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Gene Therapy to Rescue Retinal Degeneration Caused by Mutations in Rhodopsin

Brian P. Rossmiller, Renee C. Ryals, and Alfred S. Lewin

Abstract

Retinal gene therapy has proven safe and at least partially successful in clinical trials and in numerous animal models. Gene therapy requires characterization of the progression of the disease and understanding of its genetic cause. Testing gene therapies usually requires an animal model that recapitulates the key features of the human disease, though photoreceptors and cells of the retinal pigment epithelium produced from patient-derived stem cells may provide an alternative test system for retinal gene therapy. Gene therapy also requires a delivery system that introduces the therapeutic gene to the correct cell type and does not cause unintended damage to the tissue. Current systems being tested in the eye are nanoparticles, pseudotyped lentiviruses, and adeno-associated virus (AAV) of various serotypes. Here, we describe the techniques of AAV vector design as well as the in vivo and ex vivo tests necessary for assessing the efficacy of retinal gene therapy to treat retinal degeneration caused by mutations in the rhodopsin gene.

Keywords

Rhodopsin; Subretinal injection; Intravitreal injection; Autosomal dominant retinitis pigmentosa; Mouse model; Adeno-associated virus; Inverted terminal repeat; Outer nuclear layer; Electroretinography; Optical coherence tomography

1 Introduction

Mutations in rhodopsin are rarely tolerated and often lead to rod photoreceptor apoptosis causing the disease retinitis pigmentosa (RP) [1,2]. The death of the rod photoreceptors ultimately leads to the death of the cone photoreceptors causing near or total blindness [3]. Mutations in rhodopsin account for approximately 30 % of the cases of autosomal dominant retinitis pigmentosa (adRP) or approximately 10 % of the total population burden of RP [4]. In the USA, this could mean between 6,000 and 10,000 affected individuals. Fortunately, the human rhodopsin gene (*RHO*) and mouse rhodopsin gene (*Rho*) are highly conserved, suggesting that therapies validated in mouse models will have high translatability to humans (see Note 1) [5]. For these reasons, rhodopsin mutations are actively studied as a key candidate for gene therapy.

The eye is an excellent candidate for the evaluation of gene therapies because of the accessibility of the organ, the suppressed immune system of the uninjured eye, and the

¹Mouse rhodopsin is abbreviated *Rho* for the gene and *Rho* for the protein, whereas human rhodopsin gene is *RHO* and the protein is *RHO*.

numerous quantitative and qualitative vision measurements available. Ocular gene therapy using AAV has already proven partially effective in clinical trials treating Leber congenital amaurosis type 2 (LCA2) patients [6–9]. In this case, a virus expressing a normal *RPE65* gene was delivered to patients by subretinal injection [8]. While light sensitivity was increased and patients reported improved vision, photoreceptors continued to degenerate even in the treated area of the retina, suggesting incomplete protection. AAV is well suited for ocular gene therapy due to its low immunogenicity and its many characterized serotypes which successfully transduce the retina [10]. AAV has a limited carrying capacity, however. Since recombinant genomes over 4.7 kb are not efficiently packaged in the viral capsid, several groups are attempting recombination strategies to overcome this limitation using simultaneous infection with two viruses [11–14].

Gene therapy for *RHO* mutations presents a more difficult problem than gene therapy for a recessive disease like LCA2. Because the mutations are most often dominant, it may be necessary to suppress the expression of the mutant *RHO* in addition to supplementing a wild-type *RHO*. This is a two-stage process: RNA inhibitors such as ribozymes or small interfering RNAs and transcriptional inhibitors containing zinc finger DNA-binding domains have been used to suppress endogenous *Rho* in rodent models [15–18]. This approach has been reviewed elsewhere [5]. Despite its limited genome size, recombinant AAV has sufficient capacity to carry a tissue-specific promoter, *RHO*, and microRNAs, shRNAs, or ribozymes if suppression plus supplementation of *RHO* is desired [16]. In addition, if therapeutic constructs can remain smaller than 2.5 kb, the use of self-complementary AAV can greatly enhance the rate of gene expression [19].

Subretinal injections are usually employed in order to achieve efficient transduction of photoreceptors. However, capsid modifications of AAV have permitted transduction of up to 25 % of photoreceptors in mice following intravitreal injections [20].

Here, we will discuss the methods necessary to design an AAV vector for the delivery of *RHO* plus and minus RNA inhibitors and necessary in vivo and ex vivo measurements to assess visual preservation. Basic procedures for electroretinography, optical coherence tomography, and optokinetic measurements have been well described in earlier papers, but our methods are summarized below [21–24].

2 Materials

2.1 Constructs

1. Target plasmid: Luciferase assay – psiCHECK™2.1 plasmid (Promega) expressing two luciferase genes, *Renilla* and firefly, with a 100 base pair region of *RHO* under the SV40 early enhancer/promoter. Western blot – plasmid expressing *RHO* under a ubiquitous promoter such as cytomegalovirus immediate early promoter (CMV) or the CMV/chicken β -actin hybrid promoter (CBA).
2. Knockdown plasmid: pSilencer expressing RNA knockdown method of interest, shRNA or ribozyme genes (Life Technologies), and pTR-UF11 expressing miRNA.

3. Control plasmid: expressing GFP and miRNA, shRNA, or ribozyme. GFP and RNA knockdown genes are expressed from separate promoters.

2.2 Screening Constructs

1. Lipofectamine 2000 kit.
2. HEK293 cells.
3. 24 well non-pyrogenic, polystyrene tissue culture plate (Costar).
4. Dual-Luciferase Reporter Assay System kit (Bio-Rad).
5. Luminometer.
6. DC protein assay (Bio-Rad).
7. iBlot Gel Transfer System (Life Technologies).
8. Odyssey Infrared Imaging System blocking buffer (LI-COR).
9. 1× phosphate buffered saline (PBS), 0.1 % Tween 20.
10. Anti-RHO primary antibody: (Abcam antibody ab5417) 1D4 for RHO C-terminus; B6-30 for N-terminus.
11. Anti-Rabbit IRDye secondary antibody (LI-COR).
12. Odyssey Infrared Imaging System (LI-COR).

2.3 Animal Preparation

1. 1 % atropine sulfate ophthalmic solution (Akorn).
2. 1 % proparacaine.
3. 10 % phenylephrine hydrochloride ophthalmic solution (Akorn).
4. 100 mg/ml TranquiVed xylazine (Vedco).
5. 100 mg/ml ketamine (Ketved).
6. 2 mg/ml yohimbine (Yobine Injection) (LLOYD).
7. 0.9 % sterile sodium chloride injection (saline) (Baxter, US).
8. Neomycin/polymyxin B/dexamethasone ophthalmic ointment (Akorn).
9. Lo-Dose™ 1/2cc U-100 Insulin Syringe 28½G.
10. Slide warmer (Fisher Scientific).

2.4 Subretinal Injection

1. Dissecting microscope.
2. Nikon NI-150 Fiber Optic Episcopic Illuminator (Nikon Instruments).
3. Micron 4 Microsyringe pump and controller (World Precision Instruments).
4. RPE injection kit (World Precision Instruments).

5. 10- μ l NanoFil syringe (World Precision Instruments).
6. Gonak 2.5 % Hypromellose Solution (Akorn).
7. 30½-gauge disposable needles.
8. 33-gauge blunt needle.
9. 5- μ l Hamilton syringe.
10. 10 % AK-FLUOR sodium fluorescein.

2.5 Ocular Coherence Tomography

1. SD-OCT Ophthalmic Imaging System (Bioptigen).
2. Systane Ultra Lubricant Eye Drops.

2.6 Electroretinography

1. UTAS visual diagnostic system with BigShot Ganzfeld (LKC Technologies).
2. Gonak 2.5 % Sterile Hypromellose Ophthalmic Demulcent Solution (Akorn).

2.7 Fundus Imagery

1. Micron III retinal Imaging Microscope (Phoenix Research Labs).
2. Gonak 2.5 % Sterile Hypromellose Ophthalmic Demulcent Solution (Akorn).

2.8 OptoMotry

1. OptoMotry system (CerebralMechanics).
2. OptoMotry VR 1.7.7 software.

2.9 Light Damage

1. Dual Gooseneck Fiber Optic Illuminator (AmScope.com).
2. Traceable light meter (Fisher Scientific).

2.10 Assessment of Gene Replacement

1. Razor blade.
2. RNA *Later*, RNA Stabilization Reagent (Qiagen).
3. RNAeasy Mini kit for total RNA purification (Qiagen).
4. iScript cDNA synthesis kit (Bio-Rad).
5. SsoFast EvaGreen Supermix, real-time PCR mix (Bio-Rad).
6. CFX96™ Real-Time system with C1000™ Thermal Cycler (Bio-Rad).

3 Methods

3.1 Adeno-Associated Vector Design

Vector design relies on an understanding of the disease progression and the genetic causation of the retinal degeneration in the chosen animal model.

1. A typical AAV construct consists of a therapeutic gene cassette flanked by viral inverted terminal repeats (ITRs) [25, 26] (*see* Note 2).
2. A rod photoreceptor specific promoter such as the proximal mouse or human opsin promoter is cloned upstream of the therapeutic cassette to suppress gene expression to the rod photoreceptors [27]. Depending on the method of degeneration, either a wild-type or a resistant *RHO* with a segment of the 5' and 3' untranslated region will be used as the therapeutic cassette. The resistant *RHO* is used for RNA replacement by containing changes in the *RHO* nucleotide sequence but not amino acid sequence to prevent knockdown of the injected *RHO*.
3. The resistant *RHO* is accompanied by either an miRNA in the 3' UTR, an shRNA, or ribozyme under an additional promoter after the *RHO* SV40 poly-A sequence site (Fig. 1) (*see* Note 3).

3.2 Screening Constructs

To assess the efficacy of the constructs created, it is necessary to quantify the knockdown of wild-type *RHO* and *Rho* as well as the resistance of the sequence-modified *RHO*. For this purpose, we transiently transfect either HeLa or HEK293 cells (*see* Note 4). Then we perform quantification assays, luciferase assay, Western blot, or RT-PCR (*see* Note 5).

3.2.1 Transfection—Three plasmids are necessary: (1) target plasmid, either encoding *RHO* or a reporter luciferase gene with linked target region (*see* Note 6); (2) plasmid encoding the knockdown instrument: miRNA, shRNA, or ribozyme; and (3) control plasmid expressing only GFP and miRNA, shRNA, or ribozyme. The plasmid containing the RNA knockdown instrument (miRNA, shRNA, or ribozyme) is transfected in increasing molar ratios to the target plasmid, from 2:1 to 6:1. Each ratio is run in replicates of six for both time points: 24 and 48 h. To control for differences in transfection, the control plasmid, expressing GFP, is used to ensure that all wells receive the same amount of plasmid DNA.

²Maintaining the ITRs is essential for high-yield vector packaging. To maintain the ITRs, grow the cells at 30 °C and for no more than 16 h. Following each cloning step, the ITRs should be checked using digestion with an appropriate restriction enzyme (usually XmaI for AAV 2 ITRs). There is increased difficulty maintaining the ITRs during the construction of a self-complementary AAV; therefore, it may be necessary to grow the cells for as short as 10–12 h.

³Check all knockdown methods using a BLAST search on NCBI to ensure low homology with other rod photoreceptor genes. Additional genes, such as cone opsins, may need to be checked if using a nonspecific cell promoter. In addition, search engines are available for siRNA design, and these frequently identify siRNAs with “seed matches” in the 3' end of unintended target genes. Avoiding such siRNAs can avoid off-target effects caused by the miRNA pathways. Even after checking the sequence, all knockdown agents should be tested in the model organism to ensure that there are no serious off-target effects.

⁴The cell passage number can affect knockdown results. Therefore, all knockdown experiments should be performed in cells at a similar passage number. In addition, all samples of any knockdown method should be run at the same time to avoid variation in transduction efficiency.

⁵To analyze knockdown using RT-PCR assessment of changes in mRNA of *RHO* knockdown, *see* Subheading 3.10.

⁶The knockdown target regions should be placed in the center of the 100 bp region inserted into the dual luciferase plasmid. This will provide sufficient RNA structural context to properly assess knockdown in the full length mRNA. Do not exceed more than a 300 bp segment as this greatly reduces the luciferase expression.

In addition, the control plasmid is used to normalize the knockdown data in the final analysis (*see* Note 7).

1. Seed a 24-well plate with HEK293 cells (5×10^4 cells/well).
2. Begin transfection when the cells are 95 % confluent.
3. Mix the DNA to give 2 µg total DNA per well at the following ratios of target plasmid to knockdown agent (1:2, 4, 6). Fill the remaining DNA with a control plasmid, and set up a control ratio of target to control knockdown agent at (1:6). Perform all ratios with six replicates.
4. Transfection is performed utilizing Lipofectamine 2000 according to the manufacturer's instructions.
5. Collect the cells at 24 and 48 h time points by vigorously pipetting the media against the cells and transferring the detached cells into a 1.5-ml tube.
6. Pellet the cells and remove the media. Store cells at -20°C . These cells contain the item to be measured, luciferase, protein, or mRNA.

3.2.2 Quantification Assays

Luciferase Assay

1. The psiCHECKTM2.1 dual luciferase plasmid is used for the target plasmid. The psiCHECKTM2.1 plasmid is engineered to contain a 100 bp section of *RHO* including the region targeted by the knockdown agent and is ligated between the *Renilla* stop codon and the poly-A site (*see* Note 6).
2. Use Dual-Luciferase Receptor Assay System kit to perform luciferase assay.
3. Prepare psiCHECKTM2.1 transfected cell samples by resuspending the pelleted cells in 100-µl passive lysis buffer.
4. Place 20 µl of each cell lysate into an opaque 96-well plate, and then add 100 µl of buffer LAR II.
5. Read the luminosity for firefly luciferase, as no target is added to the firefly luciferase. The measured luminosity will serve as a control luciferase expression and loading control (*see* Note 8).
6. Add 100 µl of Stop & Glo to each well, and read *Renilla* luciferase activity. *Renilla* contains the *RHO* target region and will be reduced in expression by the knockdown agent (*see* Note 9). The ratio of *Renilla* to firefly luciferase normalized to control transfection will give the percent expression of luciferase.

⁷Twenty-four hours after transfection, the efficiency of transfection can be determined using the 1:6 target plasmid to control GFP as the marker for successfully transfected cells by comparing that to the total number of cells in the well.

⁸Measuring of the firefly luciferase should start 90 s after adding LAR II to allow for peak luminance.

⁹Add LAR II and Stop & Glo buffers to only six samples at a time to prevent signal loss before samples are read.

Western Blot Assay

1. To quantify the expression of introduced *RHO* using Western blot assay, first determine the total protein concentration in lysed cell extract using Bio-Rad DC™ Protein Assay according to manufacturer's instructions.
2. Run 10 µg of protein on a 12 % acrylamide SDS-PAGE gel.
3. Perform protein transfer to PVDF membrane using the iBlot Gel Transfer System.
4. Once protein transfer is finished, wash the membrane in 5 ml methanol for 5 min, and then move it into a 10-ml Odyssey Infrared Imaging System blocking buffer for 1 h.
5. Add mouse monoclonal antibody (1D4 for RHO C-terminus; B6-30 for N-terminus) at a dilution of 1:800 directly to the blocking buffer, and incubate for 2 h at room temperature before removing the buffer and applying 3 times 5 min washes with PBS, 0.1 % Tween 20 solution.
6. Add the secondary anti-Rabbit IRDye antibody, and incubate for 1 h before again applying 3 times 5 min washes with PBS, 0.1 % Tween 20 solution.
7. The Western blot is then imaged and analyzed using the LI-COR Odyssey Infrared Imaging System.

Selected vectors are used for AAV production. Methods of AAV production and purification can be found in [28, 29].

3.3 Animal Preparation

Prior to performing subretinal injections, optical coherence tomography, electroretinography, or fundus photography, mouse eyes must be properly dilated. The necessary steps to prepare animals for injections or vision tests and procedures necessary to ensure animal recovery are listed below.

1. Begin dilation by administering one drop of 1 % atropine to both eyes of all mice. In 10 min, add one drop of 10 % phenylephrine hydrochloride ophthalmic solution to each eye of all mice. Repeat 10 % phenylephrine hydrochloride ophthalmic solution drops 3 times every 5 min.
2. Anesthetize each mouse with ketamine (72 mg/kg)/xylazine (4 mg/kg) intraperitoneally (*see* Notes 10 and ¹¹).
3. Apply topical anesthetic to the cornea for procedures such as electroretinography or ocular injection.
4. Place one drop of neomycin/polymyxin B/dexamethasone ophthalmic ointment on each eye.
5. Once the procedures are finished, administer yohimbine (0.2 mg/kg) and 0.9 % saline (25 µl/g).

¹⁰An anesthetized mouse will typically afford 40–60 min toward a procedure before recovering from the anesthesia.

¹¹If a boost of anesthesia is required, use only ketamine and not the ketamine/xylazine mixture.

6. Place the mice on a 37 °C heating pad until the mouse has recovered as evidenced by full return of mobility.

3.4 Subretinal Injection

AAV transduction of rod photoreceptors relies on a successful subretinal injection. Placement of the AAV between the photoreceptors and retinal pigmented epithelium greatly increases viral uptake and gene expression in the photoreceptors. However, retinal detachment occurs which increases the risk of retinal damage (Fig. 1).

1. Load a 33-gauge blunt needle 5- μ l Hamilton syringe with 1 μ l of vector suspension (10^9 viral particles) with 0.5 % fluorescein.
2. Apply one drop 2.5 % hypromellose solution to the dilated eye to maintain ocular lubrication and clarify visualization of the eye (*see* Note 12).
3. Using forceps, place on either side of the eye, and apply gentle outward pressure to better open the eye and cause the eye to move slightly out.
4. Under a dissecting microscope, use a 30½-gauge disposable needle to puncture a hole in the sclera of the dilated eye. The needle is withdrawn once the full bevel end of the needle has entered, as placing the needle too far can damage the lens or the retina (*see* Note 13).
5. The 5- μ l Hamilton syringe is guided through the hole parallel to the table on which the mouse is resting. Once in, the syringe is moved to 45 °C to the plane of the table to better target the anterior retina.
6. Once the needle makes contact with the opposing retinal surface, the virus may be delivered via manual pushing of the plunger by an assistant or by automated injection (*see* Note 14).
7. For manual injections, have an assistant gently push down on the syringe plunger for approximately 30 s to expel the virus while resisting any movements in the Hamilton syringe. Hold the plunger for 5 s after all viruses have been expelled before gently releasing (*see* Note 15).

Alternatively, an automated injector pump and controller may be used at this step (**steps 8–11**).

8. Prime the NanoFil™ syringe by first assembling the syringe according to manufacturer's instructions and then loading the syringe with 5 μ l of saline. Attach the RPE injection kit to the syringe, and discharge the saline and attach the syringe to the Micron 4™ Microsyringe pump and controller.

¹²In light-sensitive mouse models, dilation may be avoided, and injections should be performed in lights containing red light filters. The increased difficulty of these injections invariably leads to more retinal detachments reducing the efficiency of the injections and requiring additional mice.

¹³After each mouse, discard the 30½-gauge needle.

¹⁴The fluorescein can be visualized under the microscope and serves as an aid in determining viral location. If there is an initial bright green diffuse cloud seen through the cornea, the injection has gone into the vitreous. A proper subretinal injection will show a pale green glow along the inferior portion of the eye.

¹⁵Stopping and starting of the manual injection should be very slow as to avoid either damage to the retina or a vacuum that could remove viral particles.

9. Turn on the Micron 4™ Microsyringe pump, and set up the injection parameters for the output number corresponding to the output port the syringe is connected to. Select “W” in the first field for withdrawal to load the syringe. The withdrawal rate is set to 407 nl/s. Set the volume to 1,000 nl and the device type to “L.”
10. Place the syringe tip in the virus and press the “RUN STOP” button. Once loaded, change the syringe settings to “I” for inject, rate 34 nl/s.
11. Follow **steps 2–5**. Once the needle makes contact with the posterior retina, press the foot pedal to start the injection.

3.5 Ocular Coherence Tomography

Ocular coherence tomography (OCT) uses constructive and destructive interference of a near infrared laser to obtain a live image of retina morphology without causing harm to the retina. Information gathered from the image can be used to measure changes to the outer segment layer over time (Fig. 2). Primary scans of each mouse also serve as a means to remove mice from the study that have damaged or detached retinas due to the subretinal injection (*see* Notes 16 and 17).

1. Switch on the power supply and computer.
2. For first time use, select the “Study” tab and “Add Study.” Input the study name and click “Create Treatment Arm” to specify individual treatments that will be tested before selecting “Save Changes.”
3. Select the “Patient/Exams” tab, and then “Setup Examiners & Physicians” icon to input the name of the individuals conducting the exam and the principle investigator.
4. Click the “Add Patient” icon to add mice to the study or select an individual mouse from the list that has already been added.
5. Once the mouse has been input into the database and selected, click on the “Add Exam” followed by “Begin Exam” icon. This will automatically load the “Imaging” tab.
6. Select the create custom scan icon and create two high-resolution scans for aiming and two lower-resolution scans for averaging, one each for OD (right eye) and OS (left eye). The scan parameters are as follows:
7. Click on the “Aiming” icon to ensure proper retina positioning and focus. This is achieved with the aid of one B-scan along each the X – and Y -axis. The mouse positioning is adjusted until the focus centered the retina on the X -axis, and each of the axis B-scans is horizontal.

¹⁶Given the angles used in scanning the eye, it is advisable to restrain the mouse with an elastic band across the mouse abdomen and around the biotrogen animal tube.

¹⁷Begin imaging of the retina soon after the mouse is anesthetized, and complete all scans in approximately 10 min. After this time, the cornea will begin to cloud and obscure imaging.

8. After aiming, click “Stop Aiming” and perform “Free Run” to position the optic nerve at coordinates (0, 0) using the fundus view. This is accomplished through using the different axis control knobs on the mouse platform.
9. Stop the high-resolution scan and “Abort scan.” The high-resolution scan is used for aiming and can be deleted to avoid excess data storage.
10. Click “Free Run” to start the lower-resolution averaging scan and immediately click “Stop Free Run” and “Save scan.”
11. Repeat **steps 7–10** for the left eye.
12. After all mice have been scanned, load OCT data for each mouse, and average the data in the “Imaging” tab.
13. Using the caliper tool, take measurements from four points around the optic nerve (0, 0) at (2, 0), (0, -2), (-2, 0), and (0, 2). Average all control versus experimental measurements (*see* Note 18).
14. Take several representative images of the retina with caliper and save as Bitmap files.

3.6 Electroretinography

Electroretinography (ERG) measures the electrical response generated by the retina in response to flashes of light. The resulting waves of hyper- and hypo-polarization of the retina provide information of rod, cone, and bipolar cell function (Fig. 3).

1. Place the animals to be measured in the dark for 2–16 h prior to the ERG.
2. Turn on the MGIT-100 power supply, computer, and UTAS system.
3. Unplug the UBA-4204 Patient Amplifier and Interface from the charger and power on.
4. When the operating system starts, the EMWIN software will automatically load.
5. For first time use, it will be necessary to create a database into which the waveforms and a protocol will be stored.
6. To create a database, click on “Utilities,” “Create New Database,” and “Standard Database.” Finally, enter the desired name and click “OK.”
7. For protocol creation, begin by selecting “Protocols” and “Create New Protocol.” Click “Add a Step” and add two steps for three total steps. For light-sensitive models of retinal degeneration, the light intensities are as follows, -40, -30 dB, -20 dB, and -10 dB, while non-light-sensitive models will be tested using -30 dB, -20 dB, -10 dB, and 0 dB flashes (*see* Note 19). The complete list of settings is presented in Table 1.

¹⁸Averaging the image is optional but provides for a much clearer image.

¹⁹Using the Fisher Scientific™ Traceable® light meter, the -30, -20, -10, and 0 dB instrument settings correspond to a light intensity of 1, 6, 55, and 322 cd-sr/m², respectively.

8. If all previous setup has been done, select “Utilities,” click on the existing database from the provided list, and select “OK.”
9. Anesthetize 1–3 mice 5 min before performing the ERG according to the animal preparation section.
10. While the first mouse is anesthetized, select “Tests” from the main menu, “User-Defined Protocol,” and the created protocol.
11. The mouse number must now be entered including mouse ID, treatment, and any additional information before selecting “Continue.”
12. Click “Continue on Channel Information” window to bring up the exam window.
13. Place the anesthetized mouse on the mouse platform, and apply one drop 2.5 % hypromellose ophthalmic solution to each eye. Then place the reference electrode in the mouse tail or posterior, recording electrode on the head and contact electrodes on the appropriate eye (*see* Note 20) (Fig. 3).
14. Slide the plate into the Ganzfeld dome.
15. Select the “Baseline icon” on the parameters toolbar. The two lines are recording from each eye and should be near flat. If large oscillations are observed, check and reposition electrodes as needed until the baseline is corrected (*see* Note 21).
16. Select “Record,” save icon on parameters toolbar, “Store All Waves” and “Next Step.”
17. Repeat **step 16** until all three steps are completed before clicking main menu icon on the parameters toolbar.
18. The mouse can now be removed and treated according to Subheading 3.4, and the protocol can be repeated for any subsequent mice.
19. Once completed, analysis of the raw data can be conducted using the supplied software or by first clicking “Reports” and “Export Waves” and selecting all desired waveforms before naming and saving the file. All waveforms will now be in the Exported waves folder as a CSV file and can be analyzed with spreadsheet software to find the a-wave and b-wave amplitudes (Fig. 3).
20. To complete ERG, shut down the computer and UTAS system first. Turn off the UBA-4204 Patient Amplifier and plug back into the charger. Leave on the MGIT-100 power supply to charge the UBA-4204 Patient Amplifier. Clean the acrylic contact lens electrodes with a mixture of 1:1 liquid detergent and sterile water, and then rinse with sterile water.

²⁰The anesthesia and long scan times make the mice more susceptible to hypothermia. It is, therefore, recommended to use a heating pad (37 °C) on the mouse platform during the ERG.

²¹Ensure the recording amplifier is utilizing direct current during the ERG as the alternating current can influence the ERG recordings.

3.7 Fundoscopy

Fundoscopy assesses gross changes in retinal structure due to degeneration including pigmentary deposits, yellowing of the retina, and changes in blood vessel structure. It also provides qualitative measures of viral transduction via GFP expression.

1. Power on the computer, light box, and the light.
2. Set the microscope to utilize white light by turning both filters to setting 1.
3. Open the program “Micron III imaging.”
4. For first time use, set up the program by selecting “Steam pix settings” icon and select “Works space” to change location to autosave data. Under “Recording,” select “Auto-create a sequence file in RAM” and click “OK.”
5. Prepare the mice and anesthetize one mouse at a time according to Subheading 3.3.
6. Once anesthetized, place the mouse on the positioning plate, and administer one drop of 2.5 % hypromellose ophthalmic solution (*see* Note 22).
7. Slowly bring the microscope to the eye. Adjust focus while bringing the microscope closer to the mouse eye, and reposition the mouse as needed to position the optic nerve in the center of the image.
8. Click the “Snap” icon and then the “TIFF” icon to save the image as a tiff file.
9. For qualitative measurement of viral transduction using fluorescent proteins, the light settings may now be changed. Additionally, fluorescein may be administered to observe retinal vasculature.

3.8 OptoMotry

1. Turn on computer and camera. Start the OptoMotry VR 1.7.7 software.
2. To calibrate the system, press the button with a cross on top of a circle. Once that icon appears on the screen, place the mouse cursor on the black dot of the pedestal. Move the mouse cursor from the black dot to the black circle on the pedestal. Make sure the red circle meets the black circle. Once these two circles are aligned, hit the button with a cross on top of a circle so the icon disappears.
3. To conclude the calibration process, press the button that looks like a star (five lines coming together at one point). This icon is referred to as the OptoMotry cursor. Move this cursor to different positions on the screen. While the cursor is located at different positions, look at the widths of the black and white bars that appear on the computer screens within the OptoMotry system. The bars should get thinner if the cursor is in close proximity and wider if the cursor is further away. For example, if the cursor is placed on the right side of the screen, the white and black bars that appear on the right OptoMotry screen should be thin, whereas the

²²Begin imaging of the retina soon after the mouse is anesthetized, and complete all scans in approximately 10 min. After this time, the cornea will begin to cloud and obscure imaging.

bands on the left OptoMotry screen should be wide. If the band sizes don't change as the cursor is moved, restart the machine.

4. To test spatial frequency, click the stimulus tab then the gratings tab. Set spatial frequency to 0.042c/d, contrast to 100 %, and the drift speed to 12 d/s. Then press the testing tab and the psychophysics tab. Under psychophysical methods, chose simple staircase; under directions, chose randomized/separate; and under threshold, chose frequency (*see* Notes 23 and ²⁴).
5. To test contrast sensitivity, press the stimulus tab then the gratings tab. Set the spatial frequency to 0.128c/d (or a value the mouse model being tested responds to), contrast to 100 %, and the drift speed to 12 d/s. Press the testing tab and the psychophysics tab. Under psychophysical methods, chose simple staircase; under directions, chose randomized/separate; and under threshold, chose contrast.
6. Press the compass button. The compass will indicate which direction the bars are moving on the OptoMotry screens. Customize the compass by pressing the camera tab then the overlays tab. Change compass size, cursor size, tick spacing, and tick size to a helpful setting.
7. Place the first mouse on the pedestal within the OptoMotry system. If the mouse jumps off the pedestal, pick it up and put it back on the pedestal (*see* Note 25).
8. Place the OptoMotry cursor (the compass will move too) in between the mouse's ears. It is best to line up the cursor with the nose of the mouse.
9. Once the cursor is in alignment with the mouse's head, release the computer mouse so the gratings appear, and determine if the mouse tracks. Tracking is defined as a small and steady head movement in the direction of the gratings. The mouse is tracking if the mouse's nose and ears move in the same direction of the compass. If the compass is moving clockwise, left eye function is being analyzed. If the compass is moving in a counterclockwise direction, the right eye function is being analyzed.
10. If the mouse tracks, press the Yes button. If mouse does not track, press the No button.
11. The next step in the simple staircase test will populate after pressing yes or no. Keep testing the mouse's tracking until you cannot press yes or no, and the done button is highlighted.
12. The results will populate in the results tab. A CW (right) value and a CCW (left) value will be given. CW (right) means clockwise; the gratings were moving to the right so left eye function was measured. CCW (left) means counterclockwise; the gratings were moving to the left so right eye function was measured.

²³OptoMotry is useful primarily for assessing photopic vision.

²⁴Due to the bright screens used for OptoMotry, non-light-dependent degeneration *RHO* mouse models should be used. If light-dependent models are needed, assess just before euthanasia.

²⁵Mice do not require any preparation prior to OptoMotry testing. Make sure mouse eyes have not been dilated in the past 7 days.

13. When the test of one animal is finished, take it out and place the next animal on the pedestal. Press the Reset button to start the test over for the next animal.
14. Save results, close software, and shut down machine.

3.9 Methods in Light Damage

Some mouse models, such as those containing *Rho* I307N, suffer rapid retinal degeneration in response to intense light. The following methods describe how to use a light damage model to assess the effectiveness of a gene therapy.

1. Prior to light damage, perform a pre-injury damage assessment of retinal function and structure with both an ERG and OCT using the above methods.
2. Follow the methods described in Subheading 3.3 to ensure the eyes are properly dilated and the mouse is properly anesthetized.
3. Using the Traceable light meter, dial the light intensity knob on the AmScope.com Dual Gooseneck Fiber Optic Illuminator until light intensity reaches 10,000 cd·sr/m² (see Note 26).
4. Place the mouse between the two fiber optic illuminators so each eye is perpendicular to the light source and equidistant.
5. Subject the eye to 3 min light exposure.
6. Following light exposure, treat the recovering mouse as described in Subheading 3.3.
7. One week post-light damage, assess retinal structure and function by ERG and OCT. The precise preservation of retinal function can now be calculated as a percent post- to pre-light damage.

3.10 Assessment of Gene Replacement

Real-time PCR is employed to assess changes in gene expression for validation and quantification of gene replacement. First, primers capable of distinguishing viral-mediated *RHO* expression from native *Rho* expression are designed. At postnatal day 15, 8 mice receive a subretinal injection of the experimental treatment according to Subheading 3.4. At the first month postinjection, retinas are collected. RNA extraction is performed using the Qiagen RNeasy Mini kit and the cDNA created by using the Bio-Rad cDNA synthesis kit. Finally, once the cDNA is created, the SsoFast EvaGreen[®] Supermix is used in setting up the samples for RT-PCR. After running a standard RT-PCR reaction, analyzing the results can be done using the software with the RT-PCR thermocycler (see Notes 27 and 28).

1. For removal of the mouse retina, first euthanize the mouse using CO₂ followed by cervical dislocation.

²⁶It may be necessary to test several light intensities prior to injection of the treatment as successive breeding and background strain used may influence light damage susceptibility.

²⁷Primers designed to target the 3' UTR can easily distinguish the endogenous mouse *Rho* and the human transgene *RHO*.

There are also sufficient nucleotide differences to separate human *RHO* from mouse *Rho*.

²⁸If possible, design all primers to anneal at the same temperature. This will allow all reactions to be run at the same time.

2. Cut horizontal along the border between the limbus and sclera using a razor blade.
3. Apply gentle pressure with forceps and pull and move the forceps toward the limbus.
4. The lens will come out along with a white or pinkish material which is the retina. Immediately place the retina in 30 μ l of RNA *Later*, and store in -20°C for immediate use or -80°C for long-term storage.

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Fig. 1. Vector design and injection. **(a)** Typical AAV construct consisting of a promoter, intronic region, the gene of interest, and, optionally, a knockdown method. **(b)** Proper syringe location is at 45° angle to the cornea and inserted trans-scleral to deposit the virus subretinally

Fig. 2. Optical coherence tomography. (a) Illustrated overview of the operational principle of spectral domain OCT. OCT builds an image of changes in refractive indexes and the depths of these changes in a sample using constructive and destructive interference of a broadband near infrared laser centered at 840 nm. The resulting intensity by frequency data is then converted using a Fourier transformation and graphed as intensity by time. Each point of data is called an A-scan. A linear cross section of the eye is built by compiling several A-scans to form a B-scan. (b) Proper placement of the mouse. Central animal tube rotated to 45° and base at 120° from laser. (c) Representation of the cell layers represented in an OCT B-scan. (d) The outer nuclear layer is measured using calipers to follow changes in thickness over time

Fig. 3. Electrophysiology. (a) Proper electrode placement with electrode centered and surrounding the cornea. (b) Different cell types each contribute different components of the recorded ERG. (c) Measured from the recorded ERG are the a-wave, b-wave, and the implicit time. The amplitude of the a-wave is measured from the baseline to the maximum negative deflection, while the amplitude of the b-wave is measured from the a-wave maximum to the peak of the b-wave in the positive direction.

Table 1

Electroretinography protocol design

Tab		Scan intensity	
		Low	High
<i>Averaging</i>	Number of signals to average	5	5
	Time between stimulus (seconds)	5	60
	Sweeps before update	5	5
<i>Sunburst flash</i>	Test type	Single flash	Single flash
	Intensity	−40, −30, −20, −10 dB	0 dB
	Custom color	0.329, 0.329	0.329, 0.329