Transcorneal Electrical Stimulation Rescues Axotomized Retinal Ganglion Cells by Activating Endogenous Retinal IGF-1 System

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Purpose. To investigate the effect of transcorneal electrical stimulation (TES) on the survival of axotomized RGCs and the mechanism underlying the TES-induced neuroprotection in vivo.

Methods. Adult male Wistar rats received TES after optic nerve (ON) transection. Seven days after the ON transection, the density of the surviving RGCs was determined, to evaluate the neuroprotective effect of TES. The levels of the mRNA and protein of insulin-like growth factor (IGF)-1 in the retina after TES were determined by RT-PCR and Northern and Western blot analyses. The localization of IGF-1 protein in the retina was examined by immunohistochemistry.

Results. TES after ON transection increased the survival of axotomized RGCs in vivo, and the degree of rescue depended on the strength of the electric charge. RT-PCR and Northern and Western blot analyses revealed a gradual upregulation of intrinsic IGF-1 in the retina after TES. Immunohistochemical analysis showed that IGF-1 immunoreactivity was localized initially in the endfeet of Müller cells and then diffused into the inner retina.

Conclusions. TES can rescue the axotomized RGCs by increasing the level of IGF-1 production by Müller cells. These findings provide a new therapeutic approach to prevent or delay the degeneration of retinal neurons without the administration of exogenous neurotrophic factors. (*Invest Ophthalmol Vis Sci.* 2005;46:2147–2155) DOI:10.1167/iovs.04-1339

Injury to retinal ganglion cells (RGCs) causes functional loss of vision that is irreversible because of the limited axonal regeneration of RGCs.[1] Although much research has been performed on the effect of injuries to neurons of the central nervous system (CNS) and on potential therapeutic strategies to promote axonal regeneration, a complete functional recovery has not been achieved and remains a major goal of this area of research.[2]

Axotomy of RGCs has been widely used as an experimental method to investigate whether different agents can protect the RGCs from apoptosis. In rats, axotomy of the RGCs by optic nerve (ON) transection induces apoptosis and results in rapid loss (within 2 weeks) of 85% of the RGC population.[3,4] To protect RGCs from this death, many attempts have been made to administer drugs or genes expressing various neurotrophic factors.[5–11] These trials, however, have had limited success, and many obstacles and negative side effects have arisen that have prevent widespread clinical application of these methods. Thus, it is necessary to devise other treatments using new therapeutic strategies to find a better method to protect damaged RGCs.

Recently, we discovered that direct electrical stimulation of the transected ON increases the survival of axotomized RGCs in vivo.[12] The protective effect of ON electrical stimulation (ONES) suggests that electrical stimulation of neural tissues may be a strategic approach to treat injured axons in the visual pathway. ONES is, however, too invasive to be clinically applicable, and so we tried transcorneal electrical stimulation (TES), which is known to activate inner retinal neurons and to evoke light sensations or phosphenes, in human[13] and animal[14,15] eyes. Its neuroprotective effect, however, has not been examined.

The purpose of this study was to evaluate the effect of TES on the survival of axotomized RGCs in vivo and to determine the mechanism of how TES protects axotomized RGCs. Because it has been reported that the expression of neurotrophic factors can be altered by electrical or physiological stimuli in vivo,[16–19] we hypothesized that TES upregulates some neurotrophic factors and/or their receptors in the retina. The results show that the level of insulin-like growth factor (IGF)-1 increased in the retina after TES and identified Müller cells as the source of IGF-1.

Materials and Methods

Experimental Animals

Adult male Wistar rats (230–270 g) were obtained from SLC Japan, Inc. (Shizuoka, Japan). All experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Research Committee, Osaka University Medical School. The animals were anesthetized with intraperitoneal pentobarbital (50 mg/kg body weight) for all surgical procedures.

Retrograde Labeling of RGCs

To identify RGCs from other retinal cells, they were retrogradely labeled with a fluorescent tracer (Fluorogold [FG]; Fluorochrome Inc., Englewood, CO). A small sponge soaked in 2% FG (in 0.9% NaCl containing 10% dimethyl sulfoxide) was placed on the surface of both superior colliculi after opening the skull dorsal to the lambda fissure.[3,7,12]

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ON Transection

Seven days after retrograde labeling, the left ON was transected as described in detail elsewhere.[3,4,12] Briefly, a skin incision was made through the left eyelid close to the superior orbital rim, and the orbit was opened. After the superior extraocular muscles were spread, the ON was exposed by a longitudinal incision of the orbital retractor muscle and perineurium. The ON was transected approximately 3 mm from the posterior pole of the eye, with care taken not to damage the retinal blood circulation.

Transcorneal Electrical Stimulation

For electrical stimulation, a noninvasive bipolar contact lens electrode with an inner and outer ring that served as the stimulating electrodes (Kyoto Contact, Kyoto, Japan) was used. Under corneal surface anesthesia by 0.4% oxybuprocaine HCl in addition to systemic anesthesia, the contact lens electrode was placed on the cornea of the eye in which the ON had been transected. Hydroxyethylcellulose gel (1.3%) was applied for corneal protection and for tight adhesion of the electrode to the cornea.

The electrical stimuli consisted of 20 Hz, biphasic rectangular current pulses (100 μA) that were delivered from an isolated constant-current stimulator (Stimulator, SEN-7203; Nihon Kohden, Tokyo, Japan; Isolator, A395R; World Precision Instruments, Sarasota, FL). The electrical stimulation lasted for 1 hour. To evaluate the neuroprotective effect of TES, the pulse duration of electric current was varied from 0 (sham stimulation) to 3 ms/phase. TES was commenced immediately after ON transection.

Quantification of RGC Density

Seven days after ON transection, rats received an overdose of pentobarbital and were perfused transcardially with saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Both eyes were enucleated, and the retinas were isolated and flatmounted on glass slides. The retinas were examined under a fluorescence microscope (Axioskop; Carl Zeiss, Oberkochen, Germany) with a UV filter (365 nm). The number of FG-labeled neurons was counted in 12 areas (0.5 mm^2 each) at distances of 1, 2, and 3 mm from the optic disc along the nasotemporal and dorsoventral midlines (upper, lower, nasal, and temporal direction). The density of surviving RGCs was calculated from the number of FG-labeled neurons counted in the 12 areas. The data are reported as the mean ± standard deviation.

The statistical significance of differences was determined by one-way ANOVA followed by the Tukey test. Statistical significance was set at $P < 0.05$.

RNA Extraction, RT-PCR, and Northern Blot Analysis

Eyes without ON transection underwent TES for 1 hour, and were removed at different selected time points from 1 hour to 10 days. The retinas were dissected from the eyes in a shallow bath of cold phosphate buffered saline (PBS) and were stored at −80°C until use. Total RNA was then extracted (RNeasy Mini Kit; Qiagen, Hilden, Germany) from pooled retinas and quantified (Gene Quant II; Amersham Pharmacia Biotech, Piscataway, NJ), as previously described.[20]

RT-PCR and Northern blot analysis were performed as previously described.[21] For RT-PCR, 5 μg of total RNA was reverse transcribed using oligo (dT) reverse transcriptase (Ready-To-Go You-Prime First-Strand Beads; Amersham Biosciences). The cDNAs were amplified for 25 to 30 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 60 seconds at 72°C. The sequences of the primers used were: IGF-1 forward, 5′-TGGACGCTCTTCAGTTCGTG-3′, reverse, 5′-GTTTCCTG-CACTTCCTCTAC-3′; IGF-1R forward, 5′-CAGCTGCAACCACGAGG-CTG-3′, reverse, 5′-GGTTCACAGAGGCGTACAGC-3′; BDNF forward, 5′-AGAGCTGCTGGATGAGGACC-3′, reverse, 5′-CCAGTGCCTTTTGTC-TATCG-3′; TrkB forward, 5′-CTTGGAGAAGGAGCCTTTGG-3′, reverse, 5′-CAACCCGGTAGTAGTCGGTG-3′; bFGF forward, 5′-CGGCAGCAT-CACTTCGCTTC-3′, reverse, 5′-CAGTATGGCCTTCTGTCCAG-3′; FGFR-1 forward, 5′-ACCTGATCTCGGAGATGGAG-3′, reverse, 5′-TGGTGGGT-GTAGATCCGGTC-3′; CNTF forward, 5′-TGAGGCAGAGCGACTCCA-AG-3′, reverse, 5′-GCTCTCAAGTGCTGAGATTC-3′; CNTFR forward, 5′-TTGGGTCACAACACCACGGC-3′, reverse, 5′-CCAAGGAGCTGGTG-GTGCTG-3′; and β-actin forward, 5′-TGCCCATCTATGAGGGTTA-CG-3′, reverse, 5′-TAGAAGCATTTGCGGTGCGGTGCACG-3′.

For Northern blot analysis, total RNA (10 μg) was isolated from the retina at each time point by electrophoresis on 1.0% agarose-formaldehyde gels and transferred overnight onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA). The membrane was prehybridized for 1 hour at 65°C in hybridization buffer (0.9 M NaCl, 90 mM sodium citrate [pH 7.0]) containing 5× Denhardt's solution, SDS (0.5%), and heat-denatured salmon sperm DNA (100 ng/mL). The cDNA probe was radiolabeled with [32P]dCTP (NZ522; PerkinElmer Life and Analytical Sciences, Boston, MA, with the Random Primer DNA Labeling Kit, ver. 2; Takara Bio, Shiga, Japan). After hybridization overnight at 65°C in hybridization buffer containing radiolabeled cDNA probe (5 ng/mL), filters were washed twice with 2× SSC, 0.5% SDS and 0.2× SSC, 0.5% SDS for 60 minutes at 65°C, exposed to x-ray film (Fuji Film, Kanagawa, Japan), and subjected to autoradiography. Autoradiograms were quantified by image analysis (Scion Image; Scion Corp., Frederick, MD). The relative expression levels of IGF-1 mRNA in the retinas after TES were compared with the expression in the control retina, which was normalized to 1.0. Data from three independent experiments are given as the mean ± SD.

Western Blot Analysis

Total retinal proteins were extracted from eyes at each time point after TES and were assessed by Western blot analysis, as previously described.[22,23] Total protein was extracted with lysis buffer (50 mM Tris-HCl [pH 7.4]); 0.5% deoxycholate, 1% Triton X-100, 1% NP-40, 10 mM NaF, 150 mM NaCl, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 20 μg/mL pepstatin, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM Na_3VO_4, 1 mM dithiothreitol, and 10% SDS) on ice for 30 minutes and centrifuged at 15,000 rpm for 15 minutes at 4°C. The supernatants were collected, and the protein concentration was determined by the Bradford protein assay with bovine serum albumin as a standard (Bio-Rad, Hercules, CA). Total protein (10 μg) was separated by SDS-PAGE (16% Tris-tricine gel; Invitrogen, Carlsbad, CA) and transferred to a PVDF membrane (Millipore Corp.).

Membranes were preblocked in 5% nonfat milk at room temperature (RT) for 1 hour and then incubated with primary antibodies of mouse anti-human IGF-1 (Upstate Biotechnology, Waltham, MA) at a dilution of 1:1000 in TBS and 0.1% Tween 20 (TBS-T) with 5% nonfat milk at 4°C overnight. Membranes were washed in TBS-T and incubated with HRP-conjugated goat IgG secondary antibody against mouse (Jackson ImmunoResearch, West Grove, PA; 1:1000 dilution in TBS-T with 5% nonfat milk) at RT for 1 hour. Labeled proteins were detected by chemiluminescence (ECL; Amersham, Arlington Heights, IL), and the chemiluminescence signals were captured on film (Kodak scientific imaging film; Eastman Kodak, Rochester, NY). Densitometric analyses were then performed (Scion Image; Scion Corp.). First, the relative expression levels of IGF-1 protein were compared with the expression levels of β-actin in the same retinas. Then the values were compared with that of the control retina which was normalized to 1.0. The mean ± SD of three independent experiments was used for the analyses. Experiments for RT-PCR and Northern and Western blot analyses were performed on specimens collected from three animals at each time point, and the results were repeated three times.

Immunohistochemistry

On days 1, 4, 7, or 14 after TES without ON transection, the rats received an overdose of pentobarbital and were perfused transcardially with saline, followed by 4% PFA in 0.1 M PB and the eyes immediately

enucleated. The anterior segment and the lens were removed, and the remaining eyecup was immersed in the same fixative for 30 minutes at 4°C. The eyecups including the ON were cryoprotected in 10% to 20% sucrose in PBS for 2 days, embedded in OCT compound (Tissue-Tek; Ted Pella, Inc., Redding, CA) by snap freezing in liquid nitrogen, and then sectioned (10 μm). The sections were mounted on slides and incubated with blocking buffer (PBS containing 5% goat serum, 5% BSA, and 0.2% Triton X-100) at RT for 1 hour. After three washes in 0.1 M PBS, the sections were incubated overnight at 4°C with a mouse monoclonal antibody against IGF-1 (1:300 dilution; Upstate Biotechnology) and/or a rabbit polyclonal antibody against glutamine synthetase (1:300; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS containing 0.2% Triton X-100, 5% goat serum, and 5% BSA. The sections were then rinsed three times in 0.1 M PBS and incubated with Cy3- and fluorescein isothiocyanate (FITC)-conjugated goat IgG secondary antibodies (1:200; Jackson ImmunoResearch) at RT for 1 hour, followed by three rinses with 0.1 M PBS. The sections were mounted with antifade mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) and examined with a confocal laser microscope (LSM510; Carl Zeiss Meditec).

Administration of IGF-1R Antagonist

JB-3, a selective antagonist for IGF-1R, is a cyclic D-amino acid peptide analogue of the D domain of IGF-1 (CYAAPSAYLKPC).[24,25] JB-3 was synthesized nonbiologically by Sigma Genosys Japan (Hokkaido, Japan). A subcutaneous injection of JB-3 has been shown to inhibit the activity of retinal IGF-1 action in a retinal neovascularization model.[24]

After ON transection, 200 μL of JB-3 solution was dissolved in 0.1 M PBS and was injected intraperitoneally every day for 1 week. For the control, PBS alone was injected. The dose of JB-3 was obtained from the protocol described by Smith et al.[24] This dosage schedule achieved a systemic dose of JB-3 of 10 μg/kg or 100 μg/kg per day, for 6 days.

Results

TES and the Survival of Axotomized RGCs In Vivo

Seven days after the retrograde labeling of the RGCs with FG, the left ON was transected, and TES was immediately applied for 1 hour. The rats were killed 7 days later, and the effect of the TES on the survival of axotomized RGCs was examined in flatmounts of the retina (Fig. 1). In intact control retinas, FG-labeled RGCs were recognized by the fine spots of fluorescence in the perinuclear cytoplasm and proximal dendrites (Fig. 1A). The mean RGC density in the intact control retinas was 2346 ± 175 cells/mm^2 (mean ± SD; n = 12; Fig. 2).

Seven days after ON transection and without TES, the number of FG-labeled RGCs was markedly reduced; they were irregularly shaped, and debris of dead RGCs were present (Fig. 1B). The mean RGC density had decreased to 54% of normal (n = 8).

The mean RGC density in the sham electrical stimulation was 53% (n = 6) of the control retinas. This reduction was not significantly different from that in the eyes with ON transection and without TES. In contrast, retinas that had received TES had many more surviving RGCs than those without electrical stimulation (Fig. 1C). The increase in the densities of RGCs depended on the pulse duration of electric current. TES of 0.5-ms/phase pulse duration significantly increased the number of RGCs (70% of the normal density; n = 6, $P < 0.05$). In addition, TES of 1- and 3-ms/phase pulse duration further increased the density up to 85% and 83%, respectively, of normal (n = 6, each; Fig. 2). The shapes of surviving RGCs were similar to those of the RGCs in the intact retinas. During the course of these experiments, cataracts or corneal opacities were not developed under surgical microscope in all rats. Fundus examination was performed at the end of TES, but neither retinal detachment nor vitreous hemorrhage occurred in all rats.

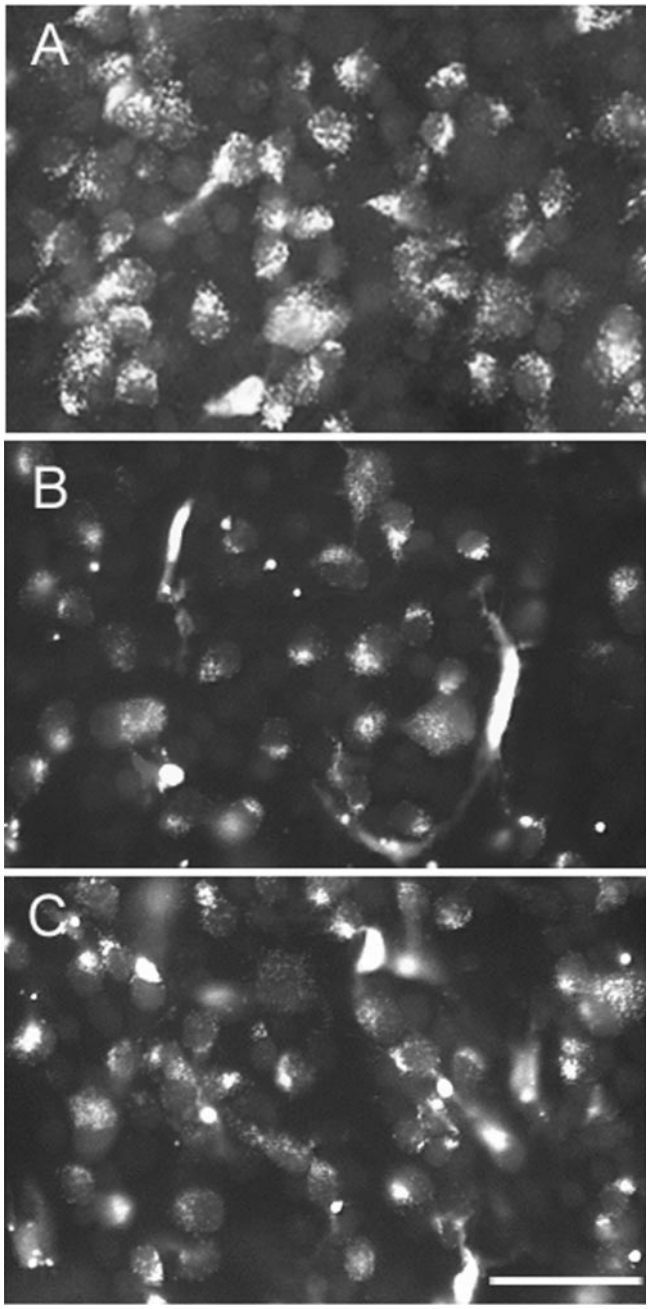

Figure 1. Representative photomicrographs of retrogradely-labeled RGCs in corresponding regions (approximately 1 mm from the optic disc) of flat-mounted retinas. (**A**) FG-labeled RGCs in intact control retina. (**B**) RGCs in the retina 7 days after ON transection without TES. (**C**) RGCs after ON transection with TES (1 ms/phase). More regularly shaped RGCs were seen in the retina after TES than in those without TES. Scale bar, 25 μm.

Increase in Level of IGF-1 after TES

We hypothesized that the neuroprotective effect of TES results from increasing the level of some neurotrophic factors or their receptors in the retina. To test this hypothesis, we examined which genes of the principal neurotrophic factors and their receptors were upregulated after TES (100 μA, 1 ms/phase, 20

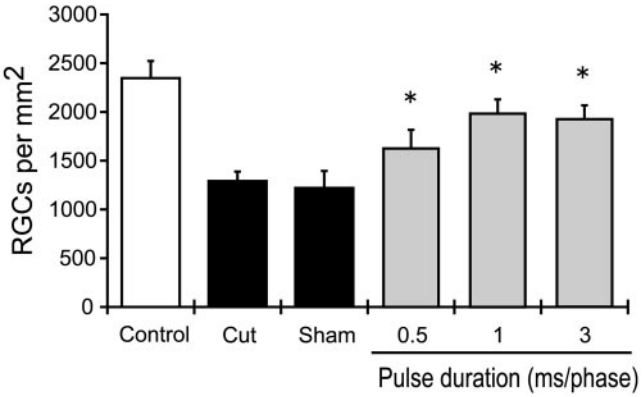

FIGURE 2. The neuroprotective effects of TES on the axotomized RGCs 7 days after ON transection depended on the pulse duration. The density of the FG-labeled RGCs per square millimeter is given as the mean ± SD. Seven days after ON transection, the density of the RGCs decreased to 54% of the control (Cut group). In the sham-treated animals (no electrical stimulation after ON transection), the density decreased to 53% of that of the intact control retina (Sham group). The RGC density in all three groups with TES (0.5, 1, and 3 ms/phase pulse duration) was significantly increased compared with that in the sham group. Statistical analysis was made by one-way ANOVA followed by the Tukey test ($P < 0.01$, $^*P < 0.05$ compared with sham).

Hz, 1 hour) without ON transection. RT-PCR was used to survey the changes in the mRNA expressions of the following neurotrophic factors and receptors: BDNF and its receptor TrkB; CNTF and CNTF receptor-α (CNTFRα); bFGF and FGF receptor-1 (FGFR-1); and IGF-1 and IGF-1 receptor (IGF-1R).

RT-PCR analysis showed that the expression level increased for only the mRNA of IGF-1, and the expression of the mRNA of the other neurotrophic factors and receptors did not change significantly (Fig. 3A). RT-PCR of IGF-1 mRNA also showed that its level of expression depended on the pulse duration of the TES (Fig. 3B). The expression of IGF-1 mRNA in the retina with 1-ms/phase pulses of TES was higher than that with 0.5-ms/phase on day 2 after TES, and this difference was maintained for at least 7 days.

A quantitative analysis of the changes of IGF-1 mRNA expression was also performed by Northern blot analysis at different times, ranging from 1 hour to 10 days after TES. Northern blot analysis showed that the level of mRNA of IGF-1 in the retina gradually increased from day 1 and reached a peak at day 7 (203% of the level of the intact control retina) and remained elevated even at day 10 after TES (Figs. 3C, 3D).

Western blot analysis was also used to determine the level of IGF-1 protein from day 1 to day 14. The level of IGF-1 protein was already increased on day 1 and reached its peak of 189% of the intact control on day 7. The elevated level was still present on day 10, confirming the results obtained from North-

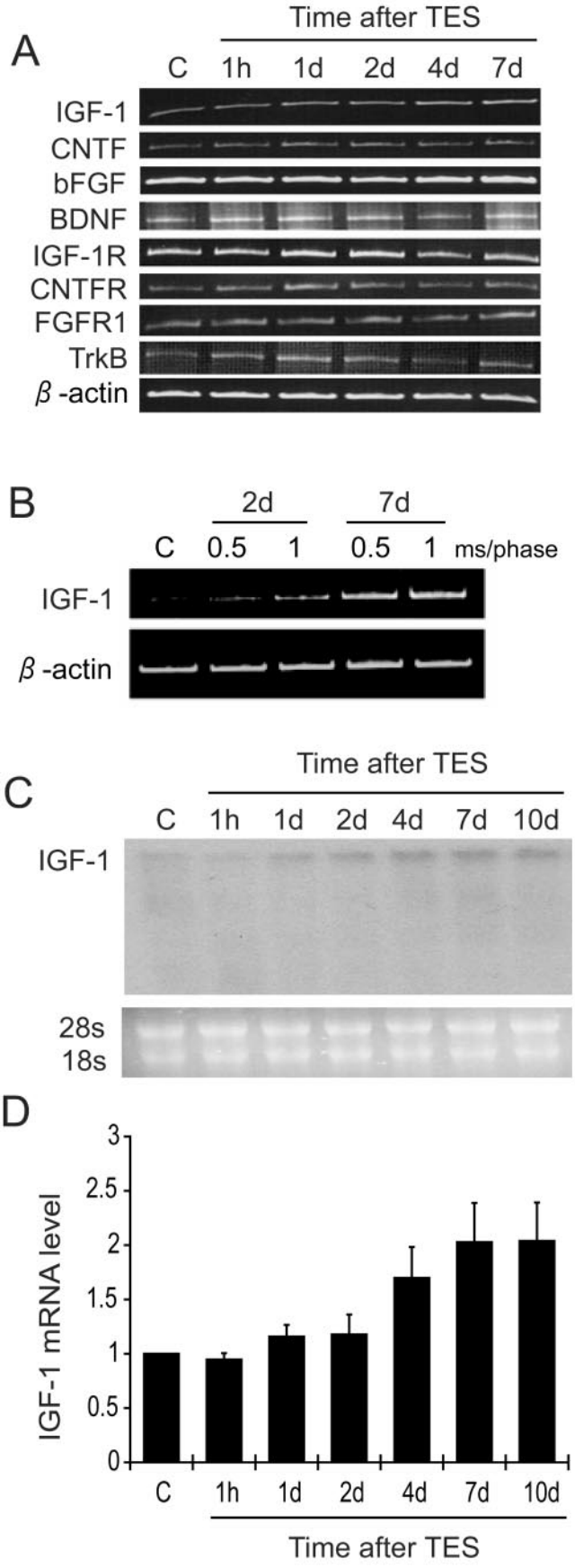

FIGURE 3. Expression of IGF-1 mRNA in the retina after TES. (**A**) RT-PCR analyses for four kinds of neurotrophic factors and receptors at different times ranging from 1 hour to 7 days after TES without ON transection. The data labeled "C" are from intact control retina. (**B**) Electrical current pulse duration-dependent upregulation of IGF-1 mRNA expression by RT-PCR analysis. RT-PCR for β-actin mRNA confirmed that equivalent amounts of RNA were used. (**C**) Northern blot analysis of IGF-1 mRNA in the retina (*top*). RNA loading was measured by gel staining with ethidium bromide (*bottom*). (**D**) Relative expression level of IGF-1 mRNA in retinas after TES compared with the control retinas (normalized to 1.0). The mean ± SD of data from three independent experiments is shown.

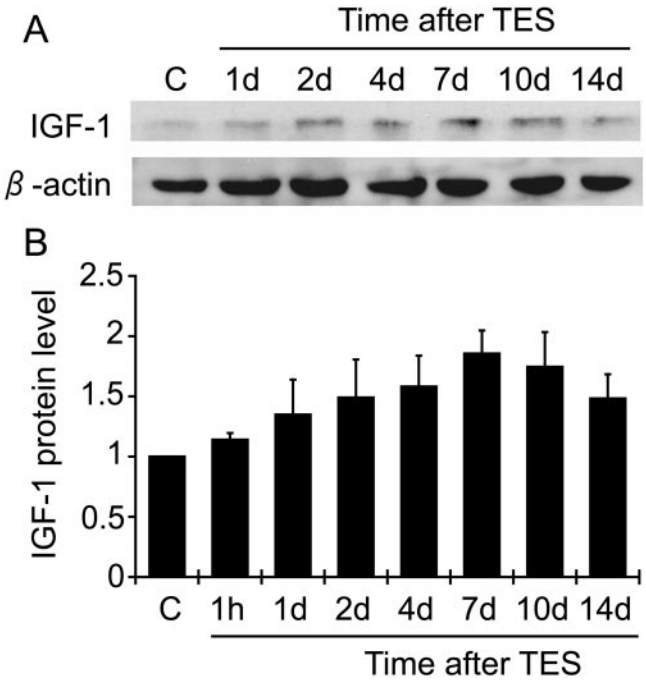

FIGURE 4. Expression of IGF-1 protein. (**A**) Western blot analysis of IGF-1 protein in the retina (*top*). (**B**) Relative expression of IGF-1 protein in retinas after TES compared with the contralateral control retinas (normalized to 1.0). Data from three independent experiments were averaged and are presented relative to that in the control retinas (mean ± SD).

ern blot analysis. However, the IGF-1 protein level then decreased on day 14 (Fig. 4).

Immunolocalization of IGF-1 in the Retina after TES

Immunohistochemical studies were performed with IGF-1 antibody, to determine the distribution of IGF-1 protein in the retina from day 1 to day 14 after TES without ON transection. In the intact control retina, IGF-1 immunoreactivity was very weak and restricted primarily to the inner limiting membrane (ILM) and the nerve fiber layer (NFL; Fig. 5A). On day 1 after TES, intense immunoreactivity for IGF-1 appeared from the ILM to the ganglion cell layer (GCL). A weak, but detectable, positive staining was also observed in the inner plexiform layer (IPL) and the inner nuclear layer (INL; Fig. 5B). On day 4, IGF-1 immunoreactivity further expanded, and the radial elements extending from the ILM to the IPL were stained (Fig. 5C). On day 7, the staining for IGF-1 was strongest within the inner retina, and intense staining for IGF-1 was seen in the radial processes of the ILM through the INL (Fig. 5D). On day 14, the immunoreactivity for IGF-1 in the inner retina decreased but IGF-1 signals in the radial processes remained within the NFL and GCL (Fig. 5E).

IGF-1 in Müller Cells

To determine whether Müller cells express IGF-1, additional immunohistochemical studies were performed on the retina with antibodies to IGF-1 and glutamine synthetase (GS), a specific marker for Müller cells. Müller cell bodies lie in a narrow band in the middle of the INL, and their processes span all cellular and plexiform layers of the retina.[26] The coimmunolocalization of IGF-1 (Fig. 5F) and GS (Fig. 5G) was not strong in the Müller cells of the intact retina. This indicates that IGF-1 was located mainly in the basal endfeet of the Müller cells in the intact retina (Fig. 5H). On day 7 after TES, IGF-1 immunoreactivity appeared in the Müller cell processes that extend from the ILM to the OLM and also in the space surrounding them within the IPL and INL (Figs. 5I–K). There was no difference in GS immunoreactivity between the control retina and the retina on day 7 after TES. Examination of the retinas at higher magnification on day 7 after TES showed that strong immunoreactivity of IGF-1 appeared in the endfeet of the Müller cells which surrounded the cells in the GCL (Fig. 5L–N).

We also performed immunohistochemical analysis for glial fibrillary acidic protein (GFAP) which is expressed in Müller cells whenever the retinal neurons are damaged.[27–30] We did not observe immunoreactivity for GFAP throughout the experimental period, suggesting that TES does not damage the retinal tissue (data are not shown). In agreement with the results from Western blot analysis, these results indicated that the IGF-1 is secreted from Müller cells and spreads throughout the inner retina and that TES increases the level of secretion.

Effect of Upregulation of IGF-1 on TES-Induced Neuroprotection of Axotomized RGCs

IGF-1 is one of the trophic factors that promote the survival of axotomized RGCs in vivo.[11] To determine whether IGF-1 is involved in the TES-induced neuroprotection, we counted the number of RGCs that survived after a combined treatment of TES and JB-3, an IGF-1 receptor antagonist.[24,25,31] JB-3 is a long-acting antagonistic peptide that inhibits interaction between IGF-1 and IGF-1R and prevents activation of tyrosine kinase of IGF-1R in a dose-dependent manner.[25,31] Daily injections of low-dose JB-3 (10 μg/kg per day) did not block the neuroprotective effects of TES, since the mean RGC density at day 7 after ON transection was 79% in the intact retina ($n = 4$), which was not significantly different from that after TES and PBS injection (86%; $n = 4$; Figs. 6A, 6B, 7).

However, a high dose of JB-3 (100 μg/kg per day) significantly inhibited the neuroprotective effects of TES, as the number of RGC was reduced to 59% of that in the control retina ($n = 4$; Figs. 6C, 7). With JB-3 alone, the number of surviving RGCs after ON transection (52%; $n = 4$), was similar to that with the ON transection without JB-3 ($n = 8$; Fig. 7). These data showed that the IGF-1 induced by TES plays a key role in TES-induced neuroprotection of axotomized RGCs.

DISCUSSION

Our results demonstrate that TES markedly increased the number of surviving axotomized RGCs in vivo, and the degree of protection was dependent on the strength of the electrical charge. Our results also show that the mRNA and protein of IGF-1 gradually increased in the retina during the 7 days after TES. Immunohistochemical analyses showed that the IGF-1 was located in the endfeet of the Müller cells, and TES led to a spread of IGF-1 in the intact retina. Thus, TES activated the Müller cells to produce more IGF-1 and release it into the inner retina. A blocking of the IGF-1R by JB-3 reduced the degree of neuroprotection by TES on the axotomized RGCs. Thus, TES activates an intrinsic retinal IGF-1 system that then rescues the axotomized RGCs.

IGF-1 as a Key Molecule for TES-Induced Neuroprotection

The upregulation of IGF-1 by the TES proved to be a crucial factor in neuroprotection. To the best of our knowledge, this is the first in vivo demonstration that a neurotrophic factor can be upregulated by electrical stimulation and can then lead to

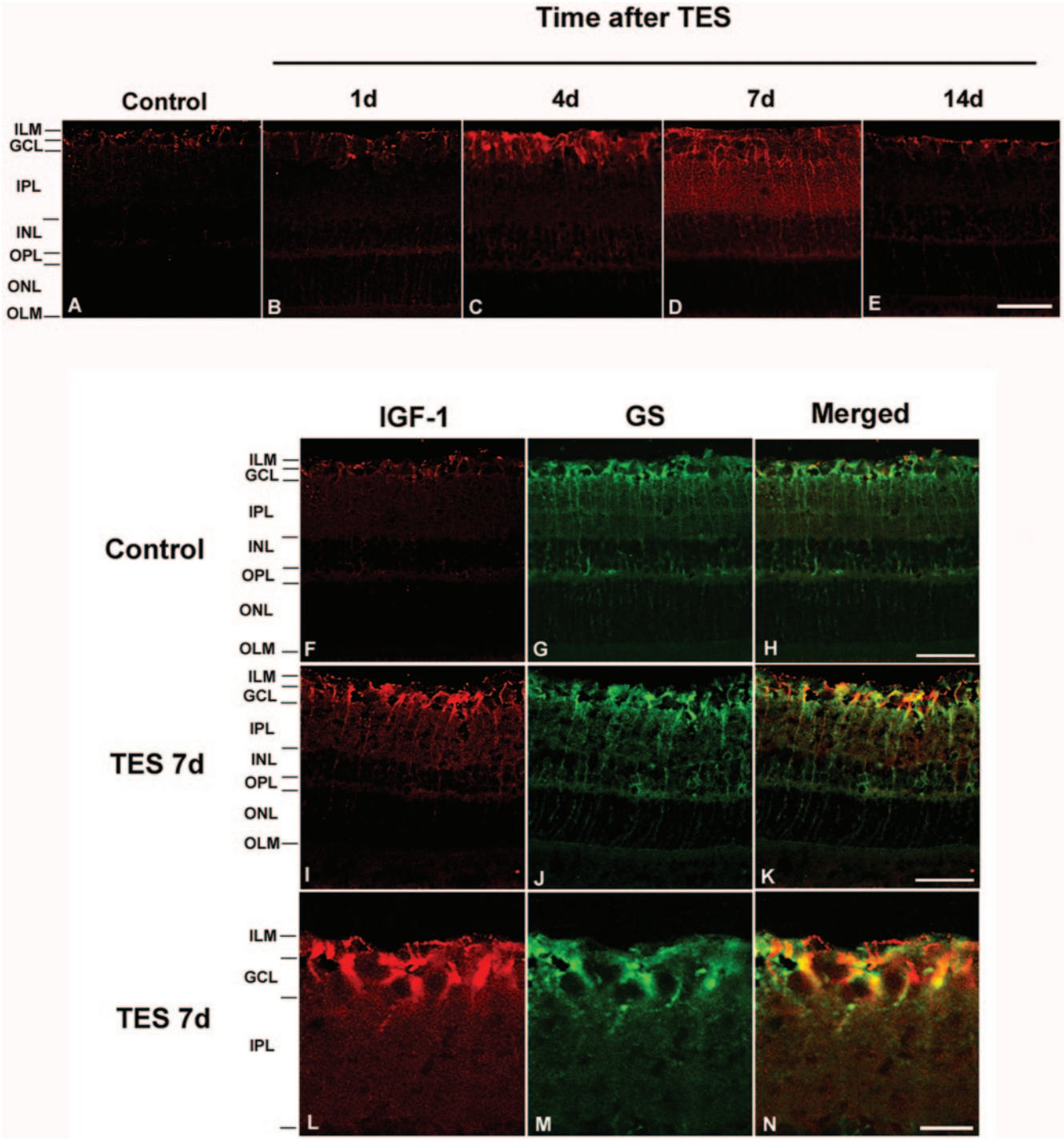

FIGURE 5. Immunohistochemical analysis of IGF-1 in the retina after TES. (**A**–**E**) Localization of IGF-1 in the retina at different time points after TES. IGF-1 immunoreactivity in the intact retina (**A**). IGF-1 staining increased on day 1 after TES (**B**) and increased more and spread from the ILM to the GCL 4 days after TES (**C**). IGF-1 immunoreactivity reached a peak on day 7 (**D**) and decreased on day 14 (**E**). (**F**–**N**) Double staining for IGF-1 (*red*) and GS (*green*), a specific marker of Müller cells in the retina. In the intact control retina, weak IGF-1 immunoreactivity was distributed from the ILM to the GCL (**F**), and GS immunoreactivity was present in Müller cells (**G**). The merged image (**H**) shows that weak signal (*yellow*) was localized in the ILM (**H**). Seven days after TES, strong immunoreactivity for IGF-1 appeared from the ILM to the IPL (**I**), and the merged image shows that IGF-1 immunoreactivity appeared in the endfeet and processes of Müller cells (**K**). A high-magnification view of IGF-1 and GS colocalization (**N**) strongly suggests that IGF-1 is produced in the endfeet of Müller cells surrounding the cells in GCL. Scale bars: (**A**–**E**) 100 μm; (**F**–**K**) 50 μm; (**L**–**N**) 20 μm.

neuroprotection. Until now, it has been reported that electrical or natural stimulation can modify the expression of neurotrophic factors or their receptors in neural tissue. Thus, electrical stimulation has been shown to upregulate BDNF and TrkB mRNA in various neurons.[16,17] The mRNA levels of BDNF or TrkB in the rat visual cortex were increased by light stimula-

tion.[18,19] In addition, it has been demonstrated that light-induced damage or mechanical injury to the retina elevates the expression of bFGF and CNTF.[27,28] Because various neurotrophic factors and their receptors are known to exist in the retina,[5,32–35] all of them can be upregulated in the retina by different types of stimuli. However, IGF-1 was specifically upregulated in the retina by TES.

We have shown an upregulation of IGF-1 by Northern and Western blot analyses and immunohistochemistry. We screened for BDNF, CNTF, and bFGF and their receptors in addition to IGF-1 and IGF-1R by RT-PCR, but other neurotrophic factors or receptors may also have contributed to the neuroprotective effects of TES. In addition to the possible upregulation of neurotrophic factors and/or receptors, TES can depolarize the RGCs directly. It has been reported that the neural activity of RGCs increases their sensitivity to peptidic neurotrophic factors.[36,37] We observed that TES of 100 μA at 1 ms/phase, which had been shown to rescue the axotomized RGCs, was also able to evoke electrical responses in the superior colliculus (data not shown). Thus, we cannot rule out the possibility that the electrical activation of the RGCs may have contributed to the effect of IGF-1. However, the systemic administration of JB-3 almost completely inhibited the TES-induced neuroprotection. This clearly shows that IGF-1 is essential for TES-induced neuroprotection, even though some other mechanisms may contribute to the effect of IGF-1.

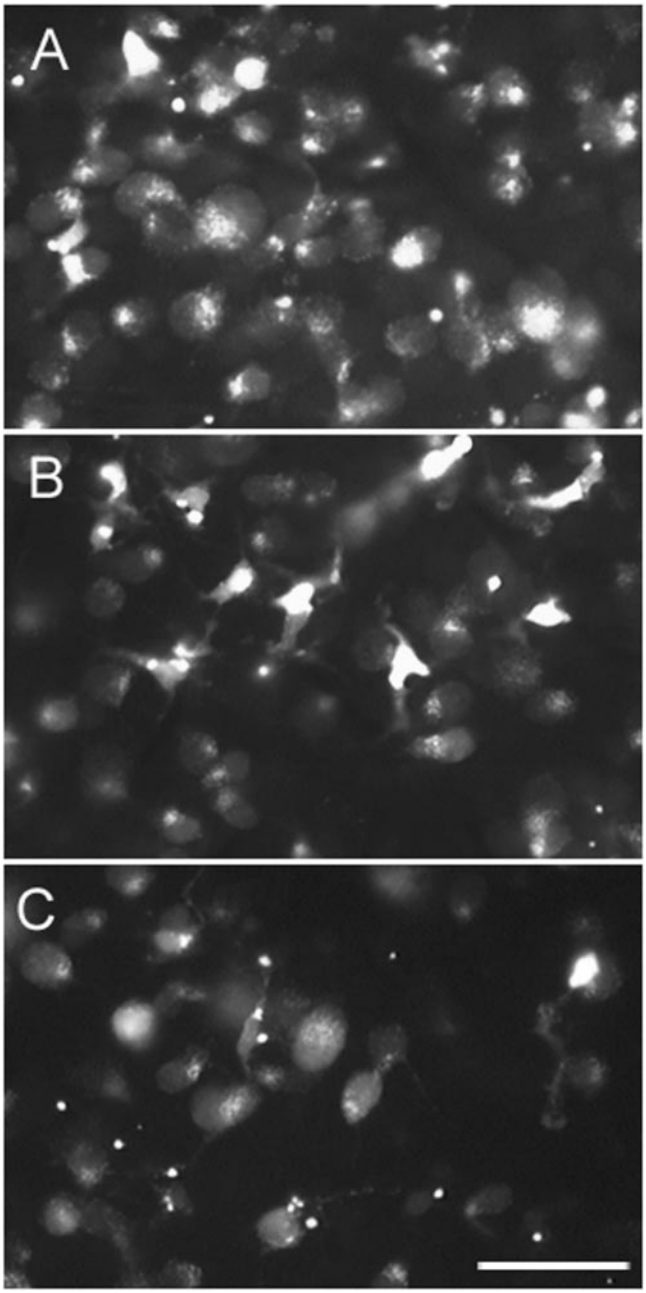

FIGURE 6. Effect of the IGF-1 receptor antagonist JB-3 on neuroprotection by TES. FG-labeled RGCs in the retina 7 days after ON transection. (**A**) Combined treatment with TES and injection of PBS. Combined treatment with TES and daily intraperitoneal injection of (**B**) 10 μg/kg or (**C**) 100 μg/kg JB-3. Scale bar, 25 μm.

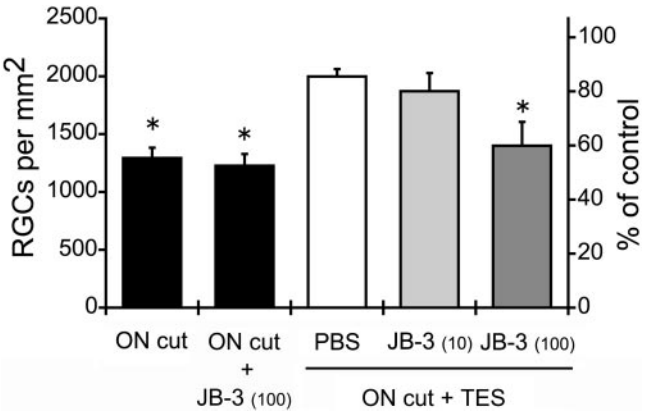

FIGURE 7. Quantitative analysis of the density of surviving RGCs after daily intraperitoneal injections of JB-3 (dose, in micrograms per kilogram per day, shown in parentheses) 7 days after ON transection (n = 4 for each group). Although daily injection of 10 μg/kg JB-3 with TES did not alter the number of surviving RGCs when compared with the treatment combining TES with daily injection of PBS, daily injection of 100 μg/kg JB-3 significantly decreased the number of RGCs. Daily injection of 100 μg/kg without TES, however, did not alter the number of RGCs, compared with ON transection without injection of JB-3 (ON cut). *Statistical significance compared with PBS ($P < 0.05$; one-way ANOVA followed by the Tukey test).

We have demonstrated that electrical stimulation of the stump of the transected ON promoted the survival of axotomized RGCs,[12] and the present study showed that TES, which is less invasive than stimulation of the transected ON, also protects axotomized RGCs from apoptosis. The effect of TES is comparable to that of electrical stimulation to the transected ON.[12] The extent of the neuroprotective effect of TES is also similar to that of intravitreal application of neurotrophic factors.[6,7] The strong effect of TES can be explained by the fact that it upregulated the expression of IGF-1 in the retina for more than 7 days.

Intrinsic Retinal IGF-1 System

IGF-1 has been reported to promote the survival, differentiation, and proliferation of retinal neurons.[38] More specifically, IGF-1 has been reported to promote the survival of injured RGCs, both in vivo[11] and in vitro.[36] In this study, we showed that IGF-1 was recruited from Müller cells by TES and was released to rescue the axotomized RGCs near the Müller cells.

Autocrine-paracrine IGF-1 systems have been reported to exist in the retina.[33,39–42] IGF-1 mRNA was shown to be localized in the GCL in the intact rat retina by in situ hybridization analysis.[33] In contrast, Müller cells express IGF-1 mRNA in vitro.[43] What retinal cells produce and how IGF-1 moves in vivo have not been determined. In the present study, immu-

nohistochemical analyses showed that Müller cells contained small amounts of IGF-1 in their endfeet before TES and that, after TES, Müller cells were activated to increase the level of IGF-1. Our study provides in vivo evidence that the intrinsic IGF-1 paracrine system is in the Müller cells.

The mechanism of the activation of production of IGF-1 by TES has not been determined. It was reported that the regulation of the expression of trophic factors in neurons is clearly linked to their electrical activity. Activation of L-type voltage sensitive Ca^{2+} channels or the non-*N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor leads to an enhancement of BDNF mRNA levels in hippocampal neurons[44,45] and in cortical neurons.[46,47] Similarly, the mechanism for the increased levels of IGF-1 in this study may be related to the electrical activity of retinal neurons and/or glial cells. Further experiments are needed to elucidate this mechanism in detail so that techniques can be designed to stimulate the control glial cells to produce more neurotrophic factors by electrical stimulation.

TES as a New Clinical Technique

Our findings allow us to propose electrical stimulation as a new therapy that activates the intrinsic neuroprotective system. Until now, intravitreal injection or gene transfer of exogenous neurotrophic factors have been used to rescue degenerating retinal neurons[5–11] to provide sustained trophic support. With these methods, however, it is still difficult to deliver exogenous neuroprotective agents chronically into retinal neurons in patients. In addition, intravitreal injection of such neuroprotective agents may cause ocular side effects such as cataract or endophthalmitis. In contrast, TES can control the synthesis of IGF-1, one of the endogenous neurotrophic factors that can then have a neuroprotective effect. This electrical stimulation therapy is simple and less invasive, and ocular side effects were not observed after TES during the course of the study. TES may also have therapeutic or preventive potential in progressive diseases of RGCs, including glaucomatous optic neuropathy. We are now designing a clinical trial using TES for optic neuropathies that are difficult to treat by present methods.

In conclusion, our results showed that TES leads to the upregulation and release of IGF-1 in Müller cells and, consequently, protects the RGCs from secondary cell death after ON transection. Müller cells play an important role in neuroprotection, as well as a housekeeping role that maintains the integrity and the normal function of the retina.

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