Restoration of Visual Response in Aged Dystrophic RCS Rats Using AAV-Mediated Channelopsin-2 Gene Transfer

Hirosi Tomita,1 Eriko Sugano,1 Hiromi Yawo,1,2 Toru Ishizuka,2 Hitomi Isago,1 Satoko Narikawa,1 Sebastian Kügler,3 and Makoto Tamai1

PURPOSE. To investigate whether the channelopsin-2 (Chop2) gene would restore visual responses in 10-month-old dystrophic Royal College of Surgeons (aged RCS; rd/y/rd/y) rats, the authors transferred the Chop2 gene into the retinal cells of aged RCS rats using the adenoassociated virus (AAV) vector.

METHODS. The N-terminal fragment (residues 1–315) of Chop2 was fused to a fluorescent protein, Venus, in frame at the end of the Chop2 coding fragment. The viral vector construct (AAV-Chop2V) for the expression of the Chop2 protein in the retina was made by subcloning into an adenoassociated virus vector, including the CAG promoter. To evaluate the expression profile of Chop2 in the retina, the rats were killed and the eyes were removed and fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline. Retinal wholemount specimens and cryosections were made. Under anesthetized conditions, electrodes for the recording of visually evoked potentials (VEPs) were implanted onto the visual cortex in aged-RCS (rd/y/rd/y) rats. AAV-Chop2V vectors were then injected into the vitreous cavity of the left eyes. As a control, AAV-Venus vectors were applied to the right eyes. VEPs were evoked by the flash of a blue, white, or red light-emitting diode (LED) and were recorded from the visual cortex of the rats at various time points after the AAV vector injection.

RESULTS. Chop2V fluorescence was predominantly observed in retinal ganglion cells (RGCs). Some fluorescence was observed in the inner nuclear layer and the inner plexiform layer neurites. A tendency of recovery was observed in the VEPs of aged RCS (rd/y/rd/y) rats after the AAV-Chop2V injection but not after the AAV-Venus injection. The visual response of AAV-Chop2V-injected aged RCS (rd/y/rd/y) rats was less sensitive to the blue LED flash than that of nondystrophic RCS (+/+). The AAV-Chop2V-injected aged RCS (rd/y/rd/y) rats were insensitive to the red LED flash, which evoked a robust VEP in the RCS (+/+).

CONCLUSIONS. The visual response of aged RCS (rd/y/rd/y) rats was partially restored by transduction of the Chop2 gene through AAV into the inner retinal neurons, mainly RGCs. These results suggest that the transduction of Chop2 would provide a new strategy to treat some retinitis pigmentosa (RP) symptoms independent of their etiology. (Invest Ophthalmol Vis Sci. 2007;48:3821–3826) DOI:10.1167/iovs.06-1501

Retinitis pigmentosa (RP) refers to a group of diseases in which a gene mutation results in the death of rod photoreceptors followed by gradual death of the cones. Approximately 1 in 4000 people is affected by this disease. Symptoms include night blindness, loss of the peripheral visual field, and loss of central vision. A number of genes responsible for RP have been identified, most of them related to phototransduction pathways (http://www.sph.uth.tmc.edu/Retnet/home.htm). However, these findings have not led to the discovery of effective treatment or prevention.

Although photoreceptor cells are often degenerated in the eyes of RP patients, other retinal neurons, including retinal ganglion cells (RGCs), are preserved. Some neurons have been made photosensitive by genetic engineering to express a Chlamidomonas-derived photoreceptor channel apoprotein, channelopsin-2 (Chop2). Recently, Bi et al. reported that the RGC expression of Chop2 restores the visually evoked cortical responses in the rd1/rd1 mouse, one of the animal models of RP. To further investigate whether this method might provide a treatment for RP, we tested the effects of the retinal expression of Chop2 on aged Royal College of Surgeons (RCS; rd/y/rd/y) rats, another classic animal model of recessively inherited RP with a different mutation. Results showed that Chop2 expression also restored the visual response in aged RCS (rd/y/rd/y) rats. It is thus suggested that the transduction of Chop2 would provide a new strategy to treat some RP symptoms independently of their etiology.

MATERIALS AND METHODS

Animals

Fourteen 10-month-old male RCS rats (10 dystrophic, rd/y/rd/y; 4 nondystrophic, +/+ ) were used in this study. The use of animals in these experiments was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Guidelines for Animal Experiments of Tohoku University. All animal experiments were conducted with the approval of the Animal Research Committee, Graduate School of Medicine, Tohoku University.

Preparation of Adenoassociated Virus Vector Carrying the Chop2 Gene Construct

The N-terminal fragment (residues 1–315) of Chop2 (GenBank accession no. AF461397) was fused to a fluorescent protein, Venus, in frame at the end of the Chop2 coding fragment described previously. The gene of Chop2-Venus (Chop2V) was introduced into the EcoRI and HindIII sites of the 6P1 plasmid (6P1-Chop2V). The synapsin promoter was exchanged for a hybrid cytomegalovirus (CMV) enhancer/chicken β-actin promoter (CAG). The pAAV-RC and pHelper plas-

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mids were obtained from an AAV Helper-Free System (Stratagene, La Jolla, CA). Semiconfluent 293T cells on 15-cm plates were cotransfected with split packaging plasmids—6P1CAG-Chop2V plasmid, pAAV-RC, and pHelper—by a calcium phosphate-based protocol according to the manufacturer’s instructions (Stratagene). The rAAV vectors (Fig. 1A) were purified by the single-step column purification (SSCP) method of Auricchio et al.16,17

AAV Vector Injection
To evaluate the expression profile of Chop2V, AAV-Chop2V vectors were intravitreally injected into both eyes of 10-month-old RCS (rdy/rdy) rats. To record the visually evoked potentials (VEPs) in the rats, the AAV-Chop2V vector was intravitreally injected into the eye, and the control AAV vector carrying the Venus gene alone (AAV-Venus) was injected into the contralateral eye. Rats were anesthetized by intramuscular injection of a mixture of ketamine (66 mg/mL) and xylazine (33 mg/kg). Under an operating microscope, an incision was made into the conjunctiva to expose the sclera. Five microliters of viral vector suspension at the concentration of 1 to 10×10^12/μL genomic particles was intravitreally injected through the ora serrata with a 10-μL Hamilton syringe with a 32-gauge needle (Hamilton Company, Reno, NV).

Retrograde Labeling of RGCs with the Fluorescent Tracer
To identify RGCs in the ganglion cell layer (GCL), retrograde labeling was performed 7 days before the rats were killed. Labeling was performed by injecting 4 μL of 2% aqueous fluorogold (Fluoro-Gold; Fluorochrome, Englewood, CO)18 containing 1% dimethylsulfoxide (DMSO) into the superior colliculus using a Hamilton syringe with a 32-gauge needle.19

Chop2V Expression Profile in the Retina
Two or 6 weeks after injection, the rats were killed, and the eyes were removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. Retinas were flatmounted on slides. Contralateral eyes were embedded in OCT compound (Sakura, Tokyo, Japan) after immersion in 30% sucrose solution with PBS. Fifteen-micrometer retinal sections were made and mounted on slides. Slides of retinal wholemount and sections were covered with medium (Vectashield; Vector Laboratories, Burlingame, CA). Venus fluorescence was visualized under a fluorescence microscope (Axiovert 40; Carl Zeiss, Oberkochen, Germany). For retinal wholemount, images from the surface of the ganglion cell layer to a depth of 10 μm at intervals of 0.5 μm were obtained in the Z-axis scan mode with an electric motor, and all images were stored.

**FIGURE 1.** Expression profile of Chop2V in the retina. (A) Construction of the AAV vector expressing Chop2V. CAG, a hybrid CMV enhancer/chicken β-actin promoter; WPRE, woodchuck posttranscriptional regulatory element; bGHpA, a bovine growth hormone polyadenylation sequence. (B) Chop2V expression in the wholemounted retina 6 weeks after Chop2V-AAV vector injection. (C) Fluorogold-labeled RGCs in the same area. (D) Merged photograph of the fluorogold and the Chop2V fluorescence. Most of the Chop2V-expressing cells were colabeled by the fluorogold. (E) Fluorescence microphotograph at a depth of 7 μm from the retinal surface in the same field as (B). Vertical section of the Chop2V-injected retina (F, Chop2V; G, fluorogold. H, merged fluorescence and Nomarski image). (I) Transduction efficiency of Chop2V in the RGCs. Fluorogold-positive RGCs with and without Chop2V fluorescence were respectively counted in the wholemounted retina under microscopy. Chop2V expression was observed in 27.7% ± 9.4% of RGCs (n = 3).
Under fluorescence microscopy, both the number of fluorogold-positive RGCs and the number of double-positive RGCs with fluorogold and Venus fluorescence counted in four distinct areas. Each area (0.144 mm²) was selected at 1 mm from the optic nerve. These numbers were used to estimate the transduction efficiency of Chop2V-AAV.

### Recording of Visually Evoked Potentials and Electroretinograms

VEPs and Electroretinograms (ERGs) were recorded using a Neuropack (MEB-9102; Nihon Kohden, Tokyo, Japan). The method of recording VEPs was derived from a combination of the protocols used by Papatheorou et al. and Iwamura et al. Briefly, at least 7 days before the experiments, recording electrodes (silver-silver chloride) were placed epidurally on each side 7 mm behind the bregma and 3 mm lateral of the midline, and a reference electrode was placed epidurally on the midline 12 mm behind the bregma. Under ketamine-xylazine anesthesia, the pupils were dilated with 1% atropine and 2.5% phentylephrine hydrochloride. The ground electrode clip was placed on the tail. Photic stimuli of 20-ms duration with various intensities were applied with a frequency of 0.5 Hz. Photic stimuli were generated by pulse activation of a blue light-emitting diode (LED) with light emitting wavelengths of 435 to 500 nm (peak, 470 nm) or a red LED of 580 to 640 nm (peak, 625 nm). A white LED (7500 K) was used for white stimuli (a white LED includes multiple wavelengths; therefore, degrees Kelvin was generally used as the unit). The high- and low-pass filters were set to 50 kHz and 0.05 kHz, respectively. One hundred consecutive response waveforms were averaged for the VEP measurements. ERG recording was performed as described previously. Briefly, rats were dark adapted overnight, the pupils were dilated with 1% atropine and 2.5% phentylephrine hydrochloride, and the corneas were anesthetized with 0.5% propacaine hydrochloride. Small contact lenses with gold wire loops were placed on both corneas, and a silver wire reference electrode was placed subcutaneously between the eyes. Flash light stimuli of 10-ms duration were generated by pulse activation of a blue or white LED. Full-field scotopic ERGs were recorded, band-pass filtered at 0.3 to 500 Hz, and averaged for five responses at each light intensity.

### Statistical Analysis

Statistical analysis was performed (GraphPad Prism; GraphPad Software, San Diego, CA). The criterion for statistical significance was \( P < 0.05 \). The statistical method of each experiment was used paired \( t \)-test, Dunnnett multiple comparison test, or unpaired \( t \)-test for the time-dependent improvement of VEP (see Fig. 3A), the intensity-response relationship of VEPs (Fig. 3B), or the summary of VEP amplitude (see Fig. 3C), respectively.

### RESULTS

#### Expression of Chop2 in the Retina

Two weeks after AAV-Chop2V was injected intravitreally into the eyes of aged RCS rats, we could observe the expression of Chop2V in the retina (data not shown). Numerous retinal cells became fluorescent 6 weeks after injection (Fig. 1B). Some of these cells were identified as RGCs, because they were labeled with the fluorogold applied to the superior colliculus of the rats 7 days before experiments (Figs. 1C, 1D). Chop2V fluorescence was intense at the RGC contour, suggesting preferential expression in the plasma membrane. At the depth of 7 \( \mu m \) from the surface of the GCL, Chop2V was seen in the neurites (Fig. 1E). When the vertical section of the retina was examined, Chop2V fluorescence was observed in the GCL, the inner plexiform layer, and even the inner nuclear layer (Figs. 1F–H). In summary, among fluorogold-positive RGCs, 27.7% \pm 9.4% expressed Chop2V fluorescence (Fig. 1I). It has previously been reported that the outer retina is specifically degenerated in aged RCS (rdy/rdy) rats. Therefore, we also examined the vertical cryosections of the retina of aged RCS (rdy/rdy) rats to determine the cellular architecture of the outer retina. The outer nuclear layer was almost lost in all specimens examined 6 weeks after injection of either AAV-Chop2V (\( n > 20 \) slides; 2 eyes from 2 animals) or AAV-Venus (\( n > 20 \) slides; 2 eyes from 2 animals).

### Recording of Visually Evoked Potentials and Electroretinograms in RCS Rats

Although the RGCs are maintained in aged RCS (rdy/rdy) rats, the VEPs are expected to be abolished because of the loss of light-evoked synchronous activities caused by the photoreceptor cells. Indeed, the VEPs were not evoked even by the maximum flash of LED in any of the aged RCS (rdy/rdy) rats or those injected with the AAV-Venus vector (Fig. 2A, upper left). On the other hand, robust VEPs were evoked by the flash of blue LED when the aged RCS (rdy/rdy) rats were injected with the AAV-Chop2V vector. The maximum flash of a white LED induced small amplitude of VEPs (Fig. 2A, lower left). White flash includes multiple wavelengths. We measured the intensity of the blue component (400–500 nm) included into a white LED. The intensity of the blue component in the white flash of the 7500 lux was approximately 1000 lux. Therefore, small amplitudes evoked by 7500 lux of the white flash were expected to be caused by the blue component. Given that channelrhodopsin-2 exhibits responses to light activity within a spectrum of 400 to 520 nm,\(^{26}\) it is expected to be insensitive to red LED light (spectrum, 580–640 nm). When the AAV-Chop2V vector-transduced eyes of aged RCS (rdy/rdy) rats were illuminated by the red LED, no indication of VEP was detected even at the maximum intensity (Fig. 2A, lower left). This is in contrast to the case of nondystrophic RCS (+/+) rats, in which the VEP evoked by red LED stimulation was as large as that by blue LED stimulation (Fig. 2A, lower right). To test the possibility that the AAV-Chop2V injection facilitated the functional recovery of the outer retina of aged RCS (rdy/rdy) rats, the flash-induced changes of ERG were investigated. As shown in Figure 2B, the response of ERG to blue or white LED stimulation was negligible in the AAV-Chop2V-transduced eyes of aged RCS (rdy/rdy) rats.

Previous studies have shown that degeneration of the outer retina progresses with age and is irreversible in aged RCS (rdy/rdy) rats.\(^{23–24}\) Nevertheless, we found that the VEPs of the treated aged RCS (rdy/rdy) rats had a tendency to recover gradually after the intravitreal injection of AAV-Chop2V (Fig. 3A). The VEP amplitude became significantly manifest as early as 2 weeks after injection and had a tendency to decrease progressively after the injection, and the difference became significant at 6 weeks (Fig. 3A).

Because increases in the intensity of LED light have been shown to have a positive effect on the Chop2-dependent photocurrent in the transduced neurons,\(^6\) its relationship to the VEP was investigated on the aged RCS (rdy/rdy) rats injected with the AAV-Chop2V vector. We found that the visual system was activated by a broad range of light intensity; the VEP amplitude had a positive relationship (Fig. 3B), whereas its latency had a negative relationship (Fig. 3B). However, even 6 weeks after AAV-Chop2V injection, the visual response of the aged RCS (rdy/rdy) rats was less sensitive than that of the nondystrophic RCS (+/+) rats (Fig. 3C).

### DISCUSSION

The present study demonstrated that Chop2 expression in the retina partially restored the visual response of aged RCS (rdy/
rdy) rats based on the following three lines of evidence. First, a large number of retinal neurons, particularly RGCs, were shown to express Chop2 after transduction with the AAV-Chop2V vector. Second, the VEPs were evoked in aged RCS (rdy/rdy) rats treated with the AAV-Chop2V vector but not in another group of animals lacking expression of Chop2. Third, the VEPs from AAV-Chop2V vector-treated aged RCS (rdy/rdy) rats showed a preference for blue light but not for red. As with the human ortholog of RP,12,13,27 the primary pathophysiology has been attributed to the inability of retinal pigment epithelium to digest the shed outer segments of photoreceptor cells.23,25 Subsequently, almost all rods are degenerated by postnatal day (P)100.24 Although the cones remain photosensitive without their outer segments,28 the light responses disappear completely in P90 to P300.29 Taken together, these results suggest that the retinal neurons avoiding degeneration become photosensitive by the expression of Chop2 and transmit visual signals to the visual cortex.

The ability of Chop2 expression in the degenerated retina to restore visual responses has been more thoroughly studied in photoreceptor-degenerated rd1/rd1 mice,9 another animal model of human retinitis pigmentosa.9,30 In the rd1/rd1 mouse, a defect in the β subunit of rod cyclic guanosine

![Figure 2](image-url)  
**Figure 2.** VEPs and ERGs from Chop2V-injected aged RCS rats. (A) Representative VEPs from RCS rats; the blue, white, or red LED flash-evoked VEPs from rats injected with AAV-Venus (upper left). AAV-Chop2V-injected rats (lower left), and a nondystrophic RCS (+/+ ) rat. Wavelengths of the emitted light are: 435 to 500 nm (peak, 470 nm) for the blue LED and 580 to 640 nm (peak, 625 nm) for the red LED. A white LED (7500°K) was used for white stimuli. LEDs were flashed for 20 ms with their maximum light intensities (blue LED, 3500 lux; white LED, 7500 lux; red LED, 2000 lux). (B) The ERG evoked by a blue or white LED flash (duration, 10 ms; light intensity, 10, 300, and 900 lux) from nondystrophic RCS (+/+ ) rats or from an aged RCS (rdy/rdy) rat injected with AAV-Chop2V or AAV-Venus.

![Figure 3](image-url)  
**Figure 3.** Recordings of VEPs from AAV-Chop2V–injected aged RCS (rdy/rdy) rats. (A) Time-dependent improvement of VEPs after AAV-Chop2V injection. Each data point is the mean ± SD (n = 4) of the blue LED responses (duration, 20 ms; light intensity, 3500 lux). Statistical significance was tested in comparison with the data 1 week after injection (paired t-test; single symbols, P < 0.05; double symbols, P < 0.01). (B) Intensity-response relationship of VEPs from AAV-Chop2V–injected aged RCS (rdy/rdy) rats. The VEP was evoked by a blue LED flash (20 ms) of various light intensities. Each data point is the mean ± SD (n = 4) from the aged RCS (rdy/rdy) rats 6 weeks after injection of the AAV-Chop2V. Statistical analysis was performed using Dunnett multiple comparison test compared with the data of 250 lux light intensity (single symbols, P < 0.05; double symbols, P < 0.01). (C) Summary of VEP amplitude (open column). Data from nondystrophic RCS (+/+ ) rats is shown as a control (filled column). Data are the mean ± SD (n = 4). Significant differences compared with the control are indicated (unpaired t-test; **P < 0.01). In all cases, VEP amplitudes were measured from positive 1 to negative 1, and VEP latency was between the flash onset and positive 1.
monophosphate phosphodiesterase is the primary cause of retinal degeneration. Significant remodeling is secondarily induced in the inner retina of rd1/rd1 mice. The inner retina is also remodeled in the RCS rat with different morphology. The synapses are generally reduced in the remodeled inner retina of the RCS rat. Therefore, both the present paper and the previous one have proved that visual signals are transmitted to the visual cortex when the retinal neurons are transformed to be photosensitive, irrespective of remodeling after the degeneration of photoreceptor cells. In other words, the Chop2 gene transfer could be applicable to restore vision for RP patients independently of their mutations.

Many investigators have examined the use of retinal prostheses to restore vision. Some of these groups have reported on the use and outcome of electrical stimulation of the retina by retinal prosthesis in patients with RP or macular degeneration. These studies showed that some degree of visual function could be achieved by applying appropriate electrical stimulation to the retina. These experiments, based on the neural network in the inner retina of RP patients, are preserved. The restoration of visual function with the Chop2 is also implemented on the same basis as retinal prosthesis. Visual signals are transmitted to the visual cortex after retinal degeneration. It has been reported that the receptive field size gradually decreases and that the threshold of light intensity increases before photoreceptor degeneration. The reason for the smaller amplitudes in the present study may be that we used 10-month-old RCS (rdy/rdy) rats. However, in the present study, the amplitudes of VEPs were gradually increased after injection. This might have been attributed to retinal plasticity because the inner retinal neurons in the aged RCS (rdy/rdy) rats remain at ages up to 12 months. When using an AAV vector, including the CAG promoter, Chop2 was mainly expressed in the RGCs. Visual signals are transmitted to the visual cortex by ON- and OFF-type RGCs, with distinct receptive field patterns in the nondegenerated retina. However, the Chop2-expressing RGCs would lose their type-specific receptive field pattern in the degenerated retina. How this would influence visual perception remains to be elucidated because the afferent connections are reorganized in the visual cortex after retinal degeneration. It has been reported that pharmacologic inhibition of the ON-channel does not severely affect the perception of shape and light decrement. Therefore, some readjustment of the visual cortical network would compensate for the loss of the type-specific receptive field pattern of RGCs. Future studies using primates will be needed to evaluate the effectiveness of the genetic endowment of RGC photosensitivity on visual perception.

We found that the visual response of AAV-Chop2 injected aged RCS (rdy/rdy) rats was less sensitive to the blue LED flash than that of nondystrophic RCS (+/+). The AAV-Chop2 inoculated aged RCS (rdy/rdy) rats were insensitive to the red LED flash, which evoked a robust VEP in the nondystrophic RCS (+/+), as suggested by the action spectrum of channelrhodopsin-2 (400–520 nm). In addition, the VEP waveform was different between the Chop2-transfected aged RCS (rdy/rdy) and the nondystrophic RCS (+/+) rats (Fig. 2A). The waveform might indicate that the feedback response of the visual pathway in the Chop2-transfected aged RCS (rdy/rdy) rat was different from that in normal rat. At present, it is unclear whether the visual pathway is induced by the Chop2 gene transfer. These critical problems must be solved before Chop2 transduction can be applied to the treatment of patients whose blindness is caused by photoreceptor degeneration. However, Chop2 transduction treatment appears to have several potential advantages over treatments using a retinal prosthesis. For example, once the surviving RGCs, other retinal neurons, or adequate cells become light sensitive by Chop2 transduction, these cells will sense the visual image topographically and at a higher resolution than is possible with prosthetic methods.

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References


