Experimental Eye Research 90 (2010) 285–291

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Optimal parameters of transcorneal electrical stimulation (TES) to be neuroprotective of axotomized RGCs in adult rats

Takeshi Morimoto [a], Tomomitsu Miyoshi [b], Hajime Sawai [b], Takashi Fujikado [c,*]

[a] Department of Ophthalmology, Osaka University Graduate School of Medicine, Japan
[b] Department of Integrative Physiology, Osaka University Graduate School of Medicine, Japan
[c] Department of Applied Visual Science, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 10 September 2009
Accepted in revised form
3 November 2009
Available online 10 November 2009

Keywords:
retinal ganglion cell
neuroprotection
electrical stimulation
optic neuropathy

ABSTRACT

We previously showed that transcorneal electrical stimulation (TES) promoted the survival of axotomized retinal ganglion cells (RGCs) of rats. However the relationship between the parameters of TES and the neuroprotective effect of TES on axotomized RGCs was unclear. In the present study, we determined whether the neuroprotective effect of TES is affected by the parameters of TES. Adult male Wistar rats received TES just after transection of the left optic nerve (ON). The pulse duration, current intensity, frequency, waveform, and numbers of sessions of the TES were changed systematically. The alterations of the retina were examined histologically seven days or fourteen days after the ON transection. The optimal neuroprotective parameters were pulse duration of 1 and 2 ms/phase ($P < 0.001$, each), current intensity of 100 and 200 μA ($P < 0.05$, each), and stimulation frequency of 1, 5, and 20 Hz ($P < 0.001$, respectively). More than 30 min of TES was necessary to have a neuroprotective effect ($P < 0.001$). Symmetric pulses without an inter-pulse interval were most effective ($P < 0.001$). Repeated TES was more neuroprotective than a single TES at 14 days after ON transection ($P < 0.001$). Our results indicate that there is a range of optimal neuroprotective parameters of TES for axotomized RGCs of rats. These values will provide a guideline for the use of TES in patients with different retinal and optic nerve diseases.

1. Introduction

Neuronal activity has a neurotrophic effect on neurons (Linden, 1994; Mennerick and Zorumski, 2000). *In vivo* and *in vitro* studies have shown that electrical stimulation (ES) or neuronal activity promotes the survival and/or neurite outgrowth of different types of neurons (Fields et al., 1990; Grumbacher-Reinert and Nicholls, 1992; Al-Majed et al., 2000a,b).

In the visual system, it has been shown *in vitro* study that ES or depolarization by KCl promotes the survival and/or neurite outgrowth of cultured retinal ganglion cells (RGCs) (Meyer-Franke et al., 1998; Shen et al., 1999; Goldberg et al., 2002). Our laboratory has shown that direct ES of a transected optic nerve (ON) in adult rats promoted the survival of the axotomized RGCs in adult rats (Morimoto et al., 2002). We also showed that transcorneal electrical stimulation (TES), which is less invasive than ES of the transected ON (ON-ES), also promoted the survival of axotomized RGCs in adult rats (Morimoto et al., 2005), and In addition, promoted the survival of photoreceptors in Royal College Surgeons rats (Morimoto et al., 2007). Miyake et al. (2007) have reported that TES immediately after crushing the ON lessened the degree of visual impairment in adult rats. In the clinic, TES has been demonstrated to improve visual function in patients with nonarteritic anterior ischemic optic neuropathy, traumatic optic neuropathy (Fujikado et al., 2006), and retinal artery occlusion (Inomata et al., 2007).

In spite of these studies, the optimal TES parameters which will result in the best neuroprotective effect and safety of the retina in the clinical situation have not been determined. There are different parameters of the TES that need to be considered, e.g., pulse duration, current intensity, frequency, duration of stimulation, waveform, and number of sessions. Different combinations of these parameters also need to be considered. There are several studies on the effect of the electrical stimulation parameters on tissue damage (Yuen et al., 1981; McCreery et al., 1990; Harnack et al., 2004; Nakauchi et al., 2007), but reports on the relationship between the ES parameters and their neuroprotective effects on injured neurons are limited (Okazaki et al., 2008).

Therefore, the purpose of this study was to determine the optimal ES parameters for the neuroprotection of axotomized RGCs. To accomplish this, we cut the ON of adult rats and stimulated the eyes with electrical pulses of different duration, current intensity,

* Corresponding author. Tel.: +81 6 6879 3940; fax: +81 6 6879 3948.
E-mail address: fujikado@ophthal.med.osaka-u.ac.jp (T. Fujikado).

0014-4835/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.exer.2009.11.002

frequency, duration of stimulation time, waveform, and number of TES sessions. The retinas were examined histologically to determine the effectiveness of the TES in protecting the retinal neurons.

2. Materials and methods

2.1. 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 Graduate School of Medicine. The animals were anesthetized with intraperitoneal pentobarbital (50 mg/kg body weight) for all surgical procedures.

2.2. Retrograde labeling of RGCs

To identify the RGCs from other retinal cells, the RGCs were retrogradely labeled with Fluorogold (FG; Fluorochrome Inc., Englewood, CO), a fluorescent tracer, before the ON transection. The anesthetized animals were held on a surgical frame with a nose clamp. Craniotomy was performed on the posterior parietal bones to expose the occipital cortex. The occipital cortex was carefully aspirated to expose the dorsal surface of the bilateral superior colliculi (SC), avoiding damage to the superior sagittal sinus and SC. A small sponge soaked in 2% FG (in 0.9% NaCl containing 10% dimethyl sulfoxide) was placed on the surface of both superior colliculi (Morimoto et al., 2002, 2005; Okazaki et al., 2008).

2.3. ON transection

Seven days after the retrograde labeling of the RGCs, the left ON was transected as described in detail elsewhere (Morimoto et al., 2002, 2005; Okazaki et al., 2008). Briefly, a skin incision was made through the left eyelid close to the superior orbital rim, and the incision was retracted to expose the globe. The superior extraocular muscles were spread apart, 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 vessels.

2.4. Transcorneal electrical stimulation

A bipolar contact lens electrode with an inner and outer ring (Kyoto Contact, Kyoto, Japan) was used as the stimulating electrodes. The corneal surface was anesthetized by 0.4% oxybuprocaine HCl in addition to systemic anesthesia, and the contact lens electrode was placed on the cornea of the eye with the transected ON. Hydroxyethylcellulose gel (1.3%) was used to protect the cornea and for making electrical contact with the cornea.

2.5. Stimulation parameters

TES was delivered with anodic first (cornea positive) biphasic square pulses from a constant current stimulator (SEN-7203; Nihonkoden, Tokyo, Japan; Isolator, A395R; World Precision Instruments, Sarasota, FL) (Morimoto et al., 2005, 2007). The stimulus parameters were: pulse durations of 0.5, 1, 2, 3, and 5 ms/phase with 100 μA, 20 Hz, and for 60 min; current intensities of 50, 100, 200, 300 and 500 μA with 1 ms/phase, 20 Hz and for 60 min; frequencies of 0.5, 1, 5, 20, 50, and 100 Hz at 100 μA, 1 ms/phase, and for 60 min; stimulation duration of 15, 30, and 60 min at 100 μA, 1 ms/phase, and 20 Hz (Table 1).

The waveform of the TES was also changed from symmetrical, asymmetrical, and symmetrical with an inter-pulse interval of 0.5 ms or 1 ms at 100 μA, 1 ms/phase, 20 Hz, and for 60 min. In addition the effect of repeated sessions of TES of 100 μA, 1 ms/phase, 20 Hz, for 60 min on days 0, 4, 7, and 10 after the ON transection was compared with a single session of TES with the same parameters of stimulation was investigated 14 days after ON transection.

Table 1
Stimulation parameters tested in this study.

Experiment	Current intensity (μA)	Pulse duration (ms/phase)	Frequency (Hz)	Stimulation duration [min]
Pulse duration	100	0.5–5	20	60
Current intensity	50–500	1	20	60
Frequency	100	1	1–100	60
Stimulation duration	100	1	20	15–60

2.6. Quantification of RGC density

Seven or 14 days after the ON transection and TES, the animals were deeply anesthetized and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). Both eyes were enucleated and small incisions were made at the dorsal pole of the eyes for retinal orientation. The eyes were stored in 4% PFA in 0.1 M PB overnight at 4 °C. The retinas were dissected from the eyes, and four radial cuts were made to flatten the retinas on a glass microscope slide.

To calculate the density of the surviving RGCs, the number of FG-labeled RGCs was counted using a fluorescence microscope (Axioskop, Carl Zeiss, Oberkochen, Germany) with UV excitation at 365 nm. The RGCs in three areas of 0.5 × 0.5 mm^2 along the naso-temporal and dorso-ventral axes (nasal, temporal, dorsal and ventral) at 1, 2 and 3 mm from the optic disc were counted using a microscanner (Sapporo Beer, Saitama, Japan). The mean density of RGCs was calculated from the number of surviving RGCs counted in the 12 areas of every retina (Morimoto et al., 2002, 2005; Okazaki et al., 2008).

2.7. Statistical analyses

Data are presented as the mean ± standard deviations (SDs) and the data were statistically analyzed using a commercial software (SigmaStat, version 3.11; Systat Software, Inc). Comparisons among the groups were made by one-way analysis of variance (ANOVA), when the equal variance test passed, and was followed by the Tukey test or by Kruskal–Wallis one-way ANOVA on ranks, when the equal variance test failed, followed by the Dunn's method. Comparisons between two groups were made by Student's t tests. The level of statistical significance was set as $P < 0.05$.

3. Results

3.1. Effect of pulse duration on neuroprotection of axotomized RGCs

The somas of the RGCs in the intact control retinas were round with the fine dots of FG in the perinuclear cytoplasm and proximal dendrites (Fig. 1A). The mean RGC density in the intact control retinas was 2357 ± 150 cells/mm^2 (mean ± SD; $n = 12$; Fig. 2A). Seven days after the ON transection and without TES, the number of FG-labeled RGCs were markedly reduced to 1290 ± 96 cells/mm^2 ($n = 8$) which is 54.7% of that of intact retinas. The RGCs were irregularly shaped and debris of dead RGCs were present (Fig. 1B). The mean RGC density in the retinas with sham electrical stimulation 7 days after ON transection, was 1221 ± 176 cells/mm^2 ($n = 6$) which was 51.8% of intact retinas. This density was not significantly different from that in the retinas with ON transection without TES, and was used as the control value to evaluate the neuroprotective effect of TES.

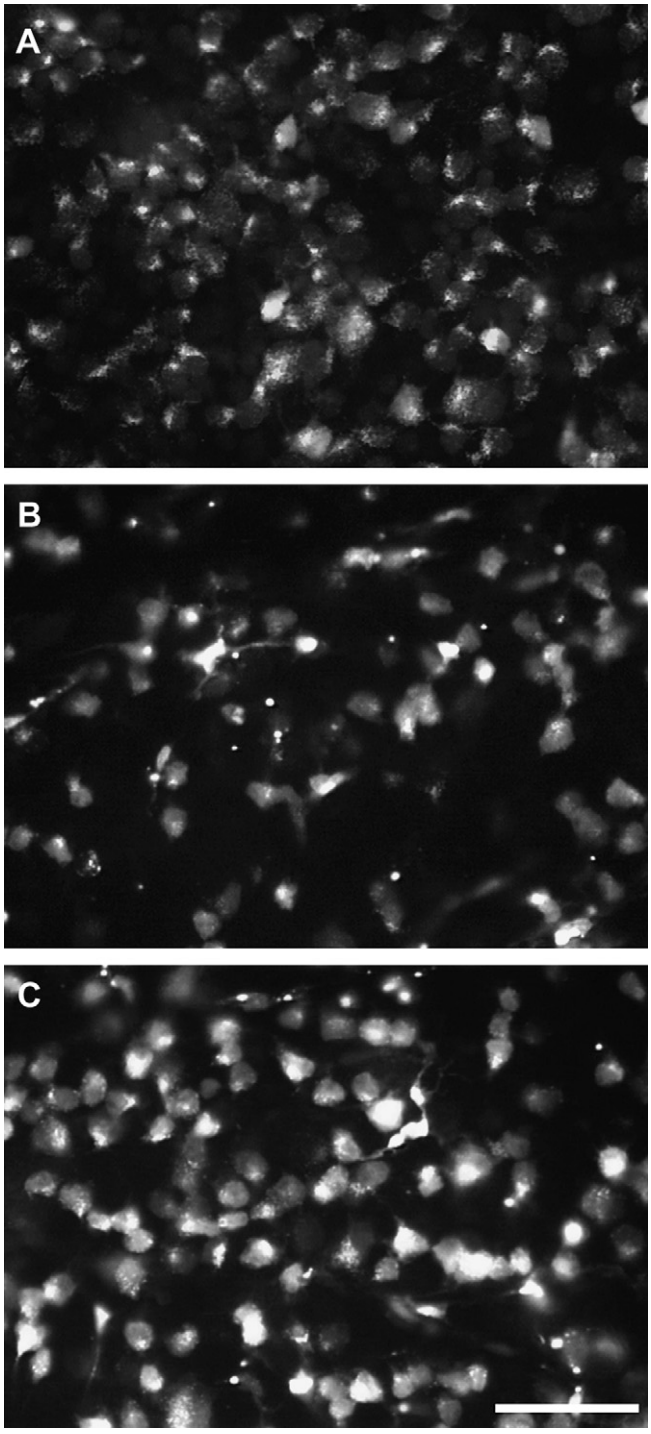

Fig. 1. Representative photographs of Fluorogold (FG)-labeled RGCs in flat-mounted retinas: intact (A), 7 days after ON transection without TES (B), ON transection with TES (C). All images were obtained from an approximately 1 mm temporal to the optic disk in each retina. A: In an intact retina, FG-labeled RGCs can be seen to have round-shaped soma with punctuate fluorescence of FG in their cytoplasm. B: In the retina 7 days after ON transection, the number of FG-labeled RGCs is markedly lower than that of RGCs in the intact retina, and debris of dead cells can be seen. C: In the retina 7 days after ON transection with TES (100 μA, 1 ms/phase, 20 Hz, 60 min), the number of surviving RGCs is strongly enhanced by TES, and many FG-labeled RGCs appeared to be similar to those in intact retinas. Scale bar = 100 μm.

Our overall results showed that TES promoted the survival of axotomized RGCs and the degree of neuroprotection depended on the pulse durations. Examination of the retinas following ON transection and TES showed many RGCs, whose shapes resembled those of the RGCs in intact retinas, had survived (Fig. 1C). The mean density of RGCs following TES of 0.5 ms/phase duration was significantly increased to 1639 ± 215 cells/mm^2 which was 69.5% of intact retinas ($P < 0.001$ vs sham stimulation; $n = 6$; Fig. 2A). TES of 1, 2 and 3 ms/phase pulse durations further increased the density up to 85.4%, 85.3% and 81.6%, respectively, of the intact retinas ($P < 0.001$; $n = 6$ each; Fig. 2A). Although TES of 5 ms/phase duration significantly increased the density of RGCs to 72.3% ($n = 6$) of that in sham stimulation, the neuroprotective effect was significantly lower than the maximum effect of 1 ms/phase ($P = 0.002$; $n = 6$; Fig. 2A).

3.2. Effect of current intensity on neuroprotection of axotomized RGCs

The mean RGC density in the retinas following TES at a current intensity of 50 μA was 1624 ± 55 cells/mm^2 ($n = 6$) which is 68.9% of intact retinas. This density was not significantly different from that in the sham stimulated retinas (Fig. 2B). However when the TES was increased to 100 μA and 200 μA, there was a significant increase in the density to 85.4% and 80.0%, respectively, of intact retinas ($P < 0.05$; $n = 6$ each). An increase of TES to 300 μA and 500 μA resulted in a decrease in the mean RGC densities to 70.0% and 64.5%, respectively, of intact retinas. These values were not significantly different from that of the sham stimulated eyes (Fig. 2B).

3.3. Effect of electric charge (intensity × duration) on neuroprotection of axotomized RGCs

The magnitude of the electric charge/phase, i.e., current intensity × pulse duration in coulombs has been identified as one of the factors that determine the effectiveness of ES in being neuroprotective in neural tissues. We compared the difference in the neuroprotective ability between increases of pulse duration to increases of current intensity. An increase in the current intensity decreased the number of RGCs more than an increase of pulse duration (Fig. 2C). There were significant differences in the number of RGCs between the survival effect with increases of current intensity and increases of pulse duration at more than 300 μC/phase ($^*P = 0.006$, $^{**}P = 0.002$; Fig. 2C).

3.4. Effect of frequency of stimulation on neuroprotection of axotomized RGCs

The mean RGC densities seven days after ON transection with sham stimulation and TES at 5 different frequencies are shown in Fig. 3. At 20 Hz, the mean RGC density was 85.0% of intact retinas which was significantly higher than that in the sham stimulated retinas ($P < 0.001$; $n = 6$). At 50 and 100 Hz, the mean RGC densities decreased to 73.1% and 68.5% respectively ($n = 6$ each), but these values were also significantly higher than that of sham stimulation. At 100 Hz the percentage of RGCs surviving after TES was significantly lower than that after 20 Hz TES ($P = 0.004$). Thus the optimal TES frequency was 20 Hz. The survival rates after TES of 5 Hz and 1 Hz were not significantly different from that at 20 Hz, 80.0% and 84.3%, respectively, of intact retinas ($n = 6$ each, $P < 0.001$ vs Sham stimulation). On the other hand, TES at 0.5 Hz did not have a significant protection, 60.8% (Fig. 3; $n = 6$).

3.5. Effect of waveform on neuroprotection of axotomized RGCs

The neuroprotective effects of the three types of waveforms with equal charge-balance were different (Fig. 4A). TES with Type II, symmetric waveforms led to a significantly increased survival of 85% compared to the sham stimulation, while asymmetrical

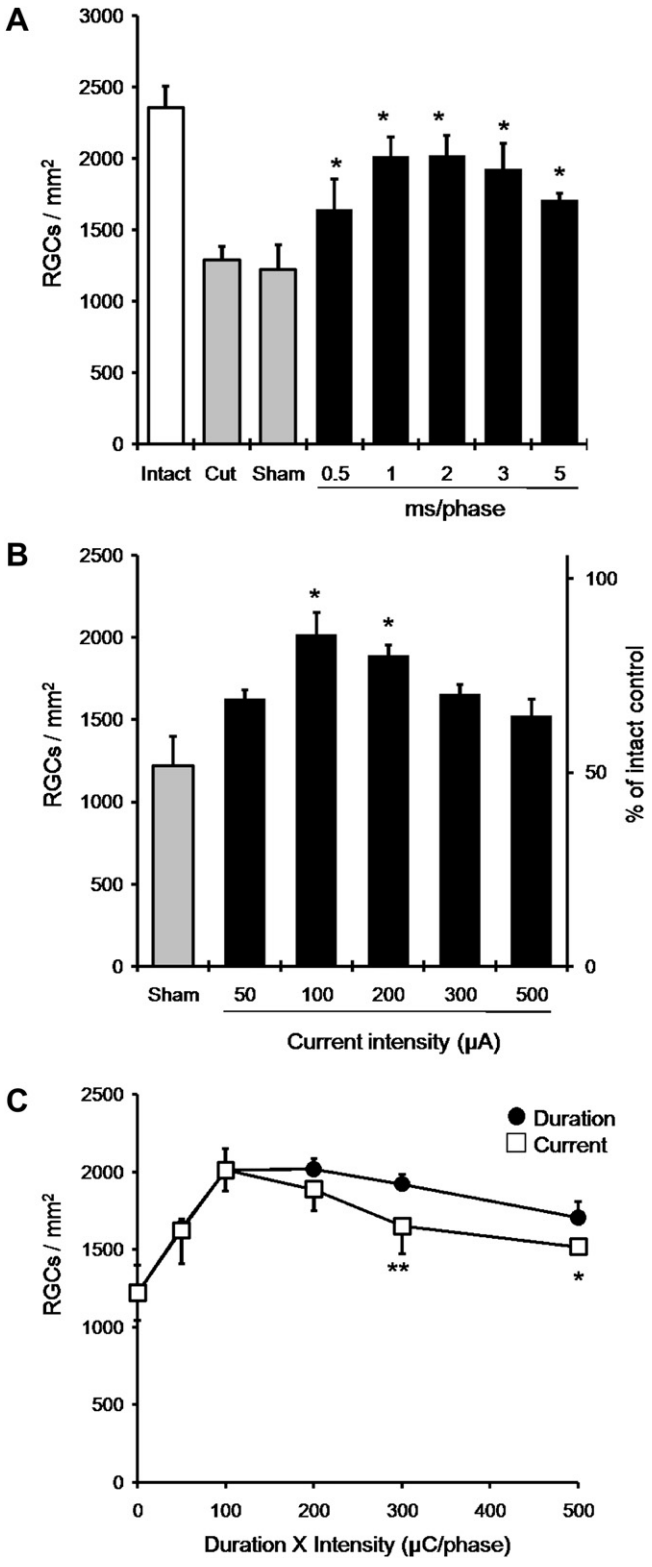

Fig. 2. Effects of pulse duration and current intensity of TES on the survival of axotomized RGCs 7 days after ON transection. A: Pulse duration-dependent neuroprotective effect of TES on RGC survival. TES (current intensity: 100 μA; frequency: 20 Hz) for 1 h was applied immediately after the ON was transected. The density of the FG-labeled RGCs/mm^2 is presented as the means ± SDs. Seven days after ON transection, the density of the RGCs is reduced 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 five groups with TES (0.5, 1, 2, 3, and 5 ms/phase pulse duration) was significantly higher than in the sham group. Statistical analysis was made by one-way ANOVA followed by Tukey test ($P < 0.001$, $^*P < 0.001$ compared with sham). B: Current intensity-dependent neuroprotective effect of TES on RGC survival 7 days after ON transection. TES (pulse duration: 1 ms/phase; frequency: 20 Hz) for 1 h was applied immediately after ON transection. TES at 100 μA and 200 μA significantly increased the mean RGC densities compared with sham stimulation. Statistical analysis was made by Kruskal–Wallis One-Way Analysis of Variance on Ranks followed by Dunn's method ($P < 0.001$, $^*P < 0.05$ compared with sham). C. Comparison of neuroprotective effect of TES with pulse duration change and that with current intensity change 7 days after ON transection. The survival effect by current intensity significantly decreases as compared with that by pulse duration (more than 300 μC/phase) (t test, $^{**}P = 0.002$, $^*P = 0.006$).

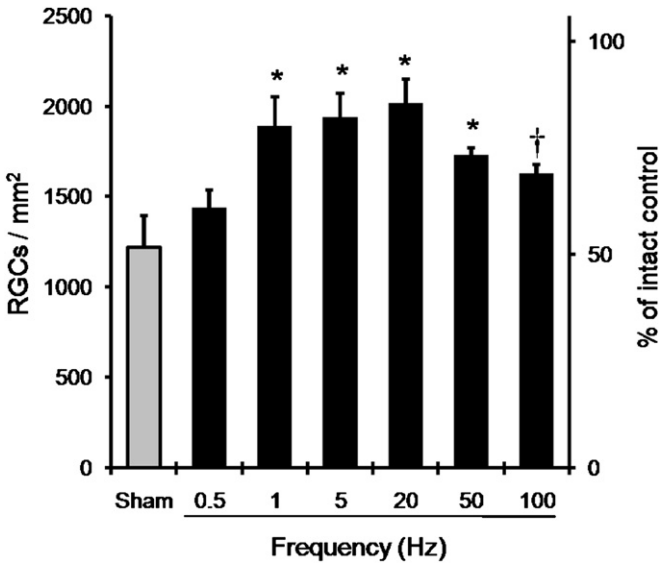

Fig. 3. Frequency-dependent neuroprotective effect of TES on axotomized RGCs 7 days after ON transection. TES (current intensity 100 μA; pulse duration 1 ms/phase) for 1 h was applied immediately after ON transection. TES at 1–20 Hz exerted the most significant effect on RGC survival; the mean densities of RGCs at 1–20 Hz exerted the most significant effect on RGC survival; the mean densities of RGCs at 1–20 Hz was significantly higher than the sham stimulation group ($P < 0.005$). Statistical analysis between the sham stimulation and ES groups was performed using one-way ANOVA followed by Tukey test ($P < 0.01$, $^*P < 0.005$, †$P < 0.001$ compared with sham).

waveforms of Type I led to a survival of 68.7% and Type III to 68.3% of intact retinas ($P < 0.001$; $n = 6$ each; Fig. 4B).

Next we examined the effect of the addition of inter-pulse interval to symmetrical pulses on the survival-promoting effect on RGCs (Fig. 4C). As the inter-pulse interval was increased, the survival rate of RGCs significantly decreased from 85% to 62% ($P < 0.001$; $n = 6$ each; Fig. 4D).

3.6. Stimulation duration on neuroprotection of axotomized RGCs

TES for 30 min significantly increased the survival of RGCs to 1802 ± 111 cells/mm^2 ($n = 6$) which is 76.8% of intact retinas ($P < 0.001$ vs sham stimulation). On the other hand, after TES for 10 min, the mean RGC density was 1398 ± 124 cells/mm^2 ($n = 6$, 59.3% of intact retinas), which was not significantly different than that of the sham stimulated retinas (Fig. 5).

3.7. Effect of repeated TES on neuroprotection of axotomized RGCs

The mean RGC density in retinas 14 days after ON transection with sham stimulation was 350 ± 216 cells/mm^2 ($n = 6$), which was 13.9% of intact retinas ($n = 6$; Fig. 6A,D). On the other hand, a single TES session (1× TES) significantly enhanced the survival of RGCs to 22.9% of intact retinas ($P < 0.001$ vs sham; $n = 6$; Fig. 6B,D). Four TES sessions (4× TES on days 0, 4, 7 and 10 after ON transection) further increased the number of RGCs to 47.1% of intact retinas ($P = 0.024$ vs 1× TES; $n = 6$; Fig. 6C,D).

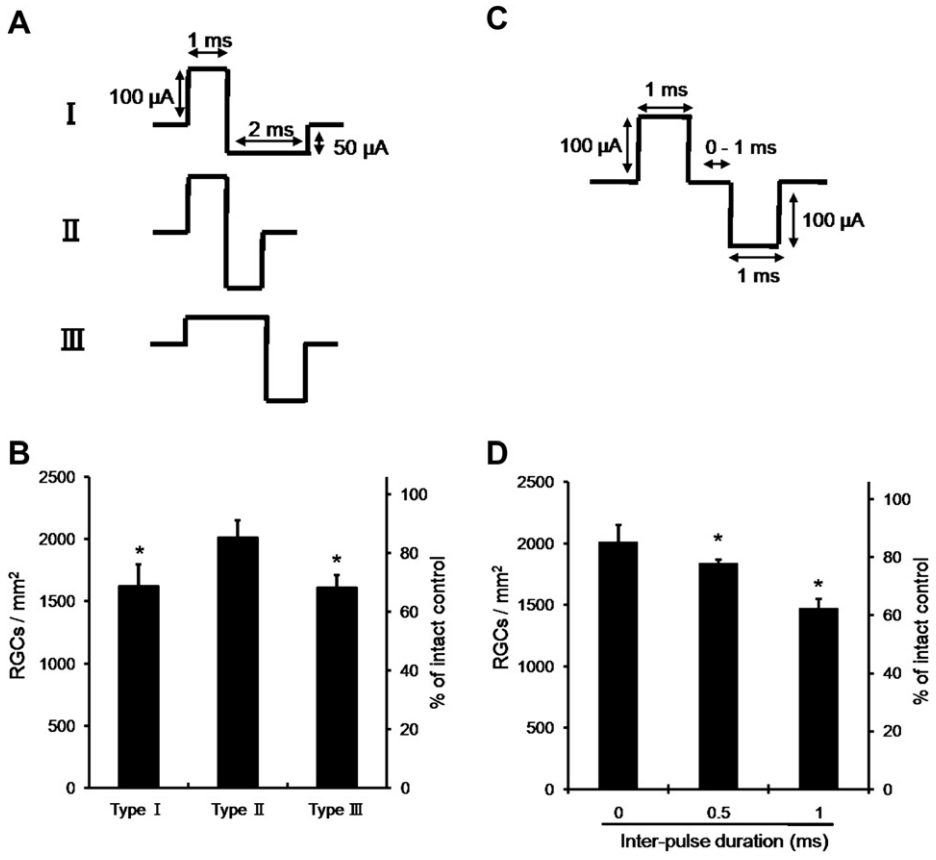

Fig. 4. Effects of waveforms of TES on the survival of axotomized RGCs 7 days after ON transection. A: Waveforms of the biphasic stimuli used in this study. Type I: Asymmetric rectangular pulse waveform (anodic first wave 100 μA, 1 ms/phase and cathodic second wave 50 μA, 2 ms/phase), Type II: Symmetric rectangular pulse waveform (100 μA, 1 ms/phase), Type III: Asymmetric rectangular pulse waveform (anodic first wave 50 μA, 2 ms/phase and cathodic second wave 100 μA, 1 ms/phase). All waves were charge-balanced. B: Neuroprotective effect of each waveform on axotomized RGCs 7 days after ON transection. TES with symmetric waveform (Type II) significantly increased the survival of RGCs more than asymmetric waveforms (Type I and Type III). Statistical analysis was performed using one-way ANOVA followed by Tukey test ($P < 0.001$, $^*P < 0.001$ compared with Type II). C: Waveform of a symmetric waveform with inter-pulse interval. D: Effect of inter-pulse interval on axotomized RGCs. The neuroprotective effect of TES on RGCs significantly decreased depending on the length of inter-pulse interval. Statistical analysis was performed using one-way ANOVA followed by Tukey test ($P < 0.001$, $^*P < 0.001$ compared with Type II without inter-pulse interval).

4. Discussion

Our results showed that the neuroprotective effect of TES on axotomized RGCs was dependent on the pulse duration, current intensity, frequency, stimulation duration, waveform, and the number of stimulation sessions.

4.1. Current charge (pulse duration × intensity)

The neuroprotective effect of TES was dependent on both the pulse duration and current intensity. There was a range of optimal pulse durations and current intensities, and an increase of the pulse duration or current intensity over the optimal range decreased the survival-promoting effect. On the other hand, the density of the electric charge, which is the production of current intensity and pulse duration, determined the extent of neural tissue damage (Yuen et al., 1981; McCreery et al., 1990; Harnack et al., 2004; Nakauchi et al., 2007). We found that there was a significant difference in neuroprotective effect of pulse duration and current intensity, on the basis of the same amount of electric charges (more than 300 μC/phase). In a retinal prosthesis study using supra-choroidal–transretinal stimulation (STS), the threshold of electric charge for the retinal safety was lower with shorter pulse durations even with the same amount of electric charge (Nakauchi et al., 2007). This is similar to our present results. Because the electric charge rate within an unit time became larger with an increase of

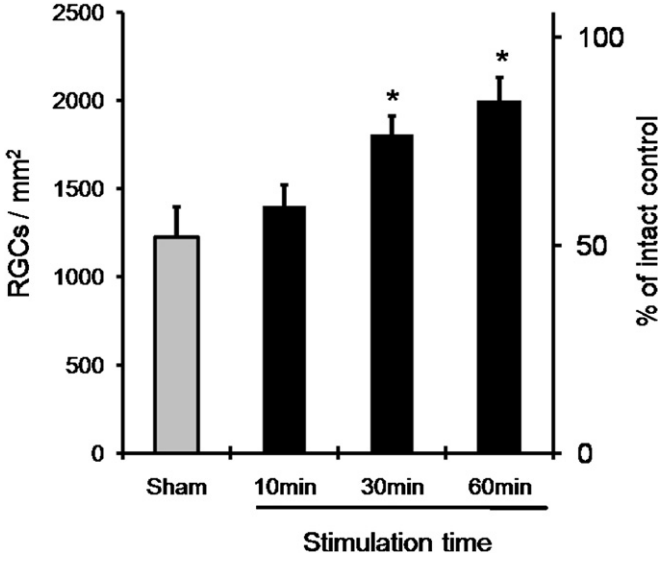

Fig. 5. Effect of stimulation duration on neuroprotection of axotomized RGCs. TES (current intensity: 100 μA; pulse duration: 1 ms/phase; frequency: 20 Hz) was applied immediately after ON transection. In the 10 min ES group, the mean density of RGCs was not significantly different from that in the sham group. In the 30 and 60 min groups, the mean density was significantly higher than that in the sham group ($P < 0.001$; each $n = 6$). Statistical analysis among the groups was performed using one-way ANOVA ($P < 0.001$) followed by Tukey test ($^*P < 0.001$ compared with the sham group).

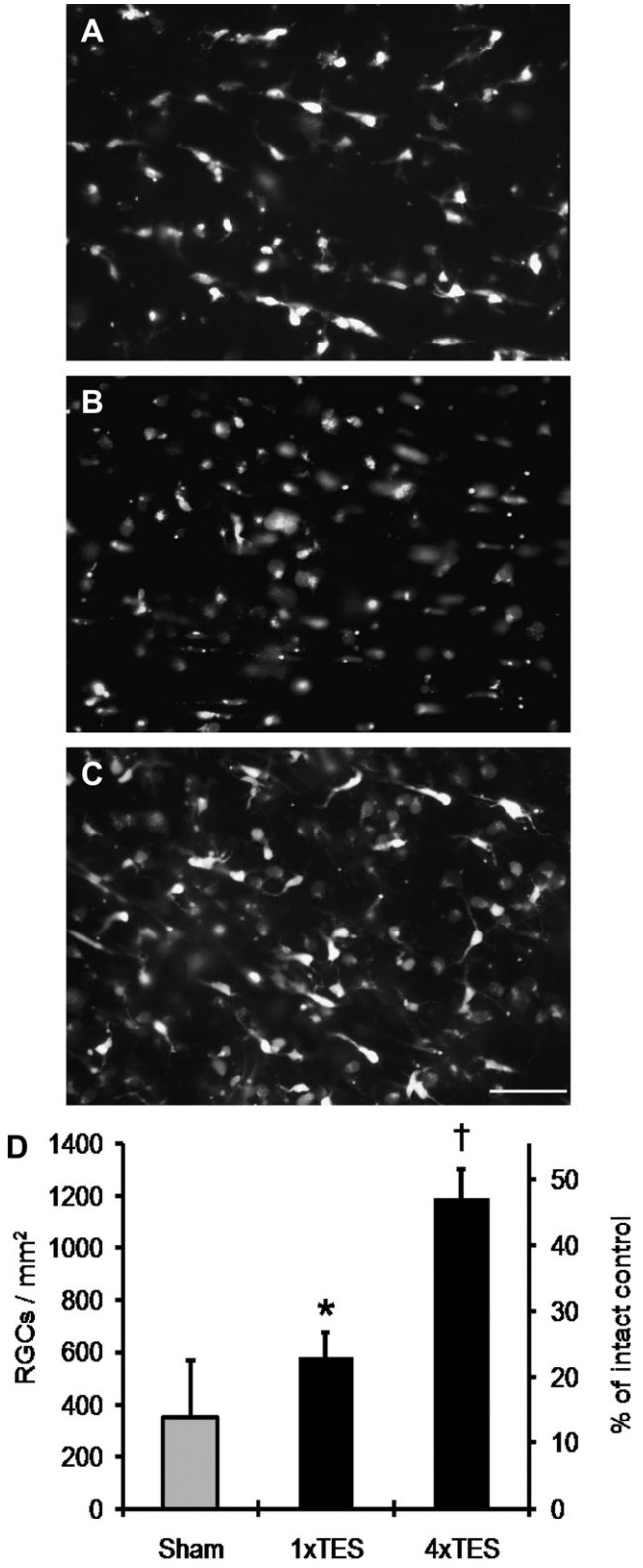

Fig. 6. Effect of repeated TES on the survival of RGCs 14 days after ON transection. Fluorescence photomicrographs of retinas 14 days after ON transection with sham TES (A), with single TES (B) and 4 times TES (C). Only a few FG-labeled RGCs are present 14 days after ON transection with sham stimulation (A). On the other hand single TES increased the viable RGCs (B), and 4× TES further increased the viable RGCs than 1× TES (C), Scale bar = 100 μm. Mean density of RGCs 14 days after ON transection with sham TES was much lower than that in the retina 7 days after ON transection. Although the mean density of RGCs 14 days after ON transection with 1× TES was also lower than that of RGCs 7 days after ON transection with TES, the density remained significantly higher than that with sham TES 14 days after ON transection ($P = 0.02$). Moreover 4× TES significantly increased the surviving RGCs more than single TES 14 days after ON transection ($P < 0.001$). One-way ANOVA: $P < 0.001$, followed by Tukey test: $^{*}P = 0.02$, $^{\dagger}P < 0.001$ comparing with sham group (D).

current intensity than with an increase of pulse duration, the increase of electric current may cause greater tissue damage even if the same amount of electric charge/phase is given. Therefore the neuroprotective effect may be lower with an increase of current intensity than that of pulse duration.

4.2. Frequency-dependent effect of TES on RGC survival

TES of 1–20 Hz frequency had the greatest neuroprotection on RGCs. On the other hand, when the ON stump is stimulated thus stimulating the axons of the RGCs, 20 Hz of ES was the most optimal frequency (Okazaki et al., 2008). It is possible that TES stimulates not only RGCs but also other retinal cells such as the Müller cells to exert its neuroprotective effects. In fact, our earlier study showed that 20 Hz TES increased the expression of IGF-1 in Müller cells *in vivo* (Morimoto et al., 2005) and direct 20 Hz ES to the cultured Müller cells also increased the expression of insulin-like growth factor-1 (IGF-1), brain derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF2) in these cells (Sato et al., 2008a,b,c). Therefore TES might have the wider range of optimal frequencies than the direct stimulation of the ON stump.

In other neural tissues, different frequencies were reported to be optimal. For example, after crushing the spinal cord *in vivo*, ES at 20 Hz also promoted axonal regeneration of the motoneurons (Al-Majed et al., 2000a,b). On the other hand, for cochlear implants high frequency (300 pps) of ES promoted a greater survival of ototoxic spiral ganglion cells (SGCs) than low frequency (30 pps) of ES *in vivo* (Leake et al., 2008). *In vitro* study, not low frequency (20 Hz) but high frequency (50 or 100 Hz) of ES promoted the secretion of BDNF from hippocampal neurons or primary sensory neurons (Balkowiec and Katz, 2000, 2002). ES of dorsal root ganglion (DRG) axons at 20 Hz promoted greater axonal regeneration than 200 Hz (Udina et al., 2008). Thus the optimal frequency of ES to have neuroprotective effects differs for different types of neurons and nervous systems. Because neurons and glial cells have various voltage-sensitive ion channels for each cells, the activation of ion channels may depend on the frequency of ES. For example, Müller cells have voltage-sensitive L-type Ca channel (Xu et al., 2002). We have demonstrated that 20 Hz ES activates voltage-sensitive L-type Ca channels to increase the expression of the mRNAs of IGF-1 and BDNF in cultured Müller cells (Sato et al., 2008a,b). In hippocampal neurons, 100 Hz ES activates the N-type Ca channels to make hippocampal neurons release BDNF (Brosenitsch and Katz, 2001). Another possibility is that high frequency of ES induces greater tissue damage. In the cat's sciatic nerve, ES at 100 Hz and 50 Hz cause the severe neural damage, although ES at 20 Hz induces little or no neural damage (McCreery et al., 1995). Thus it is important to select the suitable frequency of ES applied for each type of neuron.

4.3. Effect of waveforms on the TES-induced neuroprotective effect

TES with symmetric pulse waves increased the number of surviving RGCs more than TES with asymmetric pulse waves, although both pulse waves were charge-balanced. Moreover, longer inter-pulse intervals resulted in less surviving RGCs than TES with no inter-pulse intervals. It is possible that the electric charge might not be balanced well by asymmetrical pulses to cause the tissue damage. Or it is possible that the increase in the inter-pulse interval than the safety limit, might cause the tissue damage. In cochlear

implants, the threshold of auditory nerve response evoked by ES with asymmetric pulse waves is lower than that by ES with symmetric pulse waves and the threshold of auditory nerve response evoked by long inter-pulse intervals is lower than that by the ES without inter-pulse intervals (Macherey et al., 2006). The effects of asymmetrical or symmetrical pulses with long inter-pulse intervals were similar to monophasic pulses (Macherey et al., 2006). These pulses might lead to neural damage with the lower electric charge than the symmetric pulses.

4.4. Stimulation time-dependent effect of TES on RGC survival

TES for 10 min immediately after ON transection did not increase the number of surviving RGCs, but that for 30 min did. This result is similar to that following ES of the transected ON stump (Okazaki et al., 2008). These results indicate that ES to RGCs may influence the intrinsic survival signal or the death signal. It may take more than 30 min of intervention to obtain some survival signals. Further investigation is needed to determine what kind of signals are being induced.

4.5. Single TES vs repeated TES for long-lasting neuroprotection

Fourteen days after ON transection, the densities of surviving RGCs in the retinas with single TES (1× TES) were still higher than in the control retinas without TES. And repetitive TES (4× TES) increased the number of surviving RGCs more than 1× TES. These results indicate that repeated TES has cumulative neurotrophic effects on the long term survival of RGCs. In fact we have demonstrated that TES up-regulates the expression of the mRNA and protein of IGF-1 to rescue axotomized RGCs (Morimoto et al., 2005). Our results suggest that IGF-1 induced by TES might be cumulative and have neuroprotective effects of RGCs continuously for 2 weeks. The mechanism of repetitive TES on the longer term survival of RGCs should be investigated in the future.

5. Conclusions

We performed a systematic analysis of the neuroprotective effect of different stimulus parameters of TES on axotomized RGCs. We concluded that the optimal parameter of TES on the neuroprotection of RGCs are: current intensity of 100–200 μA, pulse duration of 1–3 ms/phase, frequency of 1–20 Hz, stimulation duration of 30–60 min, symmetrical pulse waves without inter-pulse intervals, and repeated stimulations. It is important that stimulation with these optimal parameters with low electric power, is applied for the overall period to maintain long term survival of RGCs. These findings should serve as guideline for ES in humans.

Acknowledgments

We thank Professor Yasuo Tano for helpful advice and various supports.

This work was supported by a Health Sciences Research Grant (H19-sensory-001) from the Ministry of Health, Labour and Welfare, Japan.

References

Al-Majed, A.A., Neumann, C.M., Brushart, T.M., Gordon, T., 2000a. Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration. J. Neurosci. 20, 2602–2608.

Al-Majed, A.A., Brushart, T.M., Gordon, T., 2000b. Electrical stimulation accelerates and increases expression of BDNF and trkB mRNA in regenerating rat femoral motoneurons. Eur. J. Neurosci. 12, 4381–4390.

Balkowiec, A., Katz, D.M., 2000. Activity-dependent release of endogenous brain-derived neurotrophic factor from primary sensory neurons detected by ELISA in situ. J. Neurosci. 20, 7417–7423.

Balkowiec, A., Katz, D.M., 2002. Cellular mechanisms regulating activity-dependent release of native brain-derived neurotrophic factor from hippocampal neurons. J. Neurosci. 22, 10399–10407.

Brosenitsch, T.A., Katz, D.M., 2001. Physiological patterns of electrical stimulation can induce neuronal gene expression by activating N-type calcium channels. J. Neurosci. 21, 2571–2579.

Fields, R.D., Neale, E.A., Nelson, P.G., 1990. Effects of patterned electrical activity on neurite outgrowth from mouse sensory neurons. J. Neurosci. 10, 2950–2964.

Fujikado, T., Morimoto, T., Matsushita, K., Shimojo, H., Okawa, Y., Tano, Y., 2006. Effect of transcorneal electrical stimulation in patients with nonarteritic ischemic optic neuropathy or traumatic optic neuropathy. Jpn. J. Ophthalmol. 50, 266–273.

Goldberg, J.L., Espinosa, J.S., Xu, Y., Davidson, N., Kovacs, G.T., Barres, B.A., 2002. Retinal ganglion cells do not extend axons by default: promotion by neurotrophic signaling and electrical activity. Neuron 33, 689–702.

Grumbacher-Reinert, S., Nicholls, J., 1992. Influence of substrate on retraction of neurites following electrical activity of leech Retzius cells in culture. J. Exp. Biol. 167, 1–14.

Harnack, D., Winter, C., Meissner, W., Reum, T., Kupsch, A., Morgenstern, R., 2004. The effects of electrode material, charge density and stimulation duration on the safety of high-frequency stimulation of the subthalamic nucleus in rats. J. Neurosci. Methods 138, 207–216.

Inomata, K., Shinoda, K., Ohde, H., Tsunoda, K., Hanazono, G., Kimura, I., Yuzawa, M., Tsubota, K., Miyake, Y., 2007. Transcorneal electrical stimulation of retina to treat longstanding retinal artery occlusion. Graefes. Arch. Clin. Exp. Ophthalmol. 245, 1773–1780.

Leake, P.A., Stakhovskaya, O., Hradek, G.T., Hetherington, A.M., 2008. Factors influencing neurotrophic effects of electrical stimulation in the deafened developing auditory system. Hear. Res. 242, 86–99.

Linden, R., 1994. The survival of developing neurons: a review of afferent control. Neuroscience 58, 671–682.

Macherey, O., van, Wieringen, A., Carlyon, R.P., Deeks, J.M., Wouters, J., 2006. Asymmetric pulses in cochlear implants: effects of pulse shape, polarity, and rate. J. Assoc. Res. Otolaryngol. 7, 253–266.

McCreery, D.B., Agnew, W.F., Yuen, T.G., Bullara, L., 1990. Charge density and charge per phase as cofactors in neural injury induced by electrical stimulation. IEEE Trans. Biomed. Eng. 37, 996–1001.

McCreery, D.B., Agnew, W.F., Yuen, T.G., Bullara, L., 1995. Relationship between stimulus amplitude, stimulus frequency and neural damage during electrical stimulation of sciatic nerve of cat. Med. Biol. Eng. Comput. 33, 426–429.

Mennerick, S., Zorumski, C.F., 2000. Neural activity and survival in the developing nervous system. Mol. Neurobiol. 22, 41–54.

Meyer-Franke, A., Wilkinson, G.A., Kruttgen, A., Hu, M., Munro, E., Hanson Jr., M.G., Reichardt, L.F., Barres, B.A., 1998. Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. Neuron 21, 681–693.

Miyake, K., Yoshida, M., Inoue, Y., Hata, Y., 2007. Neuroprotective effect of transcorneal electrical stimulation on the acute phase of optic nerve injury. Invest. Ophthalmol. Vis. Sci. 48, 2356–2361.

Morimoto, T., Miyoshi, T., Fujikado, T., Tano, Y., Fukuda, Y., 2002. Electrical stimulation enhances the survival of axotomized retinal ganglion cells in vivo. Neuroreport 13, 227–230.

Morimoto, T., Miyoshi, T., Matsuda, S., Tano, Y., Fujikado, T., Fukuda, Y., 2005. Transcorneal electrical stimulation rescues axotomized retinal ganglion cells by activating endogenous retinal IGF-1 system. Invest. Ophthalmol. Vis. Sci. 46, 2147–2155.

Morimoto, T., Fujikado, T., Choi, J.S., Kanda, H., Miyoshi, T., Fukuda, Y., Tano, Y., 2007. Transcorneal electrical stimulation promotes the survival of photoreceptors and preserves retinal function in royal college of surgeons rats. Invest. Ophthalmol. Vis. Sci. 48, 4725–4732.

Nakauchi, K., Fujikado, T., Kanda, H., Kusaka, S., Ozawa, M., Sakaguchi, H., Ikuno, Y., Kamei, M., Tano, Y., 2007. Threshold suprachoroidal–transretinal stimulation current resulting in retinal damage in rabbits. J. Neural Eng. 4, S50–S57.

Okazaki, Y., Morimoto, T., Sawai, H., 2008. Parameters of optic nerve electrical stimulation affecting neuroprotection of axotomized retinal ganglion cells in adult rats. Neurosci. Res. 61, 129–135.

Sato, T., Fujikado, T., Lee, T.S., Tano, Y., 2008a. Direct effect of electrical stimulation on induction of brain-derived neurotrophic factor from cultured retinal Müller cells. Invest. Ophthalmol. Vis. Sci. 49, 4641–4646.

Sato, T., Fujikado, T., Morimoto, T., Matsushita, K., Harada, T., Tano, Y., 2008b. Effect of electrical stimulation on IGF-1 transcription by L-type calcium channels in cultured retinal Müller cells. Jpn. J. Ophthalmol. 52, 217–223.

Sato, T., Lee, T.S., Takamatsu, F., Fujikado, T., 2008c. Induction of fibroblast growth factor-2 by electrical stimulation in cultured retinal Mueller cells. Neuroreport 19, 1617–1621.

Shen, S., Wiemelt, A.P., McMorris, F.A., Barres, B.A., 1999. Retinal ganglion cells lose trophic responsiveness after axotomy. Neuron 23, 285–295.

Udina, E., Furey, M., Busch, S., Silver, J., Gordon, T., Fouad, K., 2008. Electrical stimulation of intact peripheral sensory axons in rats promotes outgrowth of their central projections. Exp. Neurol. 210, 238–247.

Xu, H.P., Zhao, J.W., Yang, X.L., 2002. Expression of voltage-dependent calcium channel subunits in the rat retina. Neurosci. Lett. 329, 297–300.

Yuen, T.G., Agnew, W.F., Bullara, L.A., Jacques, S., McCreery, D.B., 1981. Histological evaluation of neural damage from electrical stimulation: considerations for the selection of parameters for clinical application. Neurosurgery 9, 292–299.