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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.

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 isoform 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

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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 6P1 plasmid (Kugler et al., 2003). The synapsin promoter was exchanged for a hybrid CMV enhancer/chicken β-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). Under an operating microscope, a small incision was made in the conjunctiva to expose the sclera, and 5 μl 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 μl 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 transmits 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-tr/2D ver.7.0, Library, 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 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