

Original Article

AAV-mediated channelrhodopsin-2 expression modified the visual function in rats via light responsiveness of visual cortex remodeling rather than improving development of retinal ganglion cells

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Abstract: Retinitis pigmentosa (RP) represents a group of hereditary diseases which possesses similar visual symptoms. Channelrhodopsin-2 (ChR2) is a kind of light-sensitive protein derived from cyanobacteria and can be stimulated by blue light. In the present study, *ChR2* gene was transduced into the retinal ganglion cells (RGCs) of the retinitis pigmentosa (RP) animal model to test its potential to remodel the visual function. Double-immunofluorescence, patch clamp technique, intracellular staining, and visually evoked potentials induction were applied to observe the impact of the *ChR2* transduction on the visual function of the model animals. It was found that the transduction of *ChR2* into RGCs could postpone the loss of RGCs in retinal degeneration and the dendrites of survival ChR2-RGCs had wider distribution than those in RP animals. Action potential of *ChR2* transduced RGCs could be induced by electrical and light stimulation. However, the frequency and amplitude were still different from that of healthy RGCs. Although the reconstruction for visual function of neuron in RP animal models was not fully achieved in the present study, the modification using ChR2 did have led to the visual function remodeling in RP animals.

Keywords: Adeno-associated virus type 2, channelrhodopsin-2, light responsive retinitis pigmentosa

Introduction

Retinitis pigmentosa (RP) is a type of retinal degenerative disorder which affects photoreceptors. Attack of RP is always associated with a progressive loss of photoreceptor cells and results in reduction of peripheral visual field and finally, blindness [1]. RP has already led to the completely loss of visual function of 15 million people worldwide. What's worse, the number of RP patients has kept increasing with the aging of the world population [2, 3]. Onset of RP can be attributed to the mutation of multiple genes which are closely related to photo-transduction pathway (RetNet; <http://www.sph.uth.tmc.edu/Retnet/>). However, even with the severe outcomes of RP and findings primarily underlying the disease mechanism, the genetic origin, the disease onset, and the progression leading to the blindness by RP are still poorly understandable [4, 5].

In most cases, the RP-related gene alternations firstly occurs in rod photoreceptors, and as the disease progressing, the cones photoreceptor will lost their light-sensitive function gradually following degeneration of rod photoreceptors without gene mutations [6]. But even in the latest stages of RP, a certain number of morphologically changed and light-insensitive cones which are a key structure for high resolution vision and reading [7, 8] still retain alive in the human fovea. Based on these findings, recent treatment strategies based on gene therapies have made considerable advance in ameliorating a relatively rare form of RP in human patients [9, 10]. However, treatment of RP with gene therapies is rendered less effective by the complex heterogeneity of retinal degeneration, retinal reorganization, and photoreceptor death [11, 12]. Development of a novel gene treatment strategy which is broadly applicable to restore vision function in RP patients regard-

less of different genetic mutation profiles is critical to improve the prognosis of RP.

In recent year, several studies have provided some promising schemes for RP patients to regain their vision function without targeting certain mutations. It is reported that although photoreceptor cells in RP patients are always degenerated, other types of retinal neurons, such as retinal ganglion cells (RGCs), are preserved [13-15]. Several groups have independently demonstrated a successful gene therapy in which spared inner retinal neurons were stimulated using channelrhodopsin-2 (ChR2) [16, 17]. ChR2 is cloned from the green algae *Chlamydomonas reinhardtii* and classified as a micro-type rhodopsin [18-20]. The molecule was firstly applied to restore the vision by Bi and his colleague in 2006 [21]. They demonstrated that ChR2 expression by adeno-associated viral vector (rAAV) reversed the vision loss due to RP of blind mice. Subsequently, other scientists have verified the potential of ChR2 in improving visual function independently as well [1, 22, 23].

To further explore the manipulation of ChR2 as a potential vision restore strategy, in the present study, ChR2 gene were transduced into RP mice models transfected with adeno-associated virus type 2 (AAV2) method. The models were cultured for different time courses in our experimental design. Double-immunofluorescence, intracellular staining, patch clamp technique, and visually evoked potentials induction were conducted to assess the impact of the *ChR2* transduction on the visual function of the model animals. Moreover, the time-dependent experimental design was also considered as an assessment on the *ChR2* function maintains in RGCs.

Materials and methods

Experimental animals

Twenty-one day old RCS-p+ (dystrophic, rdy/rdy) rats and p+ rats (non-dystrophic, +/+) were purchased from JOINN Laboratories, Inc., China. The rats were reared in cages in a light-controlled room that has a fixed lighting schedule (8:00 to 20:00). Light was generated by two fluorescent lamps that create 60 lux of intensity at the animal level. The room humidity was controlled at 50% to 60% and the temperature

was held at 22°C-25°C. All experimental protocols were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of AAV2 vector carrying the ChR2 gene construct

The construction of AAV2 vector expression ChR2 was performed as described previously [23, 24]: briefly, the sequence of residues 1-315 in N-terminal fragment of *ChR2* (H134R) gene was obtained from GenBank online (Accession No. AF461397) and fused with red fluorescent protein (RFP) at the end of the ChR2 encoding fragment. Then the ChR2 (H134R)-RFP was introduced into the AAV2/8-hSyn vector under the transcriptional control of a HSYN promoter and purified by a single-step column purification method as previously described [23, 25]. Because of the HSYN promoter, viral vectors could be easily transduced into neurons in rat retina. The packaged viruses were then purified in PBS and concentrated with a titer of 1×10^{12} AAV-chR2-RFP genome copies per milliliter.

In vivo transduction of vectors

Twenty-four RCS-p+ rats were randomly divided into two groups (12 for each group) and 12 p+ rats were employed as control: A) CTR-RGCs group, p+ rats without any administration; B) RCS-RGCs group, RCS-p+ without any administration; C) ChR2-RGCs group, RCS-p+ rats transduced with AAV2/8-hSyn-ChR2 (H134R)-RFP vector. Transduction of AAV2/8-hSyn-ChR2 (H134R)-RFP vector was conducted as follows: RCS-p+ rats in ChR2-RGCs group were anesthetized with 60 mg/kg body weight phenobarbital sodium. After administration of anesthesia, the rats were moved to an animal operating table under a microscope at the biological super-clean bench under aseptic conditions. The viral suspension was delivered to the vitreous of the eye (2 μ L) by Hamilton microinjector. Rats in ChR2-RGCs group successfully expressed RFP in RGCs (Figure S1). After transduction, all the animals were raised under same conditions and six cases in each group were sampled when age reached 60 day old (Pn60d) and 90 day old (Pn90d) for morphological and electrophysiological detection, respectively.

Immunofluorescent microscopy

After anesthesia with 60 mg/kg body weight phenobarbital sodium, eyeballs tissue of rats in different treatments were harvested and fixed in 4% paraformaldehyde for 30 min. Then the retina was separated from the eyeball and incubated in 4% paraformaldehyde for another 2 h. The tissue samples were permeabilized with 0.5% Triton X-100 for 30 min. After being washed with PBS for three cycles, 5 min for each cycle, the tissue were blocked in 10% goat serum for 2 min at 37°C. 20 µL primary anti-RFP antibodies (1:500) and primary anti-BrnIII tubulin antibodies (1:300) were then added respectively and the tissues were incubated overnight at 4°C. Afterwards, the retinas were incubated for 4 h in Cy3-conjugated goat anti-rabbit IgG (1:1000, life technologies, USA). Finally, tissues were washed and then stained with 4, 6-diamino-2-phenyl indole (DAPI) for 8 min at room temperature. After three cycles of 10-min washes with PBS buffer, the slides were fixed and imaged with the fluorescent microscopy at 200 × magnification. The RGCs densities in different treatments were also recorded at 200 × magnification.

Whole-cell patch-clamp recordings and detection of passive membrane properties

Rats from different groups were sacrificed and retina tissues were harvested, cut into 200 µm slices, and preserved in extracellular solution in an atmosphere consisted of 5% CO₂ and 95% O₂. Dissociated RGCs and retinal slice were prepared as described previously [26, 27]. The membrane currents were recorded using an Axopatch 200B patch amplifier (Axon Instruments, USA) and digitized with a Digidata 1440 A/D converter and Clampex 10.0 software (Molecular Devices, USA). Recording pipettes were pulled from borosilicate glass tubing with a horizontal puller (P-97, Sutter Instruments, USA), and these pipettes typically had a resistance of 3.0-5.0 MΩ when filled with pipette solution containing (in mM) 140 KCl, 10 EGTA, 10 HEPES, and 5 MgATP, with pH 7.3 adjusted with KOH and 290-310 mOsm in osmolarity at room temperature under an infra-red microscope. The experiments aiming to elucidate the electrical behavior of in situ RGCs were also performed: resting membrane potentials (RMP), input resistance (IR), the current change in sodium-potassium channels as well as action

potential firing were all determined. Of the series resistance, 70%-80% was compensated electronically. Signals were filtered at 2 kHz and digitized at 10 kHz. The distributions of dendrites of RGCs in different treatments were observed using a fluorescent microscopy at 200 × magnification. Each measurement was represented by at three replicates.

Recording of electroretinograms (ERGs) and visual evoked potentials (VEPs)

Rats under different treatments were grouped as described above and pre-maintained in dark room for 48 h before recording of ERGs. After anesthesia, the pupils were dilated with tropicamide and phenylephrine. FERG responses were recorded from both eyes simultaneously with gold wire loops. Retina samples and whole-cell patch-clamp were prepared as described above. 0.9% saline was frequently applied on the cornea to prevent its dehydration and to allow for electrical contact with the recording electrode. Two of the needle electrodes were inserted under the skin of angulus oculi temporalis and served as the reference electrodes, the other one was placed in the tail served as the ground electrode. Flash light stimuli of 10 ms interval were generated with a white LED of 470 nm, 19.43 × 1000 cd/m². Full-field scotopic ERGs were recorded and each sample was represented by at three responses. For recording of VEPs, the electrical activity was recorded by silver wire needle electrodes, which were placed at the visual cortex region, with the reference electrodes placed under the skin of chin, and the ground electrode was placed in the tail. The data acquisition was provided by the Reti-scan system (Roland, Germany). The stimuli intensity was -0.02 [log (cd•s•m⁻²)], the frequency was 1 Hz, and the flash duration was < 5 ms. Average times were more than 50. The bandpass of the filter was from 0.01 Hz to 300 Hz.

Retrograde labeling of RGCs with the flurogold and light stimuli on lateral geniculate body

Location of lateral geniculate body in Pn60d rats was determined by using flurogold retrograde labeling: briefly, the RGCs of rats from different treatments were retrogradely labeled seven days before scarification by injecting 4 µL of 2% aqueous fluorogold containing 1% dimethylsulfoxide into the superior colliculus. Patch-clamp in brain slices was performed as previously reported [28] and light stimuli on lat-

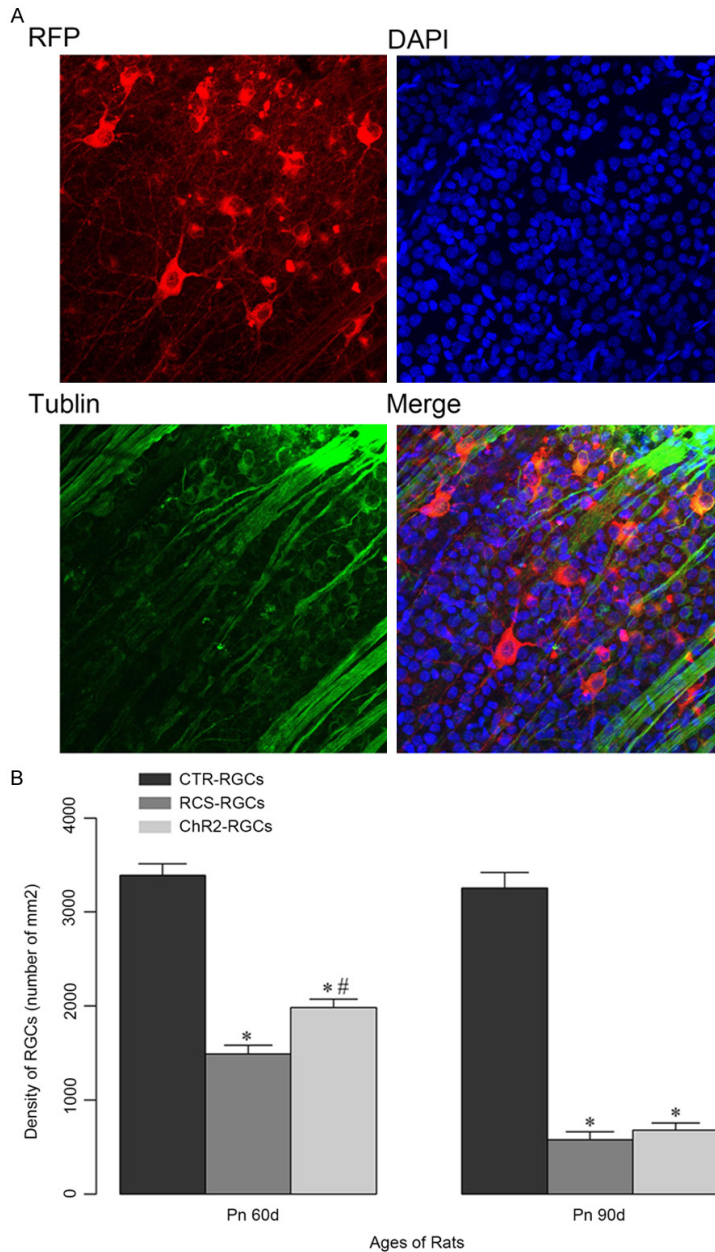


Figure 1. Effect of *ChR2* transduction on the RGC density in RP rats. A: Representative images of immunofluorescent microscopy, nuclei were stained blue and RGCs was stained red, and merge image showed clearly the distribution of the *ChR2* transduced RGCs. B: Quantitative analyses of RGC density difference between groups. “*”, significantly different from CTR-RGCs group, $P < 0.05$. “#”, significantly different from RCS-RGCs group, $P < 0.05$.

eral geniculate body were conducted as described above.

Statistical analysis

All the data were expressed in the form of mean \pm SD. Two groups comparison was performed using Student's *t* test. Multiple comparisons were conducted by LSD method using general

liner model with a significant level of 0.05. All the statistical analyses were conducted using R language version 3.2.1.

Results

Expression of ChR2 in RCS-p+ rats could attenuate the loss of RGCs

As shown in **Figure 1A**, nuclei were stained blue by DAPI and RGCs was stained green by specific antibody Tublin. Moreover, the expression of *ChR2* was detected in RGCs in rats from *ChR2*-RGCs group, which was illustrated by the expression of RFP in RGCs. The densities of RGCs in different treatments were also determined. It was found that for Pn60d rats, the density of RGCs in CTR-RGCs group was higher than that of *ChR2*-RGCs or RCS-RGCs groups, the difference was statistically significant ($P < 0.05$) (**Figure 1B**). Transduction of AAV2/8-hSyn-*ChR2* (H134R)-RFP vector increased the density of RGCs in *ChR2*-RGCs group compared with RCS-RGCs group, the difference was statistically significant ($P < 0.05$) (**Figure 1B**). However, the results of Pn90d rats were different from that of Pn60d: the improving effect of transduction of AAV2/8-hSyn-*ChR2* (H134R)-RFP vector dramatically receded and the RGCs density of *ChR2*-RGCs decreased to a level comparable to that of RCS-RGCs group (**Figure 1B**). The results indicated that up-regulation of *ChR2* in RP rats could attenuate the loss of RGCs, but this therapy might be rendered

less effective with the treating processing if only one injection was made.

Transduction of AAV2/8-hSyn-ChR2 (H134R)-RFP vector in RCS-p+ rats could improve the passive membrane properties

The RMP in CTR-RGCs reached a relatively stable level when rats grew to 60 days old and the

ChR2 restored the light responsiveness of visual cortex

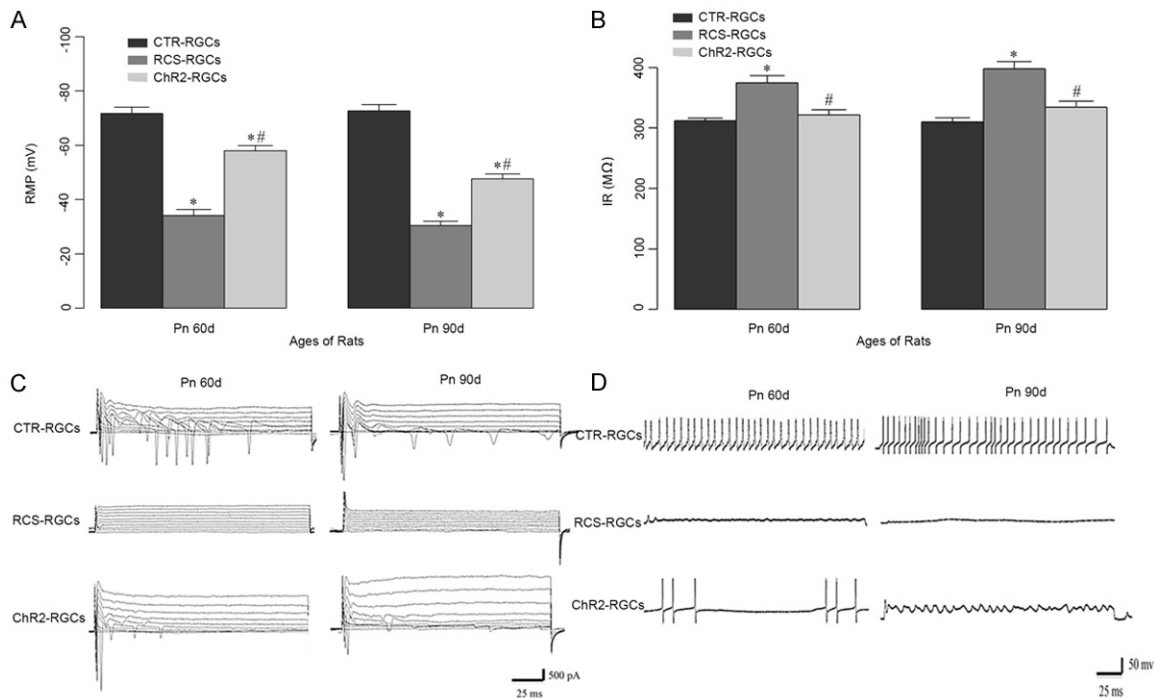


Figure 2. Effect of ChR2 transduction on the passive membrane properties, it was illustrated that all the parameters measured were improved after the transduction of *ChR2* gene. A: Quantitative analysis results of RMP difference between groups. B: Quantitative analysis results of IR difference between groups. C: Representative images of the current change in sodium-potassium channels. D: Representative images of the action potential firing. “*”, significantly different from CTR-RGCs group, $P < 0.05$. “#”, significantly different from RCS-RGCs group, $P < 0.05$.

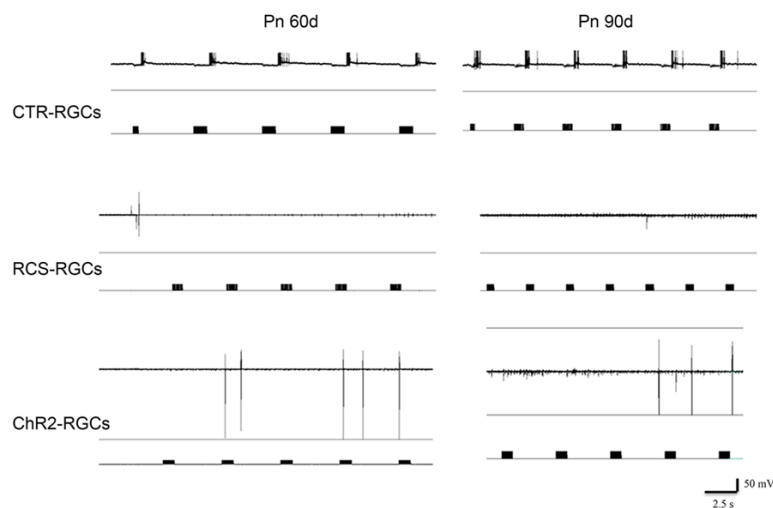


Figure 3. Representative images of the responses of ERG to light stimuli. ERG response could be observed both in Pn60d and Pn90d rats in CTR-RGCs group, more light stimuli needed to be performed for rats in ChR2-RGCs group to response. The resting time and amplitude of ERG response of ChR2-RGCs group was larger than those in CTR-RGCs group.

and RCS-RGCs or ChR2-RGCs group was statistically significant ($P < 0.05$) (Figure 2A). The *ChR2* transduction significantly decreased the value of RMP in ChR2-RGCs group compared to RCS-RGCs group ($P < 0.05$), with experiment processing, the level of RMP in ChR2-RGCs returned to a higher level. However, the difference between RCS-RGCs and ChR2-RGCs group was still statistically significant ($P < 0.05$) (Figure 2A). This results was identical with that in immunofluorescent microscopy, indicating the effect of transduction of AAV2/8-hSyn-ChR2 (H134R)-RFP vector would recede with time, as a results of that, the RGCs in RCS-p+

value was around -70 mV. For Pn60d rats, the level of CTR-RGCs was lower than rats in other two groups, the difference between CTR-RGCs

rats would degenerated to a relatively less mature state. For the detection of IR, the current change in sodium-potassium channels

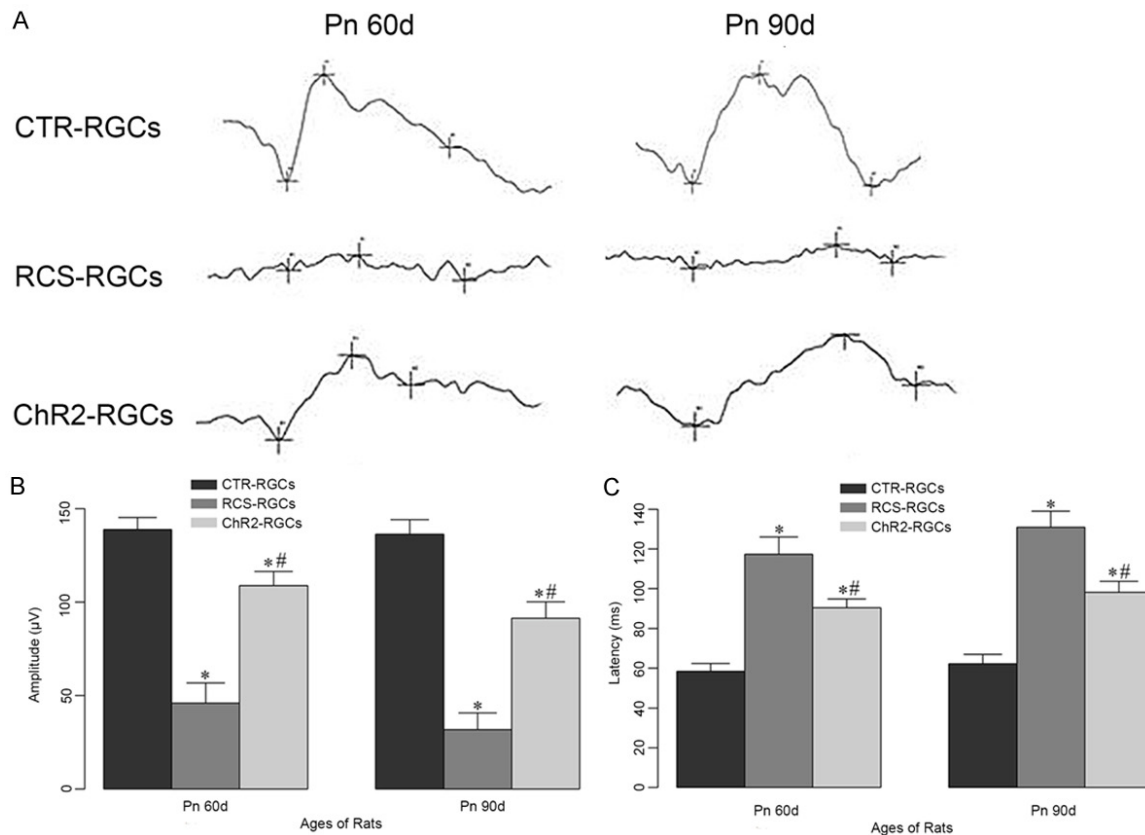


Figure 4. Effect of *ChR2* transduction on the VEP in RP rats. A: Representative images of VEP detection. B: Quantitative analysis of difference of VEP amplitude between groups. C: Quantitative analysis of difference of VEP latency between groups. “*”, significantly different from CTR-RGCs group, $P < 0.05$. “#”, significantly different from RCS-RGCs group, $P < 0.05$.

and action potential firing, similar results were also observed (**Figure 2B-D**), which confirmed our findings about the treatment pattern of *ChR2* on the retinitis. The distribution of dendrites of RGCs was shown in **Figure S2** and a wider distribution of dendrites was observed for RGCs transduced with *ChR2* compared with RGCs in RCS-RGCs group.

Expression of ChR2 improved the visual response of RGCs

To test the possibility of up-regulation of AAV2/8-hSyn-*ChR2* (H134R)-RFP vector injection facilitating the functional recovery of RGCs, the light-induced changes of ERG was investigated (**Figure S2**). As shown in **Figure 3**, the response of ERG to light stimuli in RCS-RGCs group could be negligible. For rats in *ChR2*-RGCs group, ERG response could be observed both in Pn60d and Pn90d rats, but compared with CTR-RGCs group, more light stimuli needed to be performed for *ChR2* transduced RCS rats. Interestingly, it was also found that the

resting time and amplitude of ERG response of *ChR2*-RGCs group was even larger than those in CTR-RGCs group (**Figure 3**).

For VEP detection, the amplitude in RCS-RGCs group was lower than 40 and the latency was significantly longer than the other two groups, indicating the function loss of retina (**Figure 4**). The amplitude in *ChR2*-RGCs group had returned to a relatively normal level, but the latency of VEP response was still significantly different from that of CTR-RGCs group (**Figure 4**). This phenomenon might remind us that AAV2/8-hSyn-*ChR2* (H134R)-RFP vector injection lead to the response of the visual cortex to light stimuli remodeling rather than recovered the visual function of retina.

Transduction of AAV2/8-hSyn-ChR2 (H134R)-RFP vector lead to the light response function of the visual cortex remodeling

To test the above hypothesis, we conducted the patch-clamp in brain slices and exposed lateral

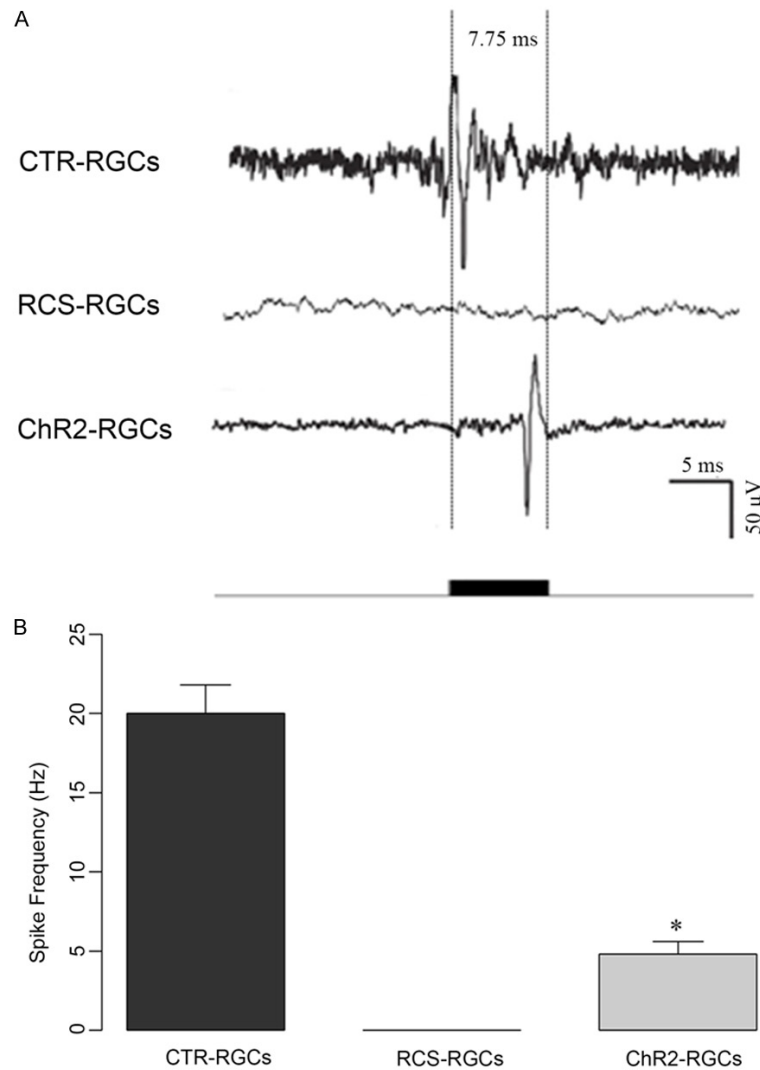


Figure 5. Effect of *ChR2* transduction on the light response function of the visual cortex in RP rats. A: Representative images of spike frequency detection. B: Quantitative analysis of difference of spike frequency between groups. “*”, significantly different from CTR-RGCs group, $P < 0.05$.

geniculate body to light stimuli. As the representative and statistical results shown in **Figure 5**, rats in RCS-RGCs group made no response to light stimuli. For rats in ChR2-RGCs group, the peak spike value was comparable to rats in CTR-ChR2 group, but the spike frequency was much lower (**Figure 5**), which might explain the reason why rats transduced with AAV2/8-hSyn-ChR2 (H134R)-RFP vector had a slow response to light stimuli.

Discussion

Photoreceptor cells in retina are obligated to convert light signals to electrical signals which

are then relayed to visual centers in the brain [29, 30]. Congenital retinal degenerative diseases such as RP often result in the severe loss of photoreceptor cells and can cause complete blindness in patients [31-33]. Even worse, retinal neurons can't be replaced if degenerated, making the treatment of RP even more complicated. Thus, development of alternative therapies for preserving or improving retinal function is imperative.

Recent studies have shown that viral-based gene therapy is a promising tool for the delivery of transgenes to non-dividing mammalian neurons [34, 35]. In the present study, AAV2/8 virus was employed to deliver a fusion construct of ChR2 and RFP into RP mice models. The transduction achieved stable expression of ChR2 in RGCs in experimental animals and improved the membrane properties of RGCs in a 60-day period. However, with experimental time processing, the improvement by ChR2 transduction on RGC membrane properties dramatically receded. Interestingly, even the degeneration of RGCs was not relieved, the experimental animals achieved

significant amelioration on visual response to light stimuli.

The high efficiency and safety of AAV mediated gene transduction into animals has been previously determined [36]. Moreover, clinical trials using AAV vectors to treat retinal degenerative diseases are also under way [37]. In the present study, w AAV2/8-hSyn were selected to construct the expression vector of ChR2-RFP, it was shown that in the retina samples from different sampling time points, stable expression of RFP could be observed, indicating the achievement of the long-term expression of ChR2. Preliminary detection of RGCs with immunoflu-

orescent microscopy showed a higher density of RGCs in Pn60d rats in ChR2-RGCs group while this improvement effect disappeared in the Pn90d rats. Additionally, the density of RGCs in RCS-RGCs group kept decreasing in the whole experiment. These findings were valuable if the gene therapy based on ChR2 was to be employed in clinic: although single injection of ChR2 expression vector would alleviate RGC loss, the effect was only to attenuate the RGC degeneration and would fade away with time. For the passive membrane properties of rats in ChR2-RGCs group, the relatively lower value of RMP as well as higher value of IR inferred the immature state of RGCs (**Figure 2A-D**), which confirmed our conclusion that overexpression of ChR2 could only lead to the relief of RGCs degeneration instead of completely restoring their biological functions. Similar patterns were also observed for the results of current change in sodium-potassium channels and action potential firing.

The effect of ChR2 transduction on the responsiveness of RGCs to light stimuli was also evaluated. ERG is an indicator of photoreceptor function and our results showed that the activity of RGCs could be recorded after transduction of ChR2. Furthermore, it was also found that VEPs could be recorded with light wavelength at 470 nm. This agreed with the earlier report that peak spectral absorption of ChR2 is approximately 460 nm [18]. However, normal rat possesses two photopigments with peak absorbances at 359 nm and 510 nm, respectively. Therefore, the spectral responsive spectrum of ChR2 rats is somewhat narrow than that of health rats. Contrary to the results of RGCs density or passive membrane properties, the effect of ChR2 on light response didn't reduce with time processing. The difference in VEP amplitude between CTR-RGCs group and ChR2-RGCs didn't change with the ages of rats: the RGCs in ChR2-RGCs group still possessed certain sensitivity to light stimuli when rats were 90 day old. Interestingly, the latency time of rats in ChR2-RGCs group was statistically longer than that of dystrophic rats. This longer latency time represented the possibility that the RGCs were still poorly developed even with the expression of ChR2, but ChR2 might help to initiate responsiveness of visual cortex to light stimuli remodeling. To test this hypothesis, brain slices patch-clamp in rats from different treatments was conducted. The results showed

that only non-dystrophic rats and dystrophic rats with ChR2 transduction responded to the light stimuli on lateral geniculate body, inferring that RGCs became photosensitive by the expression of ChR2, and signals generated in the RGCs were transmitted to the visual cortex through lateral geniculate body. But the response of ChR2-RGCs rats were much slower than those in CTR-RGCs group (**Figure 5**), which might be due to the delayed response to light of ChR2.

In summary, the findings outlined in the present study confirmed most conclusions on the gene therapy using ChR2 proposed by previous studies. However, there were still some novel findings in our study. Instead of demonstrating that ChR2 restore the function of RGCs, it was inferred that overexpression of ChR2 in RP animal models only relieved the loss of RGCs, and this relief effect of ChR2 would certainly recede with time. Thus, even ChR2 therapy was planned to be utilized in clinic, the maintain of each single injection of the expression vector would be a critical issue needed to be solved. It was also hypothesized that although ChR2 didn't completely restore the function of RGCs, it did, to some extent, lead to the light responsiveness of visual cortex remodeling, but this hypothesis was merely tested with brain slice patch-clamp in model animals. In order to further elucidate the underlying mechanism of ChR2 in improving the visual function, more comprehensive work will be conducted in our group to facilitate the practical application of ChR2-based therapies against RP.

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Disclosure of conflict of interest

None.

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ChR2 restored the light responsiveness of visual cortex

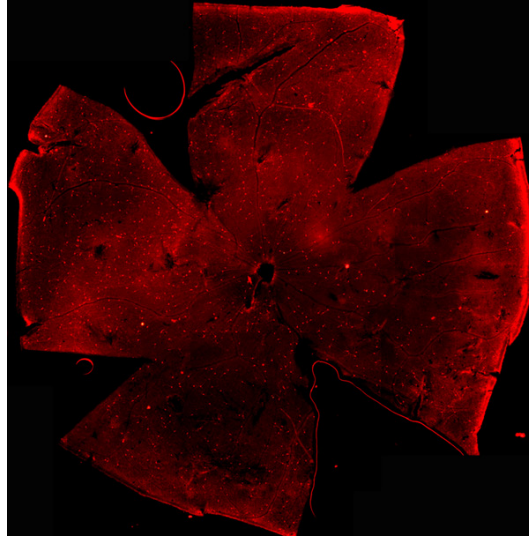


Figure S1. Expression of GFP could be detected in the whole retina.

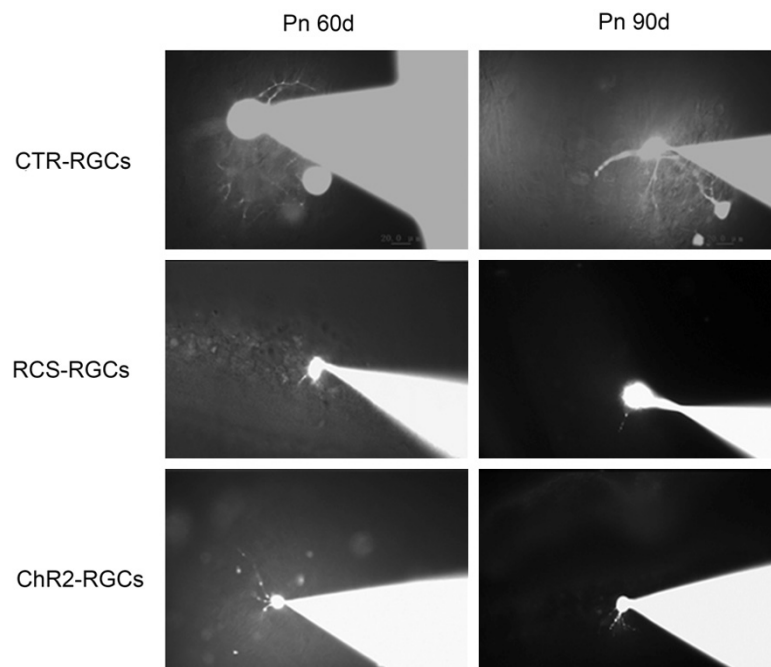


Figure S2. Distribution of dendrites in different groups. Transfected of *ChR2* gene markedly expanded the distribution of dendrites in ChR2-RGCs group.