.8	3.5

performed using a calibrated scale of the objective of the light microscope.

Statistical analysis

Statistical analysis was performed by using Student's *t*-test, paired *t*-test and one-way ANOVA with Bonferroni post test (as indicated in the text) with GraphPad (GraphPad, San Diego, CA, USA) software.

Results

Molecular profiling to determine the nature of mechanisms responsible for spontaneous functional recovery

Microarray analysis from eyes collected at 10 and 25 days post acute elevation of IOP revealed primary down-regulation of genes related to the basic neuronal and photoreceptor structure and function (Table 2), suggestive

of the continuous process of neuronal death in ischemic retinas. Analysis of up-regulated gene expression pattern revealed increase in gene activity associated with glial cell activity, acute phase protein production and inflammation (Table 3). Careful analysis of pro-survival genes and genes regulating production of growth factors revealed only increased expression of the CNTF gene, which was present at 10 (5.4-fold increase compared to control eyes) and 25 (3.4-fold increase compared to control eyes) days post induction of retinal ischemia (Table 3).

Real-time PCR analysis was used to further evaluate and confirm findings obtained by microarray analysis. PCR analysis revealed that the gene transcript for CNTF is significantly increased ($p=0.0067$, paired *t*-test) in ischemic retinas: We detected an almost 20-fold increase in the expression levels (19.7 ± 23.3 fold, mean $\pm$ SD) between the ischemic eyes and the contralateral, untreated control eyes at 10 days. Elevated expression levels could still be detected 25 days after induction of retinal ischemia, although at reduced levels (4.4 ± 1.3 fold). Analysis of the

Table 3 Genes up-regulated more than threefold in retinas exposed to acute elevation of IOP. Gene pattern is suggestive of the persistent glial activation and continuous presence of inflammatory changes in ischemic retinas. The only identified pro-survival gene with consistent up-regulation at both time points was the CNTF gene

| Gene title/sequence description	Reference sequence accession no.	Relative change 10 days	Relative change 25 days
Glial activation			
Gfap, glial fibrillary acidic protein	NM_017009	10.6	8.8
S100a4, S100 calcium-binding protein A4	NM_012618	9.6	8.7
Vim, vimentin	NM_031140	4.6	4
Acute-phase proteins			
C3, complement component 3	NM_016994	11.9	8.8
Cp, ceruloplasmin	NM_012532	7.4	5.6
A2m, alpha-2 macroglobulin	NM_012488	4.8	3.6
Inflammatory response			
Litaf, LPS-induced TNF	BI284739	4.2	3.2
Cd74, CD74 antigen (invariant polpypeptide of major histocompatibility class II antigen-associated)	NM_013069	28.5	28.4
Cd48, CD48 antigen	X13016	5.5	3.4
Lyz, lysosome	L12458	12.2	5.5
Growth factors			
Cntf, ciliary neurotrophic factor	NM_013166	5.4	3.4
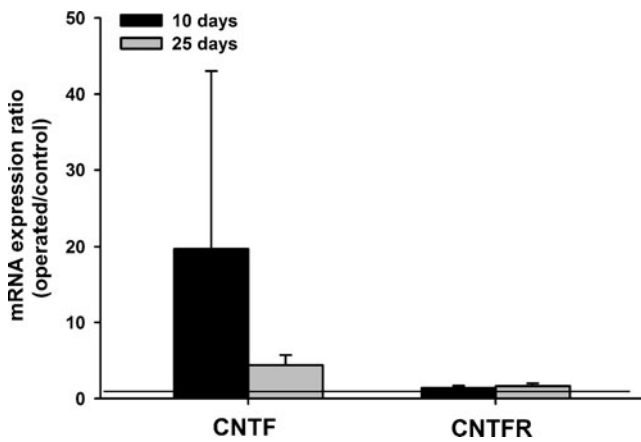
Fig. 2 Real-time PCR analysis showed significant CNTF mRNA up-regulation in operated eyes 10 and 25 days postoperatively (*horizontal line* represents ratio value 1, which corresponds to equal expression of mRNA in operated and control eyes). Analysis of CNTF receptor (CNTFR) mRNA levels did not reveal significant change between ischemic and control eyes (*bars* represent mean+SD)

CNTF receptor (CNTFR) expression showed that while this receptor is expressed in the retina, the ischemia-induced expression changes are not statistically significant when compared to control eyes ($p=0.56$, paired t-test): 1.4 ± 0.3 (mean±SD) expression ratio between the ischemic eyes and the ipsilateral, untreated control eyes at 10 days and 1.6 ± 0.3 (mean±SD) at 25 days (Fig. 2a).

Immunohistochemistry analysis of rat retinas exposed to the ischemic insult showed significantly increased CNTF protein expression at 25 days post ischemic insult, confirming results of the PCR analysis (Fig. 3).

Analysis of GDNF and its respective receptors (GFRA1, GFRA2) did not reveal significant changes in expression patterns between control and ischemic eyes ($p=0.26$; $p=0.46$; $p=0.4$ respectively): $GDNF_{10d}=0.94\pm0.3$ (mean ± SD), $GFRA1_{10d}=0.9\pm0.3$, $GFRA2_{10d}=1.2\pm0.3$; $GDNF_{25d}=$

0.74 ± 0.2, $GFRA1_{25d}=0.76\pm0.3$, $GFRA2_{25d}=0.63\pm0.3$ (Fig. 4a). Expression analysis for BDNF and its receptor Trk B showed a trend toward decreased expression in ischemic retinas, however difference was not statistically significant ($p=0.4$ and $p=0.06$ respectively, paired t-test). Values at 10 days post ischemic insult were: $BDNF=0.69\pm0.1$; $TrkB=0.4\pm0.1$ (expression ratio operated/control), and they normalized at 25 days post injury ($BDNF=0.85\pm0.3$; $TrkB=0.78\pm0.1$ expression ratio operated/control, Fig. 4b).

Assessment of optic nerve function using the pupil light reflex (PLR)

The measurement of the PLR was used as an assay to investigate possible changes in retinal and optic nerve function following acute elevation of the IOP and subsequent neurotrophic growth factor application. The reflex contraction of the pupil to a light stimulus provides an objective measure of the afferent conduction of the visual system. Damage to the retina or optic nerve reduces the amplitude of the pupil contraction to light. Since the motor output of the neuronal reflex of pupil contraction to light is distributed to both pupils, monitoring the pupil from just the non-operated eye is sufficient to assess any asymmetry of light input between the operated and non-operated eye. Because the fellow eye was used as a control at the same testing time, any defect and functional recovery was monitored longitudinally over time. All pupil parameters were calculated by comparing values from the operated and non-operated (control) eyes in the same animal (Figs. 5 and 6).

Detailed analysis of PLR amplitudes presented as the PLRratio (ratio = consensual/direct PLR) revealed that eyes which received blank microspheres or CNTF microspheres did not show significant improvement of PLR amplitudes
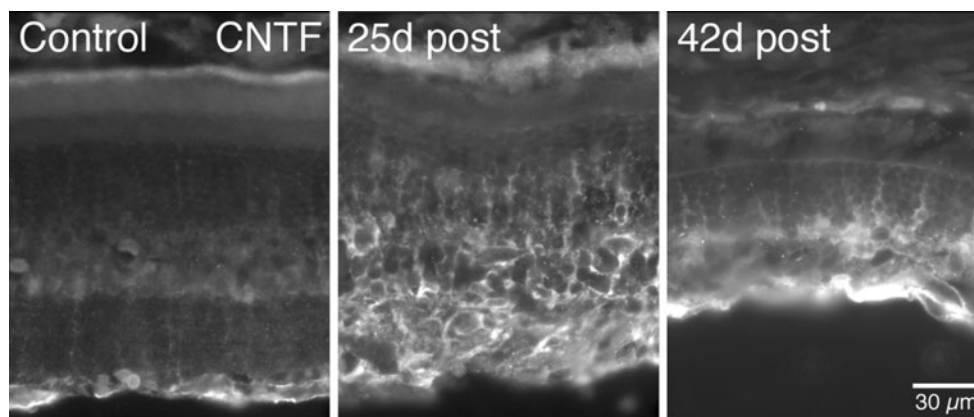

Fig. 3 Immunohistochemical analysis showed increased CNTF protein expression at 25 days postoperatively (time interval of spontaneous functional recovery). However, protein expression declined at 42 days postoperatively, which corresponded to the decline in PLR and ERG function in operated animals. Despite intrinsic growth factor production, significant inner retina thinning is present at 42 days postoperatively
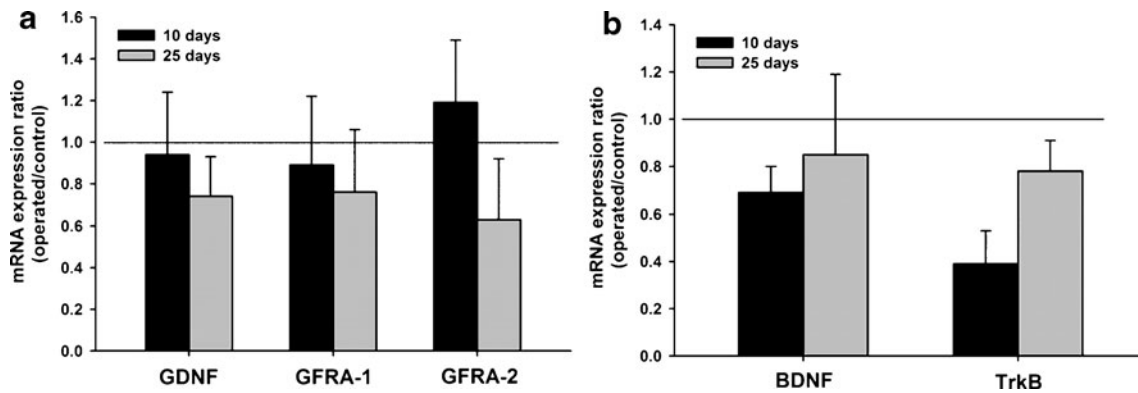
Fig. 4 a Real-time PCR analysis showed relatively unchanged mRNA expression for GDNF and its respective receptors (GFRA1 and 2) 10 and 25 days post ischemic insult (*horizontal line* represents ratio value of 1, which corresponds to equal expression of mRNA in operated and control eyes). **b** Analysis of the BDNF and its respective receptor TrkB, showed a trend toward decreased expression at 10 days; however, mRNA levels normalized 25 days post ischemic insult (*bars* represent mean+SD)

when compared to eyes which received injections of blank microspheres (Fig. 5, $p=0.051$) or eyes which did not receive any treatment ($p=0.15$).

In contrast, animals which received BDNF or GDNF microsphere injections 24 h postoperatively had significantly larger PLR amplitudes than the groups which received blank microspheres or no microspheres (ANOVA with Bonferroni's multiple comparison test). The average values for different groups were: PLRratio(BDNF)=54.5± 1.5% (mean ± SEM, $p<0.0001$), PLRratio(GDNF)=48± 3% ($p=0.037$), PLRratio(CNTF)=44.3±1.6% ($p=0.051$), PLRratio(blank)=39.1.5±1.8% ($p=0.43$), PLRratio(no microspheres)=36.4±4.4%.

Electroretinography

Electroretinography was used as an objective method to evaluate the functional status of the inner and outer retina with the exception of the retinal ganglion cells. Electroretinographic data expressed as a ratio between control and operated eyes revealed significantly better cone function (measured by flicker ERG amplitudes) in eyes which received GDNF, CNTF or blank microspheres compared to eyes which did not receive any treatment: GDNFfl$_{ratio}$=10.2±2.9% (mean± SEM, $p=0.03$, Student's *t*-test), CNTFfl$_{ratio}$=8.2±3.1% ($p=0.04$, Student's *t*-test), blankfl$_{ratio}$=9.2±3.3% ($p=0.04$, Student's *t*-test). While application of BDNF microspheres showed significant improvement in the PLR function, analysis of ERG amplitudes did not show significant improvement in any of the observed parameters when compared to non-treated eyes.

Analysis of scotopic flash a-and b-wave amplitudes and oscillatory potentials revealed no significant difference among different treatment groups for any of the observed parameters (Fig. 7). There was no significant difference between any of the neurotrophic growth factor groups and
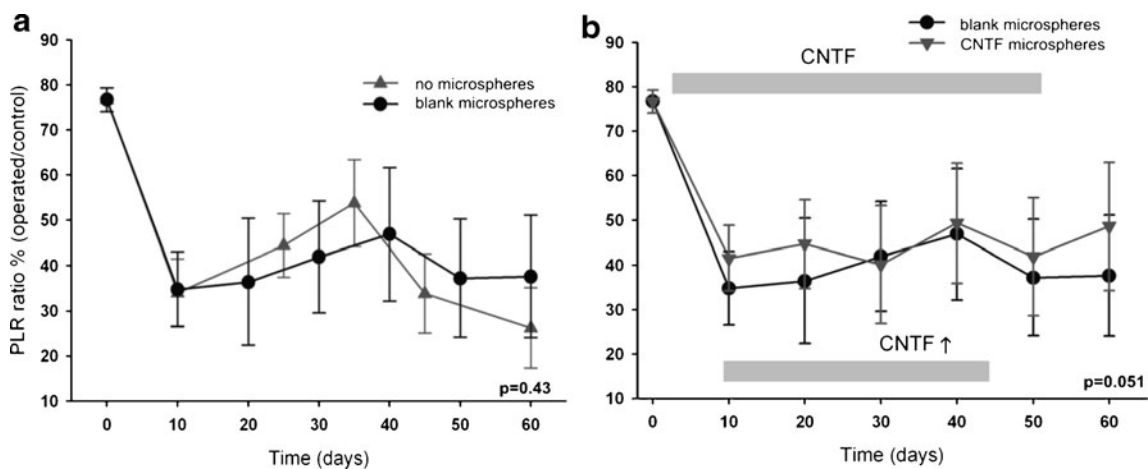

Fig. 5 a Graph shows pupil light reflex data from rodent eyes exposed to ischemic insult that received blank microspheres. There was no significant difference between the 2 groups. **b** Graph shows the group that received CNTF microspheres. While overall function in CNTF treated rats was somewhat better, difference was not statistically significant when compared to rats which received empty microspheres. Values are plotted as mean±SEM
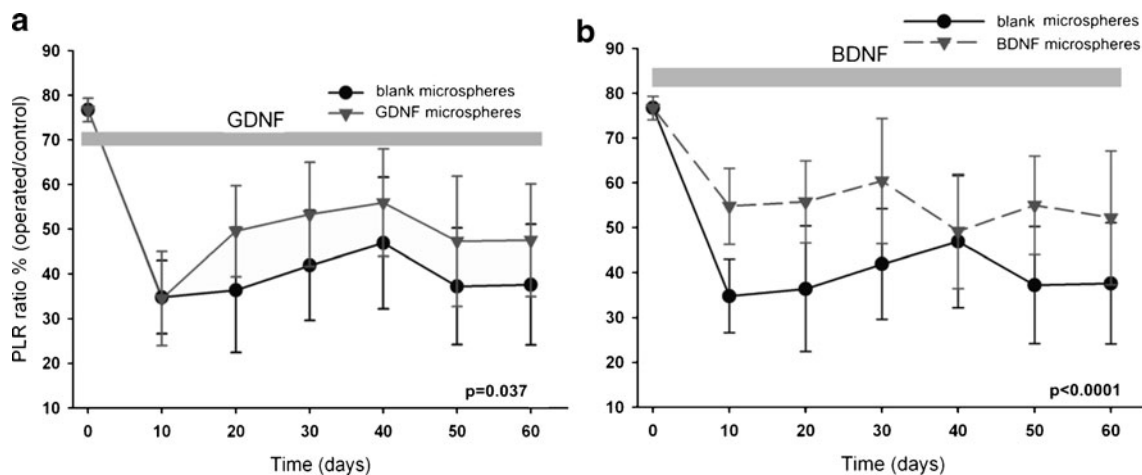
Fig. 6 a GDNF microspheres provided significant recovery of PLR function compared to control blank microspheres, starting 10 days post ischemic insult. b BDNF showed an immediate positive effect on retinal function which was sustained until the end of the experiment. Values are plotted as mean±SEM

the group which received blank microspheres when all testing parameters were compared.

Morphometric analysis

Since this model of acute retinal ischemia–reperfusion injury is characterized by severe damage of all retinal layers and particularly in the region of the central retina [17], detailed morphometric analysis was performed to determine possible preservation of retinal structure after application of neurotrophic growth factors (Fig. 8). Statistical analysis showed significant difference in the total retinal thickness and thickness of the outer nuclear layer between the GDNF-treated and non-treated (ischemia-induced but non-treated) rat eyes: $GDNF_{total}=99.7\pm6$ µm (mean ± SEM) and non-treated$_{total}=51.4\pm2.2$ µm ($p<0.0001$, Student's t-test) and $GDNF_{ONL}=46.3\pm9$ µm and non-treated$_{ONL}=20\pm1.8$ µm ($p=0.0029$, Student's t-test). Analysis of remaining parameters between all treated groups showed no statistically significant difference.

Discussion

The experimental approach used in this study allowed us to precisely monitor dynamics of functional recovery in eyes exposed to acute elevation of intraocular pressure, followed
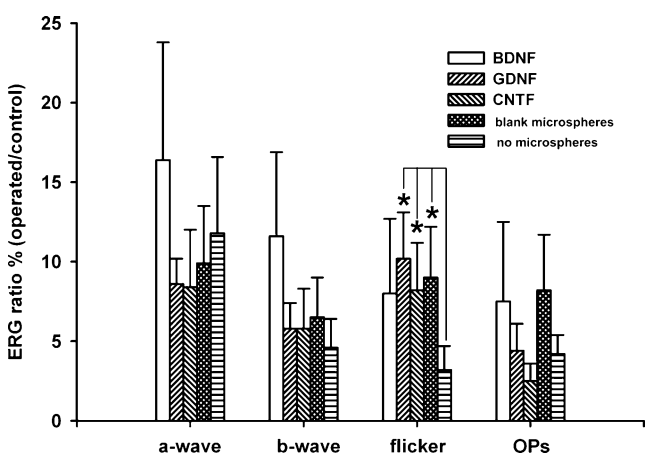

Fig. 7 Electroretinography analysis revealed significantly better cone function in those operated eyes that received GDNF, CNTF or blank microspheres than in eyes which had retinal ischemic episode but did not receive any intraocular injection (bars represent standard error of mean, * represents $p<0.05$, OPs=oscillatory potentials). Bars represent mean+SEM
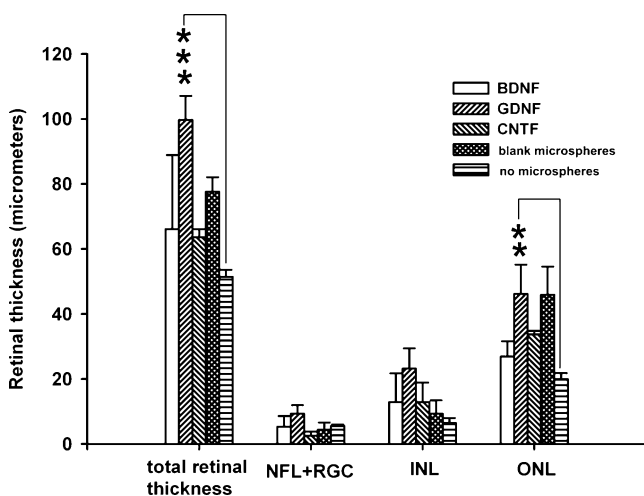

Fig. 8 Morphometric analysis showed significant preservation of retinal structure in the GDNF-treated group when compared to animals which did not receive intraocular injection. However, difference was not significant when data were compared with ischemic eyes which received blank microspheres (bars represent standard error of mean, ** represents $p<0.01$, *** represents $p<0.0001$). Bars represent mean±SEM

by chronic delivery of different neurotrophic factors for a prolonged period of time (up to 8 weeks).

It has been previously demonstrated that analysis of the PLR and ERG responses is an effective and sensitive strategy for monitoring retinal and optic nerve status after acute ocular hypertension (ischemia–reperfusion) injury and glaucoma in rodents [15–17, 24–26]. Previous studies showed that rodent retinas exposed to acute and chronic elevation of IOP have a strong intrinsic capacity to temporarily recover optic nerve function. However, the exact nature of molecular changes responsible for observed functional recovery has not been identified [15–17]. Data from this study are strongly suggestive of an intrinsic CNTF up-regulation as a possible mechanism responsible for the observed temporary functional recovery in rodent retinas exposed to acute ischemic insult, since microarray analysis of retinal tissue did not reveal up-regulation of any other molecules with possible neurotrophic or neuroprotective properties. The majority of observed gene changes were suggestive of the reactive glial status and inflammatory response, which could be a cause or just a reactive consequence of ongoing neuronal death in ischemic retinas. However, we can not absolutely exclude possible role of other molecules being responsible for observed intrinsic functional recovery in this model. Experiments with blockade of CNTF activity or use of CNTF knockout animals would be needed to absolutely confirm the role of CNTF as the major driver of the observed intrinsic functional recovery. Intrinsic up-regulation of neurotrophic factors in retinal tissue has been described as a feature of many different models of optic nerve and retinal injury [27–33]. Different studies have shown up-regulation of growth factor production predominantly in Müller glial cells in eyes with optic nerve injury or different forms of retinal degeneration [34, 35]. It has been also demonstrated that activated microglia can produce neurotrophic factors, which may promote neuronal survival [36].

This study demonstrated that exogenous supplementation with CNTF in rats with acute ischemia–reperfusion injury is not an effective strategy for improvement of functional parameters. While the reason for the observed lack of CNTF efficacy in this study can not be precisely determined from performed experiments, it can be speculated that spontaneous intrinsic production of this molecule probably occupied the majority (or all) of available receptors, and prevented any additional therapeutic effect of exogenously supplemented CNTF. Although significantly better flicker ERG function was noticed in CNTF-treated eyes, this difference was significant only when a comparison was made against non-treated operated rats.

Observed data from GDNF and BDNF treated rats may serve as additional evidence to support the hypothesis that intrinsic expression of specific growth factors can mediate

possible outcomes of exogenous neurotrophic factor application. While animals which did not receive any treatment or received treatment with blank microspheres showed temporary functional recovery at 35 days, the development of functional decline resulted in dramatic worsening of the optic nerve function at later time points. Since GDNF- and BDNF-treated eyes had overall better optic nerve function observed by PLR analysis, and functional decline after 35 days postoperatively was not observed in GDNF- and BDNF-treated eyes, it is likely that targeted therapy with specific neurotrophic growth factors resulted in effective neuroprotection in ischemic retinas. Furthermore, GDNF and BDNF mRNA increase was not detected in ischemic rodent retinas at 10 and 25 days post injury, so we can speculate that these neurotrophic growth factors do not have a role in the intrinsic spontaneous recovery of optic nerve function. Considering relatively unchanged levels of GDNF and BDNF mRNA levels (and their respective receptors), it is likely that a sufficient amount of target receptors was available in the retinal tissue, which probably allowed exogenously supplied growth factors to potentiate intrinsic mechanisms of functional optic nerve recovery observed by PLR analysis. The GDNF group also had much better preservation of cone function (flicker ERG) and retinal structure (observed by retinal morphometry) when compared to non-treated operated eyes. We have also observed positive effects of CNTF, GDNF and blank microspheres on flicker ERG; however, pupil light reflex activity showed significantly better outcome only in GDNF-treated eyes. Considering that pupil response is a summated response of the photoreceptors, inner retinal neurons and retinal ganglion cells, it is possible that CNTF and blank microspheres had some positive effects on cone function; however, lack of PLR improvement is highly suggestive of the absent neuroprotective effects on the optic nerve itself.

Based on these observations, we can speculate that neurotrophic growth factors may protect retinal function against ischemic insult only if exogenously applied neurotrophic factors are not already intrinsically produced at maximal therapeutic concentrations in the affected tissue.

Conclusion

Exogenous application of neurotrophic growth factors via polymer biodegradable microspheres has been an effective strategy for retinal neuroprotection [8]; however, different studies have also demonstrated potential negative effects of these molecules on retinal neurons [37, 38]. While the model of retinal ischemia–reperfusion injury does not represent a true clinical situation in human patients, the findings observed in this study may provide useful guide-

lines, which should be considered during selection of neurotrophic growth factors for possible therapeutic use in different ocular neurodegenerative diseases. With advancements of genomic and proteomic analysis of tissue from human donors suffering from different ocular diseases, it is likely that profiles of different neurotrophic growth factors and their respective receptors will become widely available. That data should provide excellent baseline parameters for more rational selection of specific therapeutic molecules, which may dramatically improve therapeutic outcome of growth-factor-based therapies for different ocular diseases.

References

1. Adachi M, Takahashi K, Nishikawa M, Miki H, Uyama M (1996) High intraocular pressure-induced ischemia and reperfusion injury in the optic nerve and retina in rats. Graefes Arch Clin Exp Ophthalmol 234:445–451
2. Hughes WF (1991) Quantitation of ischemic damage in the rat retina. Exp Eye Res 53:573–582
3. Quigley HA (1999) Neuronal death in glaucoma. Prog Retin Eye Res 18:39–57
4. Takahashi H, Goto T, Shoji T, Tanito M, Park M, Chihara E (2006) Diabetes-associated retinal nerve fiber damage evaluated with scanning laser polarimetry. Am J Ophthalmol 142:88–94
5. Ko ML, Hu DN, Ritch R, Sharma SC (2000) The combined effect of brain-derived neurotrophic factor and a free radical scavenger in experimental glaucoma. Invest Ophthalmol Vis Sci 41:2967–2971
6. Ko ML, Hu DN, Ritch R, Sharma SC, Chen CF (2001) Patterns of retinal ganglion cell survival after brain-derived neurotrophic factor administration in hypertensive eyes of rats. Neurosci Lett 305:139–142
7. Martin KR, Quigley HA, Zack DJ, Levkovitch-Verbin H, Kielczewski J, Valenta D, Baumrind L, Pease ME, Klein RL, Hauswirth WW (2003) Gene therapy with brain-derived neurotrophic factor as a protection: retinal ganglion cells in a rat glaucoma model. Invest Ophthalmol Vis Sci 44:4357–4365
8. Ward MS, Khoobehi A, Lavik EB, Langer R, Young MJ (2007) Neuroprotection of retinal ganglion cells in DBA/2J mice with GDNF-loaded biodegradable microspheres. J Pharm Sci 96:558–568
9. Ji JZ, Elyaman W, Yip HK, Lee VW, Yick LW, Hugon J, So KF (2004) CNTF promotes survival of retinal ganglion cells after induction of ocular hypertension in rats: the possible involvement of STAT3 pathway. Eur J Neurosci 19:265–272
10. LaVail MM, Unoki K, Yasumura D, Matthes MT, Yancopoulos GD, Steinberg RH (1992) Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. Proc Natl Acad Sci U S A 89:11249–11253
11. LaVail MM, Yasumura D, Matthes MT, Lau-Villacorta C, Unoki K, Sung CH, Steinberg RH (1998) Protection of mouse photoreceptors by survival factors in retinal degenerations. Invest Ophthalmol Vis Sci 39:592–602
12. Carwile ME, Culbert RB, Sturdivant RL, Kraft TW (1998) Rod outer segment maintenance is enhanced in the presence of bFGF, CNTF and GDNF. Exp Eye Res 66:791–805
13. Frasson M, Picaud S, Leveillard T, Simonutti M, Mohand-Said S, Dreyfus H, Hicks D, Sabel J (1999) Glial cell line-derived neurotrophic factor induces histologic and functional protection of rod photoreceptors in the rd/rd mouse. Invest Ophthalmol Vis Sci 40:2724–2734
14. Thanos C, Emerich D (2005) Delivery of neurotrophic factors and therapeutic proteins for retinal diseases. Expert Opin Biol Ther 5:1443–1452
15. Barnett NL, Grozdanic SD (2004) Glutamate transporter localization does not correspond to the temporary functional recovery and late degeneration after acute ocular ischemia in rats. Exp Eye Res 79:513–524
16. Grozdanic SD, Kwon YH, Sakaguchi DS, Kardon RH, Sonea IM (2004) Functional evaluation of retina and optic nerve in the rat model of chronic ocular hypertension. Exp Eye Res 79:75–83
17. Grozdanic SD, Sakaguchi DS, Kwon YH, Kardon RH, Sonea IM (2003) Functional characterization of retina and optic nerve after acute ocular ischemia in rats. Invest Ophthalmol Vis Sci 44:2597–2605
18. Fu K, Harrell R, Zinski K, Um C, Jaklenec A, Frazier J, Lotan N, Burke P, Klibanov AM, Langer R (2003) A potential approach for decreasing the burst effect of protein from PLGA microspheres. J Pharm Sci 92:1582–1591
19. Lavik EB, Hrkach JS, Lotan N, Nazarov R, Langer R (2001) A simple synthetic route to the formation of a block copolymer of poly(lactic-co-glycolic acid) and polylysine for the fabrication of functionalized, degradable structures for biomedical applications. J Biomed Mater Res 58:291–294
20. Ford MC, Bertram JP, Hynes SR, Michaud M, Li Q, Young M, Segal SS, Madri JA, Lavik EB (2006) A macroporous hydrogel for the co-culture of neural progenitor and endothelial cells to form functional vascular networks in vivo. Proc Natl Acad Sci U S A 103:2512–2517
21. Hermanson GT (1996) Bioconjugate Techniques. Academic Press, San Diego
22. Nkansah MK, Tzeng SY, Holdt AM, Lavik EB (2008) Poly(lactic-co-glycolic acid) nanospheres and microspheres for short-and long-term delivery of bioactive ciliary neurotrophic factor. Biotechnol Bioeng 100:1010–1019
23. Grozdanic S, Sakaguchi DS, Kwon YH, Kardon RH, Sonea IM (2002) Characterization of the pupil light reflex, electroretinogram and tonometric parameters in healthy rat eyes. Curr Eye Res 25:69–78
24. Grozdanic SD, Betts DM, Sakaguchi DS, Allbaugh RA, Kwon YH, Kardon RH (2003) Laser-induced mouse model of chronic ocular hypertension. Invest Ophthalmol Vis Sci 44:4337–4346
25. Grozdanic SD, Betts DM, Sakaguchi DS, Kwon YH, Kardon RH, Sonea IM (2003) Temporary elevation of the intraocular pressure by cauterization of vortex and episcleral veins in rats causes functional deficits in the retina and optic nerve. Exp Eye Res 77:27–33
26. Grozdanic SD, Ast AM, Lazic T, Kwon YH, Kardon RH, Sonea IM, Sakaguchi DS (2006) Morphological integration and functional assessment of transplanted neural progenitor cells in healthy and acute ischemic rat eyes. Exp Eye Res 82:597–607
27. Wen R, Song Y, Cheng T, Matthes MT, Yasumura D, LaVail MM, Steinberg RH (1995) Injury-induced upregulation of bFGF and CNTF mRNAS in the rat retina. J Neurosci 15:7377–7385
28. Cao W, Wen R, Li F, Lavail MM, Steinberg RH (1997) Mechanical injury increases bFGF and CNTF mRNA expression in the mouse retina. Exp Eye Res 65:241–248

29. Liu C, Peng M, Laties AM, Wen R (1998) Preconditioning with bright light evokes a protective response against light damage in the rat retina. J Neurosci 18:1337–1344

30. Ju WK, Lee MY, Hofmann HD, Kirsch M, Chun MH (1999) Expression of CNTF in Muller cells of the rat retina after pressure-induced ischemia. Neuroreport 10:419–422

31. Chun MH, Ju WK, Kim KY, Lee MY, Hofmann HD, Kirsch M, Oh SJ (2000) Upregulation of ciliary neurotrophic factor in reactive Muller cells in the rat retina following optic nerve transection. Brain Res 868:358–362

32. Ju WK, Kim KY, Lee MY, Hofmann HD, Kirsch M, Cha JH, Oh SJ, Chun MH (2000) Up-regulated CNTF plays a protective role for retrograde degeneration in the axotomized rat retina. Neuroreport 11:3893–3896

33. Sarup V, Patil K, Sharma SC (2004) Ciliary neurotrophic factor and its receptors are differentially expressed in the optic nerve transected adult rat retina. Brain Res 1013:152–158

34. Harada C, Harada T, Quah HM, Maekawa F, Yoshida K, Ohno S, Wada K, Parada LF, Tanaka K (2003) Potential role of glial cell line-derived neurotrophic factor receptors in Muller glial cells during light-induced retinal degeneration. Neuroscience 122:229–235

35. Harada T, Harada C, Nakayama N, Okuyama S, Yoshida K, Kohsaka S, Matsuda H, Wada K (2000) Modification of glial-neuronal cell interactions prevents photoreceptor apoptosis during light-induced retinal degeneration. Neuron 26:533–541

36. Harada T, Harada C, Kohsaka S, Wada E, Yoshida K, Ohno S, Mamada H, Tanaka K, Parada LF, Wada K (2002) Microglia-Muller glia cell interactions control neurotrophic factor production during light-induced retinal degeneration. J Neurosci 22:9228–9236

37. Beltran WA, Wen R, Acland GM, Aguirre GD (2007) Intravitreal injection of ciliary neurotrophic factor (CNTF) causes peripheral remodeling and does not prevent photoreceptor loss in canine RPGR mutant retina. Exp Eye Res 84:753–771

38. Zeiss CJ, Allore HG, Towle V, Tao W (2006) CNTF induces dose-dependent alterations in retinal morphology in normal and rcd-1 canine retina. Exp Eye Res 82:395–404