Restoring vision in mice with retinal degeneration using multicharacteristic opsin

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Abstract. Retinal degenerative diseases, such as retinitis pigmentosa (RP) and dry age-related macular degeneration (dry-AMD) and retinitis pigmentosa (RP). There is currently no treatment available to cure retinal dystrophy or halt its progress. Since these retinal dystrophies are progressive and age-related disorders, the number of patients is expected to significantly increase in the near future. In these retinal disorders, the photoreceptors slowly degenerate and are unable to produce the signals that initiate visual perception. A number of gene therapy-based trials are underway, but it is not expected to significantly increase in the near future. In these retinal disorders, the photoreceptors slowly degenerate and are unable to produce the signals that initiate visual perception.

1 Introduction

Loss of vision in millions of individuals is the result of the advanced stage of retinal dystrophies, such as dry age-related macular degeneration (dry-AMD) and retinitis pigmentosa (RP). There is currently no treatment available to cure retinal dystrophy or halt its progress. Since these retinal dystrophies are progressive and age-related disorders, the number of patients is expected to significantly increase in the near future. In these retinal disorders, the photoreceptors slowly degenerate and are unable to produce the signals that initiate visual perception. A number of gene therapy-based trials are underway, but it is not expected to significantly increase in the near future. In these retinal disorders, the photoreceptors slowly degenerate and are unable to produce the signals that initiate visual perception.

Optogenetic sensitization of retinal cells has potential as an alternative to retinal implants. In addition to higher resolution, optogenetics has several advantages over electrical stimulation, such as cellular specificity (e.g., ON bipolar cells), and does not require intraocular surgery. However, clinical translation of optogenetic-enabled vision restoration suffers from the drawback of active stimulation by a light source having an intensity an order of magnitude higher than ambient light (i.e., bright ambient light level is ~0.02 mW/mm²) owing to the low photosensitivity of opsins deployed for the purpose. Such active stimulation has resolution limited by the resolution of the near-the-eye display and requires tracking of individual cells in the retina for achieving single-cell resolution. Further, chronic stimulation with high intensity blue light leads to retinal damage. Therefore, boosting up light intensity to high enough levels to activate conventional opsin-expressing cells, especially for chronic stimulation, may substantially damage the residual light-sensing function that might exist in the diseased or impaired retina of retinal dystrophy patients. The use of red-shifted channelrhodopsin-2 (ChR2) has promise for reducing the phototoxicity of retinal cells. Recently, to enhance sensitivity of cells toward ambient white light, a fusion vector (white-opsin) containing three plasmids encoding opsins with spectrally separated activation peaks (ChR2 in blue, C1V1 in green, and ReaChR in red) was deployed. However, the use of multiple opsin constructs in same vector restricts its delivery to physical methods (e.g., optoporation or electroporation) since safe viral vectors [e.g., adeno-associated virus (AAV)] have limitations on carrying large size genes. Further, the light sensitivity still needs to be improved.

To address this difficulty in optogenetic-enabled vision restoration, we have developed an ambient light activatable multi-characteristic opsin (MCO1) encoding gene, which is activated at the bright ambient light level. The MCO1 is packaged into a safe viral vector (AAV) with a fluorescent reporter (vMCO1-mCherry). The vMCO1 is intravitreally injected into the degenerated rd10 mice retina, which is a well-established animal model of retinal dystrophy. Our results clearly demonstrate that the visually guided behavior that deteriorated with progressive retinal degeneration recovered back to a more non-degenerated level after vMCO1 treatment. More importantly,
this improved vision does not compromise the viability of vMCO1-treated retina.

2 Material and Methods

2.1 Theoretical Modeling of the Three-Dimensional Arrangement of Amino Acid Chain

We used a highly recognized web-based protocol, RaptorX, server for secondary structure and template-based tertiary structure prediction of our newly developed MCO1. The RaptorX uses a conditional neural field, a variant of conditional random fields, and a multiple template treating procedure to develop the following predicted structure of the MCO1 gene.

2.2 Virus Carried Ambient Light Activatable Multicharacteristic Opsin (vMCO1) Synthesis

The MCO1 gene was synthesized using a DNA synthesizer, and the sequence was verified. The synthesized plasmid was cloned into plasmid containing AAV terminal repeats multiple cloning site vector via its BamH1 and Sal1 sites. AAV physical titers were obtained by quantitative polymerase chain reaction using primers designed to selectively bind AAV inverted terminal repeats. TCID50 assay was conducted according to American Type Culture Collection protocol. Verification of the purity of the purified virus was confirmed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

2.3 Cell Culture and MCO1 Plasmid Transfection

HEK293 cells were transfected with MCO1 plasmids using Lipofectamine 3000 (Invitrogen). After transfection, HEK293 cells were cultured in Petri dishes and maintained in Dulbecco's modified Eagle's medium/F-12 with 10% fetal bovine serum, 0.2 mg/mL streptomycin, and 200 μg/mL penicillin. The cultures were maintained at 37°C in a 5% CO2 humidified atmosphere. Visualization of the reporter (mCherry) fluorescence was carried out under epifluorescence (Nikon Eclipse TS 100) or confocal microscopy (Olympus Fluoview FV1000).

2.4 Optogenetic Stimulation

A single-mode optical fiber coupled to a supercontinuum laser source (SuperK Compact, NKT Photonics) delivered the light to the sample via fiber optic cable (SuperK FD-2) that transmits light in the visible spectrum for in-vitro optogenetic stimulation. The light intensity was varied by a current controller. A power meter (Newport) was used to quantify the light intensity at the sample plane. The light pulse width was controlled by a shutter, synchronized with the electrophysiology recording system (Molecular Devices). Cells, transfected with MCO1, were incubated with all-trans-retinal (1 μM) for 6 h before conducting the patch-clamp experiments.

2.5 Patch-Clip Recording Setup

Inward photocurrents in MCO1-transfected cells were recorded using the patch clamp. The patch-clamp recording setup consists of an inverted Nikon fluorescence microscope platform and an amplifier system (Axon Multiclamp 700B, Molecular Devices). Micropipettes were pulled using a two-stage pipette puller (PC-10, Narishige) to attain resistance of 3 to 5 MΩ when filled with intracellular solution. The micropipette electrode was mounted on a micromanipulator. The micropipette was filled with a solution containing (in mM) 130 K-glucosan, 7 KCl, 1 MgCl2, 0.4 EGTA, 10 HEPES, 2 ATP-Mg, 0.3 GTP-Tris, and 20 sucrose. The micropipette electrode was mounted on a micromanipulator to patch onto cells. The extracellular solution contained (in mM) 150 NaCl, 10 glucose, 5 KCl, 2 CaCl2, and 1 MgCl2 was buffered with 10 mM HEPES (pH 7.3). Photocurrents were measured while holding cells in a voltage clamp at −70 mV. The electrophysiological signals from the amplifier were digitized using Digidata 1440 (Molecular Devices), interfaced with patch-clamp software (pClamp, Molecular Devices). The light pulse width was synchronized with the electrophysiology recording system, controlled by Axon Instruments Digidata system. pClamp 10 software was used for data analysis.

2.6 rd10 Mice Preparation

The advanced stage of retinal dystrophy is characterized by degeneration of photoreceptors in the eye that hinders visual ability by nonfunctional neuronal activation and transmission of signals to the visual cortex. The rd10 mice (retinal degeneration 10, spontaneous missense point mutation in Pde6b) have a later onset and progressive retinal degeneration as compared with rd11 mice. This is important for sleep restoration purposes because it gives a window of time during which the visual cortex can learn how to interpret visual signals. rd11 mice and C57BL/6J wild-type mice (control) were obtained from Jackson laboratory and bred in the animal facilities at the Nanoscope Technologies. Mice were maintained on a 12:12 light cycle.

Nanoscope Technologies’s Institutional Animal Care and Use Committee approved the experimental protocol involving animals. The methods were carried out in accordance with the relevant guidelines.

2.7 Intravitreal Injection of vMCO1 to rd10 Mouse Retina

An aseptic technique was used for all surgical procedures, and surgical tools were sterilized with autoclave. The rd10 mouse (n = 10) was anesthetized with isoflurane (2% to 3%) and mounted on a stereotaxic frame. One drop each of 1% atropine sulphate, 1% Mydriacyl ophthalmic solution, and 10% phenylephrine hydrochloride were instilled into the eye of the animal. The vMCO1 (1 μL) solution was injected by a sterilized needle of a Hamilton microsyringe inserted through the sclera into the vitreous cavity (intravitreal injection). The vMCO1 solution was injected into both sides of the eye. The cornea was kept moist with a balanced salt solution during the entire surgical procedure.

2.8 Viability of Retinal Cells Subsequent to Chronic Light Exposure

Long-term (4 weeks) viability of retinal cells in the transfected retina was evaluated after chronic light stimulation (8 h/day). The mice were placed inside a chamber containing white light sources, providing intensity of 0.1 mW/mm² on the floor of the chamber. Four weeks after light exposure, the transfected (n = 5 rd10 mice) as well as wild-type (control, n = 5) mice were euthanized, and the eye tissue was harvested for retina extraction. The explanted retina was immunostained using an apoptotic marker (cleaved caspase-3)/4',6-diamidino-2-phenylindole
(DAPI) and imaged using confocal microscopy (Olympus Fluoview FV1000). The number of total cells versus apoptotic retina cells was compared between transfected and control groups.

2.9 Water Maze Behavioral Assessment

rd10 mice (n = 5) with retinal degeneration were used inclusion criteria of age > 8 weeks. For type I error rate \( \alpha = 0.05 \) and 80% to 90% power, the required number of animals is 3 to 4/group. After vMCO1 injection, mice were tested with a radial arm water maze to determine behavioral restoration of vision in rd10 mice with vMCO1-sensitized retinal cells. Briefly, mice are placed into the center of the maze, and a platform is placed just below the water’s surface at the end of one of the arms. The mice rapidly learn to determine the location of the platform by utilizing visual cues [light-emitting diodes (LEDs) emitting light with visible spectrum]. The platform (in one of the arms) provided a reward to them where they can rest instead of having to swim. The time to reach the platform and the number of error(s) made before finding the platform were quantified for both light ON and OFF conditions. Data (video) recording was stopped once the mice found the platform or before 60 s of dropping the mice in water to prevent the mice from getting tired of swimming. For each mouse, \( n = 5 \) trials were conducted in each location (i.e., center, side arm, and edge of the water maze). The selection of dropping site (center, side, and edge) was random for each mice and each trial. The exclusion criterion consists of a mouse that does not swim (and floats).

3 Results and Discussion

3.1 Ambient Light Activatable Multicharacteristic Opsin

The present approaches for optogenetic restoration of vision have used ChR2, which is not activatable at the ambient light level and, therefore, not able to generate action potential (AP) in an ambient environment. Thus, all attempts at restoring vision by optogenetic stimulation so far has focused on the development and implementation of an active array of intense light sources (e.g., LED array) to activate opsin-sensitized cells in the retina. However, considerable concerns about viability of opsin-sensitized neurons as well as residual photosensitive cells under extended exposure to such intense light exist. We have developed an ambient light activatable MCO1. Figure 1(a) shows the domain architecture of MCO1 with reporter (mCherry). In Fig. 1(b), we show a typical circular map showing the insertion of the MCO1 gene cloned at the restriction sites (BamH I and Sal I). Figure 1(c) shows the three-dimensional (3-D) arrangement of amino acid chains of MCO1 obtained by theoretical modeling using RaptorX. Figure 1(d) shows the agarose gel electrophoresis of the mGluR6-MCO1-mCherry gene (2915 bp) after restriction enzyme digestion with BamH1 and Sal1. Multicharacteristics opsin (MCO1) has high photosensitivity with unique spectral and temporal characteristics to generate significant current in response to ambient light (Fig. 2).
3.2 Sensitivity of Multicharacteristic Opsin to Light

To determine the light-dependent inward photocurrent, the MCO1-expressing cells were exposed to pulses of light with various intensities ranging from 0.01 to 0.03 mW/mm². Figure 2(a) shows the schematic of the experimental setup for optical stimulation and electrical recording. Fluorescence image of HEK293 cells after 48 h of lipofection of MCO1-mCherry plasmids is shown in Fig. 2(b). Figure 2(c) shows the spectrum of the light used for stimulation of MCO1-expressing cells. Representative inward photocurrent profiles in HEK cells transfected with MCO1 are shown in Fig. 2(d). To obtain the activation spectrum of MCO1, the inward photocurrent was measured using stimulation light at different wavelengths (with bandwidth: 30 nm). In Fig. 2(e), we show the normalized activation spectrum of MCO1. The inward photocurrent was found to be an order of magnitude higher in MCO1-sensitized cells than that in the ChR2 expressing cells. Figure 2(f) shows a comparison of MCO1 photocurrent with ChR2 and white-opsin.21 The inward photocurrent (195 ± 32 pA) in MCO1-sensitized cells at an ambient light level of 0.024 mW/mm² is above the threshold for AP unlike that in cells sensitized with ChR2 and white-opsin.21

It may be noted that for a good fidelity of the light-evoked spiking of opsin-sensitized cells, faster response time is
required. The ON response time of an ambient light activatable MCO1 [Fig. 2(d)] is measured to be $2.94 \pm 0.70$ ms, which is similar to that measured for other fast opsins. However, the ON response time depends on the intensity of activation light and is known to increase as the light intensity decreases.

### 3.3 Transfection Using Viral Vector Carried Multicharacteristic Opsin (vMCO1)

First, delivery of MCO1 by AAV was confirmed in-vitro in HEK cells [Fig. 3(a)]. In-vivo viral transfection was conducted for delivery of the MCO1 to the bipolar cells in the retina of the rd10 mouse model. We performed intravitreal injection of $1 \mu$L solution of vMCO1 (in both eyes), in which the MCO1 was under control of a specific promoter (mGluR6), targeted for ON retinal bipolar cells. Uniformity of MCO1 expression was confirmed by the 3-D reconstruction from the confocal mCherry expression in z-slices of the whole retinal cup in rd10 mice injected with vMCO1 intravitreally [Fig. 3(b)].

### 3.4 Effect of Improved Light Sensitivity of the Retina on rd10 Mice Behavior in the Visual Water Maze

For testing the spatial memory and learning capabilities of vMCO1-treated rd10 mice toward light, a visual radial arm water maze was used. To determine behavioral restoration of vision in vMCO1-treated rd10 mice, one week before injection of vMCO1, mice were tested with light ON and OFF conditions in a radial visual water maze. The light intensity used in these behavioral studies was 7 $\mu$W/mm² at the center and <5 $\mu$W/mm² in the edge/side arms of the radial water maze. This corresponds to retinal illumination below 0.01 $\mu$W/mm². Figure 4(a) shows a time-lapse imaging of a rd10 mouse navigating the visual water maze with white LED light ON before intravitreal vMCO1 injection. The mice were again assayed with both light ON and OFF conditions after intravitreal injection of vMCO1. Figure 4(b) shows time-lapse imaging of the same rd10 mouse navigating the visual water maze six weeks after vMCO1 injection at same LED light intensity. The time traveled by the vMCO1-transfected mice before arriving at the platform was much shorter [<20 s, Fig. 4(b)] than before injection [>60 s, Fig. 4(a)].

Consistent with the number of error arms, the mean number of arms swam by the vMCO1-transfected mice before they reached the platform is significantly smaller (<1) than that of the mice before transfection (>2). Figures 4(c)–4(e) show the change in latency to find the platform by the vMCO1-transfected rd10 mouse, with and without light, dropped at the center (c), side arms (d), and edge (e). Notably, the improvement in visually guided behavior was observed at a light intensity level of ~10 $\mu$W/mm² (retinal illumination level <0.01 $\mu$W/mm²) an order of magnitude lower than that required for mice sensitized with ChR2 opsin. Figures 4(f)–4(h) show the number of error arms traversed by the mice dropped at different locations before finding the platform in the presence and absence of light: center (f), side arms (g), and edge (h). The data collected from one mouse that was found to be floating most of the time, instead of swimming, were not included.

### 3.5 Chronic Light Exposure Did Not Compromise Viability of vMCO1-Treated Retina

Chronic exposure of MCO1-transfected retina to light may raise concern about their viability. We therefore exposed vMCO1-treated rd10 mice to an order of magnitude higher intensity white light (i.e., 0.1 mW/mm²) than that of the targeted ambient light level (i.e., 0.01 mW/mm²). Long-term (4 weeks) viability of retinal cells in the transfected retina was evaluated after chronic light stimulation (8 h/day). It may be noted that, similar to the wild-type mice, the vMCO1-treated rd10 mice avoided bright light by blocking the light via the creation of a hill out of the bedding material [as shown by arrow, Fig. 5(a)].

Four weeks after light exposure, the transfected rd10 mice as well as the wild-type mice were euthanized, and the eye tissue was harvested for retina extraction. The retinal cups were immunostained using an apoptotic marker (caspase-3) and imaged using confocal microscopy. Figure 5(b) shows the caspase-3 (green)- and DAPI (blue)-stained retina of vMCO1-treated rd10 mice after chronic light exposure. Figure 5(c) shows the
caspase-3 (green)- and DAPI (blue)-stained retina of wild-type mice after chronic light exposure. The number of apoptotic retinal cells was quantified, normalized to the total number of cells, and compared between the MCO1-transfected rd10 and control (wild type) groups. No statistically significant difference was found between these two groups [Fig. 5(d)]. There is no significant cell death in either the wild type or vMCO1-injected rd10 mice, indicating that there is no compromise of cell viability of vMCO1-treated rd10 mice under chronic light exposure. While avoidance of the bright light by the vMCO1-treated mice [Fig. 5(a)] demonstrated light sensitivity of the MCO1-sensitized retina, it is possible that the mice were not fully exposed...
to chronic illumination all the time during this study (8 h/day, 4 weeks). Furthermore, since light sensitivity of MCO1-expressing cells significantly reduces the required light intensity for generating AP, the use of vMCO1 minimizes light-induced chronic damage to the retinal cells as compared with those sensitized by ChR2.

4 Conclusions

Virally delivered vMCO1 will lead to a clinical approach for treating patients with advanced retinal dystrophies by conventional intravitreal injection of vMCO1. We strongly believe that such treatment will lead to restoration of vision in RP and dry-AMD patients in an ambient light environment in a non-invasive manner without requiring surgery and/or active stimulation methods.

Disclosures

The authors Sulagna Bhattacharya and Samarendra Mohanty have equity interest in NanoScope Technologies LLC.

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References


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Subrata Batabyal has expertise that span across the area of biophysics, biochemistry, optics, biomedical instrumentation, and optogenetics. He earned his PhD in chemistry with a focus on ultrastar laser spectroscopy to investigate biomolecular interaction. During his postdoctoral fellowships, he worked on ultrastar laser-assisted gene delivery, optical stimulation, and development of label-free optical techniques to detect neuronal activity and cellular membrane fluctuations.

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