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Parameters of optic nerve electrical stimulation affecting neuroprotection of axotomized retinal ganglion cells in adult rats

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Abstract

We previously showed the enhancement of survival of retinal ganglion cells (RGCs) by electrical stimulation (ES) of the optic nerve (ON) stump in adult rats. To elucidate the mechanisms underlying the survival enhancement, we determined whether the neuroprotective effect of ES is affected by the following parameters: stimulation time, frequency of current pulses and starting of ES. ES for 10 min immediately after ON transection was not effective in increasing the number of surviving RGCs 7 days after the transection, but that for 30 min was effective. ES at 20 Hz was the most effective, when applied just after axotomy. When the starting of ES to the ON was shifted either 3 h after or 4 h before the axotomy, the neuroprotective effect of ES was not observed. These results suggest that the electrical activation of RGCs and/or the transected ON interfere with early events after axotomy that leads to RGC death.

Keywords: Retinal ganglion cells; Electrical stimulation; Axotomy; Optic nerve; Neuroprotection; Central nervous system; Adult rat; Survival

1. Introduction

Neurons of the central nervous system (CNS) in adult mammals are generally vulnerable to axonal damage. Among them, retinal ganglion cells (RGCs) are highly susceptible to axotomy-induced cell death (Villegas-Pérez et al., 1993; Berkelaar et al., 1994; Watanabe et al., 1997). Berkelaar et al. (1994) demonstrated that approximately 50% of RGCs undergoes apoptotic cell death at day 7 post-axotomy and less than 10% at day 14 after transecting the optic nerve (ON) behind the eyeball in adult rats. Thus, the ON-sectioning-induced death of rat RGCs has been used as a good model for research on the survival promotion of CNS neurons.

To enhance the survival of axotomized RGCs, several therapeutic approaches have been tested (see reviews: Isenmann et al., 2003; Miyoshi et al., 2006). For example, administration of various neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), neurotrophin-4/5 (NT-4/5) and neurotrophin-3 (NT-3), has been shown to promote RGC survival after ON transection (Mey and Thanos, 1993; Mansour-Robaey et al., 1994; Peinado-Ramon et al., 1996; Klöcker et al., 2000; Nakazawa et al., 2002). Inactivation of caspase cascades, leading to the suppression of apoptosis, also rescued axotomized RGCs from cell death (Kermer et al., 1998; Chaudhary et al., 1999; Kurimoto et al., 2003). Overexpressions of Bcl-2 and Bcl-X_L, members of the anti-apoptotic Bcl-2 family, enhanced axotomized RGC survival (Cenni et al., 1996; Inoue et al., 2002; Malik et al., 2005) conversely, inhibition of Bax, a member of the pro-apoptotic Bcl-2 family, prevented axotomy-induced RGC death (Isenmann et al., 1999; Qin et al., 2004).

We previously showed the survival-promoting effect of electrical stimulation (ES) on axotomized RGCs in adult rats (Morimoto et al., 2002). The rat ON was sectioned intraorbitally and the stump was immediately stimulated for 2 h. One week after the transection, the mean densities of surviving RGCs in the stimulated retinas significantly increased compared with those in the nonstimulated retinas. The enhancement effect of ES on RGC survival depends on the

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current intensity of the ES pulse (below 80 μA), and stronger ES has been shown to induce retinal detachment and degeneration (Morimoto et al., 2002).

The underlying mechanisms by which ES can rescue RGCs from their retrograde death after axotomy are unclear to date. Understanding such mechanisms is important for establishing a therapeutic approach of neural protection by ES. Therefore, in the present study, we examined how the survival-promoting effect of ES is affected by stimulus parameters, such as stimulation time, frequency of stimulation and starting period.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (250–350 g, Japan SLC Inc., Shizuoka, Japan) were used. The animals were cared for in accordance with the guiding principles for the care and use of animals in the field of physiological sciences of the Physiological Society of Japan and guidelines for animal experiments of Osaka University. Animals were kept in a 12-h light/12-h dark cycle room and provided feed and water ad libitum. For all surgical procedures, the animals were anesthetized via the inperitoneal injection of ketamine (75 mg/kg B.W.) and xylazine (10 mg/kg B.W.).

2.2. RGC labeling

To identify surviving RGCs after ON transection and ES, RGCs were prelabeled retrogradely with a fluorescent tracer, Fluorogold™ (FG; Fluorochrome Llc, Denver, USA). The anesthetized animals were fixed on a surgical plate with a nose clam. Craniotomy was performed on the bilateral posterior parietal bones, and the occipital cortex was aspirated gently to expose the dorsal surface of the superior colliculi (SC), avoiding damage to the superior sagittal sinus and SC. The exposed surface was covered with small pieces of Spongel™ (Yamanouchi, Tokyo, Japan) soaked in a 2% solution of FG in 0.9% NaCl and 10% dimethylsulfoxide.

2.3. Optic nerve transection and ES

One week after RGC labeling, ON transection and ES were performed. In this study, only one ES was delivered before or after cutting the ON. The animals were anesthetized and held on the surgical plate and the left orbit was exposed. In the ES delivered after ON transection, the optic nerve sheath was incised longitudinally, then the ON was transected approximately 3 mm posterior to the eyeball.

Immediately or 3 h after the transection, a pair of silver disk electrodes (each approximately, 1 mm in diameter) was attached to the proximal stump of the ON (see Supplementary Fig. 1A), and ES was delivered with monophasic square pulses (current intensity: 50 μA; pulse duration: 50 μs; frequency: 10, 20, and 50 Hz; stimulation duration: 10, 30, 60, and 120 min) from an isolated constant-current stimulator (Stimulator: SEN-7203, Isolator: SS-202J, Nihon Kohden, Tokyo, Japan).

In the case of the ES delivered before ON transection, after making a longitudinal incision on the nerve sheath, a pair of stainless paddle like-shaped electrodes (each 0.5 mm width × 2 mm length) was placed between the ON and the sheath to clip but not to compress the ON (see Supplementary Fig. 1B), and then ES was delivered (current intensity: 300 μA; pulse duration: 50 μs; frequency: 20 Hz; stimulation time: 60 min). Three hours or just after ES, the ON was cut. Sham stimulations, in which the electrodes were attached to the ON but the currents were not delivered, were performed as a control for each experiment. In these surgeries, care was taken not to disturb the retinal blood flow. When delivering ES, small pieces of parafilm™ were inserted between electrodes and neighboring tissues of the ON, then the orbit was filled with mineral oil (M8410, Sigma–Aldrich, St. Louis, USA) to prevent current leakage to surrounding tissues and to prevent drying up of the ON stump.

2.4. Tissue processing

Seven or 14 days after ON transection and ES, the animals were deeply anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PFA, pH 7.4). Both eyes were enucleated and small incisions were made at the dorsal pole of the eyes for retinal orientation. The retinas were dissected from the eyes and stored in 0.01 M phosphate-buffered saline (PBS, pH 7.4), and four radial cuts were made to flatten the retinas. The retinas were attached to pieces of filter paper and postfixed in 4% PFA, and then rinsed in PBS overnight and mounted on glass slides.

2.5. RGC densities

To calculate the mean densities of surviving RGCs, the number of FG-labeled RGCs was counted using a fluorescence microscope (Axioskop, Carl Zeiss, Oberkochen, Germany) under UV excitation (365 nm). Along the naso-temporal and dorso-ventral axes (nasal, temporal, dorsal and ventral), 3 areas (0.5 mm × 0.5 mm) at 1, 2 and 3 mm from the optic disc were selected as counting areas using a microscanner (Sapporo Beer, Saitama, Japan). The mean density of RGCs was calculated from the number of surviving RGCs counted in the 12 areas of every retina.

2.6. Statistical analysis

Density data were presented as mean ± S.D. and were statistically analyzed using SigmaStat™ software (version 3.11 for Windows, Systat Software Inc., CA, USA). One-way analysis of variance (ANOVA) and Kruskal–Wallis one-way ANOVA on Ranks were used to compare values among groups. Tukey–Kramer's procedure was used as a post hoc test of one-way ANOVA. Dunn's procedure was used as post hoc test of the Kruskal–Wallis one-way ANOVA on Ranks. The level of statistical significance of both one-way ANOVA tests was taken as $P < 0.001$, and that of both post hoc tests was taken as $P < 0.05$.

2.7. Images

Images of FG-labeled RGCs in flat mounted retinas were observed with a fluorescence microscope using a UV filter (365 nm) and imported with a cooled CCD camera and imaging software (camera: CoolSNAP, software: COOLSNAP version 1.2.0 for Macintosh, Nippon Roper, Tokyo, Japan). The resolution of imported image files was set to 300 dpi, and these images were resized and integrated into one image, then contrast was increased under the same parameter using Photoshop software (version 6.0 for Windows, Adobe Systems Inc., CA, USA).

3. Results

3.1. Densities of RGCs in intact and axotomized retinas

Fig. 1 shows fluorescence images of an intact retina (A) and a retina 7 days after ON transection (B). As shown in Fig. 1A, the somas of intact RGCs showed round-shaped outlines with puncta of FG in their cytoplasm. In the retina 7 days after ON transection (Fig. 1B), the number of surviving RGCs decreased and apoptotic cells (*arrows*), debris of dead cells (*arrowheads*) and phagocytic cells that phagocytized dead RGCs (*asterisks*) appeared with FG labeling. These FG-labeled cells were distinguished from surviving RGCs and excluded from the counting of the number of RGCs using the fluorescence microscope. The densities of RGCs in intact and axotomized retina are shown in Fig. 2. The mean ± S.D. density was 2367 ± 109 cells/mm^2 ($n = 18$) in the intact retinas, whereas that in the retinas 7 days after ON transection was 1338 ± 90 cells/mm^2 ($n = 12$), decreasing to 57% of the intact