厚生労働科学研究費補助金

障害者対策総合研究事業（感覚器障害分野）

新規開発マルチカラー化チャネルロドプシン遺伝子を用いた視覚再生研究

（Ｈ２１－感覚－一般－００８）

平成２１年度　総括研究報告書

研究代表者　富田　浩史

平成２２（２０１０）年　４月

目　　次

Ⅰ．総括研究報告

厚生労働科学研究費補助金　障害者対策総合研究事業（感覚器障害分野）

総括研究報告書

新規開発マルチカラー化チャネルロドプシン遺伝子を用いた視覚再生研究

研究代表者：富田　浩史　国際高等研究教育機構　准教授

研究要旨

我々は緑藻類より単離したチャネルロドプシン-2（ChR2）遺伝子の網膜への導入による視覚再生法を検討している。本研究では、ChR2の遺伝子導入によって得られる視覚特性を明らかにすること、ならびに遺伝子導入による副作用を調べ、臨床応用の可能性を明らかにすることを目的とする。

本年度は、ChR2を網膜神経節細胞に特異的に発現するトランスジェニックラット(ChR2V-TGラット)を用いて、ChR2によって得られる視覚特性について調べた。また、カニクイザルを用いた視力検査を実施するために、視力検査システムの開発とカニクイザルの視力検査トレーニングを行った。視細胞変性Thyl-ChR2Vラットを用いた研究から、正常と同等の空間周波数特性が得られ（青色：460nm）、ChR2の神経節細胞への遺伝子導入によって高度な視機能が作られる可能性が示された。また、カニクイザルをトレーニングすることによって、ランドルト環を用いた視力検査を実施することが可能となった。

研究分担者

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A. 研究目的

網膜色素変性の遺伝子異常保因者は日本人4000人に一人、人口で4～5万人はいると推定されている。網膜色素変性と同様に視細胞変性により失明を来す疾患である加齢黄斑変性は、アメリカでは中途失明原因の１位に位置し、日本でも高齢化社会の進行に伴い、増加の一途を辿っている。これらの疾患は現在有効な治療法は無く、厚生省特定疾患に指定されている。更に重篤な網膜はく離や増加の一途をたどる増殖糖尿病網膜症による失明者を加えると、中途失明者の数は年間16000人にも達し、失明に対する有効な治療法の開発は社会的急務である。

唯一の視覚再生法として、世界的に人工網膜が研究されており、アメリカ、ドイツを中心に臨床研究が行われている。これらの臨床研究で、網膜を電気的に刺激することによって擬似的な光覚が得られることが明らかになっている。しかし、現段階で作製できる網膜刺激電極は１００個、最大でも１０００個が限度で、１００万個存在する網膜神経節細胞の機能を代替できるかなどの様々な問題点が明らかになってきている。

そこで我々は緑藻類より単離したチャネルロドプシン-2（ChR2）遺伝子の網膜への導入による視覚再生を検討してきた。現在までに、遺伝盲ラットの光反応性を回復させることに成功し、行動学実験から実際に見えていることを確認している。しかし、ChR2の遺伝子導入によってどの程度の視力が得られるかや緑藻由来の遺伝子を眼内で発現させた場合の副作用、安全性について不明であり、ヒトへの応用に向けて、これらの点を明らかにする必要がある。

本研究は、ラットおよびカニクイザルを用いて、回復される視力を定量的に評価すること、ならび、遺伝子導入による副作用を調べ、臨床応用の可能性を明らかにすることを目的とする。

B. 研究方法

ChR2の網膜での発現が正常な視機能に及ぼす影響およびChR2によって得られる視覚特性を調べる目的で、生まれながらに、網膜神経節細胞にChR2を持つトランスジェニック（ChR2V-TG）ラットを作製した。系統ごとに網膜伸展、網膜スライス標本を作製し、網膜でのChR2遺伝子の発現部位を調べた。網膜神経節細胞にのみChR2の発現が見られた系統につ

いて、オプトモーターを用いて行動学的に空間周波数特性、コントラスト感度を調べた。ChR2V-TG ラットの視細胞のみを選択的に変性させる目的で、少なくとも 2 週間、12 時間 5-10lux、12 時間 0lux の明暗周期で飼育した後、3000lux、7 日間連続光照射を行った。視細胞変性後、オプトモーターを用いて視覚特性を調べた。また、回復される視力を定量的に評価するために、カニクイザルのトレーニングを行い、ヒトの眼科視力検査と同等のランドルト環を用いた視力検査を実施した。

（倫理面への配慮）

本研究に用いる実験動物の取扱いは、遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律を遵守し、国立大学法人東北大学遺伝子組換え実験安全管理規定のもとに、東北大学動物実験施設において、その規定に従って管理する。特に今回、サル片眼失明モデルを作製するために、サル動物実験計画書については、東北大学動物実験倫理委員会によって慎重に議論され、本研究に関わる全ての動物実験、遺伝組み換え実験は承認された。

C. 研究結果

1．ChR2 トランスジェニックラットの視覚特性　網膜神経節細胞の特異的抗原である Thy1 のプロモーターを利用して、神経節細胞特異的に ChR2 を発現するトランスジェニックラットを作製した。トランスジェニックラット、7 系統を調べた結果、系統 4 では、神経節細胞特異的に ChR2 の発現が認められ、系統 4 のラットを用いて、ChR2 によって得られる視覚特性を検討した。正常な視細胞を持つ ChR2V-TG ラットの視覚特性は ChR2 を持たない野生型ラットと同等の空間周波数特性、コントラスト感度を示した。一方、視細胞を変性させた ChR2-TG ラットでは、空間周波数特性は野生型と同等であったのに対し、コントラスト感度は顕著に上昇した。

2．カニクイザルの視力検査トレーニング

カニクイザルは約３ヶ月の馴化の後、１ヶ月間トレーニングを行うことによって、ランドルト環の視力検査を習得した。最高 0.2 までの視力について検査を行った結果、ランドルト環の大きさに関わらず、その正解率は 90%以上であった。しかし、ランドルト環の向きの違いを見分けるのに要する時間は、ランドルト環が小さくなるにつれ、増大した。

D. 考察

神経節細胞には種々の役割の異なる神経節細胞が存在し、それらの神経節細胞に一様に ChR2 が導入された時にどのような視覚が得られるか不明であった。今回のトランスジェニックラットを用いた研究から、神経節細胞の種類に関係なく、ChR2 が導入されたとしても高度な視覚が得られる可能性が示された。しかし、トランスジェニックラットでの研究であり、生まれながらにして ChR2 を発現しているために、トランスジェニックラットの視覚システムが正常な視覚システムと同じであったかどうかは疑問がある。今後はサルでの視力検査を実施し、回復される視力を評価する必要がある。

E. 結論

網膜神経節細胞は視神経を構成する細胞であり、元来、網膜から脳への視覚情報を伝達する役割を担っている。網膜色素変性症、加齢黄斑変性症患者では、網膜の光受容細胞である視細胞が変性、消失しても、網膜神経節細胞は残存していることが報告されている。緑藻類クラミドモナス由来の ChR2 は、光受容に伴い細胞内に陽イオンを透過させる、光受容陽イオンチャネルとしての機能を有する。今回、この ChR2 を網膜神経節細胞に導入することによって、青色に限定すると、正常と同等の視覚特性が得られることが明らかになり、新たな視覚再建法となる可能性が示された。

F. 健康危険情報

なし。

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3. 子供の科学　3 月号　「藻類の遺伝子で視力回復」
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【国内向け英語放送】番組名：「Japan & World Update」14:00～14:30（後半 10 分間）
【海外向け短波ラジオ放送】英、仏、中国、アラビアなど 17 言語で放送

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添付資料

チャネルロドプシン-2

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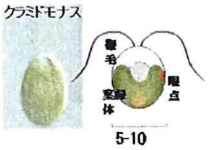
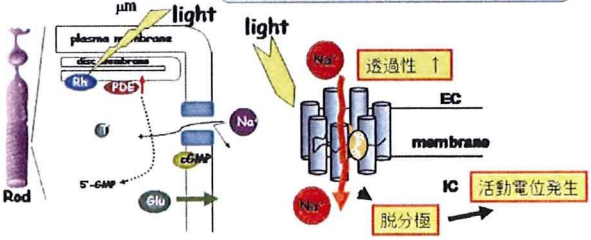


池や沼に住む走光性の緑藻類
葉緑体を持ち、光合成によりエネルギーを作る

眼点で光を受容し、鞭毛運動により移動する
眼点に存在する光受容タンパク質

チャネルロドプシン-2
光受容体 ＋ 陽イオン選択的チャネル



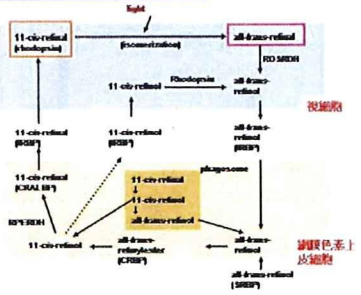
ロドプシンとチャネルロドプシン-2の相違

ロドプシン

発色団のレチナールは光吸収後、オプシンと乖離し、網膜色素上皮細胞で再合成される

チャネルロドプシン-2

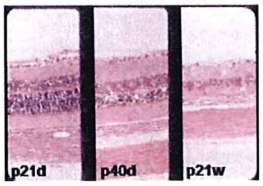
レチナールは再合成を必要とせず、暗状態でall-trans型に戻る



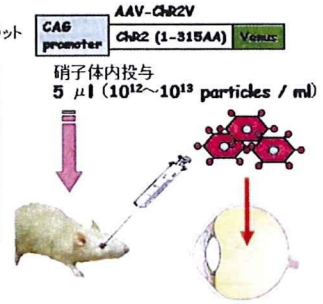
光
(発色団)
all-trans retinal → 13-cis-retinal
暗
(reversible)

方法

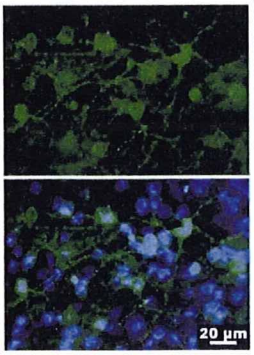
遺伝盲ラット
Royal College of Surgeons (RCS) ラット
(6 or 10 month-old, rdy/rdy)

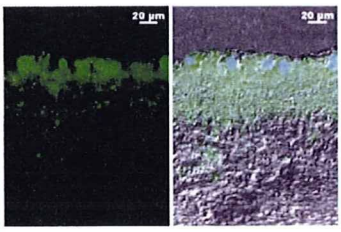


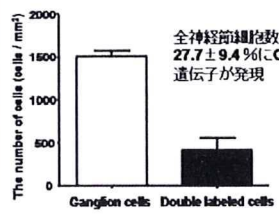
一旦、正常に網膜が形成されるが生後3週より視細胞の変性が始まり、生後3ヶ月で完全に視細胞が消失する



網膜におけるChR2Vの発現

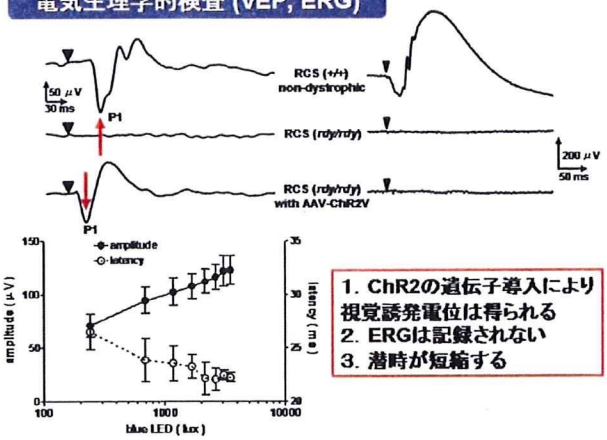






全神経節細胞数の
27.7±9.4 %にChR2V
遺伝子が発現

電気生理学的検査 (VEP, ERG)



1. ChR2の遺伝子導入により視覚誘発電位は得られる
2. ERGは記録されない
3. 潜時が短縮する

ChR2を神経節細胞に導入し、発現させることで視機能を回復できる

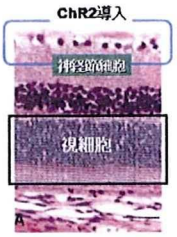
生来の役割(脳への情報伝達)に光受容能を与える

光受容
↓
情報伝達

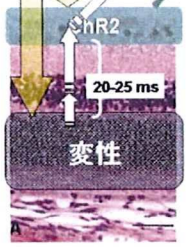
問題点

- 正常な視覚に比べ、光感受性が低い。
- 感受波長は青色領域に限定されており、見ることができるのは青色のみである。

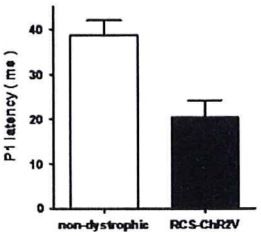
- 緑藻類由来遺伝子である。
  →異種タンパク質
- 遺伝子治療



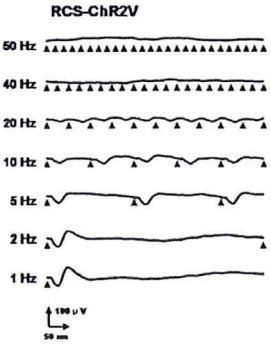
視覚野
光、映像
外側膝状体

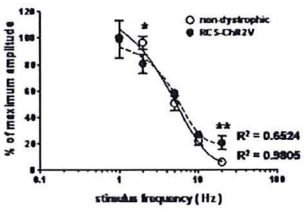


1. ChR2の遺伝子導入により視覚誘発電位が得られる
2. ERGは記録されない
3. 潜時が短縮する

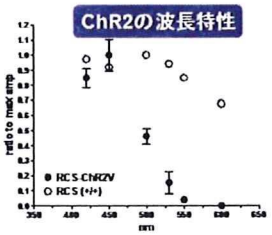


刺激に対する追従性 (正常な視覚との比較)





ChR2の波長特性



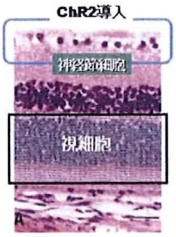
ChR2を神経節細胞に導入し、発現させることで視機能を回復できる

生来の役割(脳への情報伝達)に光受容能を与える

光受容
＋
情報伝達

問題点

・正常な視覚に比べ、光感受性が低い。
・感受波長は青色領域に限定されており、見ることができるのは青色のみである。
・緑藻類由来遺伝子である。→異種タンパク質
・遺伝子治療

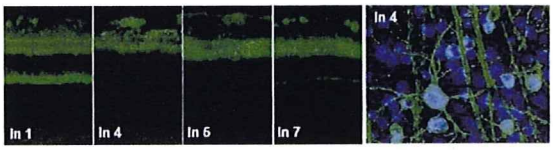


ON-, OFF-Ganglionに、ランダムにChR2が導入されたとき、どのような視覚が得られるか ？？？

Thy I-ChR2V トランスジェニックラットの作製

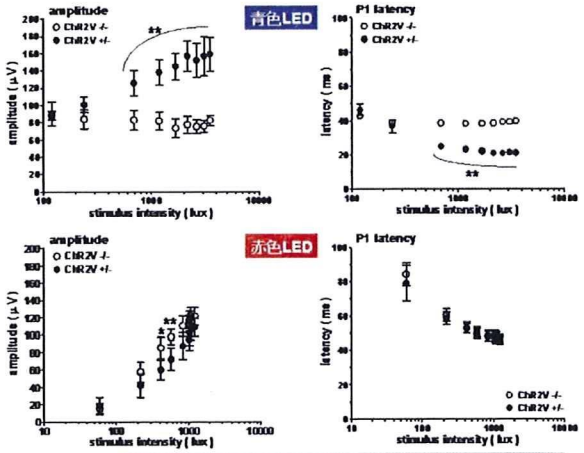
神経節細胞にChR2が導入
→ 神経節細胞が作る視覚は ？？？
(On, Off の区別無く導入される。)

Thy I 抗原 ･･･網膜では、神経節細胞に特異的に発現
Thy I プロモーターの下流にChR2遺伝子を組み込んだトランスジェニックラットを作製



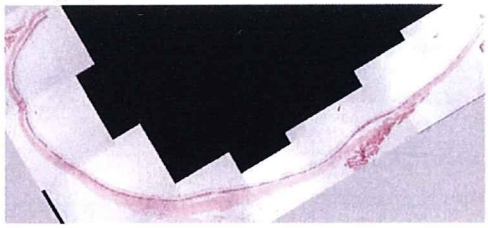
発現効率 約45%

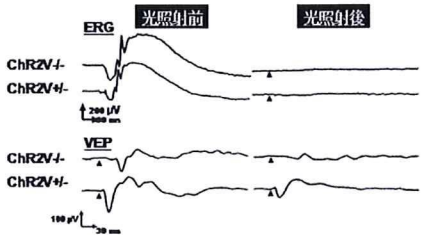
連続光照射によって視細胞を変性させ、視機能を評価



光照射前
5 lux: 12hr
Dark: 12hr

光照射条件
3000 lux
1 週間



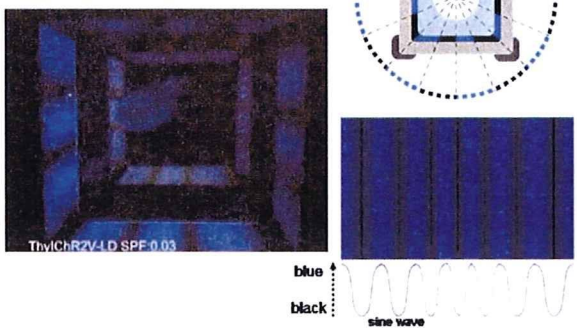


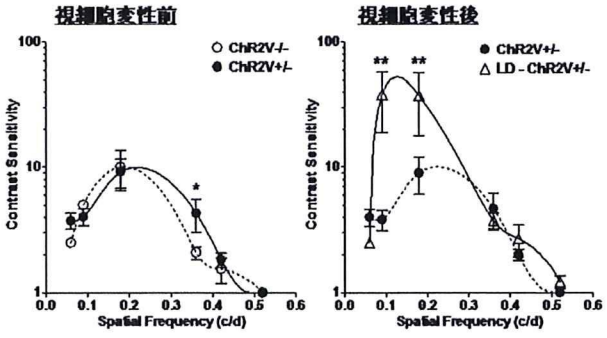
視細胞の応答:消失
→視細胞変性

・青色刺激のみ視覚誘発電位が記録される。
・潜時が短縮される。

オプトモーターを用いた視機能検査

各空間周波数におけるコントラスト感度を測定

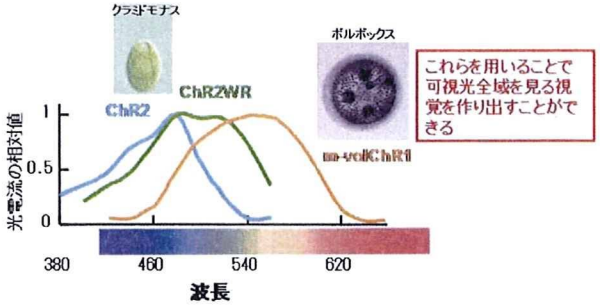




低い空間周波数で高いコントラスト感度が得られる。

問題点　限られた波長特性

ChR2
ChR2WR　(感受波長域を拡大したChR2)
m-volChR1　(黄、赤に感受性を持つ)



これらを用いることで可視光全域を見る視覚を作り出すことができる

カニクイザル視力検査

II. 研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tomita H, Sugano E, Isago H, Hiroi T, Wang Z, Ohta E, Tamai M	Channelrhodopsin-2 Gene Transduced into Retinal Ganglion Cells Restores Functional Vision in Genetically Blind Rats	Exp Eye Res	90	426-436	2010
Tomita H, Sugano E, Isago H, Tamai M	Channelrhodopsins provide a breakthrough insight into strategies for curing blindness	J Genetics	88	409-415	2009
Tomita H, Sugano E, Fukazawa Y, Isago H, Sugiyama Y, Hiroi T, Ishizuka T, Mushiake H, Kato M, Hirabayashi M, Shigemoto R, Yawo	Visual Properties of Transgenic Rats Harboring the Channelrhodopsin-2 Gene regulated by the Thy-1.2 Promoter.	PLoS ONE	4(11)	e7679	2009
Wang H, Sugiyama Y, Hikima T, Sugano E, Tomita H, Takahashi T, Ishizuka T, Yawo H	Molecular determinants differentiating photocurrent properties of two channelrhodopsins from chlamydomonas	J Biol Chem	284	5685-5696	2009
Hikima T, Araki R, Ishizuka T, Yawo H	beta-Phorbol ester-induced enhancement of exocytosis in large mossy fiber boutons of mouse hippocampus	J Physiol Sci	59	263-274	2009
Matsuzaka Y, Sakamoto K, Tanaka T, Furusawa Y, Mushiake H	Cannula-aided penetration: a simple method to insert structurally weak electrodes into brain through the dura mater	Neurosci. Res.	65	126-129	2009
Kobayashi R, Kanno S, Lee S, Fukushima T, Sakamoto K, Matsuzaka Y, Katayama N, Mushiake H, Koyanagi	Development of double-sided Si neural probe with microfluidic channels using wafer direct bonding technique	Neural Engineering, 2009. NER '09. 4th International IEEE/EMBS Conference		96-99	2009
Sakamoto K, Matsuzaka Y, Suenaga T, Watanabe H, Kobayashi R, Fukushima T, Katayama N, Tanaka T, Koyanagi M,	A simple device allowing silicon microelectrode insertion for chronic neural recording in primates.	Neural Engineering, 2009. NER '09. 4th International IEEE/EMBS Conference 2009		104-107	2009
杉山友香、王　紅霞、石塚　徹、八尾　寛	光信号を電気情報に換えるタンパク質チャネルロドプシン-オプトジェネティクスへの招待-	現代化学	459	30-36	2009

III. 研究成果の刊行物・別刷

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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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

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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

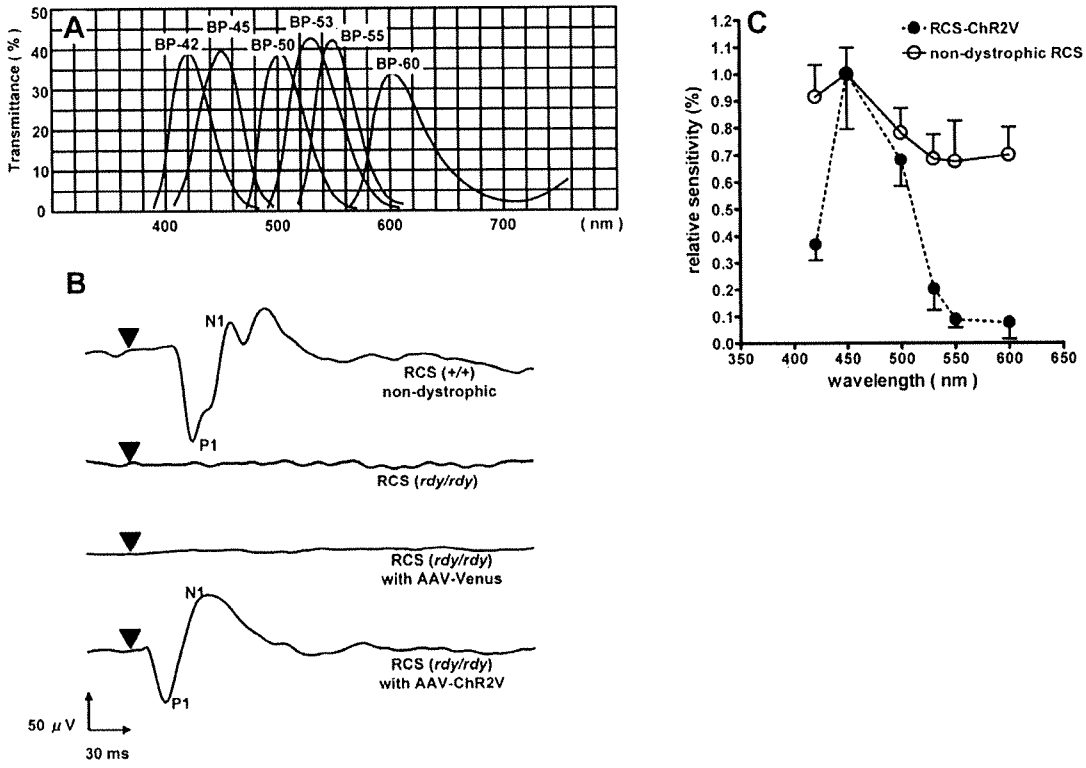

Fig. 1. Spectral responsivity of RCS rats transduced with the *ChR2V* gene. Different parts of the spectrum were isolated by six band-pass filters. A. Band passes for the six band-pass filters used to isolate different wavelengths of the visible spectrum. B. Typical waveforms of the VEPs elicited by 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

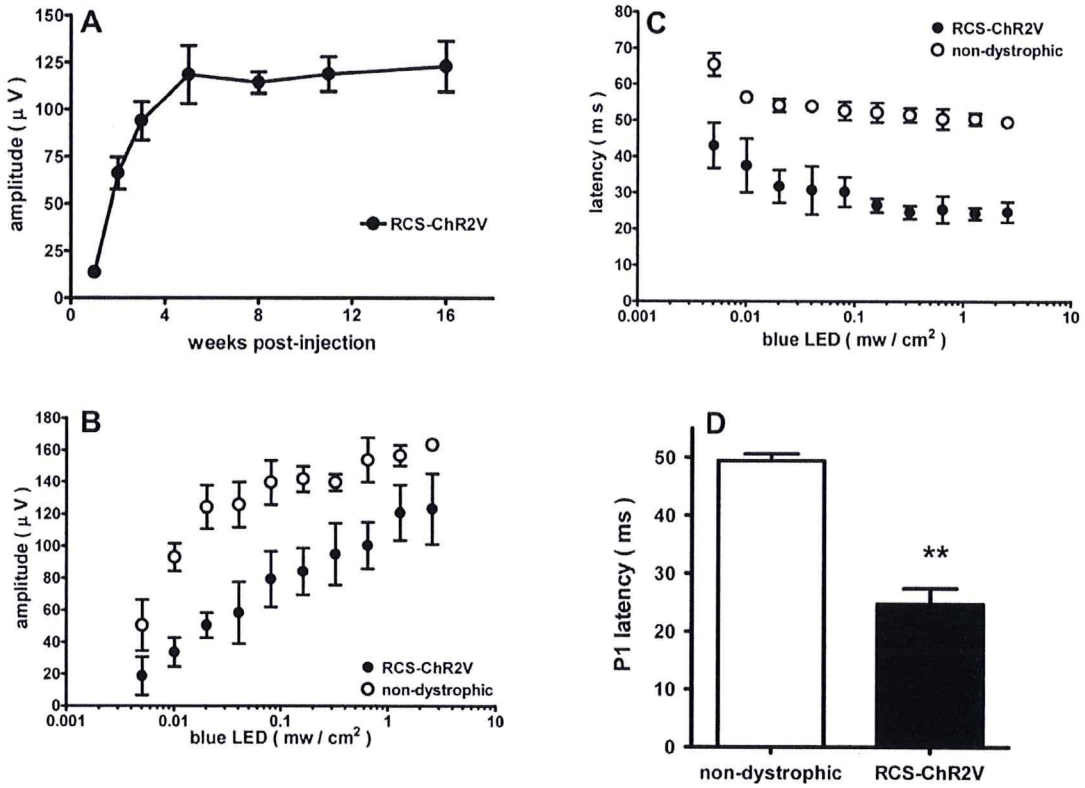

Fig. 2. VEPs recorded from RCS rats transduced with the *ChR2V* gene. A. Changes in amplitude at different weeks after the injection of AAV-ChR2V. B. Changes in amplitude (P1–N1) and latency (P1) elicited by different stimulus intensities. Blue LEDs (435–500 nm, Peak at 470 nm) were used to elicit the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

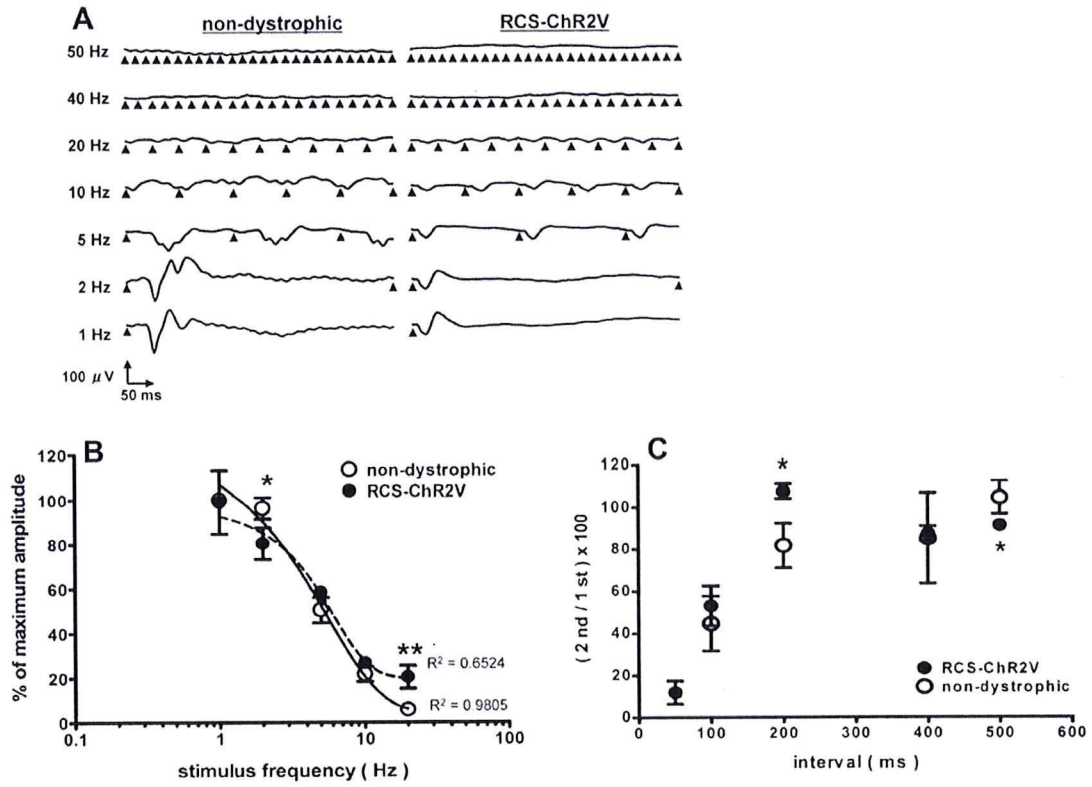

Fig. 3. Changes of responsivity in VEPs elicited by different stimulus frequencies. A. Typical VEP waveforms elicited by different stimulus frequencies. B. Changes in VEP amplitude elicited by different stimulus frequencies. Data are expressed as percentages of the amplitude at 1 Hz. The amplitude recruitment 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

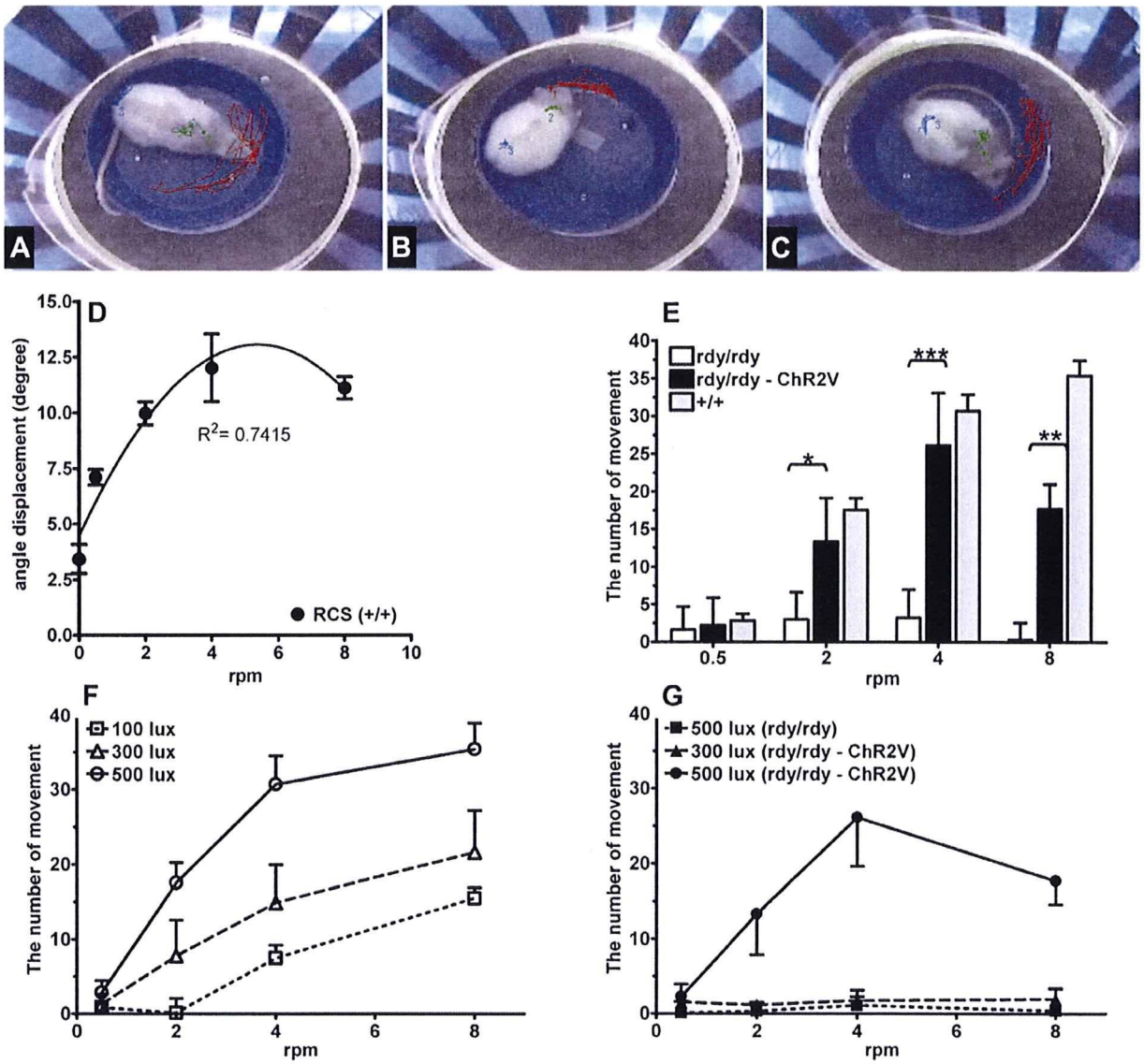

Fig. 4. Behavioral assessment of dystrophic RCS rats and *ChR2V*-transduced rats. The traces of each marked point in non-dystrophic (A), dystrophic (B) and ChR2V-transduced dystrophic (C) rats during a test at 4 rpm. The red, green and blue lines correspond to the marks on the nose, the neck and the 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

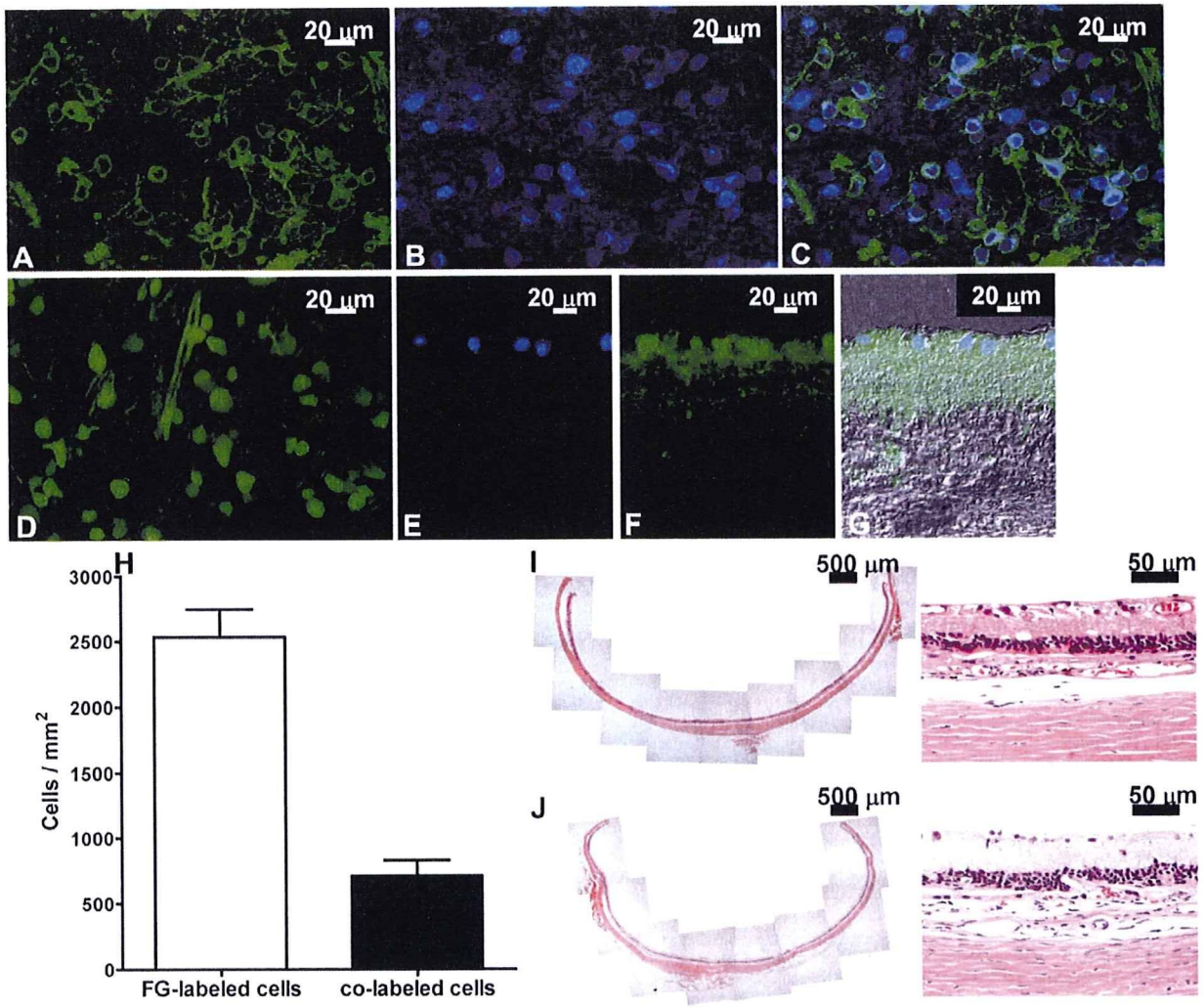

Fig. 5. Expression of ChR2V in the retina. Histological examination of retinas of rats injected with AAV-ChR2V at 16 weeks after the injection. A. Flat-mounted section showing the expression of *ChR2V* gene by green fluorescence. B. Retinal ganglion cells that were retrogradely labeled with fluorogold. 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.

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