Neuroprotective Effect of Transcorneal Electrical Stimulation on the Acute Phase of Optic Nerve Injury

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Purpose. Traumatic optic neuropathy often induces a loss of vision that proceeds rapidly within several hours, together with retinal ganglion cell death, in a much slower time course. Electrical stimulation has previously been shown to rescue injured retinal ganglion cells from cell death. The present study tests whether transcorneal electrical stimulation could preserve visual function after an optic nerve crush.

Methods. Transcorneal electrical stimulation was given immediately after a calibrated optic nerve crush. We measured visually evoked potentials (VEPs) in the visual cortex of rats before and immediately after the optic nerve crush and after the transcorneal stimulation to estimate an extent of damage and effects of stimulation in individual animals. In addition, the retinal axons were labeled with a fluorescent anterograde tracer to determine whether the transcorneal electrical stimulation can protect the retinal axons from degeneration.

Results. The optic nerve crush was made at an intensity that does not allow a spontaneous recovery of VEP for 1 week. The transcorneal stimulation immediately increased VEP amplitude impaired by the optic nerve crush, and this augmentation was often preserved after 1 week. In the stimulated animals, a larger amount of retinal axons projected centrally beyond the crushed region in comparison to the unstimulated animals.

Conclusions. Transcorneal electrical stimulation would restore the functional impairment of optic nerve by traumatic injury at a very early stage and protect retinal axons from the ensuing degeneration. (*Invest Ophthalmol Vis Sci.* 2007;48: 2356–2361) DOI:10.1167/iovs.06-1329

Traumatic optic neuropathy often induces a loss of vision that proceeds rapidly within several hours, together with retinal ganglion cell death in a much slower time course.[1–4] Electrical stimulation has been shown to exert a neuroprotective effect on retinal cells. The retinal ganglion cells were rescued from cell death after optic nerve transection by direct stimulation of the transected nerve and by transcorneal electrical stimulation (TES).[5,6] Direct retinal stimulation with an implanted prosthetic device preserves the ERG responses in the RCS rats at 4 to 6 weeks after implant surgery.[7] In addition, a recent clinical study showed a possible use of TES for treatment of optic neuropathy.[8] Although these findings suggest that electrical stimulation could be a general neuroprotective treatment for injuries in the visual pathway, the previous experiments demonstrated long-term effects of electrical stimulation on retinal cell survival over several days or weeks. It is not clear whether electrical stimulation is effective for acute impairment of visual function and conduction of neural activity, as observed in traumatic optic neuropathy.

In the present study, we determined whether TES could preserve visual function after an optic nerve crush in rats. To evaluate the effects of the stimulation on functional recovery after the optic nerve crush, we measured visually evoked potentials (VEPs) in the visual cortex before and immediately after the optic nerve crush and after transcorneal stimulation, to estimate the degree of damage and the effect of stimulation in individual animals. We found that transcorneal stimulation enhanced VEP amplitude impaired by the optic nerve crush and this augmentation was often sustained after 1 week.

Methods

Animals and Electrode Implant Surgery

Adult Long-Evans rats (>100 postnatal days) were obtained from SLC Japan, Inc. (Shizuoka, Japan). Thirty-three animals were used in the present experiments. All experimental procedures used were approved by the animal care committee of Tottori University and conformed to the standards of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

All surgeries were performed under sterile conditions. The animals were anesthetized with 3% to 4% isoflurane in 50% N_2O-50% O_2. In the long-term repetitive recording of VEP from the visual cortex, we implanted a stainless steel skull screw over the right primary visual cortex which mainly receives visual inputs from the left eye (3.7 mm lateral and 0.5 mm anterior to the lambda, reference electrode on the head skin). The skin of the head was closed by polypropylene suture (6-0 Prolene; Ethicon, Somerville, NJ) keeping the screw electrode exposed for future recording. During the same surgery, the right eye was removed by reaching behind the exposed eyeball and cutting the optic nerve to prevent a possible modification of cortical responses by binocular interactions (20 animals). The orbit was packed with sterile gelfoam and the overlying skin was closed by suture. The animals were allowed to recover completely from surgery, resting for more than 1 week in individual cages with food and water available ad libitum.

Optic Nerve Crush

After the recovery period, we recorded VEPs evoked by strobe flash stimuli under anesthesia with 3% to 4% isoflurane in 50% N_2O–50% O_2, to estimate intact visual responses of the animal. On the following day, the animals were anesthetized again under the same condition, and the left optic nerve was exposed. After a skin incision was made through the left eyelid over the superior orbital rim, the orbit was opened saving the supraorbital vein, and part of the Harder's gland was removed. Therefore, the muscle cone comprising the ocular muscles was easily accessible. The eyeball was carefully dislocated anteriorly, and

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the ocular muscles around the optic nerve were separated and held apart with forceps.

The exposed optic nerve was crushed with a calibrated pressure. A suture (6-0 polypropylene) was wrapped around the exposed optic nerve at approximately 1 to 2 mm posterior to the globe. One end of the suture was secured with forceps near the optic nerve. The optic nerve was crushed by pulling another end of the suture with a weight that was tied up to the suture (tension given to the suture: 0.01, 0.02, and 0.05 N; duration: 5 seconds). Care was taken not to give additional lateral load and dislocate the optic nerve laterally during the crush surgery. The animals were funduscopically checked for persistent retinal perfusion after the optic nerve crush. The sham-surgery animals received a similar surgery except for the step of loading a weight. We recorded VEPs immediately after the crush and 6 hours and 1 week after the crush and estimated the impairment due to the crush. The VEP amplitude did not recovered significantly even 1 week after the 0.02 and 0.05-N crush in all animals tested ($P > 0.05$, one-way ANOVA followed by Tukey-Kramer test, $n = 5$ in each group), whereas we found a significant recovery of VEP amplitude after the 0.01 N crush in four of the five animals tested. We used the crush of 0.02 N in the following experiments to examine a possible neuroprotective effect of TES.

Transcorneal Electrical Stimulation

We used a contact lens electrode for TES. Two concentric wire electrodes are attached to the inner surface of a contact lens and served as bipolar electrodes (Mayo Corp., Aichi, Japan). Under corneal surface anesthesia by 0.4% oxybuprocaine HCl, the contact lens electrode was placed on the cornea. For corneal protection, 3% sodium chondroitin sulfate was applied when necessary. The electrical stimuli was generated by a stimulator and delivered from a constant current isolator (Master-8; AMPI, Jerusalem, Israel). The stimuli consisted of 20-Hz biphasic square wave pulses (duration: 50 μs, intensity: 500 μA). The stimulus intensity was set to be twice as strong as the threshold intensity determined by recording the stimulus-evoked potentials at the LGN in separate experiments (three animals). Electrical stimulation started immediately after the postcrush VEP recording under the same anesthetic condition and was applied for 6 hours (five animals). For the sham operation, the contact lens electrode was attached to the cornea, and then the animals were kept anesthetized for 6 hours without any electrical stimulation (five animals). Throughout the optic nerve crush procedure, VEP recording, and TES, an ECG was continuously monitored, and the body temperature was maintained at 38°C with a feedback-controlled heating pad.

Electrophysiology

For VEP recording the animals were stimulated with a strobe flash (SLS-3100; Nihon Kohden, Tokyo, Japan) while anesthetized and in a dark-adapted condition. The flash stimuli were presented at 5 cm from the animal's eye, and the intensity was set at 20 J which elicited the maximum VEP. The pupils were dilated with 0.5% tropicamide. The evoked potential was recorded from the screw electrode set over the primary visual cortex using a differential amplifier (filtering: 1 Hz to 10 kHz; model 1800; A-M Systems, Carlsborg, WA) and responses to >100 flashes (interstimulus interval, 3 seconds) were stored in a computer for further analysis (MacLab; AD Instruments, Australia, Castle Hill, NSW, Australia).VEPs were recorded four times, before and after the optic nerve crush, immediately after the TES, and 1 week after the crush in individual animals. The level of anesthesia was monitored by observing heart rate, respiration rate and whether there was an increase in heart rate after a paw pinch, and adjusted to keep the level of anesthesia as constant as possible to prevent a possible variability of VEP due to a change in anesthesia. Repeated recordings of VEP from intact animals in a similar time schedule showed that VEP amplitude did not change significantly under anesthesia for several hours (data not shown).

The obtained VEP waveforms typically represented three components, the first small positive peak, the second large negative peak, and the third slow positive component. To measure the VEP amplitude in individual responses to single flash stimuli, we calculated the height between the minimum and maximum value in the latency windows set for the second and third components, respectively (peak latencies, 38 and 56 ms in intact animals). The latency window of each component had a 10-ms width and was set at the position of each component on the averaged waveform. The waveforms which showed a large deviation and exceeded the recording range were discarded from the analysis. The extent of the recovery of the VEP amplitude after the optic nerve crush was evaluated by calculating the recovery index, which is defined as the ratio of VEP amplitude at 6 hours or 1 week after the crush to the amplitude immediately after the crush. We did not find a significant change in peak latency after the crush and TES.

Histology

To assess the effect of optic nerve crush and TES on the retinal axon morphology, we labeled the retinal axons with a fluorescent anterograde tracer in nine animals (five stimulated and four unstimulated animals). After the last VEP recording, 5 μL of solution containing WGA-Alexa Fluor 594 (Invitrogen-Molecular Probes, Eugene, OR; 4% in 20 mM phosphate-buffered saline [PBS]) was injected into the vitreous body of the left eye with a syringe (Hamilton, Reno, NV). After 4 days, the animals were euthanatized with an overdose of pentobarbital (150 mg/kg, intraperitoneally) and perfused transcardially with saline followed by 4% paraformaldehyde in 20 mM PBS. The optic nerves and brains were postfixed in fixative containing 20% sucrose and sectioned at a freezing microtome in longitudinal and coronal plane, respectively.

A damage of the retinal axons was evaluated by how much tracer had been transported beyond the crushed region of the optic nerve by comparing the fluorescent intensity between the retinal side and the brain side of the crushed region. From the longitudinal sections of each optic nerve, three to five serial sections containing a clear tracer accumulation at the crushed region were selected for this analysis. Fluorescent images of the optic nerves were captured with a cooled charge-coupled device (CCD) camera (VB-7010; Keyence, Osaka, Japan). The regions of interest (ROIs) were set on both sides of the crushed region in each section at the distance of 250 to 300 μm from the crushed region. The fluorescence intensity on the brain side of the crushed region was normalized to the intensity on the retinal side to compensate for the difference in overall labeling intensity in individual optic nerves. The normalized intensity calculated for sections from the same optic nerve was averaged and referred to as the tracer transport index. A larger value of the index indicates a larger amount of tracer transported beyond the crushed region.

Statistics

The VEP amplitude recorded at four time points in individual animals was compared with one-way ANOVA followed by the Tukey-Kramer test for multiple comparisons. The comparison of VEP amplitude between four time point groups in each experimental group was performed with repeated-measures ANOVA, followed by the Dunnett test for multiple comparisons. The unpaired *t*-test was used for other comparisons between two groups.

Results

Effects of Calibrated Optic Nerve Crush

Figure 1B demonstrates the effect of a 0.02-N optic nerve crush in an animal. The amplitude of the VEP decreased immediately after the crush. The VEP showed further attenuation at 6 hours after the crush, during which the animal had been anesthetized. The animal was then recovered from anesthesia and kept in a conventional animal cage. After 1 week after the crush, the VEP had decreased to nearly undetectable levels and was sig-

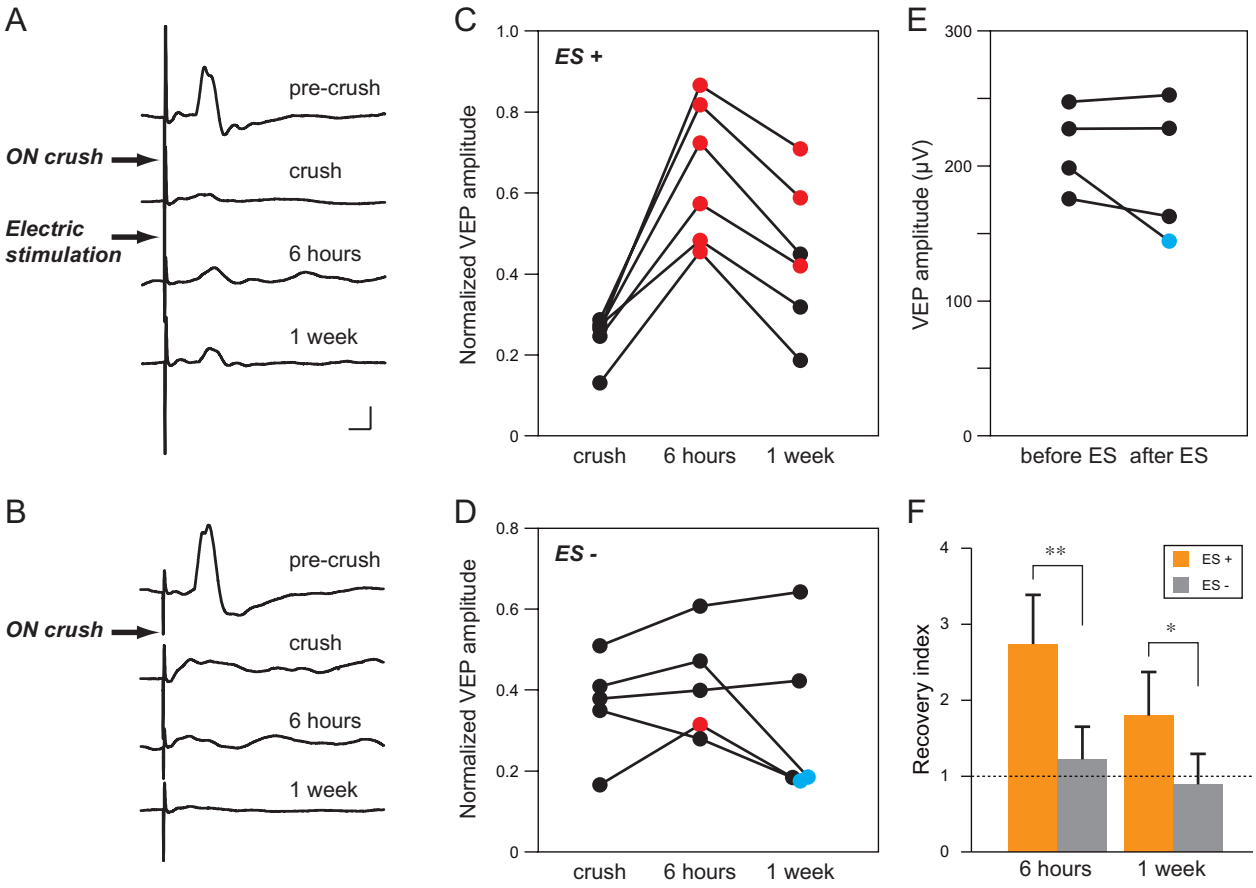

Figure 1. TES restored VEP amplitude impaired by optic nerve crush. Examples of VEP recorded in a stimulated (**A**) and an unstimulated (**B**) animal before (precrush), immediately after (crush), and 6 hours and 1 week after the optic nerve crush of 0.02 N. In (**A**) the electrical stimulation was given immediately after the recording of VEP after the optic nerve crush. Each waveform represents the average of raw recordings (number of raw recordings, **A**: 125 (pre-crush), 124 (crush), 123 (6 hours), and 127 (1 week); **B**: 122, 116, 107, and 120). Scales, 20 ms and 50 μV. (**C**, **D**) The averaged amplitude of the VEP after the optic nerve crush in all stimulated (**C**) and unstimulated (**D**) animals is plotted for each recorded time. The VEP amplitude was normalized to that before the optic nerve crush in the same animal. *Circles* representing the data from the same animal are connected by *lines*. *Circles* indicate that the amplitude is significantly larger (*red*) and smaller (*blue*) than the value immediately after the optic nerve crush (crush), respectively ($P < 0.05$, one-way ANOVA followed by the Tukey-Kramer test). (**E**) The averaged amplitude of the VEP before (before ES) and after (after ES) TES in intact animals. The *lines* connect data from the same animals and a *blue circle* indicates that the value is significantly smaller than the amplitude before ES ($P < 0.05$, unpaired *t*-test). (**F**) The average and SD of the recovery index at 6 hours and 1 week after the optic nerve crush in the stimulated (*orange*, ES+) and unstimulated (*gray*, ES−) animals. *Dotted line*: recovery index showing no recovery of the VEP amplitude after the crush. $^{*}P < 0.01$, $^{**}P < 0.001$ (unpaired *t*-test).

nificantly smaller than that immediately after the crush ($P < 0.005$; one-way ANOVA followed by the Tukey-Kramer test, $n = 5$). The VEP amplitude did not recovered significantly, even after 1 week after the crush in all animals tested ($P > 0.05$).

Effects of TES on VEP

We determined whether an electrical stimulation given to the crushed optic nerve exerts a protective effect on VEP amplitude. In an example shown in Figure 1A, the optic nerve crush immediately attenuated the VEP amplitude, as observed in Figure 1B. Electrical stimulation was given through a contact lens electrode attached to the cornea for 6 hours in rats under anesthesia. After stimulation, VEP amplitude significantly increased and was approximately 200% larger than that immediately after the crush ($P < 0.001$, one-way ANOVA followed by the Tukey-Kramer test). The significant augmentation of the VEP was maintained for 1 week ($P < 0.001$), even though no additional stimulation was given to the animal.

The increase in VEP amplitude immediately after electrical stimulation was observed in all animals tested ($n = 6$; Fig. 1C). We observed the augmented VEP even after 1 week in three cases, although the amplitude tended to be smaller than those at 6 hours in all animals. Statistical analysis confirmed that the VEP amplitude increased significantly at both 6 hours and 1 week after the stimulation ($P < 0.001$ and $P < 0.01$, respectively, repeated-measures ANOVA followed by the Dunnett test). In contrast, we did not find a significant increase of VEP in animals without the electrical stimulation, except for one case which showed a small transient increase at 6 hours (Fig. 1D). To determine the effect of electrical stimulation quantitatively, we estimated the extent of VEP recovery by calculating the recovery index (see the Methods section). The recovery index showed that the VEP amplitude was significantly larger in the animals treated with electrical stimulation than in the unstimulated animals at both 6 hours and 1 week ($P < 0.001$ and $P < 0.01$, respectively, unpaired *t*-test; Fig. 1F). The average amplitude of VEP in the stimulated animals increased to

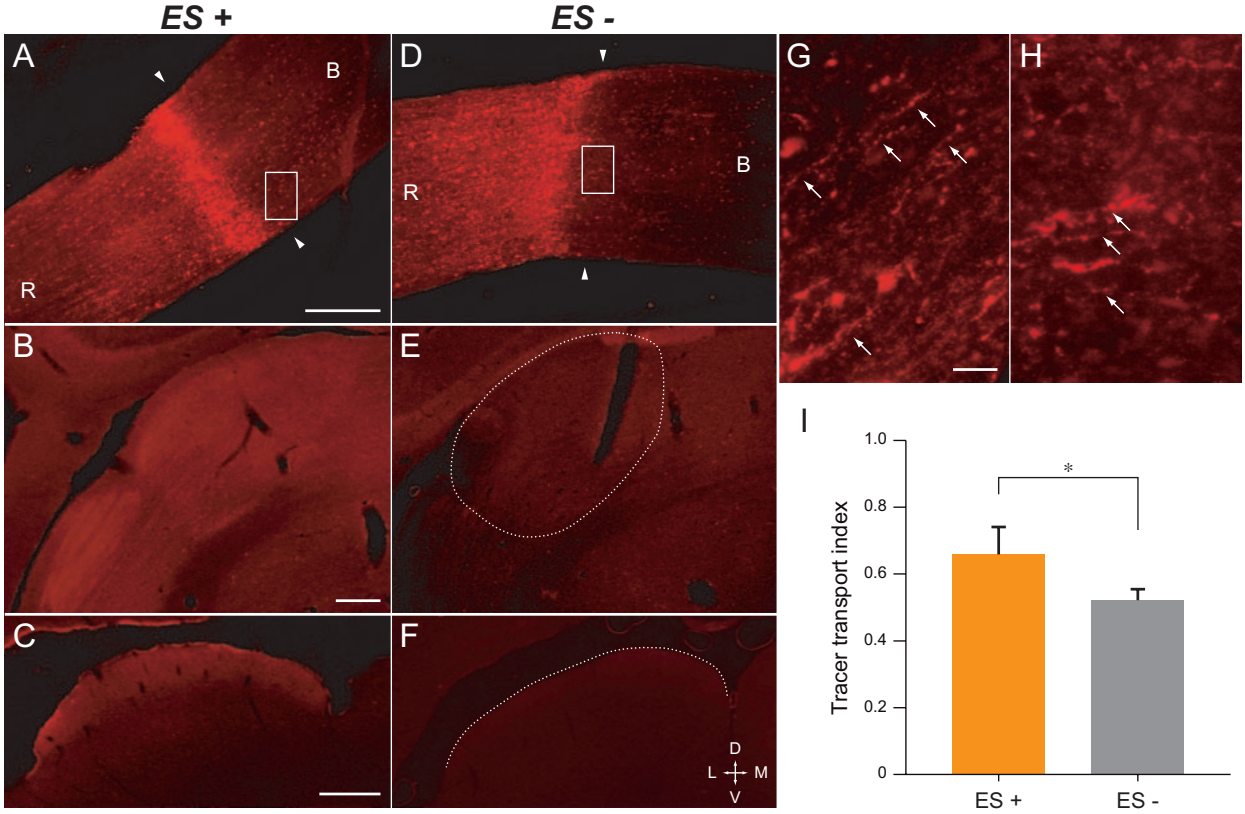

FIGURE 2. Maintained optic nerve fibers after the TES. Representative photographs of the optic nerve and its targets in a stimulated (**A–C**) and an unstimulated (**D–F**) animal after the optic nerve crush. The retinal axons were labeled with an anterograde fluorescent tracer by eye injection. (**A**, **D**) Longitudinal sections of the optic nerves including the crushed region (*arrowheads*). The direction of the nerve is indicated as the retina (R) and brain (B) side of the crushed region. Examples of photographs including the LGN (**B**, **E**) and the SC (**C**, **F**) are displayed in the coronal plane. (**E**, **F**, *white dotted lines*) location of the LGN and the SC, respectively. (**G**, **H**) High-magnification view of the labeled fibers (*arrows*) in regions indicated in (**A**) and (**D**), respectively. The directions of sections are indicated in (**F**) (applies also to **B**, **C**, **E**). (**I**) The average and SD of the tracer transport index in the stimulated (*orange*, ES+) and unstimulated (*gray*, ES-) animals. $^*P < 0.05$ (unpaired *t*-test). Scale bars: (**A**, **B**, **D**, **E**) 250 μm; (**C**, **F**) 500 μm; (**G**, **H**) 20 μm.

273% (6 hours) and 179% (1 week) of the value immediately after the crush. Therefore, TES augmented the VEP that had deteriorated because of the optic nerve crush.

There is a possibility that electrical stimulation augments the VEP regardless of whether the optic nerve is injured. To test this possibility, we measured the VEP amplitude before and after electrical stimulation in animals with intact optic nerves (Fig. 1E). We did not find an increase of VEP amplitude after stimulation in all animals tested ($n = 4$). Therefore, the augmentation of VEP after electrical stimulation should indicate the effect of stimulation on the injured optic nerve. Alternatively, the increase of VEP amplitude in the stimulated animals may simply reflect that the optic nerve crush was milder in them than that in the unstimulated animals. This is not the case, however, because no significant difference was found in the VEP amplitude immediately after the crush between the two groups (24% [stimulated] and 36% [unstimulated] of the precrush amplitude, $P > 0.05$, unpaired *t*-test).

Histologic Effects of TES

To determine whether TES has a protective effect on neural projection, we labeled the retinal axons with a fluorescent anterograde tracer and estimated how much tracer was transported beyond the crushed region of the optic nerve. In an animal that was not treated with electrical stimulation, the transported fluorescent tracer accumulated at the crushed region and labeled fibers, as exemplified in Figure 2H, were seldom observed beyond that region (Fig. 2D). In the lateral geniculate nucleus (LGN) and superior colliculus (SC) of the same animal–areas that are targets of the retinal axons—we were unable to detect a fluorescent signal (Figs. 2E, 2F). In contrast, in an animal treated with electrical stimulation, we found many labeled fibers on the central side of the crushed region (Figs. 2A, 2G). In addition, a clear fluorescent signal was observed in both the LGN and SC of the animal (Figs. 2B, 2C). We estimated how much tracer had been transported beyond the crushed region by calculating the tracer transport index, which compares the fluorescence intensity on each side of the crushed region (see the Methods section). The value of the index was significantly higher in the stimulated animals than in the unstimulated ones ($P < 0.05$, unpaired *t*-test, $n = 5$ stimulated and 4 unstimulated; Fig. 2I). Therefore, TES would rescue the retinal axons from degeneration in addition to improving its functional recovery.

DISCUSSION

The present findings demonstrated that a TES can induce a rapid functional recovery of VEPs from impairment by partial optic nerve injury. Alternatively, there is a possibility that the transcorneal stimulation may have simply affected the depth of anesthesia. The electrical stimulation may have augmented the VEP amplitude by raising the alertness of the animals rather than by restoring the impaired VEP, because depth of anesthesia by isoflurane affects visual cortical responsiveness.[9] This possibility is unlikely, because (1) we did not find a change in

the depth of anesthesia (evaluated as described in the Methods section) during the transcorneal stimulation, (2) the electrical stimulation did not have an augmenting effect on the VEP as demonstrated in intact animals (Fig. 1E), and (3) the augmentation of the VEP was observed again in recordings made 1 week later, in which the electrical stimulation was absent.

It is well known that neural activity can provoke synaptic plasticity, such as long-term potentiation of synaptic efficacy in the visual system. For example, repetitive electrical stimulation of the optic nerve was reported to induce an enhancement of cortical evoked potentials elicited by optic nerve stimulation.[10] Such use-dependent modification of synaptic function is supposed to take place at geniculocortical synapses. Therefore, the augmentation of the VEP after transcorneal stimulation may reflect an enhancement of geniculocortical transmission rather than a restoration of impaired optic nerve function. However, the finding that transcorneal stimulation did not increase the amplitude of the VEP in intact animals (Fig. 1E) strongly argues against that possibility. Furthermore, the use-dependent synaptic plasticity in the visual system is observed only in a restricted period of early postnatal life and cannot be induced in adult animals as used in the present experiments.[11–13]

Crush and transection of the optic nerve damages retinal ganglion cells, leading to apoptotic cell death.[1,2,4,14] Many attempts have been made to rescue the retinal ganglion cells from cell death by various methods—among them, inhibition of apoptotic pathways, the administration of neurotrophic factors, implantation of Schwann cells.[14] In addition, several studies have demonstrated an enhancement of retinal axon regeneration.[14] Transcorneal stimulation, as used in the present experiments, has also been reported to protect retinal ganglion cells against cell death after optic nerve transection.[5] However, the present results could not be explained by the protective effect against retinal ganglion cell death, as described earlier. The death of retinal ganglion cells is minimal during the first week and the number of those cells significantly decreases between 2 and 4 weeks after the injury.[1,2,4,14] In contrast, in the present experiments, the optic nerve crush immediately exerts a deleterious effect on the VEP, and the electrical stimulation given just after the crush restores the VEP amplitude rapidly within hours. Such a rapid improvement in the VEP cannot be explained by inhibition of retinal ganglion cell death, which has a much slower time course. Furthermore, it is highly unlikely that degenerated axons regenerated and restored the functional innervations during the electrical stimulation for 6 hours. The rapid decrease in VEP amplitude after the optic nerve crush may reflect a block of nerve conduction as reported previously,[15] because the retinal functions estimated by ERG remain almost completely intact immediately after an optic nerve injury.[16] The present results, with the previous finding,[5] indicate that electrical stimulation can reverse the acute reduction of VEP amplitude and prohibit the ensuing processes which lead to a permanent impairment of optic nerve function and the retinal ganglion cell death.

Evidence shows that axon injury immediately recruits the molecular machinery that determines later response of the injured cells. For example, the cellular processes of cell death are activated within a few hours after injury of the spinal cord and brain.[17–19] Also, cortical culture neurons exhibit neurite sprouting within a few hours after axotomy.[20,21] Of interest, the suppression of the voltage-dependent Na channel by the administration of tetrodotoxin (TTX) attenuates the damage caused by the spinal cord injury in vivo, if TTX is given at the time of injury.[22] Therefore, it is reasonable to assume that the TES applied immediately after optic nerve crush suppressed long-term damage at an early stage.

The neuroprotective effects of TTX treatment seems at odds with the restoration of impaired VEP by TES, because the two treatments would have opposite effects from each other on neural activity. However, the neural responses induced by axon injury are highly heterogeneous. For example, TTX treatment of cultured cortical neurons suppresses regenerative processes such as axonal sprouting after axotomy,[21] although TTX treatment attenuates the damage by injury as mentioned earlier.[22] Therefore, neuroprotective effects may not simply reflect the strength of neural activity. Extrasynaptic *N*-methyl-D-aspartate (NMDA) receptors mediate excitotoxicity of glutamate, whereas the operation of synaptic NMDA receptors exerts suppressive effects on apoptosis.[23] Electrical stimulation, as used in the present study, is considered to activate the synaptic NMDA receptors more effectively than the extrasynaptic ones. As a result, the neural activation by transcorneal stimulation may thus have restored the impaired VEP in a different mechanism from neuroprotection by TTX.

The transection and stretching of axons induce a significant elevation of intracellular Ca^{2+}.[21,24] Elimination of the Ca^{2+} influx by chelation disturbs the initiation of neurite regrowth in the sympathetic ganglia neurons of the rat.[25] In addition, a reduction of the stretch-induced calcium influx with calcium-free medium or MK-801 results in an increase in the death of cultured hippocampal neurons.[24] In contrast, the Ca^{2+} influx has been reported to activate a cellular pathway that prevents apoptotic cell death.[26] It seems plausible that repetitive transcorneal stimulation induces calcium influx in the retinal ganglion cells by activating the voltage-dependent Ca^{2+} channels and NMDA receptor-mediated transmission, which may therefore trigger the process of Ca^{2+}-mediated neuroprotection.

In conclusion, the present results show that a functional impairment by optic nerve crush can be restored at a very early stage and be protected from the secondary degeneration by TES. Further study to determine the optimal parameters of stimulation and the long-term stability of the effect is thus considered to be important to elucidate the cellular mechanism and to evaluate the therapeutic significance of this effect.

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