Transcorneal Electrical Stimulation Promotes the Survival of Photoreceptors and Preserves Retinal Function in Royal College of Surgeons Rats

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Purpose. To determine whether transcorneal electrical stimulation (TES) has neuroprotective effects on photoreceptors and preserves retinal function in Royal College of Surgeons (RCS) rats.

Methods. Three-week-old RCS rats received TES through a contact lens electrode on the left eye weekly for 2 to 6 weeks. The right eyes received sham stimulation on the same days. Electroretinograms (ERGs) were recorded from the rats at 3 weeks (before TES), and at 5, 7, and 9 weeks of age. After the ERG recordings, the rats were killed for morphologic analyses of the retina.

Results. Morphologic analyses showed that the mean thickness of the outer nuclear layer (ONL) at each time point was significantly thicker in eyes treated with TES of 100 μA than in eyes with sham stimulation (TES 100 μA versus sham: 5, 7, and 9 weeks of age; $P < 0.001$). ERG studies showed that TES also significantly preserved retinal function up to 7 weeks of age, but did not preserve retinal function at 9 weeks of age.

Conclusions. TES prolongs the survival of photoreceptors and delays the decrease of retinal function in RCS rats. Although further investigations are necessary before using TES on patients, these findings indicate that TES may be a therapeutic treatment for some patients with diseases of the photoreceptors such as retinitis pigmentosa. (*Invest Ophthalmol Vis Sci.* 2007;48:4725-4732) DOI:10.1167/iovs.06-1404

Electrical activity is essential for both the development and survival of neurons. For example, depolarization of neurons exerts some trophic influence on their development,[1,2] and depolarization by high KCl concentrations inhibits the death of mature retinal ganglion cells (RGCs) in culture.[3-5] In the auditory system, chronic electrical stimulation promoted the survival of spiral ganglion cells which otherwise would have degenerated from the administration of an ototoxic drug in vivo.[6-8] In motor neurons, electrical stimulation activated the cell body and accelerated axonal regeneration and increased the expression of the mRNAs of brain-derived neurotrophic factor (BDNF) and trkB.[9,10]

In the visual system, we have demonstrated that direct electrical stimulation of the transected optic nerve (ON) stump promotes the survival of axotomized RGCs in adult Wistar rats.[11] In addition, we have demonstrated that transcorneal electrical stimulation (TES), which is less invasive than electrical stimulation of the transected ON stump, also promotes the survival of axotomized RGCs in vivo.[12] We concluded that electrical stimulation may have a neuroprotective effect on injured RGCs in patients with ON diseases, such as optic neuropathy. In fact, we have applied TES on patients with traumatic optic neuropathy (TON) or with nonarteritic ischemic optic neuropathy (NAION) and have found an improvement of visual function.[13]

These findings led us to hypothesize that TES would also have a neuroprotective effect on photoreceptors in eyes with photoreceptor degeneration such as in patients with retinitis pigmentosa (RP), one of the leading causes of blindness worldwide. No established treatment is available clinically, although many experimental approaches have been tried to save photoreceptors in various animal models of RP.[14-19]

The purpose of this study was to determine whether TES would have a neuroprotective effect on the photoreceptors and preserve retinal function in RCS rats. RCS rats have been extensively used because they develop photoreceptor degeneration, and they serve as an animal model of RP.[20] In morphologic and electrophysiological analyses in the present study, TES had a neuroprotective effect on the photoreceptors and delayed the decrease of visual function in RCS rats.

Materials and Methods

Animals

All experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the procedures were approved by the Animal Research Committee, Osaka University Medical School. Tan-hooded, pink-eyed RCS rats (*rdy/rdy*) were purchased from Clea Japan Inc. (Tokyo, Japan) and inbred at the animal facilities of Osaka University. They were raised on a 12-hour dark-12-hour light cycle with an ambient light intensity of 100 lux.

Transcorneal Electrical Stimulation

The rats were anesthetized intraperitoneally with pentobarbital sodium (60 mg/kg). Only the left eye was electrically stimulated, as described in detail.[12] For the stimulation, the cornea was anesthetized with a drop of 0.4% oxybuprocaine HCl, and a contact lens electrode with inner and outer circular concentric electrodes (Kyoto Contact Lens, Kyoto, Japan) was placed on the cornea with a drop of 2.5% methylcellulose to maintain good electrical contact and prevent corneal dehydration.

Biphasic rectangular (1-ms/phase duration) current pulses were delivered at a frequency of 20 Hz from an electrical stimulation system

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(Stimulator: SEN-7320, Nihon Kohden, Tokyo, Japan; Isolator: WPI, Sarasota, FL) through the contact lens electrode. The current intensities were 0 (sham stimulation), 50, and 100 μA, and the duration of stimulation was 1 hour.

TES was applied initially on 3-week-old RCS rats (20–23 postnatal days), and was applied once a week for an hour thereafter. The TES-treated RCS rats were divided into three groups. TES was applied between 3 and 5 weeks of age in group 1 ($n = 6$), between 3 and 7 weeks of age in group 2 ($n = 6$), and between 3 and 9 weeks of age in group 3 ($n = 6$). The left eyes received TES (50 or 100 μA), and the right eyes received either sham electrical stimulation or no treatment as the control.

Electroretinography

Electroretinograms (ERGs) were recorded from RCS rats after the end of the TES-treatment (i.e., at 5 weeks of age in group 1, at 7 weeks of age in group 2, and at 9 weeks of age in group 3. For the ERGs, animals were kept in total darkness for at least 12 hours and were prepared for the recordings under dim red light. They were anesthetized intramuscularly with a loading dose of xylazine (13 mg/kg) and ketamine (86 mg/kg). The pupils were dilated with 0.1% atropine, 0.5% tropicamide, and 0.5% phenylephrine HCl. The animals were held steady with a bite bar and nose clamp in a stereotaxic frame. A heating pad maintained the body temperature at approximately 37°C.

ERGs were recorded from both eyes simultaneously with contact lens electrodes with internal and external concentric electrodes embedded in the lens (Kyoto Contact Lens, Kyoto, Japan), and the ground electrode was inserted subcutaneously, near the tail. Responses were amplified 10,000× and bandpass filtered from 0.08 to 1000 Hz with a 60 Hz notch filter. The recordings were digitized at 5 kHz. Ten to 20 responses were averaged with interstimulus intervals from 3 to 30 seconds, depending on the intensity of the stimulus.

ERGs were elicited by white light of 50 ms duration and a maximum luminance of +2.1 log cd/m^2. The luminance was attenuated with neural-density filters in 0.5- or 1.0-log-unit steps. The threshold amplitude was set at 10 μV for the b-wave and 5 μV for the scotopic threshold response (STR). During ERG recordings, the shape and amplitude of each response from the first response to the last response were monitored, and we confirmed that the form and amplitude of the first ERG did not differ significantly from the last ERG.

Histologic Analyses

Immediately after the ERG recording, the rats were killed with an overdose of pentobarbital sodium. The eyes were removed and kept overnight at 4°C in 4% glutaraldehyde in 0.1 M phosphate buffer. Eyes were trimmed and postfixed in 1% osmium for 1 hour. The epoxy-embedded tissue was cut into 1-μm sections and stained with toluidine blue for light microscopy. All sections were cut along the vertical meridian of the eye passing through the ON. Three serial sections of each eye were quantified for each experimental animal.

The degree of retinal degeneration was assessed by measuring the thickness of outer nuclear layer (ONL) and inner nuclear layer (INL). In each of the superior and inferior hemispheres, photographs of the retina were taken at nine defined points with a camera attached to a light microscope (model E800; Nikon, Tokyo, Japan). The first photograph was made at approximately 500 μm from the center of the ON head, and subsequent photographs were taken every 400 μm more peripherally. The thickness of ONL and INL were measured on the photographs (Scion Image analyzer; Scion Corp. Frederick, MD). Three measurements were made at defined points separated from the adjacent photograph by 50 μm. The three measurements were averaged for the value plotted at each point. In this way, the 54 measurements in the two hemispheres were measured which represented the thickness over almost the entire retina. Each eye was coded so that the investigator was masked to treatment of the eye.

Statistical Analyses

Data were analyzed by commercial software (SPSS, ver. 10.0J; SPSS Inc, Chicago, IL). The data are expressed as the mean ± SEM. Comparisons between two groups were made by Student's *t*-test, when the data were normally distributed, or by the Mann-Whitney rank sum test, when the data were not normally distributed. Comparisons among many groups were made by one-way ANOVA followed by the Tukey test. Statistical significance was set at $P < 0.05$.

Results

TES and Survival of Photoreceptors In Vivo

Representative retinal sections from the superior retinas from 7-week-old RCS rats that had 100 μA of TES or had sham stimulation are shown in Figure 1. The number of rows of nuclei in the ONL layer was four or five in the retina receiving TES (Fig. 1A) and two or three in the retina with sham stimulation (Fig. 1B).

Quantitative analyses showed that the ONL in the TES-treated eyes was significantly thicker than in the sham-stimulated and control eyes at 7 weeks of age (Fig. 2A). The mean thickness of ONL in control retinas of 7-week-old RCS rats was 9.8 ± 1.0 μm (mean ± SEM, $n = 6$) which was not significantly different from that in the sham-stimulated eyes at 10.9 ± 0.6 μm ($n = 6$). The mean ONL thickness in the retinas treated with TES at a current intensity of 50 μA was 13.7 ± 0.4 μm ($n = 6$), whereas that with a current intensity of 100 μA was 23.3 ± 1.8 μm ($n = 6$). The ONL with 50 μA was not significantly thicker than that of sham-stimulated eyes, but with 100 μA, the ONL was significantly thicker than that of the sham-stimulated eyes and that of the 50 μA ($P < 0.001$ versus sham, $P < 0.001$ versus 50 μA; Fig. 2A). Thus, TES at 100 μA was significantly effective on the survival of photoreceptors but TES at 50 μA was not significantly effective in RCS rats at 7 weeks of age; therefore, we used TES at 100 μA in the following experiment.

To determine whether the differences in the thickness of the ONL was localized or widespread across the retina, the mean thickness of the ONL was determined at 18 points along the superior–inferior plane of the eye in the three groups of RCS rats. The mean ONL thickness at every point in the superior and inferior hemispheres of the retinas treated with 100 μA TES was significantly thicker than that treated with sham stimulation or in the control retinas (one-way ANOVA, $P < 0.001$; Fig. 2B). Thus, TES delayed the degeneration of the photoreceptors across the retina.

To determine whether the TES affected other layers of the retina, we measured the thickness of the INL. The mean thickness of the INL was: control = 29.0 ± 0.8 μm; sham = 28.1 ± 1.1 μm; 50 μA TES = 26.9 ± 0.4 μm; and 100 μA TES = 30.1 ± 0.8 μm (mean ± SEM, $n = 6$ each; Fig. 2C). None of these differences was significant.

Time Course of Survival of Photoreceptors Treated with TES

Because 100 μA of TES prolonged the survival of photoreceptors, we used this current intensity to follow the time course of the survival of photoreceptors. The ONL of the retina of RCS rats at 3 weeks of age is composed of 10 to 12 rows of nuclei (Fig. 3A). In the sham-stimulated rats, the ONL of 5-week-old RCS rat was made up of three rows, and at 9 weeks of age, only an occasional nucleus was seen in the ONL, and the photoreceptors were scattered and disorganized (Figs. 3C, 3E). In contrast, the ONL in 5-week-old RCS rats treated with TES had five to six rows, and at 9 weeks, there were one to two rows, were organized in a line (Figs. 3B, 3D).

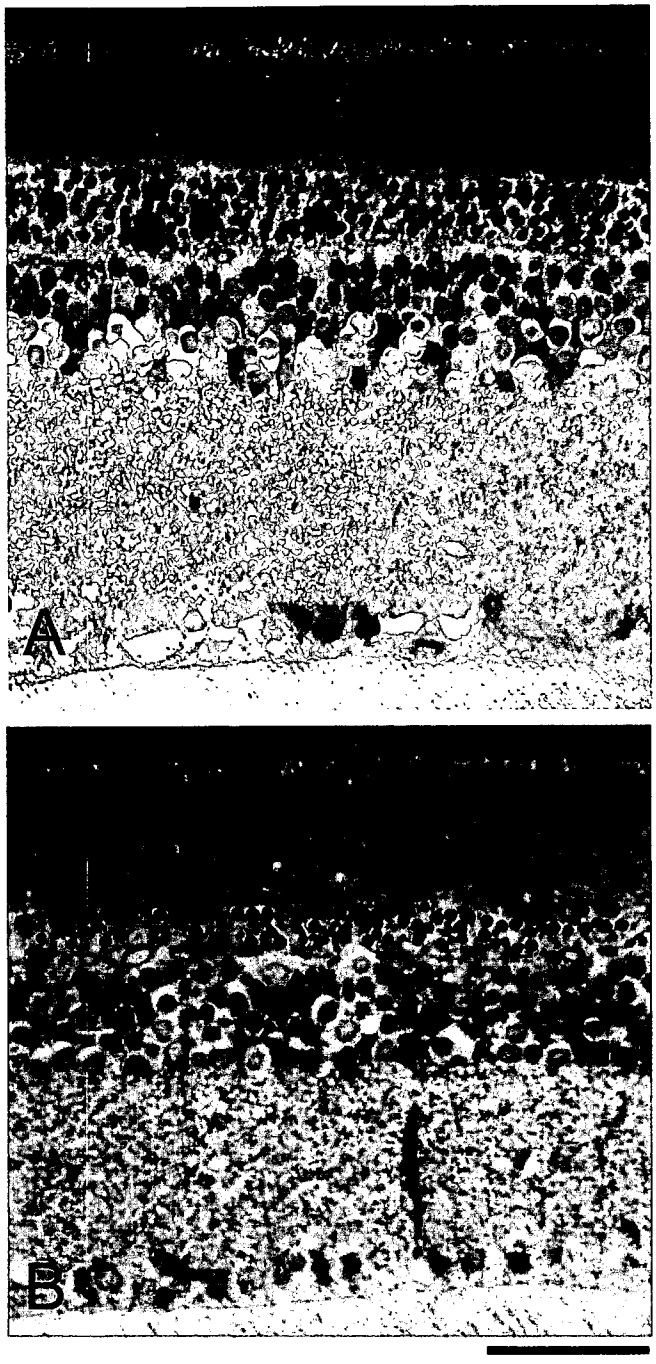


FIGURE 1. Photomicrographs of TES-treated (A) and sham-stimulated (B) retinas from 7-week-old RCS rats. TES led to better structural preservation of the photoreceptors than sham stimulated. Scale bar, 50 μm.

Measurements of the ONL thickness showed that there was a significant difference in the mean ONL thickness between TES-treated and sham-stimulated eyes in 5-week-old RCS rats (TES = 26.5 ± 2.9 μm; sham = 17.1 ± 2.7 μm; mean ± SEM, $n = 6$ each, t-test; $P < 0.001$), and at 9 weeks (TES = 8.34 ±

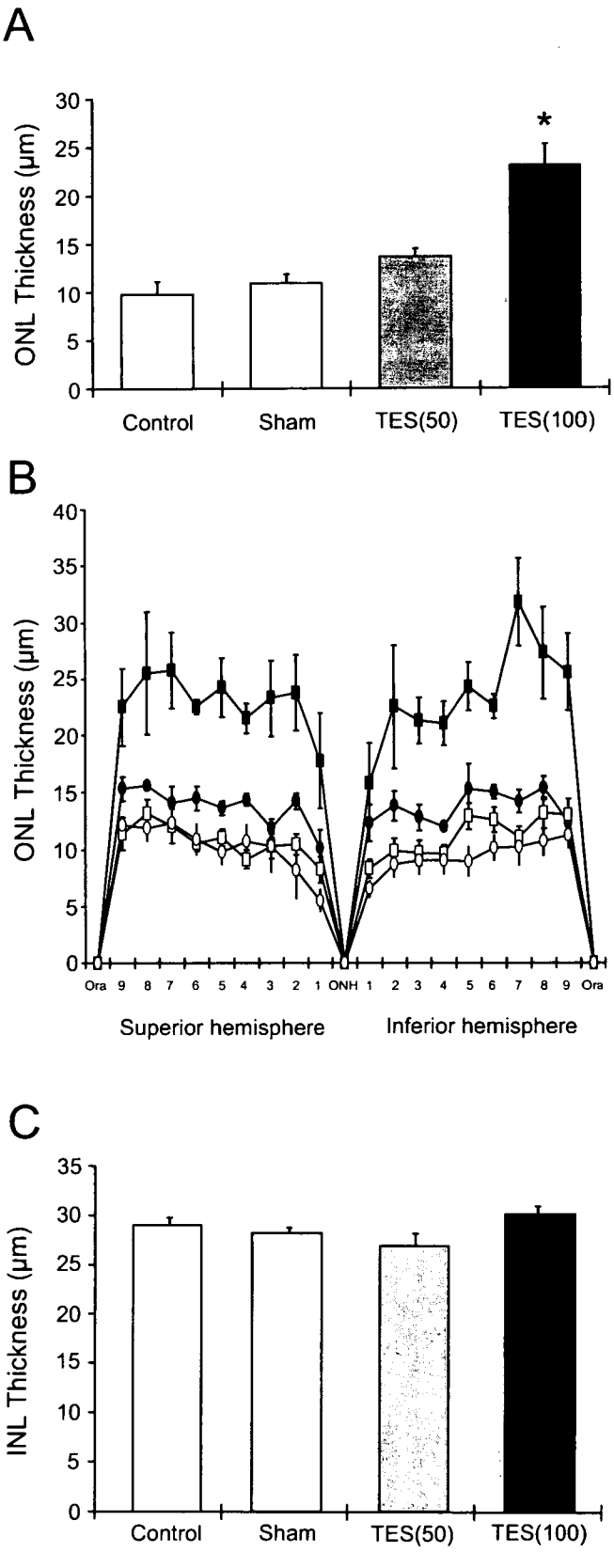


FIGURE 2. Effect of TES on the thickness of the ONL and INL of RCS rats at 7 weeks of age. Data are the mean ± SEM. (A) Mean thickness of the ONL in the retinas treated with TES (100 μA) is significantly thicker than that of control and sham-stimulated retinas (one-way ANOVA $P < 0.001$, followed by Tukey test, $^*P < 0.001$ vs. sham). (B) Mean thickness of the ONL along the vertical meridian of the retina of RCS rats. The data are the mean ± SEM. In all cases, the ONL in the eyes receiving 100 μA (■) TES was significantly thicker than that with sham stimulation (□) or controls (○) in both superior and inferior retina (one-way ANOVA, $P < 0.001$). (●) TES at the current intensity of 50 μA. (C) Mean thickness of the INL of the retinas of RCS rats at 7 weeks of age. There was no significant difference among them.

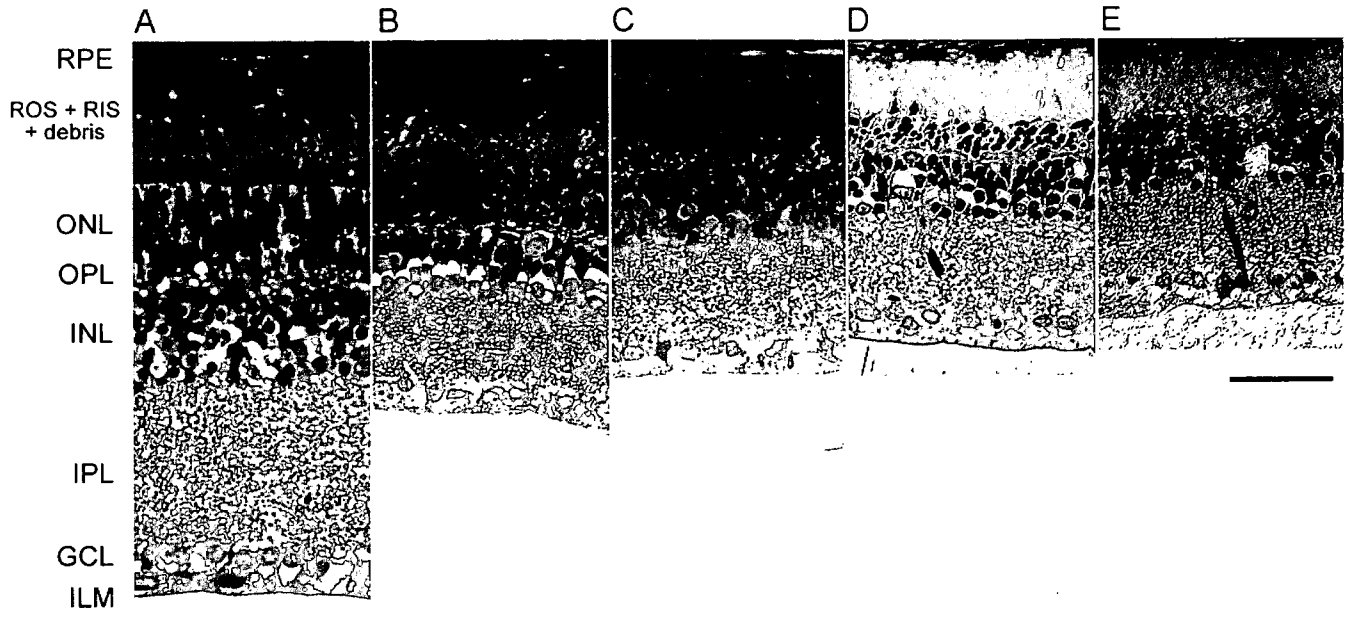


FIGURE 3. Photomicrographs of the retina from control, TES-treated and sham-stimulated RCS rats. Retinas from control RCS rats at 3 weeks of age (A) and from TES (B) and sham-stimulated (C) eyes at 5 weeks of age, from TES (D) and sham-stimulated (E) eyes at 9 weeks of age. Although the ONL thickness in the sham-stimulated retina decreases with age, the ONL was thicker in the retinas receiving TES. Scale bar, 50 μm.

0.5 μm; sham = 4.12 ± 0.5 μm; $n = 6$ each, t-test; $P < 0.001$; Fig. 4A).

As shown in Figure 4A, although TES significantly delayed the loss of photoreceptors at each time point, the rapid loss of photoreceptors occurred between 7 and 9 weeks of age. Thus, the degree of neuroprotection changed with an increase in the age of the RCS rats.

Figure 4B shows the time course of the decrease of the mean ONL thickness at 18 points along the superior–inferior plane of the eye in RCS rats at 3, 5, and 9 weeks of age. Although the mean ONL thickness decreased with an increase in the age of the RCS rats, the mean ONL thickness at every point in the superior and inferior hemispheres in the retinas treated with TES was thicker than that in the retinas treated with sham stimulation.

There was no difference in the mean INL thickness between retinas treated with TES and sham stimulation at 5 weeks and 9 weeks of ages (Fig. 4C).

The eye and fundus were examined at the end of the experiments, and neither retinal detachment nor vitreous hemorrhage was observed. In addition, cataracts or corneal opacities did not develop in all rats.

Retinal Function in RCS Rats Treated with TES

Representative ERGs recorded from RCS rats at 3, 5, and 7 weeks of ages are shown in Figure 5. In the eye of the RCS rat at 3 weeks of age, the b-wave reached the criterion amplitude at an intensity of −1.9 log cd/m^2, and the a-wave was first detected at 1.1 log cd/m^2 (Fig. 5A). In 5-week-old RCS rats with sham stimulation, a negative response dominated the ERG over the whole intensity range, and a b-wave did not appear until nearly the maximum stimulus intensity (Fig. 5C). In contrast, in the 5-week-old RCS rat treated with 100 μA TES, a b-wave appeared at −0.4 log cd/m^2 and the amplitude increased with increasing stimulus intensities. However, the amplitude of the a-wave was reduced (Fig. 5B).

Although 50 μA TES also delayed the decrease of retinal function, the effect of 100 μA was more consistent with less variation than that of TES at 50 μA (data not shown).

The b-waves were used to assess retinal function of each RCS animal at 5 weeks of age because of the absence of an a-wave in the sham-stimulated animals. There was a 1.23-log-unit difference in the mean threshold of the b-wave between TES-treated eyes and sham-stimulated eyes (TES: −0.07 ± 0.50 log cd/m^2; sham: 1.16 ± 0.58 log cd/m^2; mean ± SEM, $n = 6$, t-test; $P < 0.001$).

The intensity–response function curve for the b-wave in TES-treated eyes was shifted to the right by approximately 0.9 log units compared with the eyes at 3 weeks of age, whereas the curve from the eyes treated with sham stimulation was shifted to the right by 2.5 log units (Fig. 6A).

We also compared the mean amplitude of the b-waves elicited by the maximum intensity (2.1 log cd/m^2) from the TES-treated eyes with that from the sham-stimulated eyes at 5 weeks of age. There was a significant difference between them (TES: 47.1 ± 9.90 μV; sham: 19.2 ± 6.71 μV, mean ± SEM; $n =$ 6 each, Mann-Whitney rank sum test; $P = 0.038$).

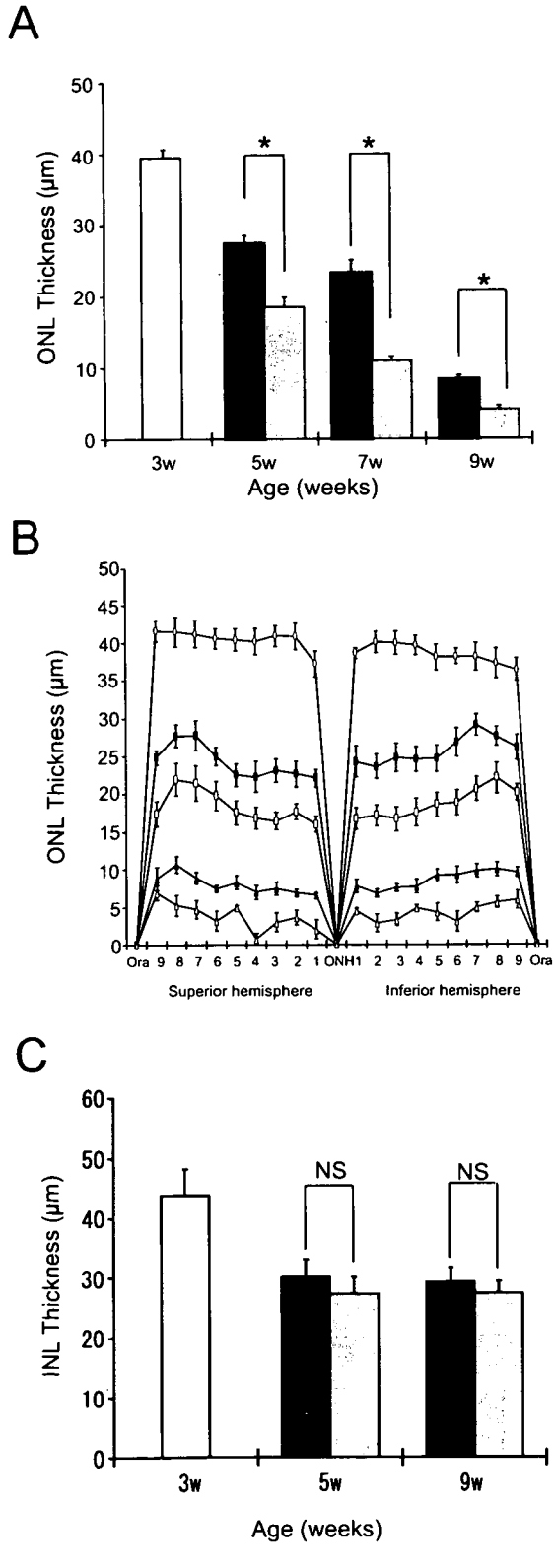
At 7 weeks of age, the retinal function of RCS rat was more depressed, the b-wave was not present, and a negative response called the "STR-like negative response" dominated the ERG over the whole intensity range. Therefore, the STR-like negative response was used to determine the neuroprotective effect of TES on the eye of 7-week-old RCS rats. In the eyes treated with sham stimulation, the STR reached the criterion amplitude at 0.1 log cd/m^2 (Fig. 5E). In the eye treated with TES, the STR appeared at −0.9 log cd/m^2, and the amplitude of the STR-like negative response increased with increasing stimulus intensities (Fig. 5D). There was a 0.88-log-unit difference in the mean threshold for the STR between the TES-treated eyes and sham-stimulated eyes (TES: −0.45 ± 0.46 log cd/m^2; sham: 0.43 ± 0.50 log cd/m^2; mean ± SEM; $n = 6$ each; Mann-Whitney rank sum test; $P = 0.027$).

In the eyes with sham stimulation, the intensity–response curve for the negative wave was shifted to the right by approximately 0.8 log units compared with the eyes with TES (Fig. 6B). The mean amplitude of the negative response at the maximum intensity in the TES-treated eyes was significantly

larger than that in the sham-stimulated eyes (TES: 25.1 ± 2.79 μV; sham: 11.9 ± 3.00 μV, mean ± SEM; *t*-test; $P < 0.001$).

We also recorded ERGs from 9-week-old RCS rats after TES or sham stimulation, but the responses from these animals were too weak to be assessed (data not shown).

Because STRs were recordable from 3- to 7-week-old RCS rats, we used the thresholds of the STR to follow the time course of loss of retinal function. The threshold for the STR as a function of age is plotted in Figure 6C. In RCS rats at 3 weeks of age, the mean threshold of STR was −2.21 ± 0.13 log cd/m^2 (mean ± SEM). Although the threshold of STR increased with age, the mean threshold in the retina at 5 weeks of age with



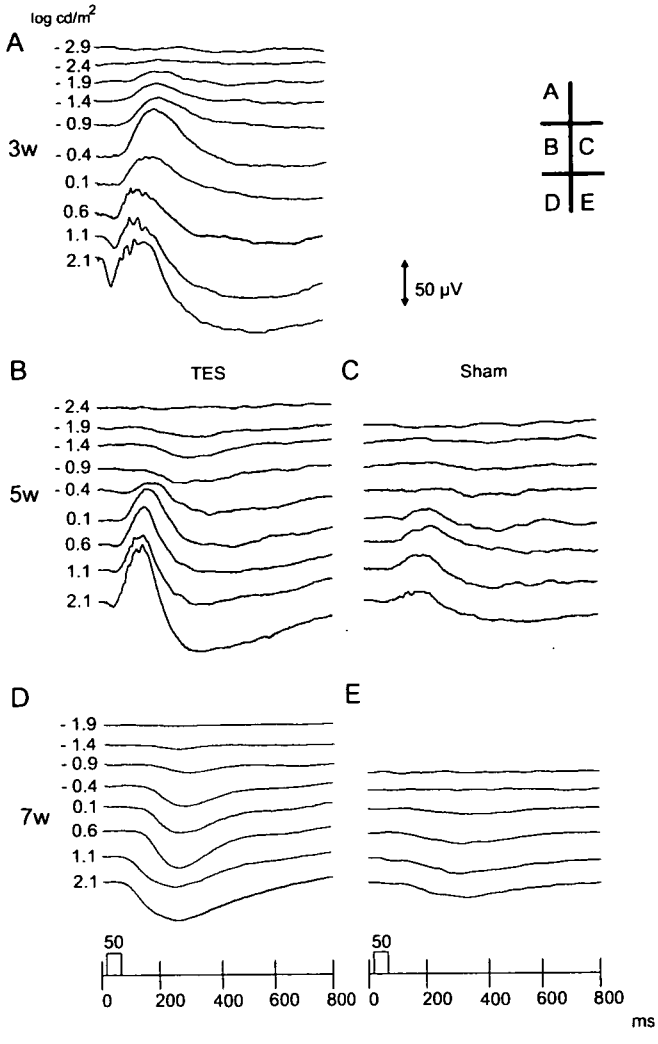
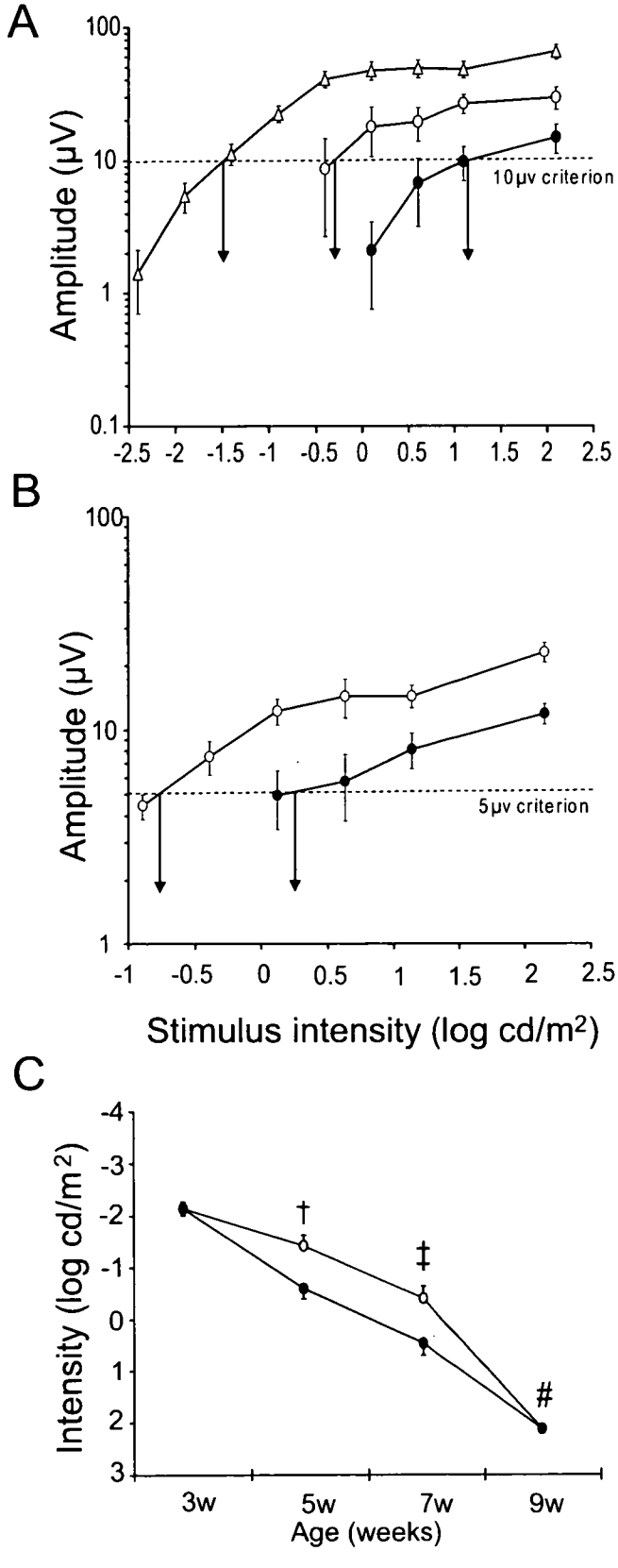


FIGURE 5. Effect of TES on the ERGs. Typical ERG responses elicited by different stimulus intensities from TES-treated and sham-stimulated eyes of RCS rats at 5 and 7 weeks of age. ERGs were recorded from both eyes simultaneously. ERGs from a 3-week-old RCS control rat (**A**), a 5-week-old RCS rat after TES (**B**) and sham stimulation (**C**), and a 7-week-old RCS rat after TES (**D**) and sham stimulation (**E**).

FIGURE 4. Time course of photoreceptor survival. (**A**) Mean thickness of ONL in the retina of 3-, 5- and 9-week-old RCS rats treated with TES or sham stimulation. The data are the mean ± SEM. The differences in the mean ONL thickness between TES-treated retinas (■) and sham stimulated retinas (▨) at 5 and 9 weeks of ages, as well as at 7 weeks of age, is significant (*t*-test, $^*P < 0.001$). (**B**) Mean thickness of ONL along the vertical meridian of the eye in RCS rats. The data are the mean ONL thickness ± SEM. In all cases, treatment with TES at a current intensity of 100 μA led to ONL thickness that was significantly thicker than that with sham stimulation in both superior and inferior retina at each time point (one-way ANOVA, $P < 0.001$). (○) 3-week-old RCS rats; (■) 5-week-old RCS rats treated with TES or (□) sham stimulation; and (▲); 9-week-old RCS rats treated with TES or (△) sham stimulation. (**C**) Measurement of mean INL thickness in the retinas in 5- and 9-week-old RCS rats treated with TES (■) or sham stimulation (▨). There was no significant difference between them.

TES was significantly lower than that in the retina with sham stimulation (TES: -1.48 ± 0.23 log cd/m^2; sham: -0.65 ± 0.21 log cd/m^2, mean ± SEM; t-test; $P = 0.027$). However, as shown in Figure 6C, TES delayed the decrease of retinal function by 7 weeks of age, but could not preserve the retinal function at 9 weeks.



Discussion

Our morphologic and electrophysiological analyses showed that 100 μA of TES prolonged the survival of photoreceptors and retinal function against the inherited photoreceptor degeneration of RCS rats. Our present study is, as best as we know, the first report to show that noninvasive electrical stimulation which only electrically stimulates the retina by using contact lens electrode, has a neuroprotective effect on the photoreceptors both functionally and anatomically, although Pardue et al.[21] had reported that electrical stimulation by using subretinally implanted electrodes had a neuroprotective effect on photoreceptor degeneration in RCS rat.

Preservation of Retinal Morphology by TES

It has been reported that intravitreal injection of neurotrophic factors,[14,15,22–24] neuroprotective genes,[16,17,25] or transplantation of cells such as RPE cells,[18,26,27] have a strong neuroprotective effects on photoreceptors in animal models of RP. However, the survival of the photoreceptors was limited to the area of the injected site. Limited and localized protection of photoreceptors in the retina of RCS rat has also been demonstrated with mechanical injury alone[22,28] and by laser burns.[29]

For TES, the neuroprotective effect on the photoreceptors extended over the entire retina (Figs. 2, 4). This suggests that with our stimulating protocol, the electrical current may spread over the entire retina to exert neuroprotection on the entire retina. This neuroprotective effect was similar to the neuroprotective effect induced by light stress, which also has a neuroprotective effect on the photoreceptors over the whole retina.[19,30]

We also measured the thickness of the INL to determine whether TES also has a neuroprotective effect on the inner retinal cells. Unlike the significant effects on the photoreceptors, the inner retina appeared less affected by TES, although we have demonstrated that TES enhances the survival of axotomized RGCs in vivo.[12] In this study, the mean thickness of INL in the retinas of 5-, 7-, and 9-week-old RCS rats with TES or without TES were not significantly different, but they were thinner than that at 3 weeks. Although our data on mean INL thickness were similar to those presented by Pardue et al.,[21] there are no reports on the time course of thickness of the INL with age in pink-eyed RCS rats, and we did not determine why the INL was thinner.

Preservation of Retinal Function by TES

We used the b-wave and the STR-like negative response, which are not direct indicators of functioning photoreceptors, to evaluate retinal function. In RCS rats, the a-wave was detected only at relatively high intensities and became unrecordable in the early stage of retinal degeneration. Therefore the a-wave was not suitable for evaluating the efficacy of the TES because of the advanced photoreceptor degeneration.[31] Sugawara et al.[32] have demonstrated that the b-wave threshold and ampli-

FIGURE 6. Intensity-response curves of the ERGs. (A) Average b-wave amplitudes versus stimulus intensity from TES-treated (○) and sham-stimulated (●) retinas of 5-week-old and untreated 3-week-old (△) RCS rats on a log-log scale. (B) Average STR-like negative wave amplitudes versus stimulus intensity from TES-treated (○) and sham-stimulated retinas (●) in 7-week-old RCS rats on a log-log scale. Error bars, SEM. Time course of the decrease of retinal function. (C) Change in mean thresholds for STR with age in retinas with TES (○) or with sham stimulation (●). Error bars, SEM. TES preserved by 7 weeks of age against retinal degeneration (t-test; †$P = 0.027$; Mann-Whitney rank sum test; ‡$P < 0.001$; #unrecordable).

tude can be reliably used to track photoreceptors cell loss due to the damaging effects of constant light. Because STR has been shown to be a prominent component of the ERG in the RCS rat retina with advanced photoreceptor degeneration, STR is more useful than the b-wave in detecting loss of light sensitivity in advanced degenerated RCS rats.[31] We therefore used the STR-like negative responses to evaluate the retinal function in RCS rats at 7 weeks of age. The STR threshold was also used to track the time course of the loss of retinal function in the advanced degenerated retina.

The amplitudes of the b-waves (5-week-old) or STR-like negative responses (7-week-old) were significantly larger in TES-treated eyes than in sham-stimulated eyes of RCS rats (Fig. 5). These functional results were consistent with the histologic results obtained by measuring the thickness of the ONL (Fig. 4).

However, at the age of 9 weeks, there was no significant difference in the amplitudes of the b-waves of TES-treated and sham-stimulated animals. These results are in agreement with the rapid decrease of ONL thickness from 7 to 9 weeks of age, although the mean thickness of ONL was still significantly thicker in the TES-treated eyes at 9 weeks. These findings demonstrated the limitation of TES for the treatment of the retina with a rapid course of photoreceptor degeneration. In humans with RP, the degeneration is relatively slow, and so other RP models such as *RPE65*-deficient mouse[33] may be more suitable to investigate the effects of TES for slowly progressing photoreceptor degeneration.

Possible Mechanism of TES-Induced Neuroprotection of Photoreceptors

We have demonstrated earlier that TES induces a significant upregulation of endogenous IGF-1, which is produced by Müller cells.[12] We also analyzed TES-induced upregulation of the mRNAs of various neurotrophic factors (e.g., IGF-1, bFGF, CNTF, NT-3, NT-4/5, GDNF, and BDNF) in the RCS rat retinas treated with TES, using real-time PCR. We also found that the mRNA of IGF-1 was also upregulated in RCS rats (Morimoto et al., unpublished data, February 2005). However, IGF-1 was reported to have a weak neuroprotective effect on the survival of photoreceptors in light-damaged retinas,[15] and thus, IGF-1 may not be the key molecule that is neuroprotective of photoreceptors.

The neuroprotective effect of TES on the photoreceptors was dependent on the intensity of the electrical current (Fig. 2A). This suggests that the increase of electrical activity of the photoreceptors exerts neuroprotective effect on photoreceptors. We did observe that TES of 100 μA at 1 ms/phase evoked electrical responses in the superior colliculus (data not shown). We could not determine whether TES activated photoreceptors, inner retinal neurons, or Müller cells to induce the rescue effect.

A subretinal implant of an artificial retina has been shown to stimulate the retina electrically and has a neuroprotective effect on the photoreceptors of the RCS rats[21] and the retinal function in patients.[34] The subretinal implantation of an retinal prosthesis has two possible mechanisms for its neuroprotective effects: one is due to the foreign body effect of the subretinal implant, and the second is the effect of subretinal electrical stimulation (SES).[21] Pardue et al.[21] showed that SES provided better preservation of retinal function than that of an inactive subretinal implant. They implied that SES had a neuroprotective effect on photoreceptors. Although the strength and stimuli of SES by the artificial retina are different from those of TES and the extent of invasion of the retina is different, both SES and TES have neuroprotective effects on photoreceptors.

All evidence considered, electrical stimulation may directly or indirectly alter the electrical activity or electrical charge balance of photoreceptors and exert a neuroprotective effect on the photoreceptors. Additional experiments are needed to determine the mechanism of TES-induced neuroprotection for photoreceptors.

TES as a Potential Clinical Technique

Our findings indicate that electrical stimulation can delay the photoreceptor loss in eyes with inherited photoreceptor degeneration. We applied TES once a week for 6 weeks to delay the retinal degeneration, and ocular side effects, such as cataracts, were not observed. In RCS rats, a mutation of the gene coding for the receptor tyrosine kinase gene *Mertk* has been identified.[35] The same gene mutation has been identified in patients with autosomal recessive retinitis pigmentosa,[36] indicating that the RCS rat is a counterpart of one type of human RP. Although photoreceptor loss was not eventually prevented against the rapid photoreceptor degeneration, the protective effect of TES on photoreceptor degeneration in RCS rats suggests that TES could delay the progression of some forms of RP. Additional studies are needed to determine the optimal electrical stimulus parameters to obtain photoreceptor survival. We must also apply TES to other animals with different genetic mutations to evaluate the neuroprotective effects before consideration of TES for clinical use.

In summary, TES prolonged the survival of photoreceptors and delayed the loss of retinal function in RCS rats. Although the neuroprotective effect of TES in treatment of the retina with photoreceptor degeneration was limited, the present results open the possibility that TES could be used to delay the photoreceptor degeneration in patients with inherited retinal degeneration such as RP.

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Threshold suprachoroidal–transretinal stimulation current resulting in retinal damage in rabbits

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Abstract

The purpose of this study is to determine the threshold suprachoroidal–transretinal stimulation (STS) current that results in retinal damage in rabbits. Biphasic STS pulses (anodic first, frequency 20 Hz) were used to stimulate the retina of pigmented rabbits ($n = 18$) continuously for 1 h using a 100 μm diameter platinum wire electrode. The STS current that induced retinal damage after 1 h was determined by ophthalmoscopy or by fluorescein angiography (FA) independently. The effect of the pulse duration on the threshold current was investigated. Histological studies were performed after electrical stimulation experiments. The threshold for a safe current to the retina was 0.6 mA for a duration of 0.5 ms. The threshold for a safe charge increased approximately linearly with an increase of stimulus duration but the threshold for a safe current decreased logarithmically with an increase of duration. The threshold for a safe electrical energy remained almost constant for all durations. Histological examination showed severe retinal damage when the current exceeded the threshold, with more damage in the inner layers compared with the outer layers of the retina. The threshold for the safe current was higher than that reported for direct stimulation of neural tissues, suggesting that the STS method was safe and able to be used with a retinal prosthesis. Because the threshold for the safe charge was lower with shorter pulse durations, care should be taken using pulses of short durations.

Introduction

Stimulating the retina electrically with an implanted retinal prosthesis in eyes with very limited vision is one of the promising approaches for providing some degree of vision to visually impaired patients. For this, suprathreshold currents are used to evoke phosphenes by stimulating still functioning neurons in the retina. Although biphasic, charge-balanced pulses are used, the retina can still be damaged if high electric currents are used. Several studies have determined the safe current for cortical cells when needle-type electrodes are used [1–4]. Epiretinal and subretinal types of electrodes are being intensively investigated as retinal prostheses, but only a few studies have reported the safe limits of the applied currents or voltages with these types of electrodes [5, 6].

We have developed a new method to stimulate the retina called suprachoroidal–transretinal stimulation (STS) [7] and have shown that STS can elicit electrically evoked potentials (EEP) from the rabbit cortex in acute experiments [8]. We have investigated the effects of chronic implantation of electrodes without electrical stimulation [9] or with stimulation by biphasic pulses of 100 μA, 20 Hz for 1 h/day [10]. EEP

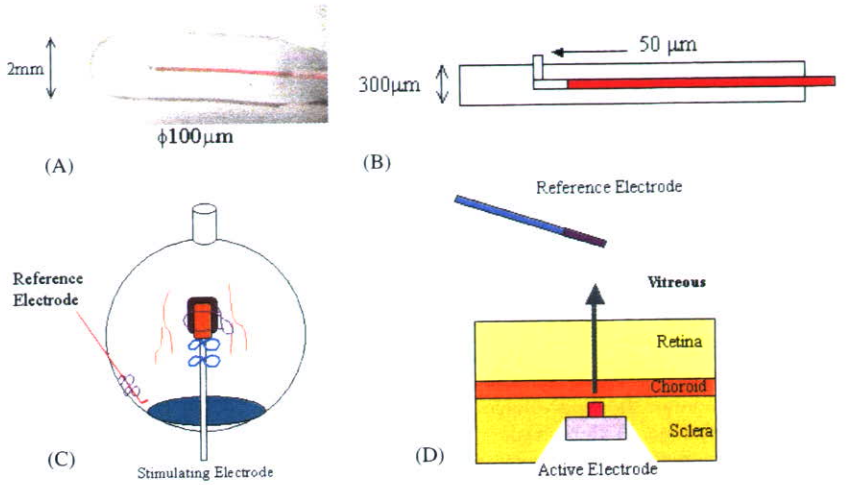

Figure 1. Photograph (A) and diagram (B) of the stimulating electrode, diagram of the placement of the electrodes in the rabbit eye (C) and diagram of suprachoroidal–transretinal stimulation used in this experiment (D). (A) and (B) A wire electrode (diameter = 100 μm) was embedded in a 2 mm × 4 mm × 0.3 mm silicone plate. The electrode wire was coated with polyurethane, and 0.5 mm of the tip was exposed. The tip protruded 0.05 mm from the plate surface. (C) Sclerotomy was performed over the visual streak of rabbits until the choroid was observed. The silicone plate with the electrode was fixed to the fenestrated sclera with 8-0 Vicryl and 5-0 Dacron sutures. The return electrode was placed in the vitreous cavity and fixed at the ora serrata with 8-0 Vicryl. (D) The stimulating electrode was set in the fenestrated sclera with a residual thickness of 50–100 μm. The return electrode was placed in the vitreous and the electric current passed through the retina.

responses could be elicited even 2 weeks after the implantation, and no retinal damage was observed under either condition.

One advantage of the STS method is that the stimulating electrode is not in direct contact with the retina as with epiretinal and subretinal electrodes. We showed that after 1 h of continuous biphasic 20 Hz stimulation (pulse duration 0.5 ms), the ophthalmoscopically determined safe current was 1 mA with a 100 μm diameter platinum (Pt) electrode and 1.5 mA with a 200 μm diameter Pt electrode [11].

Before applying the STS method to patients, the threshold current that does not injure the retina must be determined more accurately. Thus, the purpose of this study was to determine the threshold current delivered by the STS method that did not injure the retina. Ophthalmoscopy and fluorescein angiography (FA) were used to evaluate the retina.

Materials and methods

Animals

Eighteen pigmented, Dutch-belted rabbits (weight 1.5–2.2 kg) were used for the experiments. Twenty-eight retinal loci of 20 eyes (10 rabbits) were used to determine the threshold current that just caused a visible alteration of the retina as detected by ophthalmoscopy, and 30 retinal loci of 16 eyes (8 rabbits) were used to identify the threshold current using fluorescein leakage during FA as the end point. The procedures used on the animals conformed to the Institutional Guidelines of Osaka University and the ARVO Resolution on the Use of Animals in Research.

Stimulating electrodes

A 100 μm diameter platinum wire was coated with polyurethane and embedded in a 2 mm horizontal × 4 mm vertical × 0.3 mm thick silicone plate (figures 1(A) and (B)) (Unique Medical, Tokyo Japan). Then, 0.5 mm of the tip of the wire was bent to 90° and protruded from the surface of the plate by 0.05 mm. The polyurethane coating was scraped away from the tip to expose a surface area of 2.36×10^{-4} cm^2. The return electrode was made of the same polyurethane-coated platinum wire, and 2 mm of the tip was exposed and inserted into the vitreous. This electrode configuration was found to be effective in stimulating the rabbit retina by STS [8].

Surgical procedures

The rabbits were anesthetized by an intramuscular injection of a mixture of ketamine (50 mg kg^{-1}) and xylazine (20 mg kg^{-1}), and if additional anesthetics were needed, one-half of the initial dose was injected. Sclerotomy was performed over the visual streak region, and the sclera was dissected away until the choroid was visible. The stimulating electrode was fixed to the sclera with 8-0 Vicryl and 5-0 Dacron sutures. When the electrode was moved to stimulate another retinal loci, sutures were cut and re-fixed to the corresponding scleral loci. The return electrode was pushed into the vitreous cavity through the ora serrata and placed approximately in the middle of the vitreous body. The electrode was secured with 8-0 Vicryl sutures (figure 1(C)).

Electrical stimulation by STS

A diagram of the direction of current flow delivered by STS is shown in figure 1(D). The stimulus consisted of biphasic pulses (anodic first) with a frequency of 20 Hz penetrating through the retina. The biphasic pulses were generated with a signal processor (SEN-7203, Nihon Kohden, Tokyo, Japan), and the electric current was delivered through a stimulus isolation

Figure 2. Photograph of oscilloscope monitor showing voltage between the active and the reference electrode. With biphasic rectangular pulse currents, the voltage increases slowly because of the effect of capacitance.

unit (WPI-A365, WPI, Sarasota, USA). The potential changes between the active and return electrodes were monitored with a storage oscilloscope and one cycle is shown in figure 2 (TPS-2014, Tektronics, Beaverton, OR, USA).

Evaluation of voltage between the active and return electrodes

When rectangular pulses are delivered, the waveform of the response was not rectangular due to the capacitance effect (figure 2). Thus, we calculated the integrated value of the area under the pulse and found that the integrated voltage was about 85% of the peak voltage. In addition, the value of the area under the voltage trace is used to calculate the energy per phase by multiplying the area with the current amplitude.

Determination of the threshold current that does not injure the retina

To determine the threshold current that did not injure the retina, the pulse duration and current intensity were changed, while the frequency was fixed at 20 Hz. Preliminary experiments showed that 1 mA biphasic pulses of 0.5 ms duration or a charge of 0.5 μC/phase did not injure the retina [11]. Thus, total charge for the stimulation was initially kept constant at 0.5 μC/phase, i.e., for a duration of 0.1 ms, the current was 5 mA, for 0.25 ms—2 mA, for 0.5 ms—1 mA and for 1 ms—0.5 mA, respectively.

The ophthalmoscopic sign of retinal damage was defined as the appearance of a whitish spot around the electrode (figure 3). After 1 h, the retina was examined ophthalmoscopically, and if fundus changes were detected, the stimulating electrode was moved and a different retinal locus in the same eye or in the opposite eye was tested. The current was reduced by 0.1–0.5 mA units, and the procedure was repeated and the retina was examined ophthalmoscopically. This procedure was repeated until no retinal changes were detected ophthalmoscopically. When no fundus changes were detected, the electrode was moved to a different retinal locus in the same eye or in the opposite eye and repeated with 0.1–0.5 mA stronger currents and the fundus was examined.

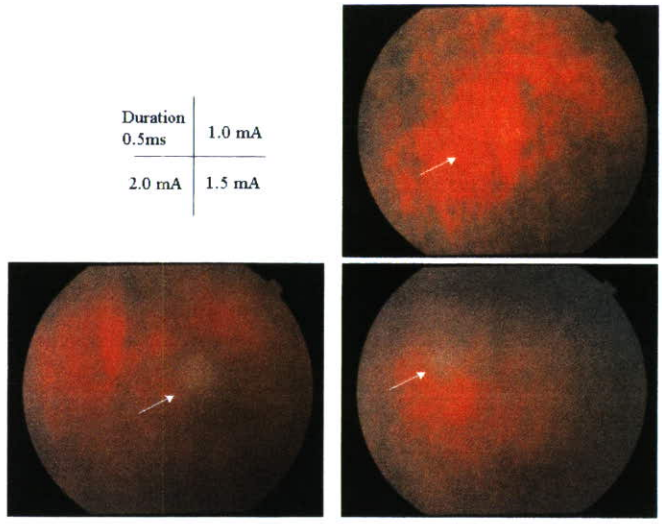

Figure 3. Fundus photograph 1 h after electrical stimulation. No change is observed with 1 mA (upper right) and a whitish spot is observed with 1.5 mA (lower right) and 2 mA (lower left). The white arrows indicate the retinal area where the electrode was attached to the sclera.

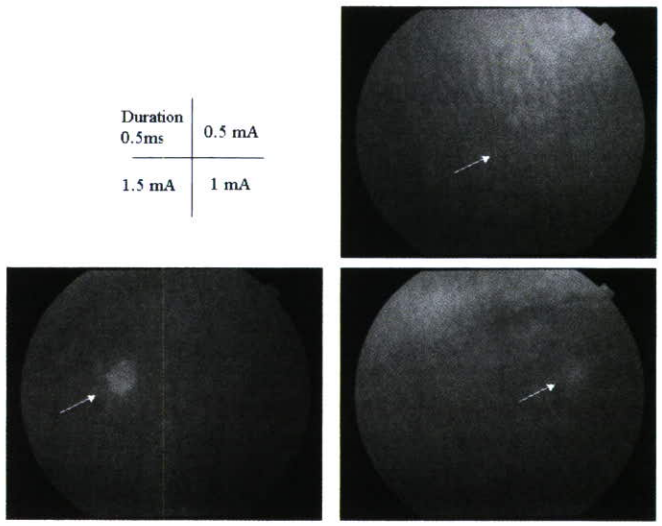

Figure 4. Fluorescein angiography 1 h after electrical stimulation. No change is observed with 0.5 mA (upper right) and leakage of fluorescein dye is observed with 1.0 mA (lower right) and 1.5 mA (lower left). The white arrows indicate the retinal area where the electrode was attached to the sclera.

This was repeated until the whitish spot on the retina was observed. The threshold current was defined as the strongest current that did not produce retinal damage as assessed by ophthalmoscopy.

For FA, the sign for electrically induced damage to the retina was the appearance of dye leakage or hyperfluorescence around the retinal area where the electrode was placed (figure 4). The baseline parameters of stimulation were similar to those used during the ophthalmoscopic assessment, and the total injected charge/phase was kept constant. After stimulation for 1 h with one current intensity and duration setting, FA was performed, and if the retinal leakage change was observed (figure 4), the current was decreased by 0.1 mA

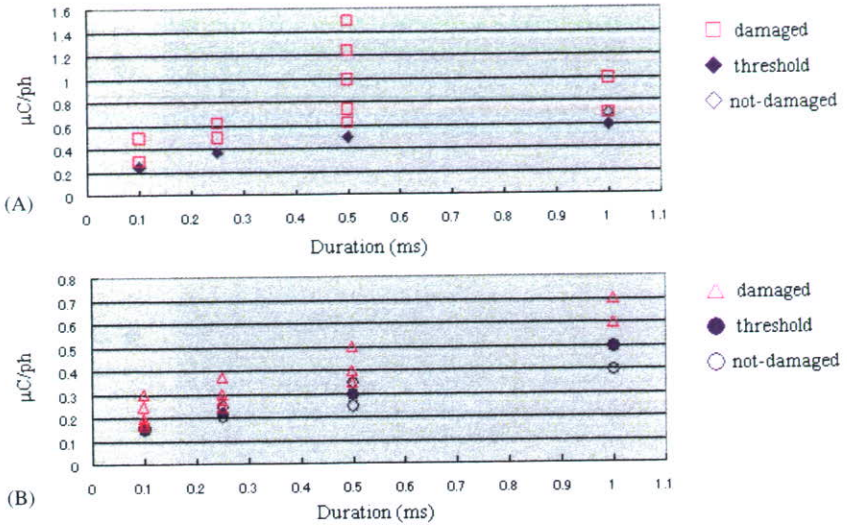

Figure 5. Graphs showing the relationship between charge per phase and duration of the pulses as assessed by ophthalmoscopy (A) or by fluorescein angiography (B). Threshold charge was defined by the highest charge that did not damage the retina. The threshold charge increases logarithmically or almost linearly with an increase of duration in both graphs.

after changing the retinal locus, and the procedure was repeated. When the current intensity that did not lead to fluorescein leakage was found, the retinal change was checked with the same current again and with a current 0.1 mA lower at a different retinal locus. If retinal changes were not observed by this current, this current was defined as the threshold current.

Histological studies

All rabbit eyes were enucleated immediately after the stimulation experiments, fixed in glutaraldehyde and observed with stereoscopic microscope.

After marking the scleral site in which the electrode was attached, eyes ($n = 8$) were embedded in paraffin, sectioned with 3 μm thickness around the marked scleral site with an interval of 20 μm and stained with hematoxylin–eosin.

Results

Threshold charge as a function of stimulus duration

Initially, we examined the effect of the pulse duration on the threshold charge (coulombs/phase or C/ph). The retina was examined by ophthalmoscopy (figure 3) and by FA (figure 4) after each set of stimuli. These data plots showed that the threshold for a safe charge increased logarithmically or almost linearly with an increase in the stimulus duration (figures 5(A) and (B)).

Threshold current as a function of stimulus duration

Next, we examined the relationship between the duration and the threshold current (mA) (figures 6(A) and (B)). These plots seemed to be lined on a logarithmic curve. When the data were plotted on a log–log scale, the threshold current decreased linearly with an increase in the pulse duration (figures 7(A) and (B)). The slope of the regression line was −0.61 by ophthalmoscopy and −0.52 by FA.

Table 1. The threshold currents for each duration and other accompanying parameters determined by ophthalmoscopy (A) and fluorescein angiography (B).

	(A)			
Duration (ms)	0.1	0.25	0.5	1.0
Current (mA)	2.5	1.5	1.0	0.6
Charge (μC/ph)	0.25	0.38	0.50	0.60
Voltage (V)	19.7	13.0	9.0	6.6
Energy consumption (μJ/ph)	4.9	4.9	4.5	4.0
	(B)			
Duration (ms)	0.1	0.25	0.5	1.0
Current (mA)	1.6	0.9	0.6	0.5
Charge (μC/ph)	0.16	0.23	0.30	0.50
Voltage (V)	11.0	5.8	6.0	4.2
Energy consumption (μJ/ph)	1.8	1.3	1.8	2.1

The charge was calculated by multiplying duration with current. The voltage was the integrated value of the area under the pulse with Excel. The energy consumption was calculated by multiplying duration with current and voltage, and remained constant at around 5 μJ/ph (ophthalmoscopy (A)) and 2 μJ/ph (fluorescein angiography (B)).

Threshold energy as a function of stimulus duration

An examination of the relationship between the threshold electrical energy (J/ph) and the duration (figures 8(A) and (B) and table 1) showed that the threshold electrical energy remained almost constant for all durations.

Histological examinations

A photomicrograph of a retinal site stimulated by biphasic pulses with a duration of 0.5 ms and a current of 1.0 mA that did not lead to an ophthalmoscopic alteration but lead to FA alterations is shown in figure 9(A). The retinal cells

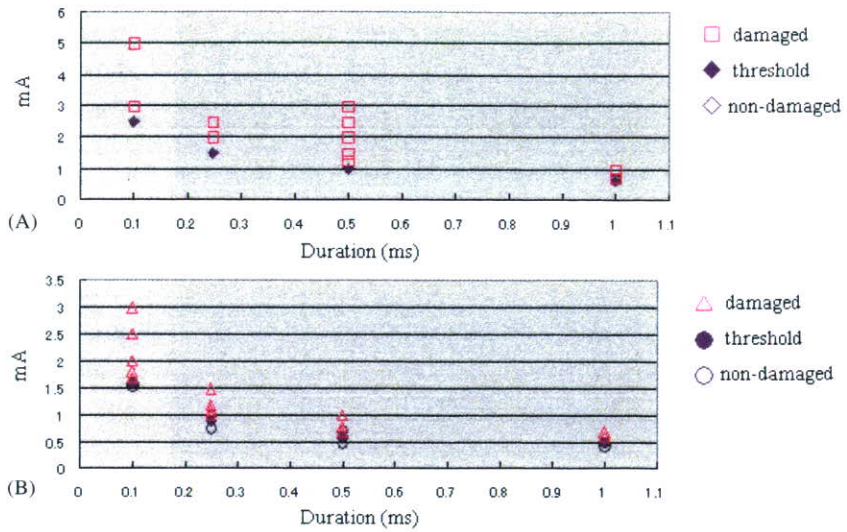

Figure 6. Graphs showing the relationship between current per phase and duration as assessed by ophthalmoscopy (A) or by fluorescein angiography (B). The threshold current decreases logarithmically with an increase of duration in both graphs.

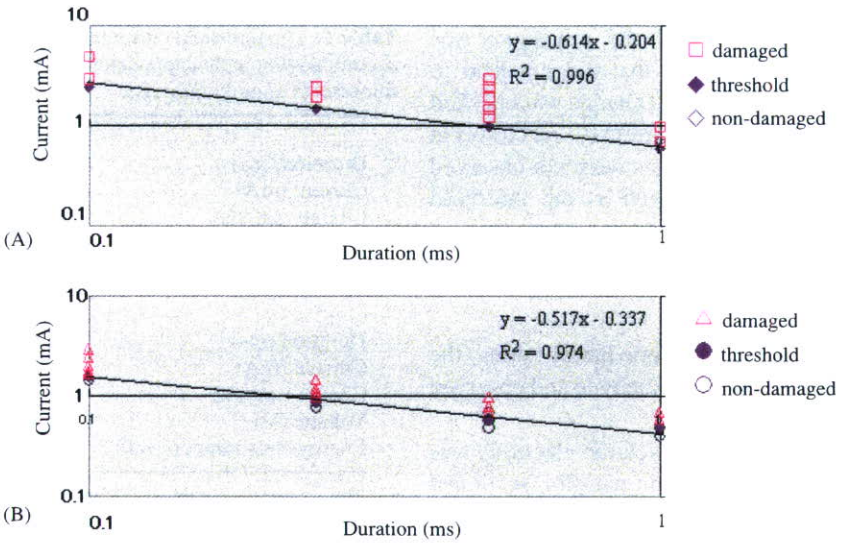

Figure 7. Graphs showing the relationship between the effect of current per phase and the duration by ophthalmoscopy (A) or by fluorescein angiography (B) with x–y axes plotted on a logarithm scale. The threshold current decreases linearly with an increase of duration in both graphs. The slope of the regression line is −0.61 by ophthalmoscopy (A) and −0.52 by FA (B).

and architecture are normal. An example of the histological damage of the retina induced by biphasic pulses (duration 0.5 ms, current 1.5 mA) is shown in figure 9(B). An enlargement of the choroidal vessels, disruption of outer nuclear layer cells, condensation of the inner nuclear layer and vacuoles in the outer plexiform, inner plexiform and nerve fiber layers can be seen.

In the most severely damaged area where the center of the electrode was attached, all retinal layers were destroyed. In the periphery of the stimulated area, the changes were less severe with relatively more damage in the inner layer than the outer layers including RPE or photoreceptors. The sclera and choroid were not altered.

Histological examination of the electrode sites showed that the scleral thickness was 50–100 μm in all preparations. When the scleral thickness was less than 50 μm, the choroid was easily ruptured in the fenestrated area, so scleral tissue with a minimum thickness of about 50 μm was needed for structural integrity.

Discussion

Our results showed that the threshold charge per phase increase with an increase of duration was similarly assessed by ophthalmoscopy and by FA, although the threshold charge was about 20% lower when assessed by FA than by ophthalmoscopy (figure 5). This difference indicates that FA is more sensitive than ophthalmoscopy in detecting retinal injury caused by electrical STS.

The threshold current with a duration of 0.5 ms, a common duration used for human STS experiments,

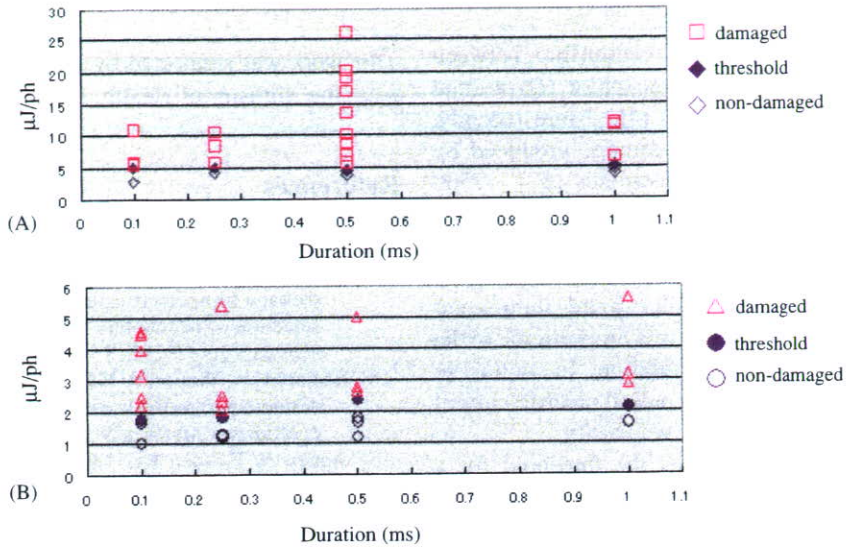

Figure 8. Graphs showing the relationship between the effect of energy consumption per phase on the retina and the duration by ophthalmoscopy (A) or by fluorescein angiography (B). Multiplying voltage with current and duration yields the electrical energy consumption per phase (J/ph). The threshold energy consumption was almost constant for all durations in both graphs.

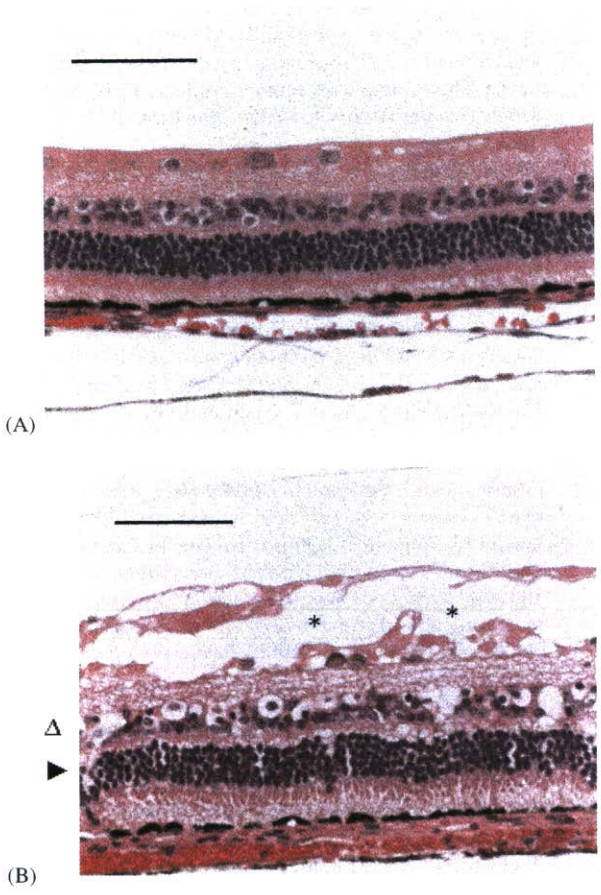

Figure 9. Histology of the retina 1 h after stimulation with currents of 1.0 mA (A) and 1.5 mA (B). (A) Except a small enlargement of choroidal vessels, no change was observed. Bar represents 100 μm. (B) Enlargement of choroidal vessels, disarray of outer nuclear layer cells (▲), condensation of inner nuclear layer cells (△) and vacuoles in the outer plexiform, inner plexiform and nerve fiber layers (∗) can be seen.

was 0.6 mA by FA (figure 6(B) and table 1(B)). This corresponds to 0.3 μC/ph for a 0.5 ms duration pulse. The safe charge density for Pt electrodes is reported to be equal to or less than 400 μC cm^{-2}/ph to avoid Faradaic reactions such as H_2O electrolysis or Pt dissolution in inorganic saline [12, 13]. This corresponds to about 0.1 μC/ph for our electrode. Although there was a report that the dissolution limit of Pt was higher if the electrodes are soaked in a protein solution than in inorganic saline [14], the safe charge at the electrode surface for the STS method is three times higher than the Faradaic or dissolution limit of the Pt electrode.

Yuen *et al* have reported that with platinum electrodes, a charge density/phase (QD/ph) of 40 μC cm^{-2}/ph did not affect cortical neural tissues even after 50 h of continuous stimulation at 50 Hz [1]. McCreery *et al* have determined the thresholds for neuronal damage at a QD/ph of 50–100 μC cm^{-2}/ph after 7 h of continuous cortical stimulation at 50 Hz [4]. Recently, Harnack *et al* also reported that a QD/ph of up to 26 μC cm^{-2}/ph did not cause any neuronal damage after 4 h of stimulation at 130 Hz with Pt/Ir electrodes [15]. Although stimulation electrode size, hours and frequency were different, these values are quite comparable, and the threshold for neuronal damage by direct stimulation may be 26–100 μC cm^{-2}/ph.

The threshold for QD/ph in our experiment was 678–2119 μC cm^{-2}/ph, which is about 20 times higher than that of previous reports. Donaldson and Donaldson reported that several factors could be the cause of tissue damage when direct electrode stimulation is used, e.g., pH changes, gas evolution, bleaching products, $Mg(OH)_2$ deposits and electrode metal dissolution [16]. The safe charge in the present work may be higher than that of previous reports because the retina is protected by the sclera against electrochemical reactions

occurring at the electrode interface. Abundant blood flow in the choroid may also protect the retina.

McCreery *et al* investigated the relationship between charge density/phase (QD/ph) and charge/phase (Q/ph) that caused neural damage by Pt electrodes [4]. More recently, Vankov *et al* reported on the cellular damage produced by an electric field using glass pipette electrodes [17]. 7500 pulses were applied at 25 Hz during 5 min of stimulation. The pulse duration ranged from 6 μs/phase to 6 ms/phase and the electrode diameter ranged from 0.1 to 1 mm. The area of cellular damage was measured by propidium iodide fluorescent staining. The threshold current density was proportional to the reciprocal of the square root of pulse duration. In contrast to earlier reports, cellular damage by the pulsed electric current was not necessarily determined by charge density.

Our results (figure 5) showed that the threshold for a safe charge was not constant but decreased with shorter pulse durations, which is consistent in terms of electrochemical reaction limit with the result of Rose *et al* [18]. This indicates that although phosphenes are elicited effectively by pulses of short duration [19, 20], care should be taken using pulses of short duration.

Our results combined with those of Vankov *et al* [17] suggested that the threshold for a safe current density/ph (mA cm^{-2}/ph) is a reciprocal of the square root of duration (figure 7). If the electrode size is fixed, current/ph (mA/ph) is also a reciprocal of the square root of duration. This relationship is expressed as

$$I^2t = A, \qquad (1)$$

where I is the current, t is the pulse duration and A is a constant.

Energy consumption (J) is expressed as

$$J = IVt = I^2Rt,$$

where I is the current, V is the voltage, R is the impedance and t is the pulse duration. The results, indicating that the threshold energy consumption is almost constant (figure 8), are consistent with equation (1), when R does not change much relative to the duration.

Donaldson and Donaldson further stated that the stimulating current produced negligible heating effect [16] and Mortimer *et al* reported that the lesion of blood–brain barrier of cortex was estimated by watt per square inch [21].

Our results that the threshold energy consumption was almost constant for all pulse durations (figure 8) may suggest that heat affects the retina with suprathreshold energy. But our histological observations showing that inner retinal layers were more damaged than the outer layers (figure 9) suggest that heat is not the main cause of retinal damage because heat may affect the proximal layers of retina (RPE, photoreceptors) more than distal layers. Mechanisms other than heat or electrochemical reaction at the electrode tissue surface may play a role in the retinal damage caused by suprathreshold STS.

In conclusion, the threshold for a safe current for 1 h of STS was higher than that reported by the direct stimulating methods for neural tissues and points to the safety of the STS system. The finding that the threshold for a safe charge was not constant but was lower with short pulse durations indicated that care should be taken using pulses with short duration although phosphenes are generated more effectively with short pulses.

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CLINICAL INVESTIGATION

Evaluation of phosphenes elicited by extraocular stimulation in normals and by suprachoroidal-transretinal stimulation in patients with retinitis pigmentosa

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Abstract
Background To determine the efficient parameters to evoke electrical phosphenes is essential for the development of a retinal prosthesis. We studied the efficient parameters in normal subjects and investigated if suprachoroidal-transretinal stimulation (STS) is effective in patients with advanced retinitis pigmentosa (RP) using these efficient parameters.
Methods The amplitude of pupillary reflex (PR) evoked by transcorneal electrical stimulation (TcES) was determined at different frequencies in eight normal subjects. The relationship between localized phosphenes elicited by transscleral electrical stimulation (TsES) and the pulse parameters was also examined in six normal subjects. The phosphenes evoked by STS were examined in two patients with RP with bare light perception. Biphasic pulses (cathodic first, duration: 0.5 or 1.0 ms, frequency: 20 Hz) were applied through selected channel(s). The size and shape of the phosphenes perceived by the patients were recorded.
Results The maximum PR was evoked by TcES with a frequency of 20 Hz. The brightest phosphene was elicited by TsES with a pulse train of more than 10 pulses, duration of 0.5–1.0 ms and a frequency of 20 to 50 Hz. In RP patients, localized phosphenes were elicited with a current of 0.3–0.5 mA (0.5 ms) in patient 1 and 0.4 mA (1.0 ms) in patient 2. Two isolated or dumbbell-shaped phosphenes were perceived when the stimulus was delivered through two adjacent channels.
Conclusion Biphasic pulse trains (≥10 pulses) with a duration of 0.5–1.0 ms and a frequency of 20–50 Hz were efficient for evoking phosphenes by localized extraocular stimulation in normal subjects. With these parameters, STS is a feasible method to use with a retinal prosthesis even in advanced stages of RPs.

Keywords Artificial retina · Retinitis pigmentosa · Phosphene · Pupillary reflex · Suprachoroidal-transretinal stimulation

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Introduction

Retinitis pigmentosa (RP) is one of the leading causes of blindness in developed countries and is characterized by a progressive degeneration of photoreceptors [15, 20]. In the advanced stage, RP patients have little or no functional vision. To restore some vision of these patients, the strategy

of stimulating the residual retinal neurons by a retinal prosthesis is being extensively studied [14, 31]. Various types of retinal or optic nerve prostheses have been developed, and these have been tested in animals [1, 4, 8, 10, 13, 25, 29, 30] and patients [2, 3, 7, 23]. A typical retinal prosthesis consists of an array of electrodes that is implanted above or beneath the retina and is used to deliver electrical stimulation to the retina to evoke a light sensation called phosphenes.

We have developed a new approach for stimulating the retina called suprachoroidal-transretinal stimulation (STS) [10, 19]. In this method, the retinal prosthesis is placed in a scleral pocket and the reference electrode is inserted into the vitreous. Although the distance between electrodes and the retina is not close compared with other types of artificial retinas, the transretinal currents can stimulate the retina effectively, and the threshold current to evoke electrically evoked potentials by STS is comparable to that by other electrodes [19].

The success of retinal prosthesis to restore vision depends on the presence of physiologically intact retinal ganglion cells (RGCs) that can transmit visual signals to the brain. Morphometric studies of the retinas in RP patients have shown that some of the RGCs are surviving [5, 24, 27]. Postmortem studies of RP eyes have shown that the number of RGC was approximately 30% of that in normal age-matched eyes in the macular area, but only 20% in extramacular regions [5, 24].

Several groups [16, 21] including our laboratory, have used transcorneal electrical stimulation (TcES) to estimate the residual function of RGCs by the threshold current to evoke phosphenes in RP patients, and our studies have shown that the threshold current to elicit phosphenes was significantly higher in a group of RP patients than in normal subjects [17].

Before investigating the effectiveness of using the STS method to stimulate the retina of RP patients, the efficient parameters to evoke phosphenes by electrical pulses should be determined in normal subjects. This was necessary because it might be difficult to determine the most efficient parameters and retinal loci that would respond to the STS stimulation in RP patients under the acute circumstances of acute experiment. Although the population of retinal neurons that are activated by electrical stimulation could be different between normal subjects and RP patients, the evaluation of efficient parameters in normal subjects should be valuable as a preclinical experiment.

For this, we first studied the effect of the frequency of the TcES stimuli on the pupillary constriction in normal subjects. From this, the efficient frequency to stimulate the retina was determined objectively.

Next, we investigated the effect of localized transscleral electrical stimulation (TsES) on eliciting phosphenes in normal subjects to determine the efficient parameters for localized transretinal stimulation. Finally, using these efficient parameters, we investigated the effectiveness of the STS method to elicit phosphenes in two legally blind patients with RP.

Subjects and methods

Normal subjects and retinitis pigmentosa patients

Eight volunteers (34±6 years; mean age ± SD) with no ocular disorders were enrolled in the TcES study, and six other volunteers (42±9 years) with no ocular disorders were enrolled in the TsES study.

Two patients (both 65 years old) with RP were studied. The diagnosis was confirmed by independent ophthalmological and ERG examinations.

All subjects and patients gave an informed consent after a full explanation of the purpose of this study and the procedures to be used. They were free to withdraw at any time. This study adhered to the Declaration of Helsinki and was approved by the Ethics Committee of Osaka University Hospital.

Transcorneal electrical stimulation

Before the TcES, the cornea was anesthetized with 0.4% oxyburocaine hydrochloride, and the cornea was covered with 3% hyaluronic acid and 4% chondroitin sulfate (Viscoat, Alcon Japan Ltd., Tokyo, Japan) to protect it from mechanical injury by the contact lens electrode. After a dark-adaptation period of 15 min, a concentric, bipolar contact lens electrode (Burian-Allen; Hansen Ophthalmic Laboratories, Iowa City, IA) was placed on the cornea, and electric current pulses (20 pulses) were delivered from a stimulator SEN-7203 (Nihonkoden, Tokyo, Japan) and stimulus isolator unit A395 (WPI, Sarasota, FL) through the two electrodes embedded in the contact lens (Fig. 1a).

The electrical stimuli were rectangular, with a train of 20 biphasic (anodic first) pulses of 10 ms duration without an interpulse delay (Fig. 1a). These parameters that elicited phophenes effectively were chosen based on the results of psychophysical experiments on normal volunteers (Matsushita K et al., ARVO abstract 2003). The current intensity ranged from 25 μA to 250 μA, and the frequency ranged from 5 Hz to 50 Hz.

Recording pupillary constriction

An infrared pupillometer, the IRISCORDER C7364 (HAMAMATSU, Hamamatsu, Japan), was used to record the pupillary responses evoked by light stimuli and by TcES. The subjects wore a goggle equipped with an

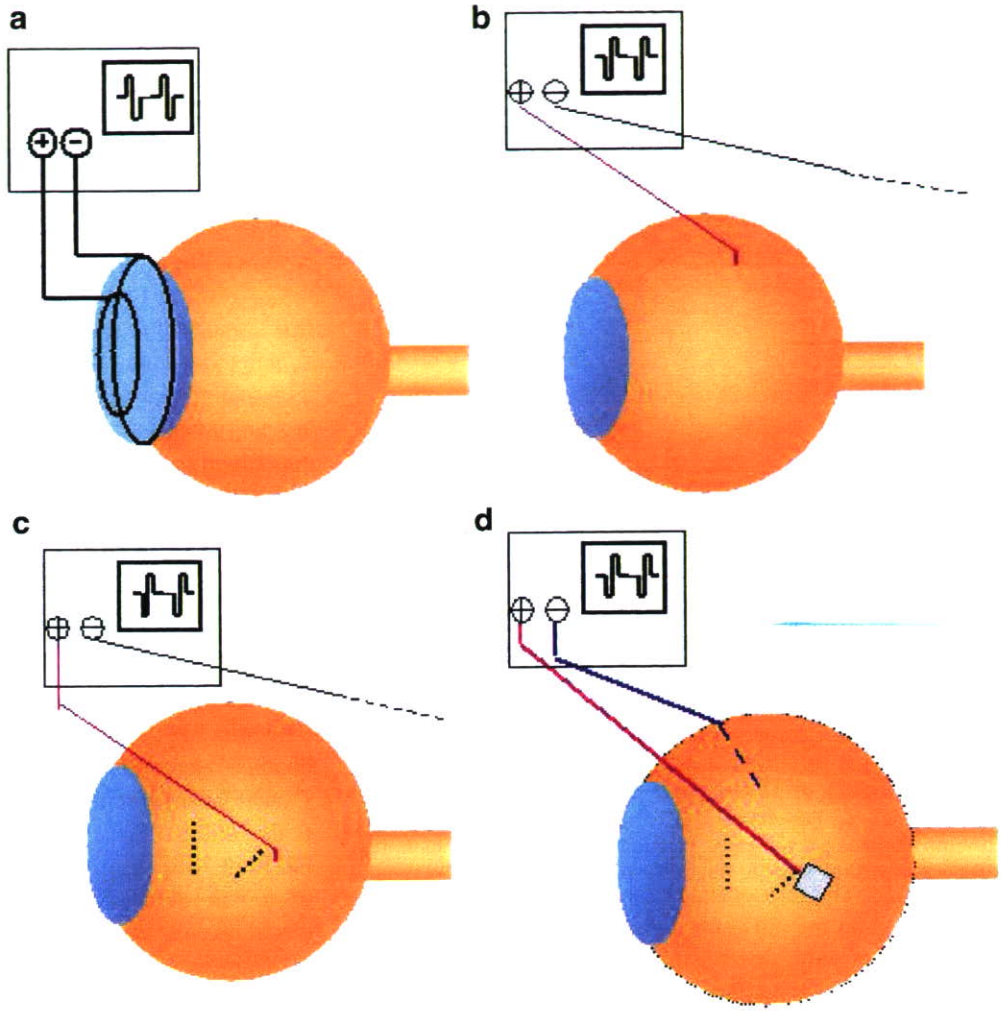

Fig. 1 Schema of different methods to stimulate the retina. **a** Trans-corneal electrical stimulation (TcES); **b** trans-scleral electrical stimulation (TsES); **c** trans-scleral monopolar stimulation in patients; **d** suprachoroidal-transretinal stimulation (STS) in patients

infrared charge-coupled device (CCD) camera and a red light-emitting diode (LED; 660 nm; maximum light power of 10±3 μW; stimulus duration of 0.1 s).

Before inserting the contact lens electrode, the indirect pupillary light reflex was recorded. After inserting the contact lens electrode, the electrically evoked pupillary responses (EEPRs) were recorded from the contralateral eye. The relative amplitude of the EEPR was determined as follows:

$$\text{Relative pupillary constriction(RPC\%)} = 100(a - b)/a$$

where a = pre-stimulus baseline pupil diameter (mm), and b = maximally constricted pupil diameter (mm).

Transscleral electrical stimulation

A stimulating electrode (platinum wire, diameter: 1.0 mm, exposed 1.0 mm at the tip, Unique Medical, Osaka, Japan) was placed on the conjunctiva in the upper temporal quadrant 16 mm to 18 mm from the corneal limbus (Fig. 1b). The conjunctiva was anesthesized with 0.4% oxyburocaine hydrochloride. A return electrode (Ag-AgCl) was placed on the ipsilateral wrist. Pulse trains with charge-balanced biphasic pulses (cathodic first) were applied through the stimulating electrodes.

We examined the relationship between the brightness of phosphenes and the pulse parameters, viz., pulse duration, interpulse delay, frequency and the number of pulse trains, using suprathreshold currents (1.0 to 1.5 mA) (Fig. 2b). Initially, the threshold current to evoke phosphenes was determined with the other parameters fixed at a pulse duration 1 ms, interpulse delay 1 ms, frequency 20 Hz and number of pulses 20. These fixed parameters were chosen based on the results of a preliminary experiment to explore the effective parameters to elicit phosphenes. The relationship between the brightness of the phosphenes and the pulse duration was examined with the injected charge per pulse constant. The pulse duration varied from 0.5 to 4.0 ms while the frequency at 50 Hz and the number of pulses at 20.

The relationship between brightness of the phosphenes and the interpulse delay was examined with the interpulse delay varying from 0 to 4 ms while the pulse duration was

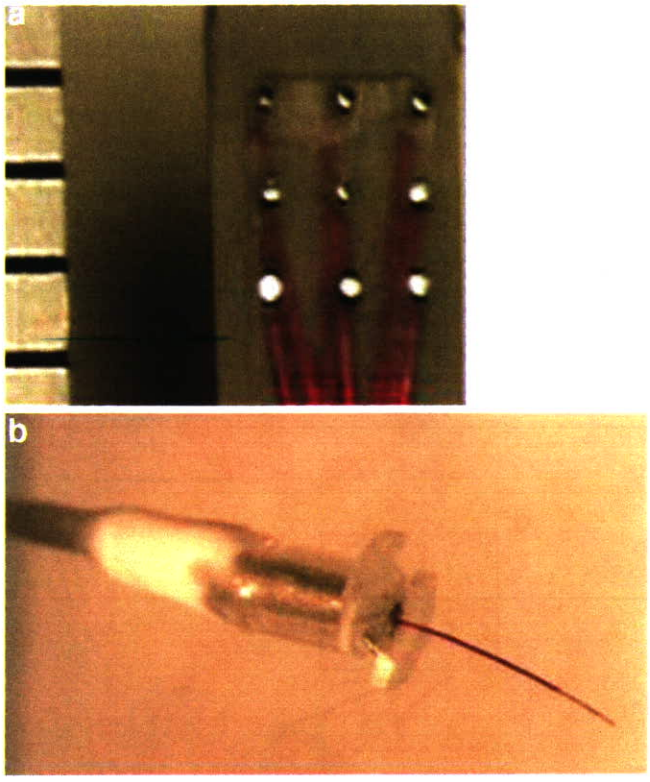

Fig. 2 Photograph of 9-channel stimulating electrode (**a**) and return electrode (**b**). **a**. The diameter of each stimulating electrode was 200 μm and the center-to-center electrode distance was 1 mm. **b** The diameter of return electrode was 100 um

fixed at 1 ms, the frequency at 50 Hz and the number of pulses at 20. The relationship between the brightness of the phosphene and the pulse frequency was examined with the frequency varying from 5 to 100 Hz while the pulse duration was fixed at 1 ms, interpulse delay at 1 ms and the number of pulses at 20. The relationship between brightness of the phosphenes and the number of pulses was also examined with the number of pulses varied from 1 to 50 while the pulse duration was fixed at 1 ms, the interpulse delay at 1 ms and the frequency at 20 Hz.

Subjects were questioned about the brightness and the size of the phosphene for each set of stimulus parameters. The brightness was classified into five grades; the brightest phosphenes during one set of experiments was assigned a value of 5 and the next brightest was 4, and so on. The experiments were conducted systematically with an increase of stimulus parameters (pulse duration, interpulse delay, frequency and the number of pulse trains) with an interval of 5 to 10 s. The number of trials in a unique set of stimulus parameters was generally once, but was two or more when subjects asked for a repetition. Subjects were masked to the test conditions, and the examiner, who was aware of the stimulus conditions, asked questions about the phosphenes. False positive trials (i.e., no stimulus presented) were included to determine the reliability of the responses.

Suprachoroidal-transretinal stimulation in patients with retinitis pigmentosa

Surgical procedure

All procedures were performed under topical anesthesia with 2% lidocaine hydrochloride drops; however, patient 1 required 20 mg of fentanest intravenously at approximately the end of the trial. The total hours including surgery and functional testing was 3 h in patient 1 and 2.5 h in patient 2.

After dissecting the lateral rectus muscle insertion, trial transscleral stimuli were given to determine the scleral area around the insertion of the inferior oblique muscle that consistently evoked low threshold phosphenes (Fig. 1c). The diameter of monopolar platinum electrode was 0.5 mm (Unique Medical, Osaka, Japan). After identifying the low threshold area by monolopar electrode, a scleralpocket of 5×5 mm was created with a crescent knife, and a nine-channel electrode array (size, 4×5 mm, Unique Medical, Osaka, Japan) was inserted into the scleral pocket and secured with sutures (Fig. 1d). The diameter of each platinum electrode was 0.2 mm, and the center-to-center separation of a pair of electrodes was 1 mm. The surface of the electrode protruded from the silicon base by 50 μm (Fig. 2a). A platinum-wire reference electrode (0.1 mm in diameter, 8 mm in length and 3 mm of tip exposed) was inserted into the vitreous cavity through the pars plana (Fig. 2b).

Functional testing

A stimulator was designed to deliver charge-balanced biphasic pulses to individual electrodes simultaneously (Fig. 3a). Biphasic pulses (pulse duration, 0.5 or 1.0 ms; frequency, 20 Hz; interpulse delay, 0.5 ms; number of pulses 20, Fig. 3b) were delivered through the selected channel(s) or combination of multiple channels.

The psychophysical testing was performed under dim room lights. The current stimulation was applied 0.5 s after a conditioning phonic stimulus by a buzzer. The threshold current to perceive a phosphene was determined by increasing the current intensity from 0.1 mA until patients recognized the localized phosphene. The maximum current was limited to 1.0 mA for safety based on the rabbit experiments (Nakauchi et al., ARVO, 2006,47, E-Abstract 3197).

The size and shape of the phosphene that the patients described were recorded. False positive trials (i.e., no stimulus presented) were included to determine the reliability of the responses. The procedure was repeated to