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1. Introduction

The retina comprises diverse differentiated neurons that have specific functions. Photoreceptor cells, the first-order neurons in the retina, have photopigments (rhodopsin and opsin) that absorb photons. Signals produced by the photoreceptor cells are transmitted to second-order neurons. Finally, visual signals are transmitted to the brain from the third-order neurons, the retinal ganglion cells (RGCs). Major diseases that cause blindness in advanced countries include glaucoma, diabetic retinopathy, retinitis pigmentosa (RP), and age-related retinopathy. Loss of vision due to these diseases is irreversible. However, with regard to glaucoma, eye drops that have the effect of reducing intraocular pressure have been developed. In diabetic retinopathy, effective surgical treatments such as vitrectomy and photocoagulation have been established. Blindness due to glaucoma and diabetic retinopathy can be prevented by administering these treatments in the early phase. On the other hand, in diseases caused by gene mutations, such as RP, effective treatments for delaying photoreceptor degeneration have not yet been established. Degeneration of photoreceptor cells results in loss of vision, even if other retinal neurons are intact [1-3].

RP is a disease that causes blindness due to photoreceptor degeneration. Symptoms include night blindness and loss of peripheral and central vision. Approximately 1 in 4,000 people are affected by this disease [4]. In 1990, Dryja et al. [5] first identified a point mutation in the rhodopsin gene from RP patients. A number of gene mutations responsible for RP has subsequently been identified. Most of these genes are associated with the phototransduction pathway in the retina. In some cases, the mutated gene exists not only in photoreceptor cells but also in retinal pigment epithelial cells. To date, 53 causative genes and 7 loci of RP have been identified (http://www.sph.uth.tmc.edu/Retnet/). Leber’s congenital amaurosis (LCA) is another retinal degenerative disease predicted to affect approximately 1/81000 individuals [6]. Most LCA patients have
severe visual defects in childhood. Histological analysis of the retinas of LCA patients shows marked retinal atrophy in the outer retinal layer, vascular thickening and sclerosis, and atrophy of the retinal pigment epithelium (RPE) [7]. Leber classified the disease as a type of RP on the basis of these characteristics. Later, Franceschetti and Dieterle differentiated it from retinal dystrophy based on the features of electroretinograms (ERGs) in these patients. Many gene mutations involved in LCA have been identified and the disease has been classified into 15 subtypes based on the affected gene [8-13]. Among these, LCA2, accounting for 10% of LCA cases [14], is due to a mutation in the RPE65 gene, which encodes all-trans retinyl ester isomerase. Deficiency in RPE65 leads to severe loss of visual function. Thus, in the case of LCA2, the cause of the disease is clearly identified as the biochemical blockade of the visual cycle caused by RPE65 deficiency [11,12]. Replacement therapy using the RPE65 gene is a candidate therapeutic strategy for LCA2. Indeed, successful results have been reported in RPE65 replacement therapy with the LCA2 animal model, Briard dogs [15]. After proof-of-principle studies [16], phase I trials using adeno-associated virus vector type 2 were conducted in 3 independent groups [17]. The results showed no adverse effects such as systemic dissemination of vector or immunological responses to the vector or transgene. Importantly, improvement of visual function as evaluated by microperimetry was observed in 1 subject [18,19]. Two other groups also reported improvement in visual function [20,21]. Continuous follow-ups for 1.5 years [22] have confirmed the safety and tolerability of replacement gene therapy [23]. The various hereditary forms of RP are as follows: autosomal dominant, recessive, and X-linked recessive. The Pro23-His gene mutation in the rhodopsin gene [24,25] occurs in 20–30% of all RP patients in Europe and the U.S. In contrast, the occurrence in Japan is only a few percent. Thus, in addition to the diversity of the gene mutations, their frequencies vary characteristically among different races. Differences in the progression, clinical findings, and development of the disease are also observed among different patients, even in those with the same mutation. A common feature of photoreceptor cell death caused by various gene mutations is eventual apoptosis via a common pathway [26]. Based on this rationale, various kinds of methods to prevent apoptosis, such as chemical treatment [27,28] and gene therapy, including gene replacement and neurotrophic factor supplementation [29-31], have been investigated. However, these strategies have not been successful in the complete prevention of cell death, although they have been shown to delay degeneration. The diversity of clinical features and gene mutations makes it difficult to develop effective treatments for RP.

A retinal prosthesis, comprising electrodes, an image processor, and a camera, is the only method to restore vision that has been studied [32-36]. Recently, a new strategy involving gene therapy for restoring vision has been developed using bacteriorhodopsin family genes [37,38]. The channelrhodopsin-2 (ChR2) gene derived from the green alga *Chlamydomonas* functions as a photoreceptor and cation-selective channel [39]. After the absorption of photons by photopigments, photon acquisition is completed by a chain reaction involving certain photoreceptor-specific proteins. Thus, the phototransduction pathway in photoreceptor cells requires not only photopigments but also certain photoreceptor-specific proteins, which complicates the reaction. Due to the inherent characteristics of ChR2, photosensitive neurons can be produced by the transfer of the ChR2 gene into neurons [40-42]. Here, we introduce new strategies for restoring vision by using channelrhodopsins.
2. Materials and methods

All the experiments performed for this report were approved by the Tohoku University Animal Care Committee, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Every effort was made to minimize the number and suffering of animals used in the following experiments.

Animals

We used 2 types of photoreceptor degeneration models: a genetically blind rat model and a light-induced photoreceptor degeneration model. The experimental design for each of these models is shown in Fig. 1.

Figure 1. Experimental design. Two types of photoreceptor degeneration models were used in this study. The photoreceptor cells of RCS rats degenerate by 3 months after birth due to the Mertk gene mutation. On the other hand, Thy-TG rats have native photoreceptors. Therefore, we subjected TG rats to continuous light exposure to induce photoreceptor degeneration. To confirm photoreceptor degeneration, ERGs were recorded before performing behavioral assessments. Finally, the eyes from all animals were subjected to histological examination.

Genetically blind rats

Royal College of Surgeons (RCS; rdy/rdy) rats [43,44] were used as model animals for photoreceptor degeneration in our experiments. The RCS rat, an animal model of recessively inherited retinal degeneration, is widely used in the study of photoreceptor degeneration. The gene responsible is the receptor tyrosine kinase gene Mertk [45], and mutations in MERTK, the human ortholog of the RCS rat retinal dystrophy gene, cause RP [46]. Photoreceptor degeneration is almost complete by 3 months after birth. We intravitreously injected the AAV-ChR2V vector into 6-month- or 10-month-old RCS rats. The rats were obtained from CLEA Japan, Inc. (Tokyo, Japan).

Thy-I ChR2 transgenic rats

We established transgenic (TG) rats harboring the ChR2 gene regulated by the Thy-1.2 promoter to investigate contrast sensitivity at each spatial frequency [47]. The rat Thy-1.2 antigen has been found to be abundant in the brain and thymus [48,49]. In the retina, the Thy-1.2 antigen is recognized as a marker specific to RGCs [50,51]. It is necessary to induce the degeneration of native photoreceptor cells in order to investigate the visual function conferred by ChR2-expressing RGCs, because the Thy-I TG rat has native photoreceptor cells. For this purpose, Thy-I TG rats were subjected to light-induced photoreceptor degeneration. Briefly, Thy-I TG rats were kept in cyclic light (12 hours ON/OFF: 5–10 lux/dark) for at least 2 weeks
prior to light exposure. The rats were then exposed to a 3000-lux intensity of fluorescent light for 7 days [28]. We used a light exposure box (NK Systems, Tokyo, Japan) to control the timing and light intensity for the induction of photoreceptor degeneration. After induction, we recorded ERGs to confirm photoreceptor degeneration.

Preparation of the adeno-associated virus vector

The adeno-associated virus (AAV) vector with the ChR2 gene was constructed as described previously [38]. Following this, the AAV Helper-Free System (Stratagene, La Jolla, CA) was used to produce infectious AAV-Venus (control) and AAV-ChR2V virions, which were purified by a single-step column purification method as previously described [52].

Recording of ERGs and visual electrophysiology (VEP)

ERGs and VEP readings were recorded using a Neuropack (MEB-9102; Nihon Kohden, Tokyo, Japan) according to methods previously described [38,53]. Briefly, rats were dark-adapted overnight, and the pupils were dilated with 1% atropine and 2.5% phenylephrine hydrochloride. Small contact lenses with gold wire loops were placed on both corneas, and a silver wire reference electrode was inserted subcutaneously between the eyes. Eyes were stimulated with flash light stimuli of 10-ms duration using a blue LED. Full-field scotopic ERGs were recorded, band-pass filtered at 0.3–500 Hz, and averaged for 5 responses at each light intensity. For VEP recordings, 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, at least 7 days before the experiments [54,55]. Under ketamine-xylazine anesthesia, the pupils were dilated with 1% atropine and 2.5% phenylephrine hydrochloride. The ground electrode clip was placed on the tail. Photic stimuli of 20-ms duration were generated under various intensities by pulse activation of a blue LED. The high- and low-pass filters were set to 50 kHz and 0.05 kHz, respectively. One hundred consecutive response waveforms were averaged for each VEP measurement.

Determination of transduction efficiency

At the end of the experiment, RCS and Thy-TG rats were sacrificed, and their eyes were resected and fixed in 4% paraformaldehyde and 0.1 M phosphate buffer, pH 7.4 [56]. The eye of each rat was flat-mounted on a slide and covered with Vectashield medium (Vector Laboratories, Burlingame, CA) to prevent the degradation of fluorescence. Then, the number of positive cells was counted.

3. Behavioral assessment

The spatial vision of each animal was quantified by its optomotor response. We used a virtual optomotor system to evaluate the contrast sensitivities of each spatial frequency. The original virtual optomotor system described by Prusky et al. [57] was modified for rats [47]. When a drum is rotated around an animal with printed visual stimuli on the inside wall, the animal tracks the stimulus by turning its head. A light-dark grating pattern was displayed on
computer monitors (ProLite E1902WS; Iiyama, Tokyo, Japan) arranged in a square around a platform. The software controlled the speed of virtual optomotor rotation, which was set at 12 degrees per second (2 rpm) in all experiments. The spatial frequency and the contrast of the grating pattern were varied but the average brightness was kept constant.

The animal was allowed to move freely on the platform in the virtual optomotor system. The grating session was started at a low spatial frequency (0.06 cycles/degree) with maximal contrast. An experimenter assessed whether the animals tracked the rotation, by monitoring the head movement and the presented rotating stimulus simultaneously on another display connected to the video camera. If head movement simultaneous with the rotation was evident, the experimenter judged that the animal could discriminate the grating, and proceeded to the next grating session. If the movement was ambiguous, the same grating session was presented again. All behavioral tests were double-blinded and performed during the first few hours of the animals’ light cycle (light on at 8 AM).

4. Results

4.1. Recording of VEP measurements in RCS rats

VEP measurements in 6- or 10-month-old RCS rats are expected to be abolished due to loss of photoreceptor cells. Generally, in RCS rats, photoreceptor degeneration is almost complete by 3 months after birth. Indeed, VEP measurements were not evoked even by the maximal LED flash in any of the aged RCS (rdy/rdy) rats (Fig. 2A). On the other hand, robust VEPs were evoked by the blue LED flash in RCS rats injected with the AAV-ChR2V vector (Fig. 2A). Initially, small VEP responses were observed at 2 weeks after AAV injection (data not shown), and the maximum amplitudes of VEP were observed 8 weeks later [58]. There were notable differences in sample waveforms from 6- and 10-month-old rats injected with AAV-ChR2V. Amplitudes and latencies of VEPs from 6-month-old rats were larger and shorter, respectively, than those from 10-month-old rats (Fig. 2B).

4.2. Transduction efficiencies of ChR2 in retinas of RCS rats

The expression of the ChR2 gene was evaluated by measuring Venus fluorescence in RCS rat retinas (Fig. 3A). The number of positive cells in rats injected at 10 months of age was significantly less than that injected at 6 months of age (Fig. 3B). The number of RGCs decreased linearly with age, following photoreceptor degeneration in the RCS rats (Fig. 3C). We have previously shown [56] that the ChR2 gene is mainly expressed in RGCs upon intravitreous injection of the AAV-ChR2V vector. Therefore, the observed decrease in the number of RGCs with age suggests that the transduction efficiencies at both ages are very similar.

4.3. Photoreceptor degeneration in Thy-1 TG rats

There were 11–12 rows of photoreceptor nuclei in the outer nuclear layer (ONL) of the Thy-1 TG rats; this is a number usually observed in rodents without retinal degeneration [59].
Following continuous light exposure, photoreceptor cells disappeared (Fig. 4A). ERGs showed no response, indicating that the photoreceptor cells degenerated in the whole retina (Fig. 4B).

**Figure 2.** VEP recordings before and after the injection of AAV-ChR2V. (A) VEP recordings from both 6-month- and 10-month-old RCS rats showed no responses. However, VEPs responses were clearly elicited 8 weeks after injection. (B) The amplitudes and latencies from rats injected with AAV-ChR2 at 6 months of age ($n = 8$) were significantly larger and shorter than those injected at 10 months of age ($n = 4$).

**Figure 3.** Transduction efficiencies of ChR2 in retinas of RCS rats. (A) Retinal whole-mount specimens obtained from rats injected with AAV-ChR2 at 6 and 10 months of age. (B) Venus-positive cells expressing the ChR2 gene were observed in whole-mount specimens. (C) The number of RGCs decreased with age.

Following continuous light exposure, photoreceptor cells disappeared (Fig. 4A). ERGs showed no response, indicating that the photoreceptor cells degenerated in the whole retina (Fig. 4B).
However, robust VEP measurements could be recorded, even though the photoreceptor cells had completely degenerated (Fig. 4B). Intense expression of the ChR2 gene was observed in the entire retina, with about 45% of RGCs positive for ChR2 (Fig. 4C).

Figure 4. Electrophysiological response of Thy-1 TG rats after photoreceptor degeneration. (A) Hematoxylin-eosin staining of the retina showed the degeneration of the native photoreceptor cells after continuous light exposure. (B) Extensive expression of the ChR2 gene was observed throughout the retina. (C) The ERG response was completely abolished following continuous light exposure, indicating that native photoreceptor cells had degenerated throughout the retina. VEP measurements could still be recorded after photoreceptor degeneration.

4.4. Behavioral assessment in photoreceptor degenerated-Thy-1 TG rat

In our virtual optomotor system, a stimulus of blue stripes over a black background was produced according to a sine wave function with variable amplitude and frequency (Fig. 5A). All the photoreceptor-degenerated Thy-1 TG and wild-type (normal) rats tracked the virtual rotating blue/black gratings (Fig. 5B). However, tracking stopped when the contrast was reduced below a specific threshold. We observed that contrast sensitivity was small at the minimal spatial frequency of 0.06 cycles per degree (CPD), increased with an increase in spatial frequency, and was negligible at spatial frequencies over 0.52 CPD. Therefore, the relationship followed an inverted U-shaped curve, as noted in previous reports [57]. In photoreceptor-degenerated Thy-1 TG rats, no reduction of contrast sensitivity was observed at any spatial frequency. Unexpectedly, the contrast sensitivity was instead somewhat enhanced at low spatial frequencies such as 0.09 or 0.18 CPD (Fig. 5C).

5. Discussion

The photo-acquisition system of mammalian photoreceptor cells, which mediates various photoreceptor-specific proteins, is very complicated. In contrast, the corresponding system in green algae such as *Chlamydomonas* and *Volvox* is simpler. ChR2 contains a 13-cis retinal that absorbs a photon, inducing a conformational change. The ChR2 functions as a cation-selective ion channel. For this reason, the transfer of a single gene, ChR2, to RGCs allows the generation
of photosensitive RGCs. In the normal visual pathway, the light incident upon the eyes is first received by photoreceptor cells located at the end of the retinal layers. The photoreceptor cells control neurotransmitter release, and second-order neurons located in the inner nuclear layer respond to the neurotransmitter. Finally, RGCs produce action potentials and transmit to the lateral geniculate nucleus (LGN) via the optic nerve (Fig. 6). In RP, the photo-acquisition system is damaged due to the degeneration of photoreceptor cells, even if the other retinal layers remain intact. RGCs that are rendered photosensitive by the transfer of the ChR2 gene can directly respond to light and transmit signals to the brain. In this newly organized photo-acquisition system, the other retinal neurons besides the RGCs are not required for the perception of light.

Although VEP responses recovered after ChR2 gene transfer, the amplitudes and waveforms were different between rats injected with AAV-ChR2V at 6 and 10 months of age. One possibility is that RGC activity decayed after photoreceptor degeneration. However, our data show that the number of RGCs decreased after photoreceptor degeneration (Fig. 3C). The calculated RGC transduction efficiencies in 6-month-old rats were the same as those in 10-month-old rats. The differences in the recorded amplitudes and latencies shown in Fig. 2 appear to be due to differences in the number of photosensitive RGCs. We previously reported that the RGC transduction efficiency in 10-month-old rats was about 28% [38]. Subsequently, Isago et al. showed that the RGC transduction efficiencies in 6- and 10-month-old rats were 28.3 and 27.7%, respectively [56]. The data clearly indicates that the transduction efficiency is the same, although the number of ChR2-expressing cells was lower, corresponding to the decrease in the number of RGCs.
To investigate visual acuity resulting from ChR2-expressing RGCs, we established a TG rat model expressing the ChR2 gene in RGCs. Photoreceptor-degenerated TG rats clearly tracked the rotation of blue-black stripes in a virtual optomotor. However, RCS rats that received the ChR2 gene in the AAV vector did not track the rotation of the virtual optomotor at any spatial frequency. Recently, we tested the behavior of RCS rats using a mechanical optomotor system and showed that the intensity of luminosity the rat received was the most important factor influencing their tracking of the rotation of the column [53]. A luminosity of over 500 lux was needed to induce head tracking in ChR2-expressing RCS rats. However, the maximum luminosity of the virtual optomotor was about 100 lux. It was therefore too low to induce head tracking in RCS rats. The question then arises: what is the difference between the TG and RCS rats? We do not have a reasonable explanation for this. One possibility is that the number of ChR2-expressing RGCs in the TG rat is greater than that in the RCS rat. About 45% of the RGCs expressed ChR2 in the TG rat. Compared to the TG rat, the transduction efficiency in the RCS rat is about 28% independent of the age of the animal. This may affect the light sensitivity. As the another explanation, in the case of TG rats, ChR2 is expressed after birth; therefore, there is a possibility that retinal organization and function might be altered, that cannot be ruled out.

RGCs are merely one of the candidate cell types that could receive the ChR2 gene. Lagali et al. [60] succeeded in transferring the ChR2 gene into ON-bipolar cells in the retina and confirmed the restoration of visual and behavioral responses. ON- and OFF-bipolar cells receive synaptic input from photoreceptors. Considering that ChR2 can elicit light-on responses, ON-bipolar cells seem to be the most appropriate cells for the transfer of the ChR2 gene. However, 2 questions arise in this regard. First, how can we deliver the ChR2 gene into ON-bipolar cells for human gene therapy? Lagali et al. [60] transferred the ChR2 gene into neonatal mice by electroporation of the plasmid vector. It is generally difficult to transfer a gene into the depths
of the retina via intravitreous injection of AAV vectors, in spite of the development of various serotypes of AAV vectors for retinal gene therapy [61-65]. Second, does synaptic transmission remain intact after photoreceptor degeneration? Some studies have reported that retinal remodeling is triggered in bipolar cells and horizontal cells following photoreceptor degeneration [66-70]. Recently, Doroudchi et al. [71] succeeded in transferring the ChR2 gene into ON-bipolar cells by the subretinal injection of a modified AAV vector (AAV8-Y733F) [72] that included a specific promoter for ON-bipolar cells (mGRM6-SV40), and demonstrated the behavioral recovery of the light response. These 2 questions could be resolved by these attractive methods used the specific promoter and the modified AAV vectors if the recovered visual acuity is investigated using a behavioral approach.

Since the discovery of ChR2, bacteriorhodopsins that have similar functions as that of ChR2 derived from Chlamydomonas have been identified. Channelrhodopsin-1 from the green alga Volvox [73] is a light-activated cation channel that has a different wavelength sensitivity from that of Chlamydomonas-derived ChR2. Halorhodopsin, which functions as a light-activated chloride channel, has been identified in Halobacterium salinarum [74,75]. Researchers have attempted to discover new light-activated ion channel genes, or to artificially design more functional ones [76-78]. In the future, more effective gene therapy strategies for restoring vision in RP might be developed using newly developed genes and vectors.

6. Conclusion

Target diseases for gene therapy were previously restricted to lethal and severe diseases that lead to death. In our country (Japan), the gene therapy guidelines were updated in 2002, whereby diseases in which bodily functions are severely impaired, such as loss of arms or legs, blindness, and deafness, were added to the list of target diseases for gene therapy. Based on these guidelines, people suffering from impaired vision caused by RP are eligible for gene therapy. However, gene therapy using genes derived from living organisms other than humans has not previously been tested in clinical trials. Safety studies, especially immunological reactions, using appropriate animal models in ChR2-based gene therapy is important before proceeding to clinical trials.

Acknowledgements

This work was partly supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 24390393 and 23659804) and the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO). We express our heartfelt appreciation to Dr. Ichiro Hagimori in Narita Animal Science Laboratory Co. Ltd., whose enormous support and insightful comments were invaluable during the course of this study.
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