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Authors
Gaub, Benjamin M
Berry, Michael H
Visel, Meike
et al.

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Optogenetic Retinal Gene Therapy with the Light Gated GPCR Vertebrate Rhodopsin

Benjamin M. Gaub[1], **Michael H. Berry**[2], **Meike Visel**[2], **Amy Holt**[2], **Ehud Y. Isacoff**[2,3,4], **John G. Flannery**[5,6,7]

[1]Department of Biosystems Science and Engineering (D-BSSE), Eidgenössische Technische Hochschule (ETH) Zürich, Basel, Switzerland. [2]Department of Molecular and Cell Biology, University of California Berkeley, Berkeley, CA, USA. [3]Physical Bioscience Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA. [4]Helen Wills Neuroscience Institute, University of California Berkeley, Berkeley, CA, USA. [5]Vision Science Graduate Group, School of Optometry, University of California, Berkeley, CA, USA. [6]The Helen Wills Neuroscience Institute, University of California, Berkeley, CA, USA. [7]Department of Molecular and Cell Biology, University of California, Berkeley, CA, USA.

Abstract

In retinal disease, despite the loss of light sensitivity as photoreceptors die, many retinal interneurons survive in a physiologically and metabolically functional state for long periods. This provides an opportunity for treatment by genetically adding a light sensitive function to these cells. Optogenetic therapies are in development, but, to date, they have suffered from low light sensitivity and narrow dynamic response range of microbial opsins. Expression of light-sensitive G protein coupled receptors (GPCRs), such as vertebrate rhodopsin, can increase sensitivity by signal amplification, as shown by several groups. Here, we describe the methods to (1) express light gated GPCRs in retinal neurons, (2) record light responses in retinal explants in vitro, (3) record cortical light responses in vivo, and (4) test visually guided behavior in treated mice.

Keywords

Retinitis pigmentosa; Congenital blindness; Retinal gene therapy; Optogenetics; Translational medicine; Visual prosthetics; Light-gated receptors

1 Introduction

Inherited retinal degenerative diseases are a significant unmet medical problem, affecting one in 3000 people worldwide [11–13]. There is currently only one FDA approved treatment for retinal degeneration, the Second Sight Electronic prosthesis [14]. There are currently a few Phase I/II gene therapy clinical trials started, nearly all for patients in the early stages of monogenic disease where there are surviving photoreceptors to treat. These trials, which are encouraging, are mostly limited to “gene replacement” for disease resulting from recessive null mutations in causative genes with a coding region below 8 kb, the size cutoff for AAV or lentiviral vectors.

In retinal disease, many retinal interneurons survive in a physiologically and metabolically functional state for long periods (years), providing an opportunity for treatment by genetically adding a light sensitive function to these cells [15, 16]. One approach used by several groups is to add a light-receptive function to the surviving inner retinal neurons by expressing a microbial opsin [1, 2, 3, 5, 7–10]. In 2014, the FDA approved a clinical trial for RST-001 (RetroSense Inc.), an intravitreal AAV2 vector to transfer Channelrhodopsin-2 (ChR2) to retinal ganglion cells [17]. GenSight, Inc., is beginning a clinical trial using intravitreal AAV to deliver ChrimsonR to cone inner segments [4] in patients with severe vision deficit from rod cell death, and loss of the light-sensitive cone outer segments.

Poor light sensitivity is one of the biggest challenges to the optogenetic vision restoration. The threshold intensity required to activate ChR2-sensitized bipolar cells and ganglion cells is 10^{15} photons/cm^2 s [9, 10] (*see* Fig. 1). Halorhodopsin (NpHR) expressed in cones has slightly better light sensitivity, requiring a minimum of 10^{13} photons/cm^2 s to generate a measurable photocurrent [3]. In comparison, activation threshold of the endogenous photopigments in rods (10^6 photons/cm^2 s) and cones (10^{10} photons/cm^2 s) is substantially lower [9].

Furthermore, microbial opsins have limited sensitivity range—ChR2 and NpHR only adapt to intensity changes of 2–3 orders of magnitude, whereas rods and cones adjust their responses to 8–9 orders of magnitude [3, 4]. ChR2 and NpHR operating as a single unit (without a GPCR cascade), require very high intensity light for stimulation. Additionally, the bright light intensity must be carefully regulated so as to not saturate the cell response, which could cause failure of the cells to follow high frequency stimulation [18, 19].

Recently, Acucela, Inc. has licensed a human rhodopsin based optogenetic gene therapy [20] for treatment of RP from the Univ. of Manchester [21], which promises to have higher sensitivity than ChR2 variants. In their study, AAV-mediated expression of rhodopsin into both RGCs and inner nuclear layer neurons generated reproducible responses to light pulses at the intensities as low as 10^{12} photons/cm^2 s, within the range of the irradiance encountered in our daily life. We have found very similar sensitivities by AAV mediated expression of rhodopsin in specific retinal cell classes (*see* Fig. 1) [22].

In all these approaches, the early responses from patients will tell us much about what is needed for a workable retinal prosthetic. While awaiting those exciting results, it is clear that these approaches have shortcomings: (1) microbial gene expression in a mammalian tissue is a potential concern, (2) once expressed, the opsin cannot be silenced in case of adverse reaction, and (3) microbial opsins operate over a narrow range of light and only at very high intensity, an intensity that is likely to cause light damage to the surviving retinal cells over time.

Here, we describe a gene therapy based method to deliver the light gated mammalian GPCR vertebrate rhodopsin to the retina of blind mice. We show how to verify expression of rhodopsin in retinal tissue and how to probe the treated retina for light responses in vitro. Further, we describe in detail how to test light responses in vivo, first by recording visually

evoked responses in the visual cortex and second, by testing visually guided behavior in treated mice.

2 Materials

2.1 Intraocular AAV Injection

1. Retinal degeneration mice: rd1, rd10 strains. Control mice with wildtype retina on similar genetic background: C57/BL-J.
2. 1 μl of AAV >10^{13} vg/ml per eye to be injected.
3. 72 mg/kg Ketamine and 64 mg/kg xylazine for general anesthesia.
4. 0.5% Proparacaine, 2.5% phenylephrine, 1% tropicamide for topical anesthesia of the cornea.
5. Sharp 30-G needle (disposable tuberculin syringe).
6. Blunt 32 G 1-cm Hamilton syringe.
7. Operating Microscope with foot-controlled motorized focus.
8. 1% phenol red in PBS.
9. Heating pad.
10. See video of injection procedure at JOVE [23].

2.2 Verification of Gene Expression

1. Microscope slides and coverslips.
2. 4% paraformaldehyde (PFA).
3. Phosphate Buffered Saline (PBS).
4. Fine forceps.
5. Fine scissors.
6. Agarose.
7. Vibratome sectioning device.
8. Vectashield mounting medium with DAPI.
9. Fluorescent microscope with digital imaging camera, computer and software.
10. Blocking buffer: 10% normal goat serum (NGS), 1% BSA, 0.5% Triton X-100 in PBS (pH 7.4).
11. Appropriate combinations of primary and secondary antibodies (optional).

2.3 In Vitro Multielectrode Array (MEA) Recordings of Retina Response

1. Dissection stereomicroscope.
2. Darkroom with dim red light.

3. Fine scissors, forceps and scalpel.
4. Small incubator.
5. Organotypic cell culture inserts (Millicell, Millipore).
6. 60-channel perforated MEA1060 multi electrode array system with constant vacuum pump (Multi Channel Systems).
7. MEA chamber pMEA 100/30iR-Tpr (Multi Channel Systems).
8. Mesh weight (Slice grids, Scientific Instruments).
9. Ames media (Sigma).
10. Constant perfusion system to supply oxygenated Ames media (32 °C).
11. Heating system for media.
12. Illumination system for optogenetic stimulation (DG-4, Sutter Instruments).
13. Green band pass filter 510/50 nm (Thorlabs).
14. Upright microscope.
15. MCS rack software (Multi Channel Systems).
16. Analysis software: Offline Sorter (Plexon).

2.4 In Vivo Visually Evoked Potential (VEP) Recordings of Cortical Response

1. 2 mg/kg Chlorprothixene.
2. 1.5 g/kg Urethane for general anesthesia.
3. Isoflurane for general anesthesia.
4. Temperature controller, rectal probe, and heating pad.
5. 1% Tropicamide.
6. Silicone oil.
7. Syringe with 25 G needle.
8. Fine forceps and scissors.
9. Acrylic glue (Ortho-Jet BCA).
10. Betadine solution.
11. Marker pen.
12. Small ruler.
13. Handheld dental drill with foot pedal control.
14. Custom made metal head plate.
15. Borosilicate glass (1.5 mm OD, 1.16 mm ID, Warner Instruments).
16. Horizontal puller (Sutter Instruments).

17. Artificial cerebrospinal fluid (ACSF): 124 mM NaCl, 2.5 mM KCl, 2.0 mM $MgSO_4$, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, 4 mM sucrose, 2.5 mM $CaCl_2$. Note: When the components are first mixed, the ACSF solution may be slightly milky, but it should clear when aerated with 95% O_2, 5% CO_2, after which the pH of the medium should be 7.4.
18. Microscope and micromanipulator.
19. LED light source with fiber optic light guide (455 nm, 15 mW/cm^2, Thorlabs).
20. Axoclamp 200B amplifier (Axon Instruments).
21. I/O board (Lab-PC-1200, National Instruments).
22. Custom made acquisition software written in MatLab.

2.5 Visually Guided Behavior: Open Field Test

1. Plastic box (dimensions l = 60 cm, w = 40 cm, h = 30 cm) with a light compartment (l = 25 cm, w = 40 cm, h = 30 cm) with white walls and a dark compartment (l = 35 cm, w = 40 cm, h = 30 cm) with black walls and small area between the two compartments (h = 5 cm, w = 10 cm).
2. Ethanol.
3. Light source (custom 5 × 6 LED array (447.5 nm Rebel LED, Luxeon star).
4. Video camera.

2.6 Visually Guided Behavior: Forced 2-Choice Water Maze Task

1. Y-maze (or modified radial arm maze).
2. Transparent escape platform.
3. Ethanol.
4. Light source (custom LED array (5 × 6 LEDs, 447.5 nm Rebel LED, Luxeon star).
5. Video camera.
6. 6 cm diameter glass beaker.

2.7 Visually Guided Behavior: Light Cued Fear Conditioning

1. Coulbourn shock chamber Habitest with test cage.
2. Ethanol.
3. Light source (custom 5 × 6 LED array (447.5 nm Rebel LED, Luxeon star).
4. Freeze-frame acquisition software.

3 Methods

3.1 DNA Preparation and AAV Production

1. For targeted expression of vertebrate rhodopsin in ON-bipolar cells, prepare a plasmid containing a 4× repeat of the metabotropic glutamate receptor 6 promoter (4×grm6) followed by the rat rhodopsin gene tagged C-terminally with a yellow fluorescent protein. The DNA cassette should be flanked on either side by inverted terminal repeat (ITR) domains. Package and purify Adeno associated virus serotype 2 with the rAAV2/2(4YF) capsid following the protocol of Visel et al. [24]

2. For further instructions on DNA preparation and AAV packaging and purification, *see* Notes 1–3 and refer to the methods paper by Visel et al. [24]

3.2 Intraocular AAV Injection

1. To prepare for intraocular injection, anesthetize mice by intraperitoneal injection of 72 mg/kg ketamine and 64 mg/kg xylazine. When the mice are not responding to toe pinch anymore, anesthetize the eyes with a drop of 0.5% proparacaine and dilate the pupils with a drop of 2.5% phenylephrine followed by a drop of 1% tropicamide. Wait for >1 min between drug applications.

2. For intravitreal injections (*see* Note 4), make an incision posterior of the ora serrata using a sharp 30-G needle. Then feed a 1–2 μl volume containing about 5×10^{11} viral genomes of AAV diluted in PBS (with 1% phenol red as contrast agent) through the incision site and inject into the vitreous using a blunt 32 G Hamilton syringe. It is critical to avoid damaging the lens with the syringe as this will lead to cataracts. Leave the Hamilton needle tip in the eye for >60 s to allow homogenization and reduce the efflux.

3.3 Tissue Preparation and Verification of Expression

1. Verify gene expression 4–6 weeks post injection by fluorescent imaging (*see* Note 5).

2. To isolate the retina, sacrifice mice, enucleate the eyes and fix in 4% paraformaldehyde for 1 h. Remove the cornea by making a circular incision around the ora serrata using scissors. Place two forceps around the edges of the eyecup and carefully tear the retina from the sclera. Make radial cuts to flatten the retina thereby forming the typical clover-leaf shape. Then use retinal whole mounts for antibody staining (*see* Note 5). Alternatively, mount whole mounts directly on a glass slide using Vectashield mounting medium with DAPI to stain cell nuclei. Now these retinas are ready for imaging, ideally with a confocal microscope.

3. To prepare retinal sections, embed whole mount retinas in agarose and make transverse sections of 150 μm thickness using a vibratome device at medium speed with maximum vibration. Mount retinal slices on glass slides using Vectashield or stain with antibodies.

4. For antibody staining (*see* Note 5), incubate whole mounts or sections in blocking buffer for 2 h at RT on a shaker. Apply primary antibodies over night at 4 °C or for 4 h at RT and apply secondary antibodies for 2 h at RT. It is important to wash the tissue 3 × 10 min with PBS between antibody applications and before imaging to reduce fluorescence background.

3.4 In Vitro Multielectrode Array (MEA) Recordings of Retina Response

1. For MEA experiments, use mice 6–10 weeks after AAV injection. First, prepare whole mounts as described in Subheading 3.3.2 with modifications. Place the cloverleaf retina onto an organotypic membrane (Millipore) instead of glass slide and do not fix the samples as this would kill biological activity. Place the excised retina photoreceptor cell side down on the Millipore membrane, add a drop of Ames medium on the retina and transfer the filter to an incubator to let the retina settle for >30 min prior to experiments. Perform dissection and experiments in a dark room with minimal red light to prevent bleaching of 11-*cis*-retinal (*see* Note 6).

2. Take the Millipore membrane with the whole mounted retina and use a scalpel to cut around the edges of the retina and leave ~5 mm of margin. Use forceps to place the retina ganglion cell side down in the center of the MEA array. In order to prevent the retina from floating and to improve electrode contact and signal-to-noise, place a mesh weight on the retina. The contact of tissue and electrodes can be further improved by applying vacuum to the base of the retina through the MEA chamber using the constant vacuum pump. All the tubing to and from the MEA chamber and vacuum pump must be preloaded with buffer. It is important that no air bubbles enter the tubing as they interfere with the constant vacuum. During recording, provide a constant perfusion of oxygenated Ames media (32 °C) to the recording chamber to support neural activity.

3. The retina is now ready to be stimulated with light. Optogenetic stimulation requires strong light sources that emit only the desired wavelength of light. This can be achieved with an LED system which emits monochromatic light or bandpass filtered white light from an arc lamp. If possible, couple the illumination through the imaging objective and calibrate light intensities using a handheld power meter.

4. Acquire data with MCS rack software at 25 kHz frequency and filter data below 300 Hz and above 2000 Hz. Simultaneously record light stimulation parameters as TTL pulses. When finished with acquisition, convert voltage traces to spike trains off-line. Apply principal components analysis using an offline sorting software (e.g., Plexon) to sort spikes recorded at one electrode into single units, and define them as “cells.” Export single unit spike clusters to MatLab for further analysis.

3.5 In Vivo Visually Evoked Potential (VEP) Recordings of Cortical Response

1. To prepare for VEP recordings, anesthetize mice. Induce anesthesia with 5% isoflurane for 3 min and then reduce to 0.5–1%. Inject mice with chlorprothixene (2 mg/kg, intraperitoneally), wait 5 min and then inject mice with urethane (1.5 g/kg, intraperitoneally). Supplement the anesthesia throughout the surgery with 0.5–1% isoflurane. Dilate pupils with 1% tropicamide, then add a drop of silicon oil to prevent the eyes from drying out. Maintain body temperature of anesthetized mice using a DC temperature controller with rectal probe and a heating pad set to 37 °C.
2. Clean mouse head with betadine solution for disinfection. Grab the scalp with forceps at center of head half way between eyes and ears and remove scalp with scissors. Then use a syringe with 25G needle to clean the skull by scratching to remove the muscle and membrane. This step is very important otherwise the glue will not hold. With a small ruler, locate the visual cortex by coordinates (1.7 mm lateral to midline and 0.7 mm anterior to lambda) and mark the location with a pen. Apply glue around the marked location and attach a small custom made metal head plate by pressing for ~30 s. Wait 10 min for the glue to cure. Use a handheld drill with foot pedal control to make a small craniotomy and durotomy over the primary visual cortex. Supply a drop ACSF every 2–3 min to prevent the tissue of drying out. Take care to avoid damaging blood vessels.
3. Pull electrodes with a resistance of 3 MΩ from borosilicate glass (1.5 mm OD, 1.16 mm ID) using a horizontal puller and fill them with ACSF.
4. Transfer mice to the microscope and place them on a heating pad. Then, position the ACSF-filled electrode over the craniotomy and slowly lower it to a final depth of 400 μm, to layer 4 of the visual cortex.
5. To stimulate the retina, use an LED system or a band pass filtered white light source coupled to a fiber optic guide. Position the end of the fiber optic guide 1 cm away from the contralateral eye.
6. Stimulate the eye and record visually evoked potentials. Amplify responses at 10 kHz frequency, band pass filter at 2 kHz, digitize using an I/O board in a computer and average responses over multiple trials of optogenetic stimulation. Export, analyze and graph the voltage traces in MatLab.

3.6 Visually Guided Behavior: Open Field Test

1. Begin by spraying the box meticulously with ethanol to prevent odor from previous experiments to impact visually guided behavior.
2. Position the light source (*see* Note 7) over the light compartment.
3. Bring mice into the testing room in their home cages, transfer them to the open field box (*see* Note 8) with their littermates and allow them to habituate to the new environment for 45 min. Then, put mice back in their home cage and test them individually.

4. First, place a mouse in the light compartment of the box in a randomized starting position and allow a maximum of 3 min for the mouse to discover that there is a second compartment.

5. Start the trial (5 min) as soon as the mouse crosses into the dark compartment, and record the time spent in the light compartment. Mice that cross the opening only once and stay in the dark compartment for entire time (5 min) are disqualified.

6. Use permanent records (video) to analyze the percent of time spent in the light and dark compartment.

3.7 Visually Guided Behavior: Forced 2-Choice Water Maze Task

1. First, spray the maze meticulously with ethanol to prevent odor from previous experiments to impact visually guided behavior.

2. Fill the Y-maze with water at 20 °C (*see* Note 9).

3. Place a light source (*see* Note 7) at the end of one of the “escape arms” that cues the location of the escape platform. Add the platform directly under the light source.

4. Position a video camera above the maze to make permanent records of the experiment. A wide angle lens is preferable (e.g., GoPro camera).

5. Bring mice into the testing room in their home cages and transfer them into new cages located on a heating pad and next to a space heater to warm the mice after every trial. Keep the room dark for the entire time of the experiment.

6. Habituate mice to the maze one day before the start of an 8-day trial. Place mice onto the platform for 1 min. Then release the mice at increasing distances from the platform and finally release them from the chute for ten trials. Repeat with the platform on the opposite side.

7. Additionally, habituate the mice each day before the start of the experiment by placing them onto the platform for 1 min on both sides and returning them to the cage.

8. For each trial, remove mice from their cage, place them in a glass beaker (6 cm diameter) and then slowly (10–60 s) lower them into the water opposite of the divider. It is important that the mouse decides when to exit the beaker. If the beaker is lowered too fast, mice are stressed and will not perform well. Give the mouse a maximum of 60 s to find the platform. Trials in which mouse found the hidden platform without entering the alternative arm first are counted as correct trials. Trials in which the mouse explored the alternative arm first or takes longer than 60 s to find the platform are counted as failed trials.

9. After the trial, dry the mouse with paper towels, place it into a warm chamber with a space heater and allow it to rest for at least 3 min before the next trial. All mice perform ten trials per session with two sessions a day, with a total of 20 trials per mouse per day for 8 consecutive days. Move the platform and the light

cue between trials according to the following pattern: LRRLRLLRLR and RLLRLRRLRL on alternating days.

3.8 Visually Guided Behavior: Light Cued Fear Conditioning

1. Prior to the experiment, inject control mice with sham injections (PBS) to control for the virus treatment.
2. The experiment is done over three consecutive days: habituation on day 1, training on day 2 and testing on day 3.
3. Start every day by spraying the Habitest chamber meticulously with ethanol to prevent odor from previous experiments to impact visually guided behavior.
4. On day 1, bring the animals into the testing room in their home cages and then acclimatize them individually to the Coulbourn shock chambers for 30 min.
5. On day 2, train the mice by subjecting them to paired or unpaired light cued fear conditioning. Training consists of 5 min habituation to the chamber followed by three shock trials at 0.7 mA. For paired trials synchronize the 20 s light cue (*see* Note 10) with 3× 2 s foot shocks at 4 s inter-shock-interval with 40 s inter trial interval. For unpaired trials, provide the same amount of foot shocks and light cues but in a random order without synchronization. These brief, low current shocks provide the minimal aversive stimulus to create a fearful memory associated with a light cue.
6. On day 3, test the animals in a fear probe trial. Change the floor of the chamber from the shock grid to a solid floor. Then allow the mice to habituate to the chamber for 5 min, and then present the same light stimulation protocol as on day 2, but without shock, while recording their movement and behavior with the Freeze-frame software.
7. Use the video recordings to analyze conditioned fear behavior (time spent freezing, a typical rodent fear response) associated with the learned light cue.

4 Notes

1. In order to construct a genomic plasmid for gene therapy, begin with a plasmid containing internal terminal repeat domains (ITR) and add promoter element, gene of interest and fluorescent reporter gene. If expression should be limited to one target cell type, use cell specific promoter elements (e.g., 4×grm6). Otherwise use generic promoter elements (CMV, EF1-alpha, hsyn). For DNA preparation it is important to transform the DNA into competent bacteria that lack recA (e.g., SURE2 or STBL 3) and grow the bacteria for <12 h to minimize homologous recombination which can render the ITRs nonfunctional.
2. Package and purify AAVs following the protocol described by Visel et al. [24]. The choice of serotype is critical as this will determine the cells that the virus is targeting. For targeting ON-bipolar cells, we have found best results with the quadruple tyrosine mutant AAV2(4YF). It is also important to consider the route

of delivery (subretinal vs. intravitreal) when choosing a serotype. AAV serotypes that penetrate deep into the retina like 7 m8 [25] or AAV2(4YF) [26] are crucial when choosing the route or intravitreal injection.

3. Determine the titer of AAVs via qPCR relative to inverted repeat domains (ITR) standard. Titers for these viruses should range between 10^{12}-10^{14} viral genomes/ml.

4. Two routes of intraocular AAV delivery are commonly used: subretinal and intravitreal injections. Intravitreal injections are preferred over subretinal injections as they are technically easier to deliver and produce pan-retinal expression in contrast to subretinal injections which produce a very local expression around the site of virus deposition covering less than a quarter of the retina.

5. Antibody staining is required if the gene of interest is not tagged with a fluorescent reporter, otherwise no staining are required. In case the gene of interest is expressed in the distal layers (RGC or photoreceptor layer), whole mount imaging may be sufficient. For expression in inner retinal neurons, agarose sections are required.

6. After removal of the retina from the retinal pigment epithelium RPE, the ligand for rhodopsin, 11-*cis*-retinal cannot be replenished. To prevent bleaching of 11-*cis* retinal, retinas must be handled in dark rooms under red light.

7. For visually guided behavior experiments, it is important to have very strong light sources in order to have a homogenous illumination in a large area (e.g., light compartment of open field test: $A = 0.1\ m^2$). This can be achieved by custom built LED arrays (we used a 5 × 6 LED array).

8. It is extremely important to handle the mice gently to minimize stress. Stress-induced fear masks any learned behavior and will overwrite what the mice may have previously learned.

9. Warm water will be less aversive leading to floating of mice. Cold water will put too much stress on the mice.

10. Using this behavioral paradigm, one can test for several levels of visual restoration. Begin with simple light recognition (light on vs. off) and then increase the difficulty of the task by testing for spatial pattern recognition (moving vs. static stimuli) or temporal pattern recognition (flashing vs. static stimuli).

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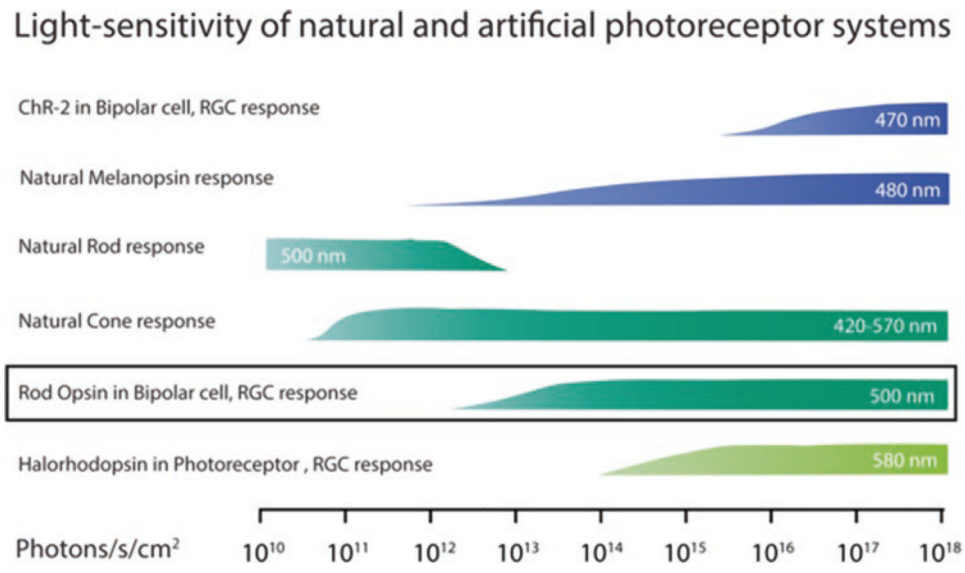

Fig. 1.

Comparison of the light sensitivities of natural and artificial photoreceptor systems. Rod and cone photoreceptor sensitivities and melanopsin-expressing retinal ganglion cell sensitivity are compared with those of halorhodopsin (NpHR), wild-type channelrhodopsin 2 (ChR2), and vertebrate rhodopsin installed in various cell types. Light sensitivities are given in photons/cm^2 s The wavelengths for the respective therapeutic approaches are color-coded and indicated as numbers in white. All optogenetic channel light sensitivities are based on in vitro retinal whole-mount recordings from retinal ganglion cells (Figure modified from [27])