Human melanopsin–AAV2/8 transfection to retina transiently restores visual function in *rd1* mice

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Abstract

• **AIM:** To explore whether ectopic expression of human melanopsin can effectively and safely restore visual function in *rd1* mice.

• **METHODS:** Hematoxylin –eosin staining of retinal sections from *rd1* mice was used to detect the thickness of the outer nuclear layer to determine the timing of surgery. We constructed a human melanopsin–AAV2/8 viral vector and injected it into the subretinal space of *rd1* mice. The Phoenix Micron IV system was used to exclude the aborted injections, and immunohistochemistry was used to validate the ectopic expression of human melanopsin. Furthermore, visual electrophysiology and behavioral tests were used to detect visual function 30 and 45d after the injection. The structure of the retina was compared between the human melanopsin –injected group and phosphate buffer saline (PBS)–injected group.

• **RESULTS:** Retinas of *rd1* mice lost almost all of their photoreceptors on postnatal day 28 (P28). We therefore injected the human melanopsin –adeno –associated virus (AAV) 2/8 viral vector into P30 *rd1* mice. After excluding aborted injections, we used immunohistochemistry of the whole mount retina to confirm the ectopic expression of human melanopsin by co –expression of human melanopsin and YFP that was carried by a viral vector. At 30d post –injection, visual electrophysiology and the behavioral test significantly improved. However, restoration of vision disappeared 45d after human melanopsin injection. Notably, human melanopsin –injected mice did not show any structural differences in their retinas compared with PBS–injected mice.

• **CONCLUSION:** Ectopic expression of human melanopsin effectively and safely restores visual function in *rd1* mice.

• **KEYWORDS:** human melanopsin; retinal degenerative diseases; visual restoration

INTRODUCTION

Retinal degenerative diseases (RDDs) are the leading cause of vision loss and blindness. With the development of medical technology, an increasing number of mutant genes that cause inherited retinal diseases have been identified, and most of these genes are related to photo transduction pathways [1-3]. As a rapid retinal degeneration model, *rd1* mice have a mutation in the phosphodiesterase type 6 (PDE6)-β subunit that causes a complete loss of photoreceptors by postnatal day 30 [4]. This animal model has similar gene mutations and phenotypes as some human RDDs[5] and is therefore commonly used as a model to test potential treatments of RDDs. More recently, experimental efforts have explored new drugs [6-7], cell therapies [8-9] and light-sensitive proteins[10-12]. However, drugs can only provide functional benefits in the early stage of RDDs, and the efficacy of cell therapy is dependent on remnant photoreceptor cells. By comparison, light-sensitive proteins show a great potential in the treatment of late RDDs due to their independence on photoreceptor cells. Channelrhodopsin (ChR) and melanopsin, two types of light-sensitive proteins, have raised much attention for restoration of vision. However, ChR originates from Chlamydomonas reinhardtii and is less sensitive to light compared with melanopsin[13].

Melanopsin, which exists in the retinal ganglion cells of mammalian retina, is a G-protein coupled receptor that couples to the canonical transient receptor potential channels *via* Gq-type G protein activation [14]. As a result, melanopsin can absorb photons by itself and melanopsin-containing photosensitive ganglion cells have been directly linked to brain functions [15]. It is plausible that melanopsin can restore visual function in the advanced stages of RDDs. Some

researchers have demonstrated that melanopsin plays important roles in the circadian rhythm[16], depression[17] and pupil light reflex [18]. In addition, we have found that ectopic expression of mouse melanopsin can restore visual function in *rd1* mice[11]. This work suggests that melanopsin may be a candidate therapeutic method for advanced stage RDDs. However, human melanopsin is a prerequisite to advance clinical applications of melanopsin. Therefore, in this study, we constructed a human melanopsin-adeno-associated virus (AAV) 2/8 vector (AAV-hMel-YFP) to transfect *rd1* mice retinas.

MATERIALS AND METHODS

Animals Twenty-five healthy SPF-grade *rd1* mice and three C57 normal control mice of both sexes were provided by the Experimental Animal Center of Southwest Hospital, Third Military Medical University. Three C57 mice were used for hematoxylin-eosin staining, ten randomly selected *rd1* mice were used for the flash electroretinogram (FERG) and flash visually evoked potentials (FVEP) experiments and fifteen randomly selected *rd1* mice were used for the behavioral tests. The mice were reared in a light-controlled room that has a fixed lighting schedule (8:00 to 20:00). Light was generated by two fluorescent lamps that created 60 lx of intensity at the animal level. The room humidity was controlled at 50% to 60%, while the temperature was held at 22℃ -25℃ . All experimental protocols were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research[19].

Anesthesia Animals were anesthetized *via* an intraperitoneal injection of 4% chloral hydrate at a dose of 1 mL/kg. Oxybuprocaine eye drops (0.4%) were used for superficial anesthesia (Santen Pharmaceutical Co., Ltd., Osaka, Japan).

Construct of Viral Vectors Full-length human melanopsin was cloned into an AAV 2/8 vector under the transcriptional control of a mCMV promoter. Because of the mCMV promoter, viral vectors easily transfected all retinal neurons in the retina of *rd1* mice. The constructs were packaged at GENECHEM biological company's virus production core in Shanghai, China. The packaged viruses were concentrated and purified in phosphate buffer saline (PBS) with a titer of 3.5×10^{12} AAV-hMel-YFP genome copies per mL.

Subretinal Injection of Adeno −associated Virus After administration of anesthesia, *rd1* mice were moved to an animal operating table under a microscope. Then, 1 μL of a viral suspension was delivered to the subretinal space using a Hamilton micro-injector. To minimize individual differences in the FERG and FVEP testing, AAV-hMel-YFP was injected into the right eye, and the left eye was used as a PBS-injected control (*n*=10). For behavioral testing, ten *rd1* mice received an AAV-hMel-YFP injection in both eyes and the control group (*n*=5) received PBS injections. After 30d of subretinal injections, we used phoenix Micron IV system

(Phoenix company, USA) to observe the retinas of the injected mice to exclude mice with retinal puncture or cataracts due to aborted injections.

Immunohistochemistry Frozen sections were air-dried, washed in PBS for 5min, and then stained with hematoxylin and eosin. For fluorescence immunohistochemistry, whole-mount retinas were blocked in 3% bovine serum albumin (BSA) and 10% normal goat serum in 0.5% Triton X 100 (Sigma-Aldrich, USA) for 1h at room temperature (25℃). Then, the whole retinas were incubated with a rabbit polyclonal antibody against human melanopsin (1:500, Abcam, England) overnight at 4℃ . The following day, after washing in PBS for 45min (3 ×15min), the retinas were incubated for 4h in Cy3-conjugated goat anti-rabbit IgG (1:1000, life technologies, USA). Finally, the nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen, USA) for 4h. Confocal images were acquired using Zeiss LSM 510 microscope (Carl Zeiss Co. Ltd., Oberkohen, Germany).

Flash Electroretinogram Recording For FERG recordings, *rd1* mice were dark adapted for nearly 12h and prepared for recording under dim red light. After administration of anesthesia, the pupils were dilated with tropicamide and phenylephrine. FERG responses were recorded from both eyes simultaneously with gold wire loops. Saline (0.9%) 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 the angulus oculi temporalis and served as the reference electrodes. Another electrode was placed in the tail and served as the ground electrode. Data were acquired by the phoenix Micron IV system (Phoenix company, USA). Dark-adapted intensity responses were 0 Log (cd•s/m²). To avoid any adapting effect from the previous flash, the flash interval was set between 60-120s, depending on stimulus intensity. Data were exported and processed by Igor.

Flash Visually Evoked Potentials Recording The mice were reared in a normal, light-controlled room (8:00 to 20:00). After administration of anesthesia, the electrical activity was recorded by silver wire needle electrodes, which were placed in the visual cortex region, with the reference electrodes placed under the skin on the chin and the ground electrode was placed in the tail. The data acquisition was provided performed by the Reti-scan system (Roland, Germany). The stimulus intensity was -0.02 Log(cd•s/m²), frequency was 1 Hz and the flash duration was <5ms. The bandpass of the filter was between 0.01 Hz and 300 Hz. Data were exported and processed by Igor.

Open Field Test The open field test was performed according to previous studies[11,20]. The open field test box was 45×30×40 cm. It was divided into a white open field and a dark zone with a door (10×10 cm) between the two areas.
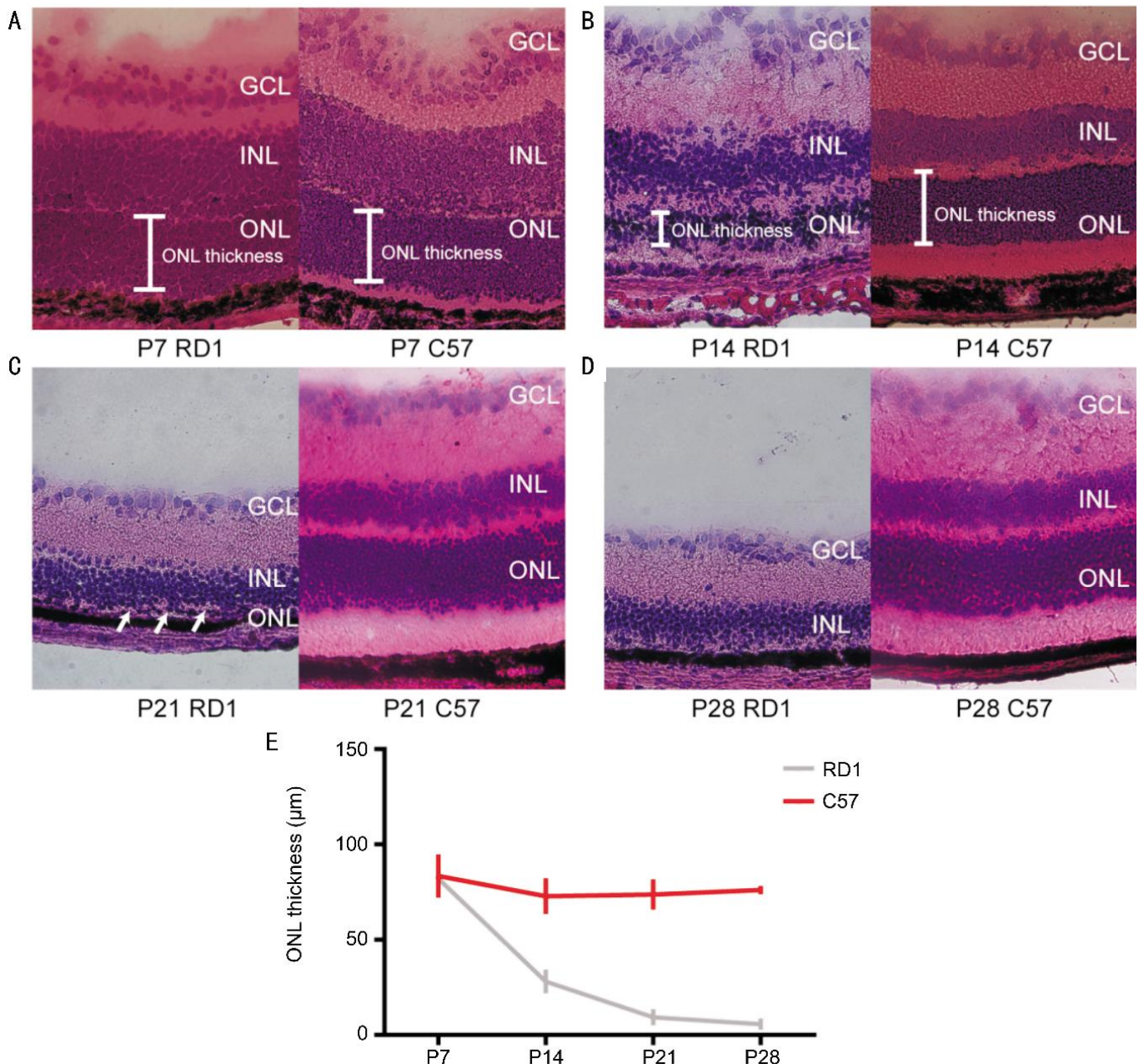
Figure 1 Hematoxylin−eosin staining of retinal sections from C57 and *rd1* mice A-D: Representative hematoxylin-eosin staining of retinal sections from C57 and *rd1* mice on postnatal days 7, 14, 21 and 28. Arrows indicate monolayer of ONL. E: Quantification of ONL thickness in C57 and *rd1* mice. ONL thickness in *rd1* mice gradually decreased. ONL: Outer nuclear layer; INL: Inner nuclear layer; GCL: Ganglion cell layer.

The mice were adapted in the dark room for 2min, and then, the door was opened for behavioral observations. The amount of time spent in the dark zone and white open field was recorded. The open field test was performed under 100 lx light intensity and recorded using a video camera to enable subsequent evaluation. The total time of the test was 300s.

Statistical Analysis Data analysis was carried out using the SPSS 13.0 statistical package (SPSS, Chicage, IL, USA). Data are expressed as the mean±standard deviation (SD) and were analyzed using the independent-samples *t* -test to compare the treated and control groups in both the electrophysiology recording and behavioral testing. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Ectopic Expression of Human Melanopsin Protein in Retina of *rd1* Mice Using hematoxylin-eosin staining, we confirmed that the retinas of *rd1* mice lost almost all of the photoreceptors on postnatal day 28 (P28) (Figure 1). To study whether human melanopsin protein could restore visual function in advanced retinal degeneration, we used AAV to ectopically express human melanopsin in the retina of P30 *rd1* mice.

First, we excluded mice with cataracts or retinal puncture due to aborted injections (Figure 2). Subsequently, immunohistochemistry of the whole mount retina was used to investigate the ectopic expression of human melanopsin.
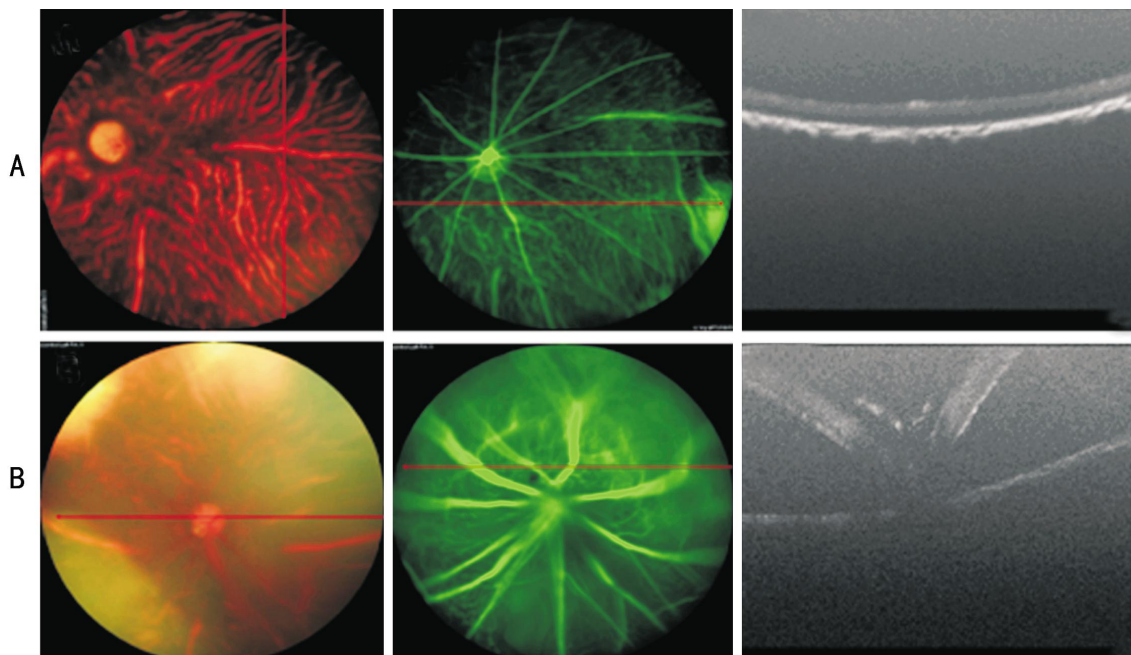
Figure 2 Screening the hMel subretinal injected mice by phoenix Micron IV system A: Successfully injected mice showing normal fundus, fundus fluorescein angiography (FFA) and optical coherence tomography (OCT); B: Aborted injections caused diffusion of fluorescence in FFA and retinal puncture in OCT.

Thirty days post-injection, more than 80% of the retina displayed hMel-YFP fluorescence (Figure 3A). Furthermore, we scanned the mount retina at a higher magnification. In three random fields, co-expression of hMel and YFP fluorescence was observed in the ganglion cell layer (GCL) (Figure 3B), the inner plexiform layer (22 μm depth to GCL) (Figure 3C) and the inner nuclear layer (42 μm depth to GCL) (Figure 3D). However, 45d post-injection, the expression of human melanopsin significantly decreased (Figure 4).

Assessment of Visual Function After Ectopic Expression of Human Melanopsin Protein in the Retina of _rd1_ Mice Visual function was assessed using FERG, FVEP and behavioral tests. Previous studies have indicated that the b-wave and P1 wave amplitude of _rd1_ mice were abolished quickly after postnatal P30 due to a complete loss of photoreceptors [21]. However, the b-wave of FERG and P1 wave of FVEP in P60 _rd1_ mice were well restored 30d post-injection, with the injected eye demonstrating significantly higher amplitude compared to the control eye (Figure 5). Forty-five days post-injection, the amplitude of b-wave and P1 wave in post-injection 45d _rd1_ mice decreased to control levels (Figure 5).

To further assess visual function, we used an open field test, which is a behavioral test of visual function. Many researchers have demonstrated that normal mice avoid open, brightly lit spaces and that this innate tendency depends on their ability to distinguish light from dark [11,20]. Mice were placed in the apparatus shown in Figure 6A. We showed that hMel-injected mice spent more time in the dark zone compared with PBS-injected mice 30d post-injection (P =

0.000). However, 45d post-injection, there was no significant difference in the amount of time spent in the dark zone between hMel-injected mice and PBS-injected mice (P = 0.126).

Safety Assessment of Ectopic Expression of Human Melanopsin Protein in _rd1_ Mice Retina To validate the safety of a human melanopsin transfection, we examined the morphology of the retinas and the optic nerve from hMel-injected mice. Importantly, no structural differences were observed between hMel-injected mice and PBS-injected mice using hematoxylin-eosin staining. In addition, the critical organs, such as the heart, liver, spleen, lung and kidney, all showed normal morphology, just as in the control mice (data not shown).

DISCUSSION

In this study, we demonstrated that ectopic expression of human melanopsin in the degenerated retina of _rd1_ mice transiently restored visual function.

To study the late stages of retinal degeneration, we used P30 _rd1_ mice based on the results that almost all of the photoreceptors were absent in P28 _rd1_ mice. We then injected human melanopsin into the subretinal space of P30 _rd1_ mice and confirmed the ectopic expression of human melanopsin. Consistent with our previous work [11], we found that the AAV-hMel-YFP virus not only transduced retinal ganglion cells but also transduced other retinal neurons. Furthermore, we used FERG and FVEP to evaluate the effect of hMel treatment because FERG and FVEP are the most effective standard methods used for evaluation of visual function[21-23]. FERG is a method that reflects the whole retinal
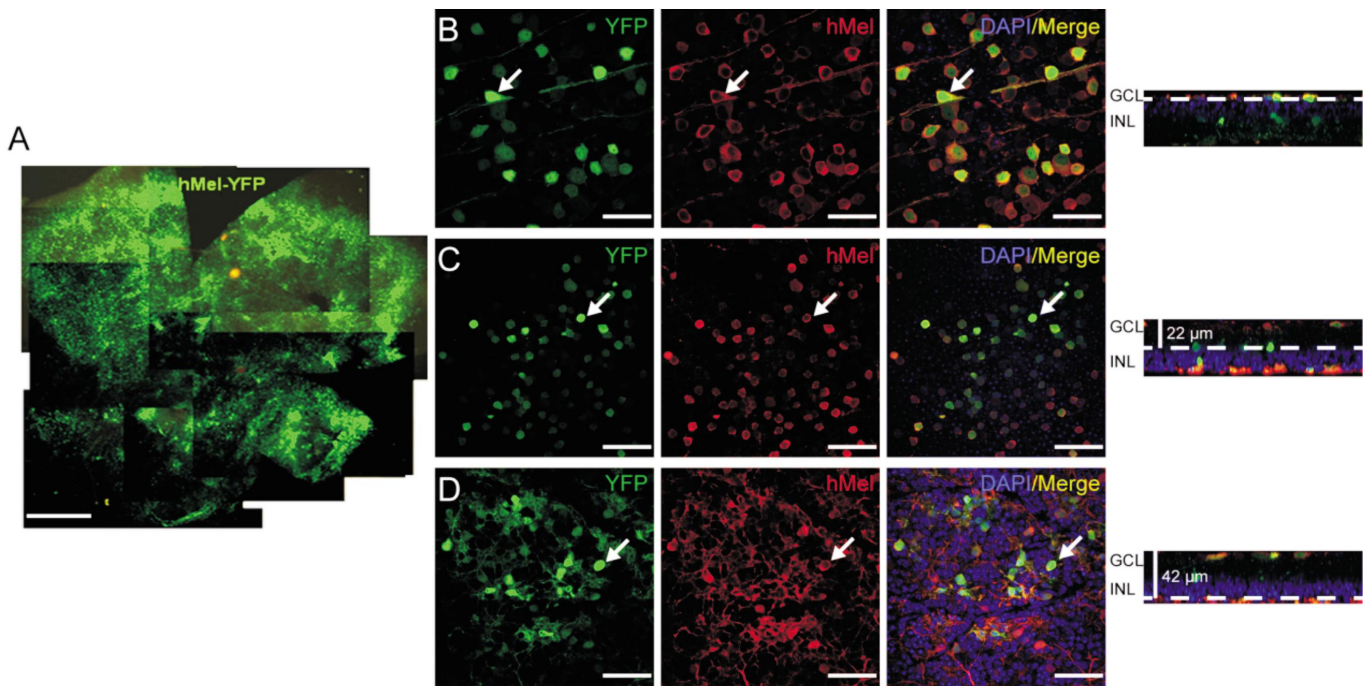
Figure 3 Ectopic expression of human melanopsin protein in the retina of *rd1* mice 30d post−injection A: YFP expression of the whole mount retina. Scale bar: 500 μm. B-D: Co-expression of YFP and human melanopsin in the whole mount retina from superficial ganglion cell layer (B) to deeper layers (C, D). Arrows indicate colocalization of YFP and hMel. Scale bar: 50 μm.
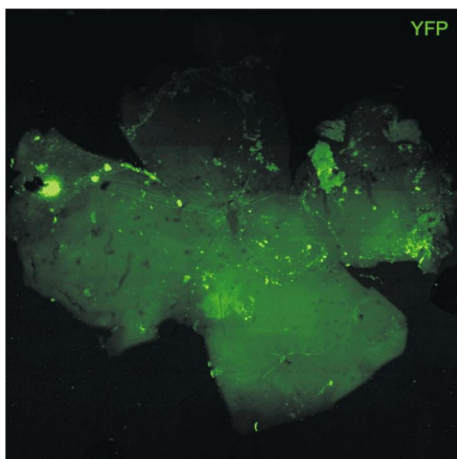

Figure 4 Ectopic expression of melanopsin protein in the retina of *rd1* mice 45d post−injection.

function, including many neuronal types in the retina, such as photoreceptor cells, bipolar cells and amacrine cells [21-22]. FVEP reflects the function of visual pathways that connect ganglion cells to the visual cortex [23]. To minimize surgery damage due to aborted injections, we used the Phoenix Micron IV system to exclude mice with cataracts or retinal puncture. We showed that the amplitude of the b-wave in FERG and the P1 wave in FVEP were significantly higher in the hMel-treated mice 30d post-injection compared with PBS-treated mice. Furthermore, we showed that the hMel-treated *rd1* mice showed better behavioral aversion to light compared with PBS-treated mice 30d after the injection. This finding suggests that hMel-treated *rd1* mice had a restored visual function on postnatal day 60. However,

transplantation of retinal pigment epithelial (RPE) cells, neural stem cells (NSCs), and mesenchymal stromal cells (MSCs) can only provide photoreceptor preservation for 21d in P14 *rd1* mice [24]. In a previous study, we intravitreally injected mouse melanopsin in P80 *rd1* mice and used a behavioral test to confirm that it could restore visual function 4wk post-injection [11]. Furthermore, to advance the clinical application of melanopsin, we used FERG, FVEP and behavioral tests to validate that ectopic expression of human melanopsin could also restore visual function in RDDs at the late stage. Although human melanopsin has been found to be effective in control of wakefulness[25], this is the first time that human melanopsin has been shown to restore visual function in late retinal degeneration.

However, we found that the restoration of visual function disappeared 45d after hMel injection, suggesting that ectopic expression of human melanopsin alone could only transiently restore visual function in late retinal degeneration. We inferred that the short-lived rescue effects were due to the loss of hMel expression, which might result from the species differences between human and mouse. When applied to human retinas, hMel might restore visual function for a longer period. Nevertheless, further studies are needed, such as the use of a better vector for ectopic expression of hMel, to accomplish the long-term restoration of visual function. Notably, ectopic expression of human melanopsin did not induce any serious immune reaction or toxicity.

In conclusion, human melanopsin is a candidate for clinical treatment of retinal degeneration patients. However, the
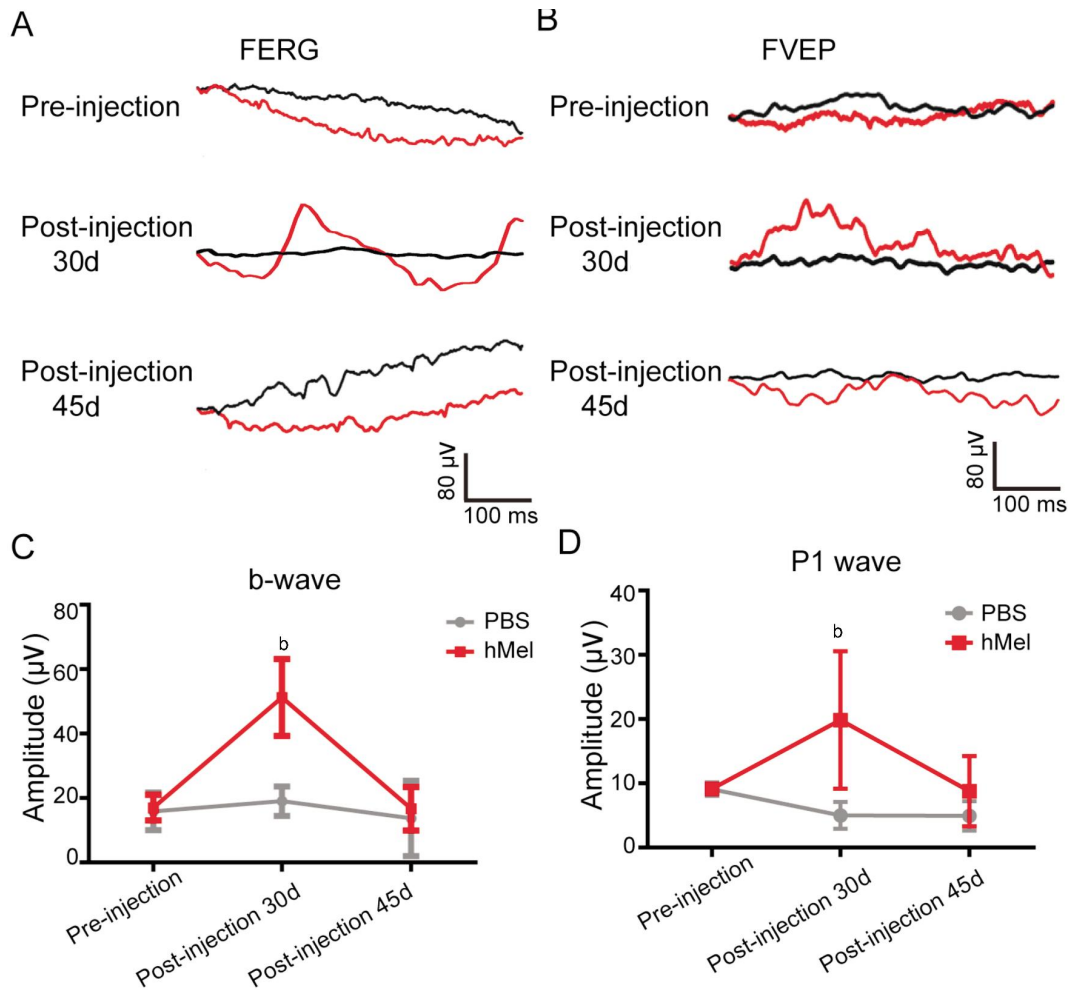
Figure 5 The FERG and FVEP test of subretinal hMel injections in *rd1* mice A, B: Representative traces of FERG and FVEP before the injection, 30d post-injection and 45d post-injection. The black line represents the left eye (PBS-injected eye), red line indicates the right eye (hMel-injected eye). C: The mean amplitude of b-wave before the injection, 30d post-injection and 45d post-injection (mean±SD, n=4). [b]P<0.01. Thirty days post injection, the mean amplitude of the b-wave in the hMel-injected eye was significantly higher than that in the PBS-injected eye (P=0.008) (mean±SD, n=4). D: Thirty days post-injection, the mean amplitude of the P1-wave in the hMel-injected eye was significantly higher than that in the PBS-injected eye (P=0.008) (mean±SD, n=6). However, there was no significant difference in the P1-wave amplitude pre-injection (P=0.916) and 45d post-injection (P=0.203).
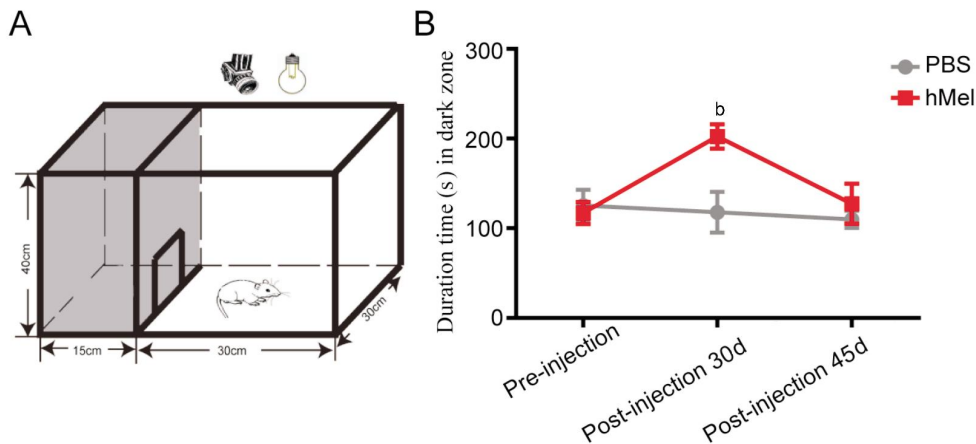

Figure 6 Behavioral test of *rd1* mice with subretinal hMel injections A: Schematic diagram of the open field test equipment. The testing chamber was divided into a white open field and a dark zone. Mice could move freely through a door (10×10 cm) between the white and dark zones; B: The amount of time spent in the dark zone by hMel-injected and PBS-injected mice pre-injection, 30d post-injection and 45d post-injection (mean±SD, n=8). [b]P<0.01.

molecular mechanism of the visual restoration by human melanopsin is not clear, and further studies are needed to elucidate the mechanism of visual restoration by human melanopsin.

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