**Channelrhodopsin-2** gene transduced into retinal ganglion cells restores functional vision in genetically blind rats

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**Abstract**

To test the hypothesis that transduction of the channelrhodopsin-2 (ChR2) gene, a microbial-type rhodopsin gene, into retinal ganglion cells of genetically blind rats will restore functional vision, we recorded visually evoked potentials and tested the experimental rats for the presence of optomotor responses. The N-terminal fragment of the ChR2 gene was fused to the fluorescent protein Venus and inserted into an adeno-associated virus to make AAV2-ChR2V. AAV2-ChR2V was injected intravitreally into the eyes of 6-month-old dystrophic RCS (rdy/rdy) rats. Visual function was evaluated six weeks after the injection by recording visually evoked potentials (VEPs) and testing optomotor responses. The expression of ChR2V in the retina was investigated histologically. We found that VEPs could not be recorded from 6-month-old dystrophic RCS rats that had not been injected with AAV2-ChR2V. In contrast, VEPs were elicited from RCS rats six weeks after injection with AAV2-ChR2V. The VEPs were recorded at stimulation rates < 20 Hz, which was the same as that of normal rats. Optomotor responses were also significantly better after the AAV2-ChR2V injection. Expression of ChR2V was observed mainly in the retinal ganglion cells. These findings demonstrate that visual function can be restored in blind rats by transducing the ChR2V gene into retinal ganglion cells.

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**1. Introduction**

Channelrhodopsin-2 (ChR2), cloned from the green algae *Chlamydomonas reinhardtii*, is classified as a microbial-type rhodopsin that can be activated by specific wavelengths of light (Nagel et al., 2003; Sineshchekov et al., 2002; Suzuki et al., 2003). ChR2 is similar to bacteriorhodopsin (Oesterhelt and Stoeckenius, 1973), which uses an attached chromophore to absorb photons. A reversible photoisomerization of the all-trans isomer of retinaldehyde changes its conformation, and this directly induces ion movement through the membrane (Oesterhelt, 1998). It is this specific feature that allows ChR2 to function as a cation channel after exposure to light (Nagel et al., 2003).

Retinitis pigmentosa (RP) is a retinal degenerative disease that is associated with a progressive loss of photoreceptor cells resulting in a loss of peripheral visual fields, then central vision, and finally blindness. Mutations of a number of genes have been shown to cause RP, and these genes are mainly related to the phototransduction pathway (RetNet: http://www.sph.uth.tmc.edu/Retnet/). Unfortunately, these findings have not led to a successful way to treat or prevent RP. A new strategy for restoring vision has been recently investigated, viz., transduction of the *channelrhodopsin-2* (ChR2) gene into genetically blind mice (Bi et al., 2006). These experiments have been performed on animals that have the same mutation as humans with retinitis pigmentosa (Bowes et al., 1990; Pittler and Baehr, 1991). We have also reported that the intravitreal injection of the ChR2 gene into older dystrophic Royal College of Surgeons (RCS) rats (Mullen and LaVail, 1976), an animal model of recessively inherited retinitis pigmentosa (D’Cruz et al., 2000; Gal et al., 2000), restored functional vision (Tomita et al., 2007). These observations suggested that transduction of the ChR2 gene would provide a new method for treating eyes with RP that is independent of the etiology of the retinal degeneration.

Flannery and Greenberg (2006) reported that behavioral testing would be necessary to determine if the use of ChR2 was a viable...
strategy for restoring functional vision to blind animals. Lagali et al. (2008) reported that ON-bipolar cells that were engineered to be photosensitive by the transfer of the ChR2 gene restored behavioral responses to genetically blind mice. When the ChR2 gene was transduced into ON-bipolar cells, the retinal ON pathway was selectively activated by light. This is a reasonable way of activating the normal retinal ON pathway, although some methodological difficulties are still present when clinical applications are considered, e.g., the mechanism of gene transfer into ON-bipolar cells. Retinal ganglion cells are good candidates for receiving the ChR2 gene because target genes can be easily transduced into them. We have shown that a single injection of an AAV vector including ChR2 made it possible to change about 30% of all retinal ganglion cells to photosensitive ganglion cells. Recently it was reported that the ectopic expression of melanopsin in the retinal ganglion cells of retinal degeneration mice results in functional vision (Lin et al., 2008). In the same way, it is important to determine whether the ChR2 gene can restore functional vision when transferred retinal ganglion cells.

Thus, the purpose of this study was to determine whether transduction of the ChR2 gene into retinal ganglion cells of blind RCS rats can restore functional vision. We used visually evoked responses and optomotor responses to assess the functional condition of the visual system. We found that AAV2-mediated ChR2 transfer can lead to recovery of not only electrophysiological but also optokinetic responses.

2. Materials and methods

The procedures used on the animals in these experiments were 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.

2.1. Experimental animals

The experiments were conducted on 6-month-old male RCS rats; 18 dystrophic (rdy/rdy), and 4 non-dystrophic (+/+) The rats were obtained from CLEA Japan, Inc. (Tokyo, Japan).

2.2. Vector construction

The construction of the vector expressing ChR2 and the preparation of the vector for injection have been described in detail (Sugano et al., 2005; Tomita et al., 2007). In brief, the N-terminal fragment (residues 1–315; GenBank Accession No. AF461397) of the ChR2 gene was fused to a fluorescent protein, Venus, in frame at the end of the ChR2 coding fragment. Then ChR2-Venus (ChR2V) was introduced into the EcoRI and Hind III sites of the 6Pl plasmid (Kugler et al., 2003). The synapsin promoter was exchanged for a hybrid CMV enhancer/chicken \( \beta \)-actin promoter (CAG) (Niwa et al., 1991). The AAV2-Chr2V vector was purified by a single-step column purification method of Auricchio et al. (Auricchio et al., 2001; Sugano et al., 2005).

2.3. AAV vector injection

The method used to inject the AAV-Chr2V vector into the vitreous of both eyes of 6-month-old RCS (rdy/rdy) rats has been described in detail (Tomita et al., 1999, 2007). In brief, rats were anesthetized by an intramuscular injection of a mixture of ketamine (66 mg/ml) and xylazine (33 mg/kg). Using an operating microscope, a small incision was made in the conjunctiva to expose the sclera, and 5 \( \mu l \) of a viral vector suspension at a concentration of 1–10 \( \times 10^{12} \) genomic particles/ml was injected into the center of the vitreous cavity through the ora serrata with a 32 gauge needle on a 10 \( \mu l \) Hamilton syringe (Hamilton Company, Reno, NV).

2.4. Recording visually evoked potentials (VEPs)

VEPs were recorded before and at one week after the injection of AAV-Chr2V vector with a Neuropack system (MEB-9102; Nihon Kohden, Tokyo, Japan) as described in detail (Tomita et al., 2007). The method of recording was derived from a combination of the protocols used by Papathanasiou et al. (2006) and Iwamura et al. (2003). Briefly, at least seven days before the recordings, silver–silver chloride electrodes were implanted epidurally 7 mm behind the bregma and 3 mm lateral to the midline of both hemispheres. A reference electrode was implanted epidurally on the midline 12 mm posterior to the bregma.

Under ketamine–xylazine anesthesia, the eye was stimulated with 20 ms duration 0.5 Hz photic stimuli. The photic stimuli were generated by pulse activation of a blue light-emitting diode (LED) with light-emitting wavelengths of 435–500 nm (peak at 470 nm). A white LED was used to determine the spectral responsiveness (white LEDs include all wavelengths). The high and low band-pass filters of the amplifier were set to 50 kHz and 0.05 kHz, respectively. One hundred consecutive responses were averaged for each VEP. We also investigated the changes of the VEP responses elicited by a train of stimulus frequencies of 1–50 Hz with a pulse duration of 10 ms.

The stimulus light intensity was measured by a laser power meter (Lasercheck, Edomond Optics, Japan).

2.5. Spectral responsivity of eye after transduction of ChR2V

To investigate the spectral responsivity of the retinas transduced with Chr2V, VEPs were elicited by different wavelength stimuli of 1 mW/cm\(^2\). The wavelengths were isolated by band-pass filters (FUJIFILM Japan, Tokyo, Japan; Fig. 1A).

2.6. Behavioral assessments

The behavioral assessments were performed in a head-tracking instrument (Hayashi Seisakusyo, Kyoto, Japan). The instrument consisted of a circular drum rotating around the animal (Cowey and Franzini, 1979; Haruta et al., 2004; Lund et al., 2001). We covered the circular rotating drum with a transparent blue filter (Ultra color filter #67, Toshiba, Japan; filter transmission wavelengths <560 nm) because of the spectral absorption of ChR2. The vertical blue and black stripes subtended an angle of 10°, and the rotation speed was changed from 0 to 0.5, 2, 4, and 8 rpm. The spatial frequency corresponds to 0.05 cycle/degree, but the stimulus spatial frequency will change slightly with rat head position because the animal can freely move on the platform. The luminosity at the center of the holding chamber was set to 500 (1 mW/cm\(^2\)), 300 (0.55 mW/cm\(^2\)), and 100 lux (0.19 mW/cm\(^2\)). Dystrophic and control RCS rats were tested for 4 min at each speed before and after the ChR2 gene transfer.

The head movements of the animals were recorded by a video camera mounted above the apparatus. All movements were recorded at a rate of 29.95 frames/s. The number of movements was analyzed with movement-sensitive software (Move-t/2D ver.7.0, Libray, Tokyo). We made three marks; on the nose, the neck, and the waist of the rat on the software. The marked points were selected in the area that had a distinct color. To contrast to make it easy to trace them automatically. The software produced the angle of the three marked points. All of the angular movements >5° were considered to be tracking movements if the direction corresponded with the movement of the rotating stimulus. Large movements were counted as ‘rotating responses’ and optomotor responses to assess the functional condition of the visual system. We found that AAV2-mediated ChR2 transfer can lead to recovery of not only electrophysiological but also optokinetic responses.
with movements of the body of the animal were not counted. The number of movements at 0 rpm was subtracted from that at each rotation speed.

2.7. Retrograde labeling of retinal ganglion cells (RGCs) with fluorogold

To identify the RGCs in the ganglion cell layer (GCL), the RGCs were retrogradely labeled seven days before the rats were sacrificed. The labeling was done by injecting 4 μl of 2% aqueous fluorogold (FG; Fluorochrome, Englewood, CO; Brecha and Weigmann, 1994) containing 1% dimethylsulfoxide into the superior colliculus with a 32 G needle on a Hamilton syringe.

2.8. ChR2V expression in retina

Sixteen weeks after the injection of AAV-ChR2V, rats (n = 4) were sacrificed and the eyes were removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. The ipsilateral retinas were isolated and flat-mounted on microscope slides. The fluorogold-labeled and ChR2-expressing cells were counted in 12 distinct areas of the retina (three areas in each quadrant starting 1 mm from the optic nerve) to evaluate the transduction efficiency.

Two of the contralateral eyes were embedded in OCT compound (Sakura, Tokyo, Japan) after immersion in 30% sucrose solution in PBS. Fifteen micrometer retinal sections were cut and mounted on slides. The slides of retinal whole mounts and sections were covered with Vectashield medium (Vector Laboratories, Burlingame, CA). The Venus fluorescence was examined with a fluorescence microscope, Axiovert40 (Carl Zeiss).

2.9. Histological studies of the retina

Another two of the eyes were used for paraffin-embedded sections to examine histological changes induced by the expression of ChR2. Analyses of the retinal morphologies in ChR2V+/− and ChR2V+/+ rats were performed as described Li et al. (2007). In brief, rats were sacrificed by asphyxiation with carbon dioxide after the induction of photoreceptor degeneration. The eyes were enucleated, fixed, and embedded in paraffin. Three-micrometer thick sections of retinas were cut along the vertical meridian and stained with hematoxylin and eosin to allow examination of the retina in the superior and inferior hemispheres (LaVail et al., 1992).

2.10. Statistical analyses

Statistical analyses was performed using GraphPad Prism software (GraphPad Software, San Diego, CA). The criterion for statistical significance was P < 0.05.

3. Results

3.1. Spectral responsivity of ChR2V-expressing retinas

To investigate the spectral responsivity of ChR2V-expressing retinas, visually evoked potentials were elicited by light filtered
through six band-pass filters that isolated different parts of the spectrum (Fig. 1A). Typical waveforms elicited by light filtered through the BP-450 nm filter in rats with or without ChR2V are shown in Fig. 1B. Large amplitude VEPs were recorded from 6-month-old non-dystrophic RCS rats, but no response was elicited from untreated 6-month-old dystrophic RCS rats (Fig. 1B). However, six weeks after the injection of AAV-ChR2V, large (123.0 ± 13.5 μV) VEPs were recorded when the eye was stimulated with a stimulus intensity of 3500 lux (Fig. 1B). The largest amplitude was elicited by the wavelength of 450 nm (Fig. 1C), and VEPs were evoked by stimuli whose wavelengths were ≤550 nm.

3.2. Changes in VEP amplitude at different times after injection of AAV-ChR2V

VEPs in RCS rats injected with AAV-ChR2V were first detected two weeks after the injection (Fig. 2A). Thereafter, the amplitude progressively increased up to five weeks post-injection when the mean amplitude was 118.4 μV (Fig. 2A). In dystrophic RCS rats of the same age, VEPs were not detected with the same stimuli (noise level 5 μV). With increasing stimulus intensities, the amplitudes of the VEPs increased and the latencies of P1 decreased (Fig. 2B). Interestingly, the latencies of P1 in the ChR2-transduced RCS rats (24.68 ± 2.78 ms) were shorter than those in non-dystrophic RCS rats (49.43 ± 1.21 ms; P < 0.0001; un-paired t test; Fig. 2C).

3.3. Changes of VEPs responses by different frequencies of light stimulation

VEPs elicited by different frequencies of light stimulation were recorded from wild-type and dystrophic rats transduced with the ChR2V gene. VEPs were recorded from both types of rats when the stimulus frequencies were <20 Hz (Fig. 3A). Responses could not be detected in either type of rat when the stimulus frequencies were 40 Hz and 50 Hz. The responses from both rats were well fit by the Boltzmann fitting curve (Fig. 3B). The amplitudes of the VEPs in rats with ChR2V were not affected by a 200 ms interval of a train of light stimuli (Fig. 3C). These results indicated that the responsivity to light allowed by the transduction of ChR2V is similar to that in wild-type rats.

3.4. Behavioral assessment by optomotor responses

To determine whether transduction of the ChR2 gene restored functional vision, optomotor responses were recorded from non-dystrophic normal (Fig. 4A), dystrophic (Fig. 4B) and ChR2-transduced RCS rats (Fig. 4C). Preliminary experiments showed that when the angle of the neck moved over 5°, the movements were well correlated with the rotation speed in the non-dystrophic RCS (+/-) rats (Fig. 4D). Therefore, we counted the number of neck movements over 5°. The score in 30-week-old uninjected dystrophic rats at 2 rpm was 3.00 ± 3.64, while that in 30-week-old rats six weeks post-injection was significantly higher 13.31 ± 5.82 (P < 0.0006; Fig. 4E). Although non-dystrophic rats (+/+) responded to the rotation even at speeds of 2 rpm at 300 lux and to 4 rpm at 100 lux (Fig. 4F), the rats with the transduced ChR2V gene did not respond to the lower light intensities (Fig. 4G).

3.5. ChR2V expression in retina

Histological examination of flat mounts of the retina showed cells over a wide area of the retina had been retrogradely labeled

![Fig. 2. VEPs recorded from RCS rats transduced with the ChR2V gene. A. Changes in amplitude at different weeks after the injection of AAV-ChR2V. B. Changes in amplitude (P1–N1) and latency (P1) elicited by different stimulus intensities. Blue LEDs (435–500 nm, Peak at 470 nm) were used to elicit the VEPs. C. Differences of the P1 latency between non-dystrophic and ChR2V-transduced dystrophic rats. Error bars represent the standard deviation of the mean. The statistical evaluation was performed using the un-paired t test (dystrophic RCS with ChR2V; n = 8, non-dystrophic RCS; n = 4, **P < 0.0001).](image-url)
with Fluorogold (Fig. 5A). These cells were considered to be RGCs (Fig. 5B). Merged images showed that the expression of ChR2V was mainly in the RGCs (Fig. 5C). When the AAV-Venus vector was injected, Venus fluorescence was also observed in the RGCs, but the Venus protein was localized in the cell body, which was completely different from those injected with AAV-ChR2V (Fig. 5D). Cryosections showed that the labeled cells were observed in the ganglion cell layer (Fig. 5E and F) and some of them were in the inner nuclear layer (Fig. 5F). Photoreceptor cells were not seen in the retinas of the RCS rats (Fig. 5G). The number of fluorogold-labeled cells, which are most likely retinal ganglion cells, was 2531.8 ± 214.8. The number of double-labeled cells was 710.6 ± 117.7. Thus, the transduction efficiency was about 28.3% (Fig. 5H). Paraffin sections also showed no difference in the thickness of the photoreceptor layer between non-injected and AAV-ChR2-injected retinas (Fig. 5I and J).

### 4. Discussion

Our results demonstrated that VEPs can be recorded from genetically blind RCS rats that expressed the ChR2 gene, and the maximum response was elicited by stimuli with a peak wavelength at 450 nm. This agrees with an earlier report that the peak spectral absorption of ChR2 is approximately at 460 nm (Nagel et al., 2003). In addition, VEPs were elicited by stimuli up to 550 nm, whereas non-dystrophic RCS rats responded to wavelengths over 600 nm. This ability of normal rats to respond to longer wavelengths is probably because they have two cone photopigments with peak absorbances at 359 nm (Deegan and Jacobs, 1993; Yokoyama et al., 1998) and at 510 nm (Neitz and Jacobs, 1986). Therefore, the spectral responsivity spectrum of rats transduced with the ChR2 gene is somewhat narrower than that of non-dystrophic rats, and this is due to the presence of only channelrhodopsin-2 in the retina.

Distinct VEPs were first recorded at two weeks post-injection. The amplitudes of the VEPs of dystrophic RCS rats carrying the ChR2 gene in their RGCs gradually increased up to six weeks post-injection. Interestingly, the implicit times (ITs) of the VEPs were shorter than those of non-dystrophic rats. The cause of the shorter ITs was most likely because the neural signals were transduced in the RGCs, and the signals did not have to pass through the inner retinal network. These results suggest that the retinal ganglion cells became photosensitive by the expression of the ChR2 gene, and the signals generated in the ganglion cells were transmitted to the visual cortex from the RGCs.

We compared the responsivity to different frequencies of light stimulation between non-dystrophic RCS rats and ChR2V-injected rats. The RCS rat with the ChR2 gene responded up to 20 Hz, which was same as that from non-dystrophic RCS rats. Jehle et al. (2008) reported that steady-state VEPs could be elicited by a stimulus frequency of 38 Hz and distinct amplitudes were observed at 19 Hz. The responsivity was slightly higher than our results (20 Hz). The maximum amplitude evoked from RCS rats with ChR2 was about 50% of that from non-dystrophic RCS rats at 1 Hz. The lower amplitude from rats with ChR2V was probably due to the gene transduction efficiency in the retinal ganglion, which was about 30% of the retinal ganglion cells in this study. We previously
reported that the transduction efficiency of 10-month-old RCS rats was about 30% (Tomita et al., 2007). The transduction efficiency in the 6-month old rats we used in this study was approximately the same. The AAV we used in this study required host-cell synthesis of the complementary strand for transduction. The failure to undergo viral second-strand synthesis leads to a lower efficiency of transgene expression (Ferrari et al., 1996; Fisher et al., 1996). The use of self-complementary AAV (scAAV) vectors that do not require synthesis of the complementary strand for transgene expression can circumvent this problem. Thus, this method has the possibility of being more efficient and acting more rapidly (Andino et al., 2007; Jayandharan et al., 2008; McCarty et al., 2001).

To determine the functional visual capabilities of ChR2-transduced RCS rats, we investigated their optomotor responses (Haruta et al., 2004; Lund et al., 2001). The a-wave of the ERG is an indicator of photoreceptor function, and it disappears by 80–100 days in dystrophic RCS rats (Bush et al., 1995; Sauve et al., 2004). However, the activity of single ganglion cells could be recorded from the optic tract of RCS rats even after the electroretinogram (ERG) could not be recorded (Cicerone et al., 1979). Assessments of their visual sensitivity as determined by electric potentials recorded from the superior colliculus indicated that the sensitivity progressively decreased to reach a plateau at 180–240 days (Sauve et al., 2001). Therefore, we chose 8-month-old RCS rats (2 months after the injection of AAV-ChR2V) for the behavioral assessments. The behavioral scores of the ChR2-transduced RCS rats were significantly higher than those of untreated rats. We also found that the scores of the ChR2-transduced RCS rats were affected by the light intensity in the drum (Fig. 4F).

Lagali et al. (2008) reported that ON-bipolar cells that were engineered to be photosensitive by the transduction of the ChR2

Fig. 4. Behavioral assessment of dystrophic RCS rats and ChR2V-transduced rats. The traces of each marked point in non-dystrophic (A), dystrophic (B) and ChR2V-transduced dystrophic (C) rats during a test at 4 rpm. The red, green and blue lines correspond to the marks on the nose, the neck and the waist, respectively. Each score was calculated by subtracting the number of movements at 0 rpm. The angular displacement of each movement in the non-dystrophic rats (D). The luminosity at the center of the holding chamber was set to 500 lux (E). Effects of light intensity on the movements of non-dystrophic (F) and dystrophic RCS rats with ChR2V (G). The score of the non-dystrophic rats increased with increasing light intensities. The drum with black and transparent blue stripes was rotated at speeds of 0, 0.5, 2, 4 and 8 rpm. Error bars represent standard deviations of the means (un-paired t test; n = 8, *P < 0.05, **P < 0.01, ***P < 0.001).
gene restored visual function to eyes with retinal degeneration. ON and OFF bipolar cells receive synaptic input from photoreceptors. The ON-bipolar cells are one of the candidate cells for receipt of the ChR2 gene because ChR2 can elicit light-on responses. However, some reports have been published that retinal remodeling is triggered in bipolar cells and horizontal cells following photoreceptor degeneration (Marc et al., 2003, 2007; Strettoi and Pignatelli, 2000; Strettoi et al., 2002, 2003). Therefore, the function of the inner retinal layers, including the ON-bipolar pathway, might have some differences from that in normal eyes.

We found that behavioral responses could not be elicited by stimulus intensities <300 lux, although rats could respond at 500 lux. The 500 and 300 lux intensities correspond to about 2.25 × 10^{15} and 1.24 × 10^{15} photon/cm^2, respectively. The critical light intensity that elicited behavioral responses in rats with ChR2 transduced into their RGCs was expected to be 2.25 × 10^{15} photon/cm^2 s, which was close to the light level (3 × 10^{15} photon/cm^2 s) (Lagali et al., 2008) reported in the behavioral experiments performed on mice with ChR2 transduced into their ON-bipolar cells.

Our findings that ChR2 transduced-ganglion cells could restore visual function both electrophysiologically and behaviorally demonstrate that ganglion cells should also be considered as promising candidates cells for restoring vision via transfer of the ChR2 gene.

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References

