A. RT-PCR法に使用したプライマー

- Igf1-RT-F1: ccacccctcaaaagaactga
- Igf1-RT-R1: aacgagggaaccaaatatc
- Igf2-RT-F1: gtcccacatttgcagttct
- Igf2-RT-R1: ctggatgacatggacagtgg
- Igfbp1-RT-F1: ccagggatccagctgccgtgcg
- Igfbp1-RT-R1: ggcgttccatcaggatgggctg
- Igfbp2-RT-F1: caactgtgacaagcatggccg
- Igfbp2-RT-R1: caccagtctcctgctgctgt
- Igfbp3-RT-F1: gacaccagaaacttctcctcc
- Igfbp3-RT-R1: catacttgtccacacaccagc
- Igfbp4-RT-F1: cgtcctgtgcccctagggttcct
- Igfbp4-RT-R1: gaagcttcaccctgtcttccg
- Igfbp5-RT-F1: gtttgcctcaacgaaaaagaggct
- Igfbp5-RT-R1: ctgctttctcttgtagaatcctt
- Igfbp6-RT-F1: ccccgagagaaacgaaagagacg
- Igfbp6-RT-R1: ctgcgaggaaacgacactgctg
- Hdac7a-RT-F1: gaagctggctgaagtgatcc
- Hdac7a-RT-R1: ccaagggctcaagagttctg
- Folr1-RT-F1: acgaattcctgctgttccac
- Folr1-RT-R1: tcggggaacactcatagagg
- Bmp2-RT-F1: tgaggattagcaggtctttg
- Bmp2-RT-R1: cacaaccatgtcctgataat
- Cckbr-RT-F1: ctcccttctcaacgcagtag
- Cckbr-RT-R1: tgcatgcactgcagtattcga
- Mc2r-RT-F1: cctctgcccaaataaccctta
- Mc2r-RT-R1: tgggctccgaaaggcatata

B. クロマチン免疫沈降法に使用したプライマー

- Bmp2-ChIP-F01: cacacacacacacacacatcacta
- Bmp2-ChIP-R01: ccgatacacacatctgagtgcattat
- Folr1-ChIP-F01: caccctttctctagtggttcttct
- Folr1-ChIP-R01: aggtatggtggaggagaaaagttta

C. バイサルファイトシークエンス法に使用したプライマー

- Bmp2-BS-F01: agagttaggttgaggatgtagagtg
- Bmp2-BS-R01: aaattcaaaaaaatctccaaccaaatacta

表3 使用したプライマーのリスト

A. 脳神経関連遺伝子群(96) Ache-S/Adcyap1r1/Adora1/Adora2a/Adora2b/Anxa8/ Apoe/Artn/Ascl1/Bdnf/Bmp2/Bmp4/Bmp8b/Bmp15/ Brs3-1/Brs3-2/Cckar/Cckbr/Cd40/Cdk5r1-1/Cdk5r1-2/ Cdk5rap1-1/Cdk5rap1-2/Cdk5rap2-1/Cdk5rap2-2/ Cdk5rap3-1/Cdk5rap3-2/Chrm1-1/Chrm1-2/Chrm2-1/ Chrm2-2/Chrm3-1/Chrm3-2/Chrm4-1/Chrm4-2/Chrm5-1/ Chrm5-2/Cntfr/Crh/Crhbp1/Crhbp2/Crhr1-1/Crhr1-2/ Crhr2-1/Cxcl1/Cxcr4/Dll1/Drd1a/Drd2/Drd3/Drd4/Drd5/ Egf/Ep300/Erbb2/Fas/Fgf2/Fgf13/Fgfr1/Flna/Frs2/Frs3/ Galr1/Galr2/Gfap/Gfra1/Gfra2/Gfra3/Gmfb/Gmfg/Gnao1/ Gp1/Grin1/Grpr/Hcrt/Hcrtr1/Hcrtr2/Hdac1/Hdac4/Hdac7a/ Hdac8/Hdac9/Hes1/Hey1/Hey2/Heyl/Il3/Inhba/Mc2r/Mdk/ Mef2c/Mt3/Ncoa6/Nestin/Pdgfra/Vnn1
B. 発生発達遺伝子群(11) Igf1/Igf2r/Igfbp1/Igfbp2/Igfbp3/Igfbp4/Igfbp5/Igfbp6/ Gr/Acox1/Pparg/Ppara
C. 葉酸関連遺伝子群(14) Slc19a1/Slc19a2/Slc19a3/Slc46a1/Folr1/Folr2/Folr4/ Rtbdn/Gbas/Gnmt/Mthfs/Dmgdh/Ftcd/Nipsnap1
D. インプリンティング遺伝子群(8) Igf2/H19/Dlk1/Mash2/Rian/Sgce/Phlda2/Cdknlc

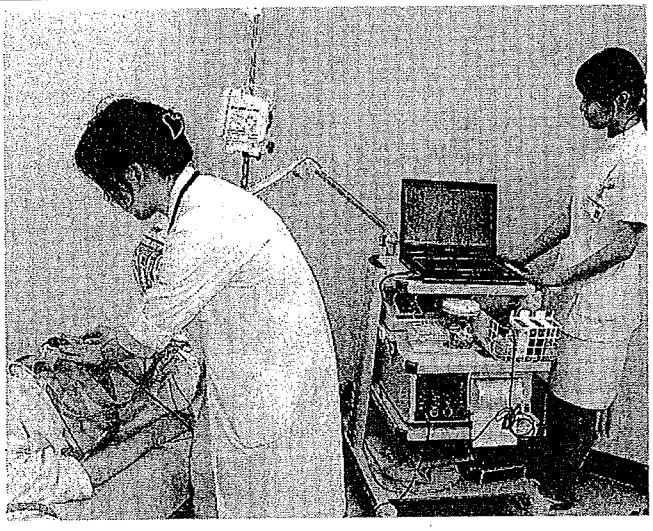
表 4　遺伝子発現解析に用いた129遺伝子のリスト

医療機器産業を東北に

大学・官の支援態勢 来月から本格始動

旬感 経済

医療機器産業への参入を目指す東北地域の企業を支援する取り組みが今春、本格化する。東北大は各大学の技術を実用化につなげる支援センターを整備。東北経済産業局は企業の要望を聞く「企画運営委員会」を組織し、5月21日に初会合を開く。（斎藤義浩）



実用化に近い胎児心電図＝仙台市青葉区の東北大病院

「大丈夫。赤ちゃんの心臓は正常です」。東北大病院の周産母子センターで、胎児の心電図を取る研究が行われている。

心電図は心臓から出る電気信号を記録したもの。胎児の心電図が取れれば「胎内死」を招く心臓の不調や脳内出血の早期発見と治療に役立つ。しかし、胎児の信号は弱すぎて母の信号にかき消されるため、正確な測定はできないというのが常識だった。

この難題に東北大の木村芳孝教授（周産期医学、生体情報工学）らが挑戦した。信号から胎児の部分だけを抜き出す新しい情報処理技術を05年に考案。医療機器メーカーのアトムメディカル（本社、東京都文京区）と共同で、胎児心電図を3年で開発した。

臨床研究としてこれまで200人近い胎児の心電図を取ることに成功。正式な臨床試験を経て、3年以内に実用化する予定だ。国内の市場規模は年に50億円と試算する。

「おなかに巻くだけで心電図が取れるシートを開発し、自宅にいる妊婦と胎児の健康状態を病院に送信するシステムにすることが目標」と木村教授。

◆

胎児心電図は、大学が苦手な基礎研究から応用への「橋渡し」がうまくいった例だ。

木村教授らは、医療現場でどう使うかという「出口」のビジネスデザインを描きつつ「入り口」の情報理論の研究を進めた。当初から企業と協力し、特許や薬事法の規制など個人では対処が難しい問題を検討したことも大きい。

このような成功例を受けて文部科学省は2年前、全国7カ所に橋渡し研究の拠点を選定。東北大の「未来医工学治療開発センター」はその一つだ。東北地方の各大学や国内外の企業の情報を集め、出口の設定や質の高い臨床試験の実施などを支援して、先端医療機器を東北から世界に発信する。

専用の実験施設や臨床試験のデータを一括管理する施設がこのほど完成し、支援体制がほぼそろった。副センター長の西田幸二・医学系研究科教授（眼科学）は「医療機器の実現には中小企業の力が欠かせない。これから研究開発をしようという企業の相談にも応じたい」と話す。

◆

東北地方には、微細加工や光工学で高い技術をもつ企業が少なくない。多くは薬事法については素人だが、国の「産業クラスター計画」で東北経産局が昨年、医療機器分野を重点分野に決めたことから、同分野への新規参入の機運が高まり、各地に開発グループ＝表＝ができた。

同局が組織する企画運営委は、これらの企業の要望を大学に伝える「実動部隊」で、東北に立地している医療機器メーカーや開発グループの代表などから委員を選定中だ。

同局産業支援課の遠藤司課長補佐は「まず医療機器の部品を受注することから始めるのが現実的」とみる。

●東北の医療・福祉機器開発グループ

- 北東北ナノ・メディカルクラスター研究会（青森、秋田、岩手）
- あおもりウェルネスランド構想
- 秋田メディカルインダストリ・ネットワーク
- いわて医療機器事業化研究会
- 置賜メディカルテクノ・ネット（山形）
- 先進予防型健康社会・仙台クラスター
- 仙台フィンランド健康福祉センター
- 福島県医療福祉機器研究会

Experimental Eye Research 90 (2010) 429–436

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

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

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ARTICLE INFO

Article history:
Received 17 July 2009
Accepted in revised form 10 December 2009
Available online 27 December 2009

Keywords:
channelrhodopsin-2
Royal College of Surgeons rat
retinal ganglion cells
photoreceptor degeneration
visually evoked potential
retinitis pigmentosa
adeno-associated virus vector

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

☆ Grant Information: This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education Science and Culture (No. 20791241, 21791664, 21200022), Ministry of Health, Labor and Welfare, Comprehensive Support Programs for Creation of Regional Innovation Science and Technology Incubation Program of the Japanese Government, Japanese Retinitis Pigmentosa Society, and Sumitomo Foundation.

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doi:10.1016/j.exer.2009.12.006

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

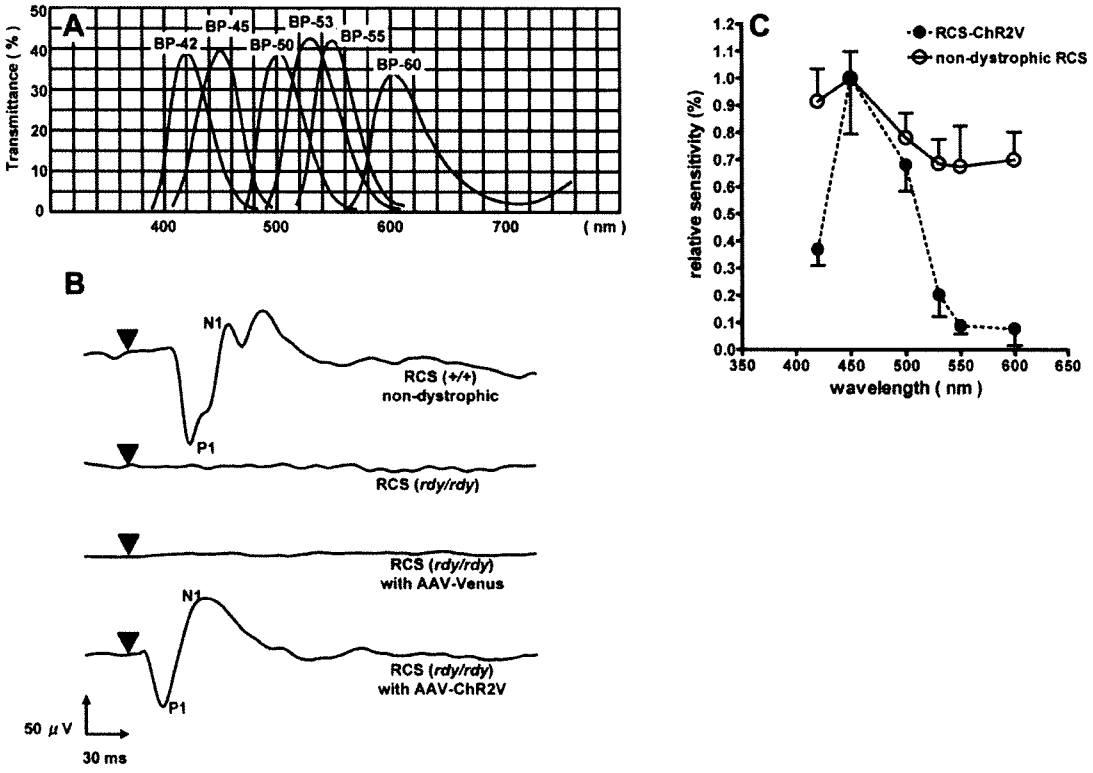

Fig. 1. Spectral responsivity of RCS rats transduced with the *ChR2V* gene. Different parts of the spectrum were isolated by six band-pass filters. A. Band passes for the six band-pass filters used to isolate different wavelengths of the visible spectrum. B. Typical waveforms of the VEPs elicited by 3500 lux stimuli emitted by blue LEDs (435–500 nm, peak at 470 nm). Upper: VEPs from a non-dystrophic rat; VEPs from a dystrophic rat without *ChR2V*; VEPs from a dystrophic rat with *Venus*. Lower: from a dystrophic rat with the *ChR2V* gene. C. Spectral responsiveness of eyes after transduction of *ChR2V* and of eyes of non-dystrophic rats. Amplitudes of VEPs elicited at the different wavelengths at the intensity of 1 mW/cm^2. The relative responses to the amplitude of the stimuli with a 450 nm band-pass filter were plotted. VEPs were recorded by stimuli delivered through each band-pass filter (open circles): non-dystrophic RCS rats ($n = 4$), (closed circles): dystrophic RCS rats with ChR2V ($n = 8$). Error bars represent standard deviations.

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 AAV2-ChR2V

VEPs in RCS rats injected with AAV2-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

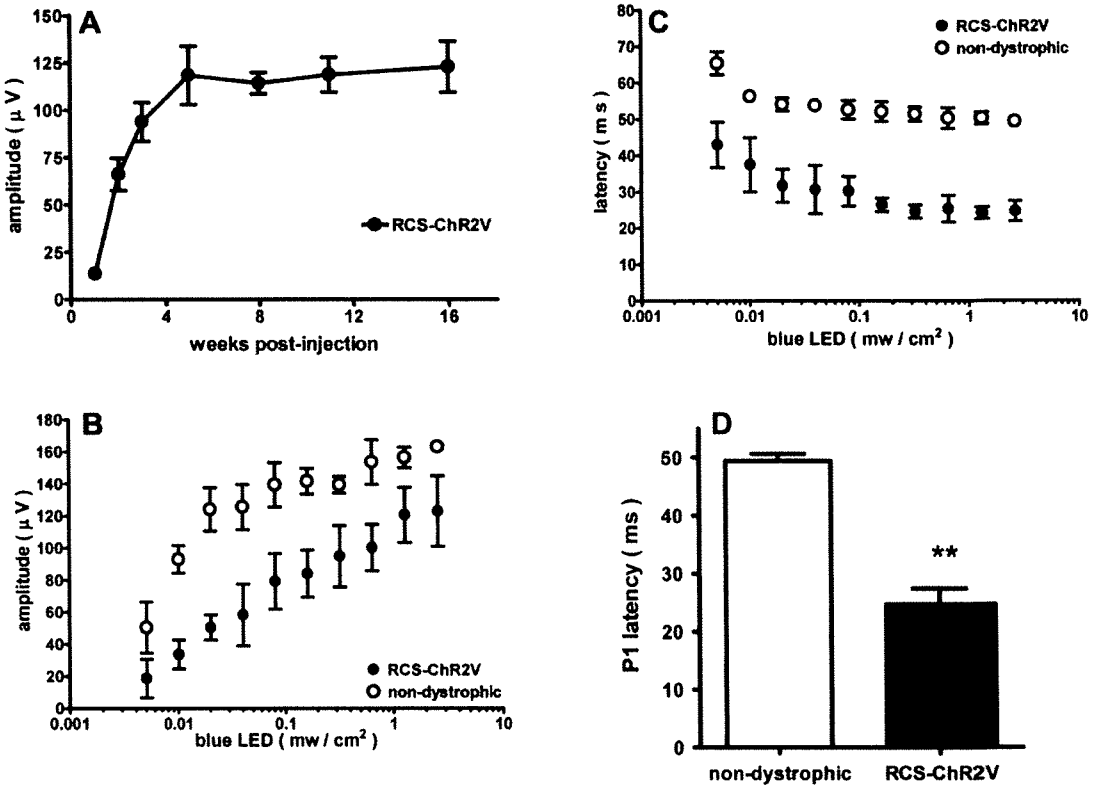

Fig. 2. VEPs recorded from RCS rats transduced with the *ChR2V* gene. A. Changes in amplitude at different weeks after the injection of AAV-ChR2V. B. Changes in amplitude (P1–N1) and latency (P1) elicited by different stimulus intensities. Blue LEDs (435–500 nm, Peak at 470 nm) were used to elicit the 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; $n = 4$, $^{**}P < 0.0001$).

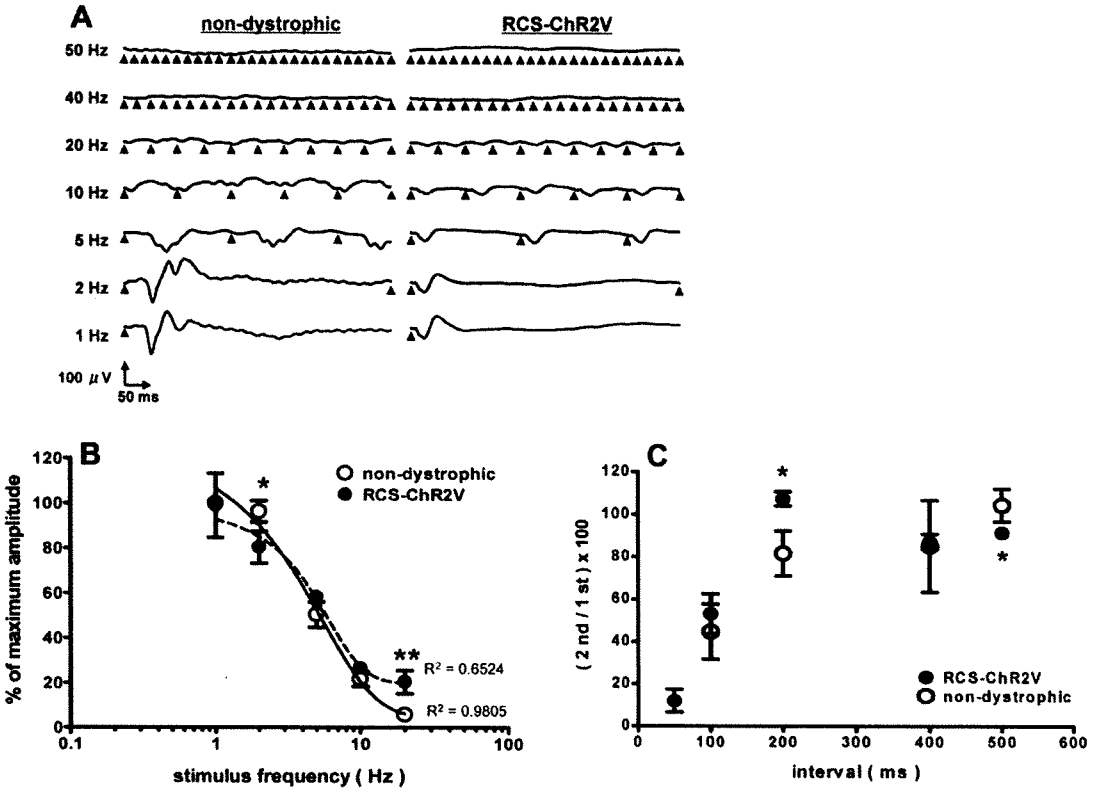

Fig. 3. Changes of responsivity in VEPs elicited by different stimulus frequencies. A. Typical VEP waveforms elicited by different stimulus frequencies. B. Changes in VEP amplitude elicited by different stimulus frequencies. Data are expressed as percentages of the amplitude at 1 Hz. The amplitude recruitment curve was fitted to the Boltzmann model. C. Changes in VEP amplitude elicited by different stimulus trains. Data are expressed as a percentage of the amplitude elicited by the first stimulus. Photic stimuli were generated by a blue LED (435–500 nm, peak at 470 nm) at 3500 lux. The statistical evaluation was performed using the un-paired *t* test (dystrophic RCS with ChR2V; $n = 8$, non-dystrophic RCS; $n = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$).

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 rat. Jehle et al. (2008) reported that steady-state VEPs could 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

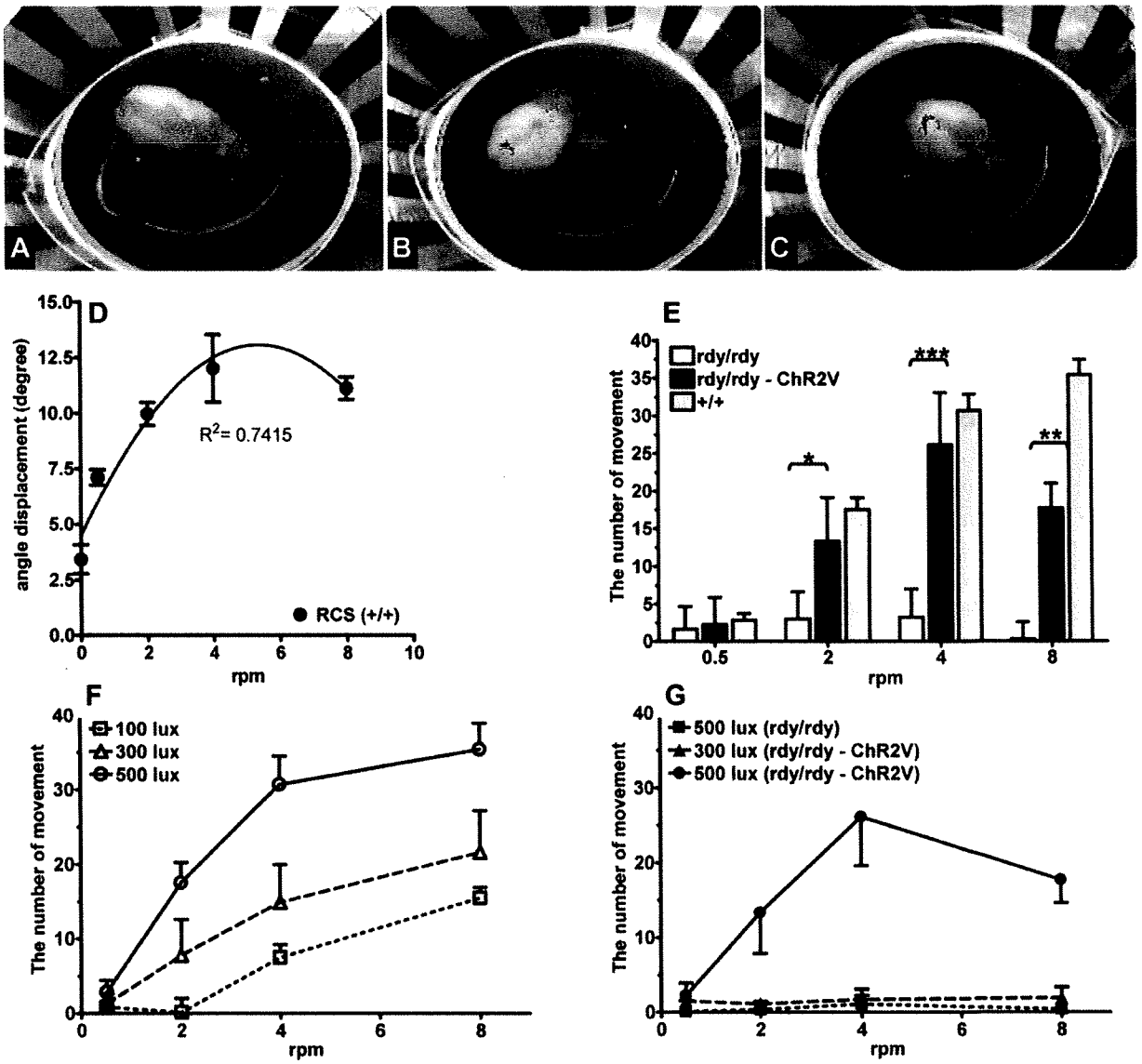

Fig. 4. Behavioral assessment of dystrophic RCS rats and *ChR2V*-transduced rats. The traces of each marked point in non-dystrophic (A), dystrophic (B) and ChR2V-transduced dystrophic (C) rats during a test at 4 rpm. The red, green and blue lines correspond to the marks on the nose, the neck and the 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; $n = 8$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

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

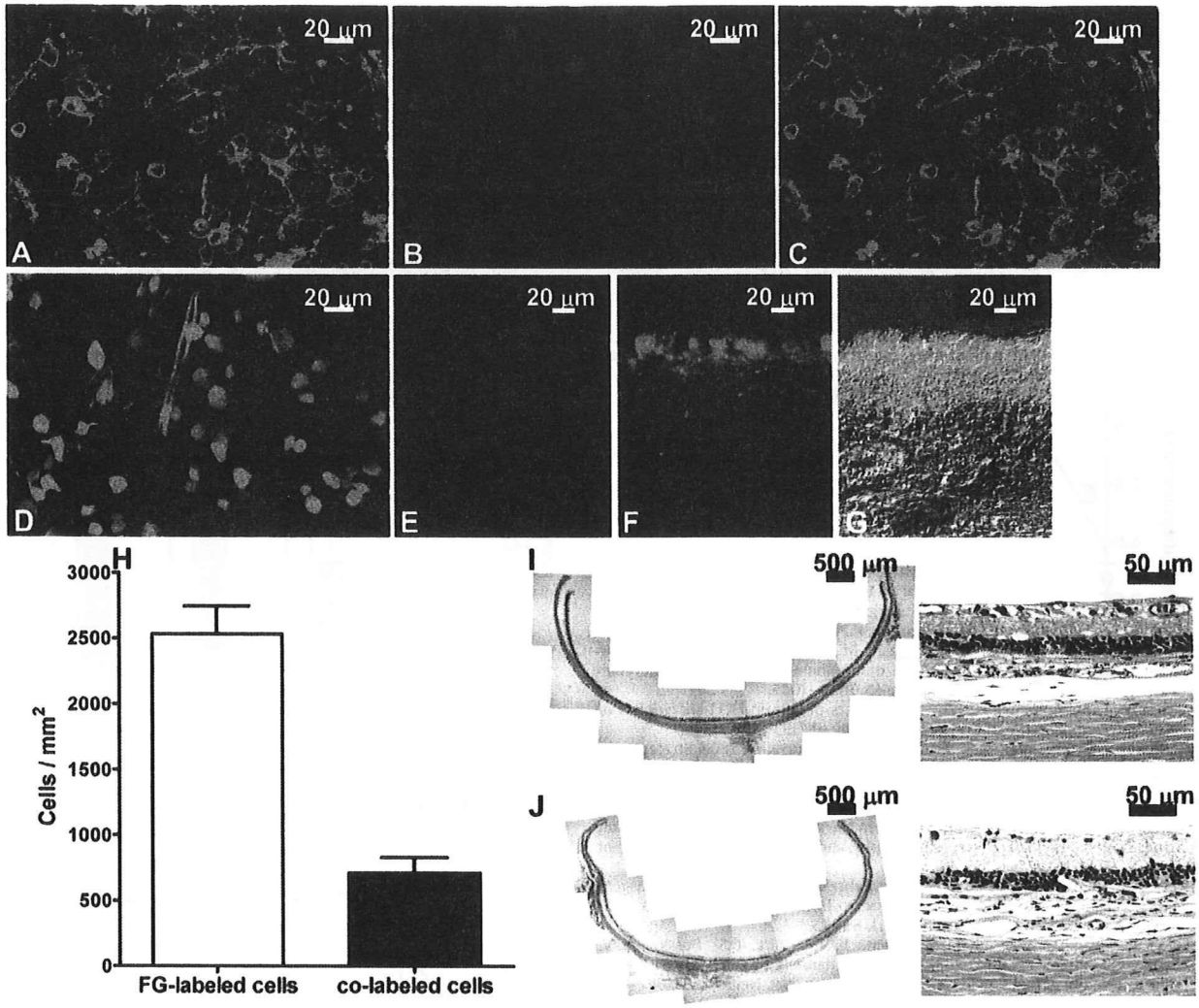

Fig. 5. Expression of ChR2V in the retina. Histological examination of retinas of rats injected with AAV-ChR2V at 16 weeks after the injection. A. Flat-mounted section showing the expression of *ChR2V* gene by green fluorescence. B. Retinal ganglion cells that were retrogradely labeled with fluorogold. C. Merged photograph showing both fluorogold and ChR2V. Many cells are double-labeled. D. Flat-mounted section from a rat transduced with AAV-Venus as a control vector. E. Merged photograph of the Nomarski image, Fluorogold (F) and ChR2V (G). H. The transduction efficiency of ChR2 gene into RGCs ($n = 8$). Hematoxilin–eosin sections from both non-injected RCS rats (I) and AAV-ChR2V-injected RCS rats (J) revealed a loss of photoreceptors in the entire retina.

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.

Acknowledgements

We thank Prof. Duco Hamasaki for discussions and editing the manuscript.

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REVIEW ARTICLE

Channelrhodopsins provide a breakthrough insight into strategies for curing blindness

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Abstract

Photoreceptor cells are the only retinal neurons that can absorb photons. Their degeneration due to some diseases or injuries leads to blindness. Retinal prostheses electrically stimulating surviving retinal cells and evoking a pseudo light sensation have been investigated over the past decade for restoring vision. Currently, a gene therapy approach is under development. Channelrhodopsin-2 derived from the green alga *Chlamydomonas reinhardtii*, is a microbial-type rhodopsin. Its specific characteristic is that it functions as a light-driven cation-selective channel. It has been reported that the channelrhodopsin-2 transforms inner light-insensitive retinal neurons to light-sensitive neurons. Herein, we introduce new strategies for restoring vision by using channelrhodopsins and discuss the properties of adeno-associated virus vectors widely used in gene therapy.

[Tomita H., Sugano E., Isago H. and Tamai M. 2009 Channelrhodopsins provide a breakthrough insight into strategies for curing blindness. *J. Genet.* **88**, 409–415]

Phototransduction pathway

Retinitis pigmentosa (RP) is the most common type of inherited disease that leads to blindness; it has an expected prevalence of one in 4000 cases and its symptoms include night blindness, loss of peripheral visual field, and loss of central vision (Hartong *et al.* 2006). A number of genes responsible for RP – most of them associated with phototransduction pathways – have been identified and their functions elucidated (http://www.sph.uth.tmc.edu/Retnet/home.htm).

In the vertebrate retina, phototransduction is initiated by activation of rhodopsin. In the dark, rhodopsin contains 11-*cis*-retinal as the chromophore, embedded in its protein moiety. Light-induced isomerization of 11-*cis*-retinal to all-*trans*-retinal initiates a G protein-coupled signalling cascade, involving the hydrolysis of cyclic guanosine monophosphate (cGMP) by upregulated phosphodiesterase, which causes closure of a cGMP-regulated cation channel (figure 1). The photoreceptor cells are hyperpolarized, and they transmit light signals to second-order neurons such as bipolar cells. After the isomerization of 11-*cis*-retinal to all-*trans*-retinal by absorption of photon(s), the latter travels from retinal photoreceptor outer segments to the retinal pigment epithelium (RPE) for regeneration of the chromophore. Thus, visual cycle requires sequential propagation of steps, including enzymatic reactions (Lamb and Pugh 2004); any loss of function causes various retinal disorders.

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Gene therapy for retinal disorders

Gene therapies have attempted to compensate the loss of function for the treatment of retinal disorders, mainly hereditary diseases. Various types of viral vectors such as retrovirus (Sakamoto *et al.* 1995), lentivirus (Miyoshi *et al.* 1997; Lotery *et al.* 2002), adenovirus (Reichel *et al.* 2001), and adeno-associated virus (AAV) (Ali *et al.* 1996) have been investigated for an efficient transfer of the relevant gene into target cells, and they have been selected specific to each disorder or purpose, e.g. for transient or long-term gene expression according to the target cell type. Viral vector-based gene transfer may cause undesirable side effects such as systemic dissemination of the vector, immune responses, and overexpression of the gene. To deal with these complications, the eyes specifically have the blood-retinal barrier and

Keywords. channelrhodopsin-2; retinitis pigmentosa; adeno-associated virus vector; blindness.

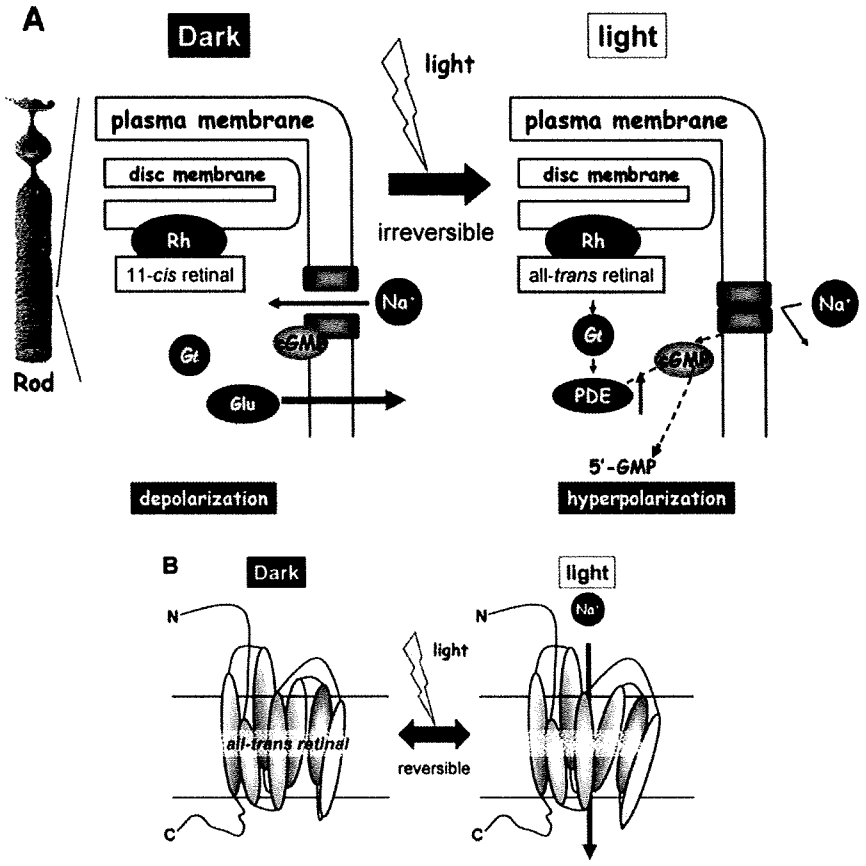

Figure 1. Schematic representation of light-induced phototransduction pathways in (A) rhodopsin, and (B) channelrhodopsin-2. (A) Light induces isomerization of 11-*cis*-retinal to all-*trans*-retinal, which initiates a G protein-coupled signalling cascade. (1) Photons induce isomerization; (2) activated rhodopsin (Rh) activates the guanosine triphosphate (GTP)-binding protein transducin (Gt); (3) activated Gt further activates cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE); (4) PDE hydrolyses cGMP, coupled to the sodium channel, to 5'-GMP; (5) cGMP hydrolysis leads to closure of the sodium channel; (6) photoreceptor cells are hyperpolarized by the closure of the sodium channel. The isomerization of 11-*cis*-retinal is irreversible, and many enzymatic reactions are needed for the regeneration of 11-*cis*-retinal. (B) Light directly induces conformational change in channelrhodopsin-2 after isomerization, which is a reversible reaction.

the specific immune surveillance system. The blood-retinal barrier is expected to play a role in preventing systemic dissemination of the viral vector, and the eye-specific immune surveillance system may minimize the antibody reaction to the capsid proteins of the viral vector (antigens). Owing to these characteristics of the eye, gene therapy may be better indicated for retinal disorders than systemic disorders.

AAV, which is a small and nonenveloped virus belonging to the parvovirus family, has been well investigated as a vector in studies conducted on gene therapy for retinal disorders (Ali *et al.* 1996; Flannery *et al.* 1997; Jomary *et al.* 1997; Lewin *et al.* 1998). Further, it is used in clinical trials (Bainbridge *et al.* 2008; Hauswirth *et al.* 2008; Maguire *et al.* 2008). Various serotypes of AAV have been identified and modified for use as vectors in gene therapy, each vector having individual characteristics. There are many types of cells in the retina. Each AAV serotype shows different transduction efficiency as a vector, and the time it takes for transgene expression depends on the types of cells in the retina or the species under investigation, such as human, monkeys, or mice. On intravitreal injection of AAV2/2 vector, gene expression is observed mainly in retinal ganglion cells (Ali *et al.* 1998; Guy *et al.* 1998; Martin *et al.* 2002; Qi *et al.* 2007). On subretinal injection of AAV2/2 vector, gene expression is observed in the photoreceptor cells and RPE (Ali *et al.* 1996; Sarra *et al.* 2002); the same is the case with subretinal injection of AAV2/1 vector. However, the time taken for gene expression differs. AAV2/2 and AAV2/1 vectors take 6–8 weeks (Sarra *et al.* 2002) and 3–4 days (Auricchio 2003), respectively, for transgene expression. For each AAV serotype,

the site of and time taken for gene expression are shown in table 1.

Gene therapy for protection of photoreceptor cells

Leber congenital amaurosis (LCA) is a rare retinal dystrophy with a prevalence of one in 30000 (Koenekoop 2004) to one in 81000 (Stone 2007) cases. It is characterized by severe visual loss in the early stages of life, which progresses to blindness. The *RPE65* gene encodes an isomerase enzyme in the RPE, which catalyzes a critical step in the visual cycle, permitting the photoreceptor visual pigments to absorb photons and maintain sight; mutations in this gene have been identified to be responsible for LCA. Recently, two clinical trials of AAV-mediated gene therapy for patients with LCA have been performed (Bainbridge *et al.* 2008; Maguire *et al.* 2008), and successful results have been reported (Bainbridge and Ali 2008; Bainbridge *et al.* 2008; Cideciyan *et al.* 2008; Hauswirth *et al.* 2008; Koenekoop 2008; Maguire *et al.* 2008; Smith *et al.* 2009).

Gene therapy for restoration of vision

Degeneration of photoreceptor cells leads to blindness, even in the case of survival of other retinal neurons. Indeed, inner retinal neurons such as bipolar, horizontal and ganglion cells survive in the retina of patients with RP (Humayun *et al.* 1999; Santos *et al.* 1997); however, some synaptic remodelling occurs (Marc *et al.* 2003; Strettoi *et al.* 2003). Since late 20th century, some approaches such as use of retinal prostheses (Margalit *et al.* 2002; Javaheri *et al.* 2006) and transplantation of retinal cells (Gouras and Lopez 1989; Lopez *et al.* 1989; Sheedlo *et al.* 1991; Lavail *et al.* 1992; Lund *et al.* 1998; Seiler and Aramant 1998; Abe *et al.* 1999; Kaplan *et al.* 1999; Humayun *et al.* 2000; Aramant and Seiler 2002) or stem cells (Schraermeyer *et al.* 2001; Yang *et al.* 2002; Lund *et al.* 2003; Haruta *et al.* 2004) have been employed to restore vision by making use of surviving retinal neurons. Various types of retinal prostheses such as epiretinal (Majji *et al.* 1999; Humayun 2001), subretinal (Chow and Peachey 1998; Peyman *et al.* 1998; Chow and Peachey 1999; Zrenner *et al.* 1999; Zrenner 2002) and suprachoroidal (Sakaguchi *et al.* 2004; Nakauchi *et al.* 2007) implants have been under development worldwide and are being progressively used in clinical trials (Hayes *et al.* 2003; Humayun *et al.* 2003). The discovery of channelrhodopsin-2 (ChR2) (Nagel *et al.* 2003) has provided a new insight into the strategies for restoring vision.

Channelrhodopsin-2

ChR2 is a microbial-type rhodopsin derived from the green alga *Chlamydomonas reinhardtii* (Sineshchekov *et al.* 2002; Nagel *et al.* 2003). Bacteriorhodopsin is a classical example of microbial-type rhodopsin, and functions as a light-driven-proton pump (Subramaniam and Henderson 2000); its structure and functions have been well investigated (Grigorieff *et al.* 1996; Kimura *et al.* 1997). Microbial rhodopsins are 7-transmembrane proteins like retinal rhodopsin, containing all-*trans*-retinal, and not 11-*cis*-retinal, as the chromophore (Tsuda *et al.* 1980). ChR2 functions as a light-driven cation-selective channel (Nagel *et al.* 2003) (figure 2). The reaction occurring with ChR2 after absorption of photons (figure 2) is completely different from that occurring with vertebrate

Table 1. Transduction efficiency of each AAV serotype. All the serotypes, except AAV5/5, contained the genome of AAV2.

Serotype	Intravitreal	Subretinal	Reference
AAV2/1	N. D.	RPE in mice (3–4 days)	Auricchio *et al.* (2001).
AAV2/2	GC in mice, rats	Photoreceptor & RPE in mice (6 weeks)	Ali *et al.* (1998); Martin *et al.* (2002); Sarra *et al.* (2002); Tomita *et al.* (2007)
AAV2/3	N. D.	N. D.	Yang *et al.* (2002)
AAV2/4	N. E.	RPE in dogs and mokeys	Weber *et al.* (2003).
AAV2/5	N. D.	Photoreceptor & RPE in mice, rod in monkeys (3–4 days)	Auricchio *et al.* (2001); Lotery *et al.* (2003)
AAV5/5	N. D.	Photoreceptor & RPE in mice (3–4 days)	Auricchio *et al.* (2001)
AAV2/6	N. E.	RPE in mice	Yang *et al.* (2002a)
AAV2/7	N. D.	Photoreceptor & RPE in mice	Allocca *et al.* (2007)
AAV2/8	N. E.	Photoreceptor & RPE in mice (3–5 days)	
AAV2/9	N. E.	Photoreceptor, RPE and Müller in mice	

N.D., not detected; N.E., not examined. The time taken by each serotype for initial expression of the transgene is given in parentheses.

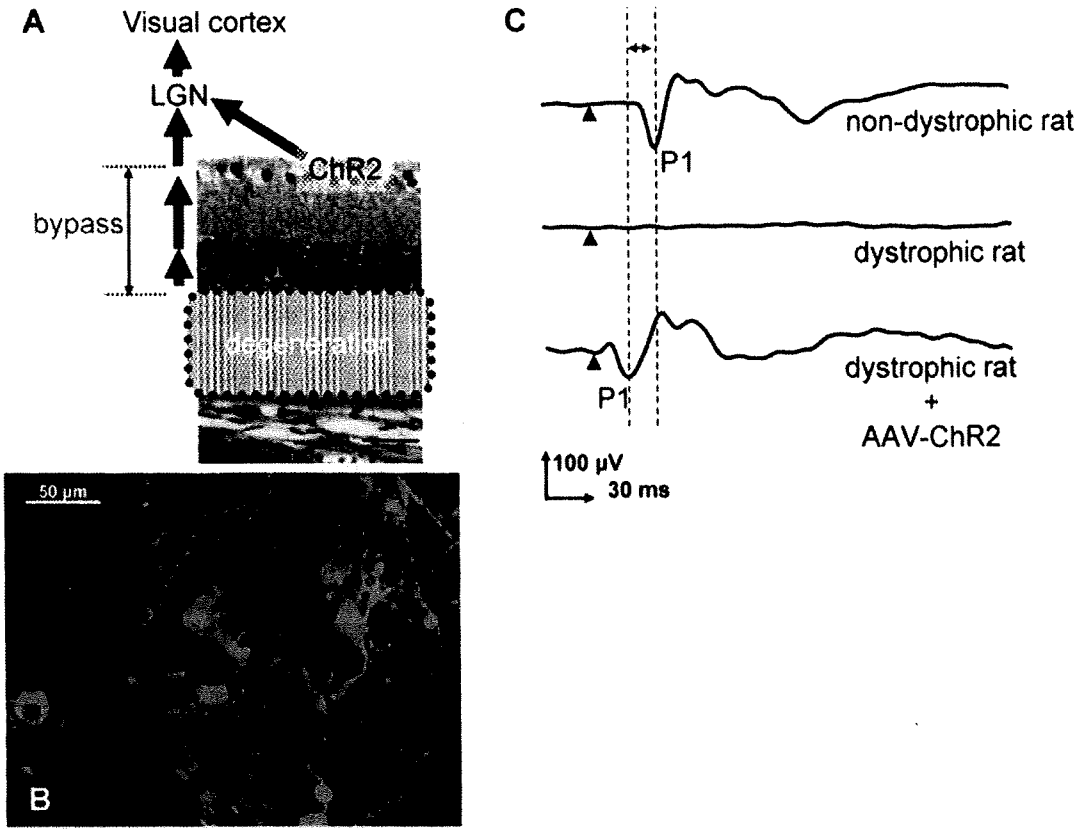

Figure 2. Channelrhodopsin (ChR2)-expressing retinal ganglion cells produce the visually evoked potential. (A) Schematic representation of the visual pathway. In general, a photon is absorbed by photoreceptors, and signals produced by the photoreceptors are transmitted to retinal ganglion cells via second-order neurons such as bipolar cells. However, retinal ganglion cells expressing ChR2 directly absorb the photon. Thus, the visual pathway mediated through the second-order neurons is bypassed by ChR2 expression in retinal ganglion cells (red arrow). (B) About 30% of the total number of retinal ganglion cells expressed ChR2 after a single intravitreal injection of AAV-*ChR2*. (C) The robust amplitude of the visually evoked potential was recorded in the dystrophic rat, which received an intravitreal injection of AAV-*ChR2*. P1 latency of the dystrophic rat is shorter than that of the non-dystrophic rat because of the direct response of retinal ganglion cells to light (red arrow).

rhodopsin (figure 1). The function of ChR2 offers a possibility that ChR2 expression may maintain light sensitivity of neuronal cells (Boyden *et al.* 2005; Ishizuka *et al.* 2006).

ChR2 gene-based strategy for restoring vision

Two *ChR2* gene-based strategies for restoring vision in blind rodents with the photoreceptor degenerative disease RP have been studied. One strategy used ON bipolar cells as the target cells for *ChR2* gene transfer (Lagali *et al.* 2008), whereas the other used retinal ganglion cells (Bi *et al.* 2006; Tomita *et al.* 2007). When the *ChR2* gene is transduced into ON bipolar cells, retinal ON pathway is selectively activated by light. This is a rational way of activating the normal retinal ON pathway, although some methodological difficulties were encountered, such as those pertaining to the mechanism of gene transfer into ON bipolar cells. Lagali *et al.* (2008) successfully expressed the *ChR2* gene in ON bipolar cells by using the mGluR6 promoter, which specifically express in these cells. However, from the perspective of clinical applications, selection of an appropriate vector for transfer of the gene into bipolar cells remains a problem.

On the other hand, it is easy to transfer the gene into retinal ganglion cells. A single intravitreal injection of AAV2/2 vector carrying the *ChR2* gene enables the transfer of the gene into retinal ganglion cells. It is expected that the *ChR2*-expressing retinal ganglion cells directly respond to light and transmit signals to the lateral geniculate nucleus (LGN) without any involvement of the bipolar cell-mediated pathways (figure 2A). We observed that about 30% of the total number of retinal ganglion cells expressed *ChR2* (figure 2B). Royal college of surgeons (RCS) rats are established models of inherited retinal degeneration, becoming blind about three months after birth. Their vision was restored by a single intravitreal injection of AAV-*ChR2*, as determined electrophysiologically (Tomita *et al.* 2007) as well as behaviorally. P1 latency of the *ChR2*-injected rat was

clearly shortened as compared to that of the normal rat (figure 2C). However, it remains unclear what degree of vision is restored by ChR2-expressing retinal ganglion cells. The results of the above mentioned studies give rise to two important questions. There are mainly three types of retinal ganglion cells: ON-ganglion, OFF-ganglion and ON-OFF ganglion cells (Levick 1967; Schiller 1992). Intravitreal injection of AAV vector randomly transfers the gene into all the three types of retinal ganglion cells. The visual signal produced by each type is expected to be different from that produced by nontransduced retinal ganglion cells in the normal visual pathway. Further, primate retinas have the fovea, which lacks ganglion cells. Therefore, only the images obtained from the *ChR2*-expressing retinal ganglion cells may be distorted. Further studies using nonhuman primates, who can undergo the morphological cognition test, are needed to elucidate the images obtained from *ChR2*-expressing retinal ganglion cells.

Prospects of channelrhodopsins

The human eye contains light-sensitive visual pigments—rhodopsin in rods for monochrome dim-light vision and three colour visual pigments in cones for daylight vision; these pigments are sensitive to wavelengths between 350 and 750 nm. The sensitivity of ChR2 is limited to wavelengths of <540 nm, with the peak at 450 nm (Nagel *et al.* 2003). Therefore, even if *ChR2*-expressing ganglion cells can provide useful vision to patients, they can only recognize wavelengths corresponding to blue colour and not green and red colours.

Recently, a few reports have indicated that properties of *ChR2* such as sensitivity to light and its different wavelengths can be improved by modifying ChR2 (Lin *et al.* 2009; Sugiyama *et al.* 2009; Tsunoda and Hegemann 2009; Wang *et al.* 2009). During the efforts to improve different properties of ChR2 by using molecular engineering techniques, a new channelrhodopsin was identified from an unknown microbial rhodopsin—the red-shifted 'ChR2', named as 'VChR1,' since it was identified from the spheroidal alga *Volvox carteri*; it has a ChR2-related sequence and shows a robust wavelength shift towards red. VChR1 can also be useful for restoring vision (Zhang *et al.* 2008).

The central nervous system consists of numerous subtypes of excitatory, inhibitory and modulatory neurons. Signal activities (activation or inhibition) in retinal neurons are bidirectionally controlled by the input information. Channelrhodopsins such as ChR2 and VChR1, transduced into retinal neurons, function as light-gated cation-selective channels and cause depolarization of the neurons by absorbing specific wavelengths of light. Halorhodopsin derived from *Natronomonas pharaonis* (NpHR) is a light-driven chloride pump (Lanyi 1990) and shows peak sensitivity to the wavelength of ~580 nm (yellow). Thus, bidirectional control of neuronal firing can be possible by the transduction of both ChR2 and NpHR in the neurons, because of the difference in the excitation wavelength of the two ion pumps (ChR2, blue; NpHR, yellow) (Evanko 2007; Han and Boyden 2007; Zhang *et al.* 2007a,b).

Channelrhodopsins have generated considerable interest with regard to restoration of vision, and investigation of New World 'channelrhodopsins' is gaining momentum. In the clinical field, expectations are rising with respect to restoration of vision. In the near future, channelrhodopsins might contribute to restoration of lost vision in patients with RP.

Acknowledgements

This work was supported, in part, by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (nos. 17390465, 17791217 and 17659541); Ministry of Health, Labour and Welfare of Japan; Japanese Retinitis Pigmentosa Society; and Suzuken Memorial Foundation. We thank Prof. Duco Hamasaki for discussions and editing the manuscript.

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Received 3 August 2009; accepted 7 September 2009
Published on the Web: 31 December 2009

OPEN ACCESS Freely available online PLoS one

Visual Properties of Transgenic Rats Harboring the Channelrhodopsin-2 Gene Regulated by the Thy-1.2 Promoter

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Abstract

Channelrhodopsin-2 (ChR2), one of the archea-type rhodopsins from green algae, is a potentially useful optogenetic tool for restoring vision in patients with photoreceptor degeneration, such as retinitis pigmentosa. If the ChR2 gene is transferred to retinal ganglion cells (RGCs), which send visual information to the brain, the RGCs may be repurposed to act as photoreceptors. In this study, by using a transgenic rat expressing ChR2 specifically in the RGCs under the regulation of a Thy-1.2 promoter, we tested the possibility that direct photoactivation of RGCs could restore effective vision. Although the contrast sensitivities of the optomotor responses of transgenic rats were similar to those observed in the wild-type rats, they were enhanced for visual stimuli of low-spatial frequency after the degeneration of native photoreceptors. This result suggests that the visual signals derived from the ChR2-expressing RGCs were reinterpreted by the brain to form behavior-related vision.

Citation: Tomita H, Sugano E, Fukazawa Y, Isago H, Sugiyama Y, et al. (2009) Visual Properties of Transgenic Rats Harboring the Channelrhodopsin-2 Gene Regulated by the Thy-1.2 Promoter. PLoS ONE 4(11): e7679. doi:10.1371/journal.pone.0007679

Editor: Eric Warrant, Lund University, Sweden

Received July 16, 2009; **Accepted** October 8, 2009; **Published** November 5, 2009

Funding: This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (No. 17390465, 17791217, 17659541), Science and Culture and Special Coordination Funds for Promoting Science and Technology of the Japanese Government, Strategic Research Program for Brain Sciences (SRPBS), Japanese Retinitis Pigmentosa Society (JRPS), and Suzuken Memorial Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Retinitis pigmentosa (RP) is a genetically heterogeneous disease characterized by degeneration of the retinal photoreceptor cells. A number of genes responsible for RP have been identified, most of them related to the phototransduction pathways. Patients who have such mutations experience night blindness, loss of their peripheral visual field, and loss of central vision [1]. Although the photoreceptor cells are degenerated in the eyes of RP patients with vision loss, other retinal neurons, including retinal ganglion cells (RGCs), are still preserved [2,3,4].

Channelrhodopsin-2 (ChR2), a rhodopsin identified in the green algae Chlamydomonas reinhardtii, is unique in that it acts as a directly light-gated cation-selective ion channel [5]. Several studies have revealed that neurons became photosensitive when transfected with the ChR2 gene [6,7]. In addition, Bi et al. reported that the transfer of ChR2 restored visually evoked cortical responses in blind mice [8]. We also observed restoration of visual response in genetically blind rats [9]. Following on the study of Bi et al. and our own research, we believe that, in addition to their native function of transmitting visual signals to the brain, RGCs are endowed with a photoreceptor-like function by the ChR2 gene. There are three types of RGCs in the mammalian retina: ON, OFF, and ON-OFF [10,11]. Since the transfer of the ChR2 gene into RGCs was not regulated according to RGC type in these studies, it is possible that all RGC types became photosensitive. Thus, RGC-derived signals must be reinterpreted by the brain in order to organize effective vision. Transgenic rats that express ChR2 in RGCs provide a useful experimental model with which to evaluate quantitatively the visual function of an animal in which RGCs are made photosensitive by the expression of ChR2.

The Thy-1.2 antigen is a glycoprotein found on the cell surface of a variety of cell types [12,13]. Rat Thy-1.2 antigen has been found to be abundant in the brain and thymus [14,15]. In the retina, the Thy-1.2 antigen is recognized to be a marker specific to RGCs [16,17]. Thus, the Thy-1.2 promoter is an effective regulator of a gene that is expressed exclusively in the RGCs [18,19,20]. In the present study, we generated transgenic rats in which the ChR2 transgene was driven by the Thy-1.2 promoter. One of them, line 4 (W-TChR2V4), expressed ChR2 specifically

in the RGCs of the entire retina. We found that contrast sensitivities of optomotor responses in W-TChR2V4 rats were equivalent to wild-type rats, even when native photoreceptor cells were degenerated by continuous light exposure. However, contrast sensitivities at low spatial frequencies were enhanced after photoreceptor cell degeneration. This suggests that the visual signals derived from the ChR2-expressing RGCs are reinterpreted to form behavior-related vision.

Results

Generation of Transgenic Rats

The Thy-1.2 vector derived from a 6.5-kb fragment of the murine Thy-1.2 gene has been reported to promote gene expression in RGCs and in neurons in the brain [21] (Fig. 1A). We analyzed the genomic insertion of a ChR2V cDNA fragment by performing polymerase chain reaction (PCR) on tail DNA and subsequently detected a PCR product of 324 bp in eight founder rats (Fig. 1B). We termed these transgene positive lines "Wistar-Thy-1.2 promoter-Channelrhodopsin 2-Venus rats" (W-TChR2V). Among these 8 lines (W-TChR2V1-8), 6 lines, which were capable of reproduction and transgenerational propagation of the transgene, were evaluated further for expression of the ChR2V protein in the retina.

Under fluorescence microscopy, ChR2V was shown to be expressed in the retina of the heterozygous rat (ChR2V +/−) in four of six lines of transgenic rats: W-TChR2V1, W-TChR2V4, W-TChR2V5, and W-TChR2V7 (Fig. 2A–D). As shown in Fig. 2, ChR2V expression was extensively observed in the flat-mounted retina. Vertical sections indicated that cells expressing ChR2V were distributed differently in each transgenic line. In the case of W-TChR2V1, the "Venus" marker fluorescence (see Methods section) was observed in the RGC layer (GCL), inner plexiform layer (IPL), and outer plexiform layer (OPL). The W-TChR2V4 strain showed ChR2V expression in the GCL and IPL. In addition to expression in these layers, strains W-TChR2V5 and W-TChR2V7 showed intense fluorescence in the inner nuclear layer (INL). When the flat-mounted retina of the W-TChR2V4 rat was vertically examined using the z-axis scanning mode of the microscope, the Venus fluorescence was colocalized with Fluorogold, which retrogradely labeled the RGCs (Fig. 2E).

Direct Photoactivation of ChR2V-Expressing RGCs

We expected that the ChR2V-expressing RGCs in the W-TChR2V4 rat retina would be sensitive to light. To test this hypothesis, we investigated the light-evoked responses of ChR2V-expressing RGCs, while all the synaptic inputs derived from the photoreceptor cells were pharmacologically blocked by 1 mM kynurenic acid, a nonselective glutamate receptor blocker. In a ChR2V-expressing RGC placed under whole-cell voltage clamp at −60 mV (Fig. 3A), a blue light-emitting diode (LED) light pulse evoked an inward current whose amplitude was dependent on the light power density (Fig. 3B). The light-evoked current has similarities to a ChR2 photocurrent [6], i.e. rapid onset without detectable latency, peak-and-plateau biphasic kinetics, and a rapid offset. The onset time constant was dependent on the light power density, but 4–9 ms in this case. The offset time constant was less dependent on the light power density and was 15–18 ms. Under current-clamp configuration, membrane potential was depolarized by an LED light pulse with an undetectable delay and was accompanied by action potentials (Fig. 3C). Action potentials were evoked by a 100-ms LED pulse with a power density as low as $3.7 \pm 2.3\ \mu W/mm^2$ ($n = 14$). We found that the action potential could also be evoked by an LED light pulse as short as 10 ms (Fig. 3D).

Degeneration of Photoreceptor Cells

There were 11–12 rows of photoreceptor nuclei in the outer nuclear layer (ONL) of the transgenic rats; this is a number usually

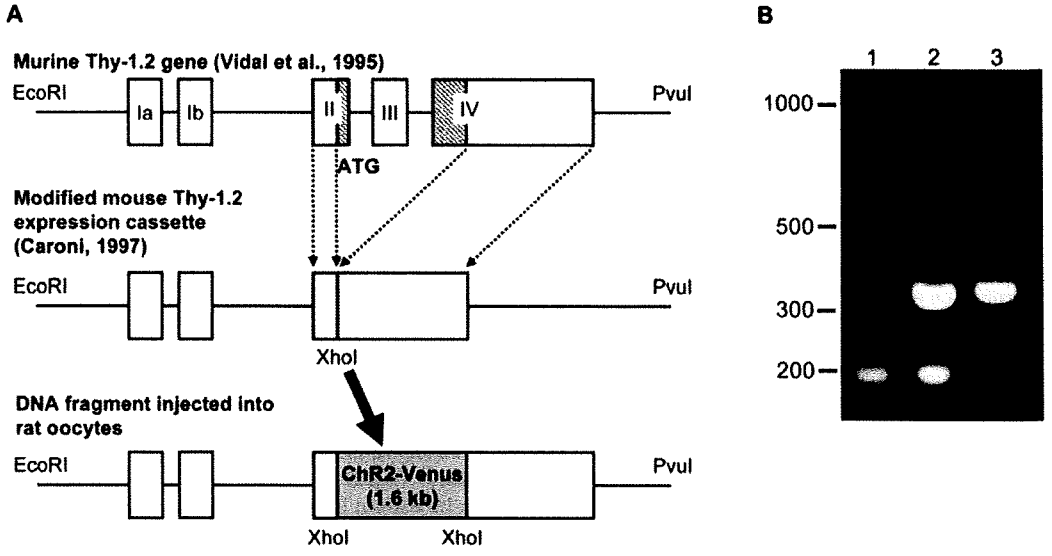

Figure 1. Generation of Thy-1.2 ChR2V transgenic rat. Schematic drawing of DNA fragment injected into rat oocytes. (A) The cDNA coding channelrhodopsin-2 (ChR2) tagged with Venus was inserted at XhoI site of the modified mouse Thy-1.2 expression cassette. A linearized DNA fragment (7.5 kb) prepared by digestion with EcoRI and PvuI restriction enzymes was injected into rat oocytes. (B) Examples of PCR analysis of genomic DNA from transgenic founder rats injected with the transgene shown in A. Genomic DNAs from the injected DNA fragment (lane 1), a transgenic founder (lane 2) and a non transgenic founder (lane 3) were amplified by PCR. DNA bands at 173 bp and 324 bp correspond to amplified DNA fragments for the transgene (ChR2-Venus, ChR2V) and the T cell receptor gene as an internal control, respectively.
doi:10.1371/journal.pone.0007679.g001