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Restoration of visual function by expression of a light-gated mammalian ion channel in retinal ganglion cells or ON-bipolar cells

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Most inherited forms of blindness are caused by mutations that lead to photoreceptor cell death but spare second- and third-order retinal neurons. Expression of the light-gated excitatory mammalian ion channel light-gated ionotropic glutamate receptor (LiGluR) in retinal ganglion cells (RGCs) of the retina degeneration (rd1) mouse model of blindness was previously shown to restore some visual functions when stimulated by UV light. Here, we report restored retinal function in visible light in rodent and canine models of blindness through the use of a second-generation photoswitch for LiGluR, maleimide-azobenzene-glutamate 0 with peak efficiency at 460 nm (MAGO460). In the blind rd1 mouse, multielectrode array recordings of retinal explants revealed robust and uniform light-evoked firing when LiGluR-MAGO460 was targeted to RGCs and robust but diverse activity patterns in RGCs when LiGluR-MAGO460 was targeted to ON-bipolar cells (ON-BCs). LiGluR-MAGO460 in either RGCs or ON-BCs of the rd1 mouse reinstated innate light-avoidance behavior and enabled mice to distinguish between different temporal patterns of light in an associative learning task. In the rod-cone dystrophy dog model of blindness, LiGluR-MAGO460 in RGCs restored robust light responses to retinal explants and intravitreal delivery of LiGluR and MAGO460 was well tolerated in vivo. The results in both large and small animal models of photoreceptor degeneration provide a path to clinical translation.

Microbial opsins, like channelrhodopsin and halorhodopsin, have been successfully tested as visual prosthetics in animal models of human blindness (10–15). Genetically encoded light-gated proteins can be exogenously expressed in retinal cells using viral or nonviral gene delivery vehicles, imparting a light-sensitive function to cone photoreceptors that have become insensitive to light from loss of their outer segments (14), but also to ON-BCs (12, 13), as well as RGCs (10, 15, 16), leading to rescue of basic aspects of visual function in mice. Microbial opsins are appealing for this application due to the bioavailability of the light-sensitive ligand retinal. However, there are potential drawbacks to this approach. Xenotransplantation is generally concerning, because it might lead to immune responses and inflammation potentially spreading to the brain via the optic nerve.

**Significance**

We restored visual function to animal models of human blindness using a chemical compound that photosensitizes a mammalian ion channel. Virus-mediated expression of this light sensor in surviving retinal cells of blind mice restored light responses in vitro, reanimated innate light avoidance, and enabled learned visually guided behavior. The treatment also restored light responses to the retina of blind dogs. Patients that might benefit from this treatment would need to have intact ganglion cell and nerve fiber layers. In general, these are patients diagnosed with retinitis pigmentosa and some forms of Leber congenital amaurosis. Patients diagnosed with other types of blindness, for example, age-related macular degeneration or diabetic retinopathy, would not be candidates for this treatment.


Conflict of interest statement: E.Y.I. is an author on US Patent US 8,114,843 B2 on the design of protein photoswitches and a cofounder of Photoswitch Biosciences, Inc., which employs such switches in cell-based assays for drug screening and is developing an alternative method for restoration of vision that employs noncovalent photoswitches which endow light sensitivity onto native channels.

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Inherited retinal degenerative diseases affect 1 in 3,000 humans worldwide (1). Retinitis pigmentosa (RP) describes a family of over 50 different gene mutations that cause progressive loss of rod photoreceptors (1, 2). Rod loss is followed by degeneration of cone photoreceptors, ultimately leading to complete blindness in many patients (3). Despite the complete loss of photoreceptors in the outer nuclear layer, many interneurons of the inner retina survive in a functional state for long periods, providing an opportunity for treatment (4, 5).

Direct electrical stimulation of the surviving inner retina has proven to be successful in restoring useful vision (6–8). One approach employs surgically implanted photovoltaic or electrode arrays to stimulate retinal ganglion cells (RGCs) (8) or bipolar cells (BCs) (6, 7) directly in the inner nuclear layer (INL) of the degenerated retina, and promising results in clinical trials have led to US Food and Drug Administration approval for the Argus II device (Second Sight Medical Products, Inc.) (8). The electrical implants demonstrate that inner retinal neurons in blind patients can respond to appropriate stimulation and lead to a useful visual percept allowing simple navigation and object recognition. These electronic designs are under continual development to increase the resolution, improve the surgical implantation procedures, and increase the sophistication of their signal-encoding algorithms (9).
Additionally, once expressed, it is impossible to silence the system in case of adverse reactions in patients.

One promising alternative to microbial opsins is an optopharmacological strategy that uses synthetic azobenzene-based photoswitches to endow light sensitivity either to native ion channels of neurons (17, 18) or to engineered mammalian receptors and channels that, like the microbial opsins, allow for genetic targeting to specific cells (19–22). We previously showed that an engineered light-gated ionotropic glutamate receptor (LiGluR) restores light responses to blind retinal degeneration (rd1) mice (23). The gene encoding for LiGluR was delivered to RGCs by intravitreal injection of adeno-associated virus (AAV), and the photoswitchable tethered ligand maleimide-azobenzene-glutamate (MAG) was delivered in a subsequent intravitreal injection (23). LiGluR contains a single Cys substitution in the kainate receptor, GluK2(439)C, which serves as an anchoring site for MAG close to the ligand binding site. Upon illumination at 380 nm in the near-UV range, the azobenzene linker in MAG photoisomerizes from trans to cis, shortening the molecule and allowing the glutamate to bind into the ligand binding pocket to activate and open the channel.

The first-generation MAG photoswitch suffered from two major limitations for vision restoration: (i) The UV light needed for activation is absorbed by the human lens and can damage the retina, and (ii) MAG is bistable, requiring a second pulse of light at a longer wavelength for deactivation. We recently developed a second-generation photoswitch, maleimide-azobenzene-glutamate 0 with peak efficiency at 460 nm (MAG0460), to overcome these problems (24). MAG0460 is activated by white light and spontaneously turns off in the dark. In the present study, we compare retinal light responses and both innate and learned visually guided behaviors. To demonstrate efficacy in a larger animal model, we targeted LiGluR-MAG0460 to RGCs in a canine model of human blindness and restored light-activated retinal responses in vitro. Because LiGluR-MAG0460 is functional in both the mouse and dog, it is an attractive candidate for a genetically encoded retinal prosthesis for the blind.

Results

Restoration of Light Response to the Retina of the rd1 Mouse by LiGluR in RGCs or ON-BCs. Our first-generation MAG photoswitch for LiGluR had limited utility for vision restoration because it required 380-nm UV light stimulation for activation and a second pulse of light at a longer wavelength for deactivation (19, 25). We therefore turned to a recently developed second-generation compound, MAG0460, which is activated by blue or white light and spontaneously turns off in the dark (Fig. 1). In the present study, we compare retinal light responses and both innate and learned visually guided behaviors in the rd1 mouse model of retinal degeneration when LiGluR-MAG0460 is targeted to either RGCs or ON-BCs. We find that both cell types support robust light-induced retinal activity and visually guided behavior. To demonstrate efficacy in a larger animal model, we targeted LiGluR-MAG0460 to RGCs in a canine model of human blindness and restored light-activated retinal responses in vitro. Because LiGluR-MAG0460 is functional in both the mouse and dog, it is an attractive candidate for a genetically encoded retinal prosthesis for the blind.

We tested the expression and function of LiGluR-MAG0460 in the retina of the rd1 mouse, a small animal model of human blinding disease. The rd1 mouse has a null mutation in the phosphodiesterase type 6 (PDE6)-beta subunit causing complete loss of rod and cone photoreceptors by postnatal day 90 (p90) (26). This phenotype is comparable to patients in the early stages of retinal degeneration who may still have a functioning network of all retinal cell types except for the photoreceptors. At later stages, however, only the RGCs may survive (4, 27). To address both early and late stages of the disease, we examined the effect of targeting LiGluR to either the RGCs or ON-BCs (Fig. 2 C and E).

Good restriction of LiGluR expression in RGCs was achieved using an AAV vector combining the human synapsin promoter (hsyn-1) and the AAV 2/2 capsid as previously described (23). The gene expression cassette (Fig. 2 A) in a volume of 2 μL containing 10^12–10^14 viral genomes was injected into the vitreous of rd1 mice. Expression was visualized >4 wk after injection using an antibody against the GluK2 subunit from which LiGluR is composed (19). Intravitreal injection of the vector resulted in expression in the RGC layer (Fig. 2 D and G and Fig. S2 A). Due to the limited retinal penetration of the AAV2/2 serotype (15) and the lack of syn-1 expression in choline acetyltransferase-positive amacrine cells (28), these transduced cells are likely to be predominantly RGCs. LiGluR expression was panretinal and localized to somata and dendrites of both ON- and OFF-RGCs, as seen by stratification of the dendritic terminals in both on- and off-sublayers of the inner plexiform layer (IPL) (Fig. 2 G and Fig. S2 A).

Because ON-BCs required the use of an AAV capsid with deeper tissue penetration and an ON-BC–specific promoter. To achieve deeper penetration, we turned to the tyrosine mutant AAV2/2 capsids (29), which are protected from proteasome degradation, leading to better transduction in the inner retina. We restricted LiGluR expression to ON-BCs with a four-copy concatemer of a minimal version of the cell-specific metabolotropic glutamate receptor 6 promoter (mGluR6) promoter (4xgmb6) (12, 30). This gene expression cassette (Fig. 2 B) was packaged into AAV2/2(4YF) (29) and injected subretinally in rd1 mice (1–2 mo old), creating a bleb covering ∼25% of the retinal surface (Fig. S2 B). Subretinal injections were used instead of intravitreal injections because this route enabled us to deposit the AAV closer to the ON-BC target cells. Expression was confirmed >6 wk after virus injection by staining with the anti-GluK2 antibody, and it was predominantly found in ON-BCs, as seen by stratification of the
and ON-BCs: 4 retinas, 416 cells) and presence (green) (K retinas in RGCs and ON-BCs reliably followed 4-Hz 3 s of blue light and 8 s dark. (10 retinas with rd1 trans a polyadenylation signal sequence D Fig. S5 n H Fig. S2 B L expressed in ON-BC and RGC 62.87 ms, SEM was able to drive light responses at moderate m o u s e r e t i n a w i t h J M retinas expressing LiGluR in RGCs (Fig. 2 K and M). Similarly, rd1 retinas expressing LiGluR in ON-BCs were insensitive to blue light (Fig. 2 F) until they were exposed to MAG0 (Fig. 2 L and N). Stimulation with broad-spectrum (white) light triggered similar responses (Fig. S2G), as demonstrated previously in HEK cells (24). Both in RGCs and ON-BCs, LiGluR-MAG0 was able to drive light responses at moderate intensities (0.3 mW/cm² or 7.1 × 10¹⁴ photons per cm⁻² s⁻¹) for RGCs and 0.2 mW/cm² or 4.7 × 10¹⁴ photons per cm⁻² s⁻¹ for ON-BCs, respectively, Fig. 3 A and B), representing an ~10-fold improvement in sensitivity compared with the published values for the first-generation MAG photoswitch (23).

**Kinetics and Frequency Detection Supported by Light-Sensitive RGCs and ON-BCs.** Ideally, a visual prosthetic should have low variability, elicit RGC firing similar to wild-type (wt) conditions, and show fast dynamics to restore natural vision after loss of photoreceptor cells (31–33). LiGluR-MAG0 expressed in ON-BC and RGC showed low cell-to-cell variability (Fig. S3) similar to wt retinas. The firing rate above baseline (Fig. S3A) and the type of response (Fig. S3B) to a full-field flash of light was comparable. This was also true for retina-to-retina variability (Fig. S4 and Table S1) measured in vitro. We found that light pulses as short as 35 ms in duration were sufficient to trigger robust RGC firing when LiGluR-MAG0 was expressed in either RGCs or ON-BCs (Fig. 3 C and D). In both cases, the responses reached a peak firing rate similar to the peak firing rate observed with much longer pulses of light (compare Fig. 2 K and L with Fig. 3 C and D).

The responses had a short delay following light onset and terminated rapidly from peak response to baseline as shown by the decay constant tau (τ) (τoff = 51.96 ms, SEM = 3.48 with LiGluR-MAG0 in RGCs; τoff = 62.87 ms, SEM = 5.26 with LiGluR-MAG0 in ON-BCs), with values from steady state to baseline (LiGluR-MAG0 in RGCs: response inactivation time (Δt off) = 40 ms (Fig. S5C), LiGluR-MAG0 in ON-BCs: Δt off = 80 ms (Fig. S5D)). The similarity between RGC-driven (Fig. 3C and Fig. S5C) and ON-BC–driven (Fig. 3D and Fig. S5D) termination suggests that the decay kinetics are mostly governed by ligand inactivation from the cis to trans state, and the speed (Fig. 3 C and D) suggests that these systems should enable retinal output to follow high-frequency modulation of light intensity. We tested this hypothesis by measuring RGC firing in response to trains of short light pulses given at either 4 Hz (50-ms flashes at a 200-ms interstimulus interval) or 10 Hz (50-ms flash with a 50-ms interstimulus interval) with LiGluR-MAG0 in RGCs (Fig. 3 E and G) or in ON-BCs (Fig. 3 F and H). In both cases, at 4 Hz, the individual RGCs responded to every flash of light. With increasing frequency, the light-induced firing rate decreased (Fig. S5F) and individual cells stochastically missed some responses (Fig. S5A). At the population level, however, rd1 retinas with LiGluR-MAG0 in RGCs and ON-BCs reliably followed 4-Hz (Fig. 3 E and F) and 10-Hz stimulation frequencies (Fig. 3 G and H). LiGluR-MAG0 elicited graded changes in the RGC firing rate in response to graded changes in light intensity (Fig. S5G) similar to what we saw in HEK cells (Fig. S1C).
More than 20 different subtypes of RGCs have been reported in the mouse retina, with 1047 cells; in ON-BCs (n = 65 cells). In contrast, rd1 mice, RGCs with LiGluR-MAG0460 showed uniform responses, with similar onset delays and decay rates (Fig. 4 B and F). The photoswitching index (PI; normalized difference in firing rates in the light vs. dark) was positive for every cell (Fig. 4F) as expected for its direct excitation by LiGluR-MAG0460. In contrast, rd1 RGCs that received synaptic input upstream from ON-BCs with LiGluR-MAG0460 showed more diverse responses (Fig. 4C). Some cells were excited by light, and others were inhibited (Fig. 4 C and G). The onset, offset, and duration of the light response also varied among cells (Fig. 4C) similar to wt retina (Fig. 4 A and E).

The diversity of responses seen in single units prompted us to ask if this effect could be seen more globally at the level of the retina, taking into account all of the cells from one recording. Rather than averaging across cells, and thus masking cell-to-cell variation, we wanted to analyze all responses systematically from each cell individually to understand the relationship between the RGC firing patterns generated by LiGluR-MAG0460 installed in the RGCs themselves or in the ON-BCs. To this end, we correlated the PSTH (the firing rate of a given cell over time) of all cells with one another and constructed a correlation matrix in which each data point represents the correlation value (r). Responses during the 1-s light flash and responses 100 ms before the light vs. dark) was positive for every cell (Fig. 4F) as expected for its direct excitation by LiGluR-MAG0460. In contrast, rd1 RGCs that received synaptic input upstream from ON-BCs with LiGluR-MAG0460 showed more diverse responses (Fig. 4C). Some cells were excited by light, and others were inhibited (Fig. 4 C and G). The onset, offset, and duration of the light response also varied among cells (Fig. 4C) similar to wt retina (Fig. 4 A and E).

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To explore our hypothesis in vitro using the MEA, we examined single-unit RGC responses to 1-s flashes of light for four different conditions: wt (Fig. 4A), rd1 LiGluR-MAG0460 in either RGCs (Fig. 4B) or ON-BCs (Fig. 4C), and rd10 LiGluR-MAG0460 in ON-BCs (Fig. 4D). Nonpatterned light was used as a stimulus to allow comparisons to be made without concern for variability of LiGluR expression levels and density (compare Fig. 2D and F and Fig. S2 A and B). Peristimulus time histograms (PSTH) were plotted for each recording (Fig. 4 E–H). In rd1 mice, RGCs with LiGluR-MAG0460 showed uniform responses, with similar onset delays and decay rates (Fig. 4 B and F). The photoswitching index (PI; normalized difference in firing rates in the light vs. dark) was positive for every cell (Fig. 4F) as expected for its direct excitation by LiGluR-MAG0460. In contrast, rd1 RGCs that received synaptic input upstream from ON-BCs with LiGluR-MAG0460 showed more diverse responses (Fig. 4C). Some cells were excited by light, and others were inhibited (Fig. 4 C and G). The onset, offset, and duration of the light response also varied among cells (Fig. 4C) similar to wt retina (Fig. 4 A and E).

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LiGluR Restores Innate and Learned Associative Light-Guided Behavior. We next asked whether LiGluR-MAG0 could restore basic visually guided behavior. Mice naturally avoid brightly lit open spaces (37). This preference is absent in adult rd1 mice that have lost all rod and cone photoreceptors (14, 16). To test for restoration of light avoidance, we placed wt mice and rd1 mice with LiGluR in RGCs or ON-BCs in an open field test (Fig. 5A) and tested their behavior before and after treatment with MAG0. Mice were first habituated to the testing environment, which consisted of an open-topped plastic box with dark and light compartments connected by a small opening (Fig. 5A), and then allowed to explore the box for 5 min. The percentage of time spent in the light compartment was recorded (16). Following intravitreal injection of MAG0, mice with LiGluR in RGCs (n = 18) or ON-BCs (n = 13) showed a strong light avoidance, which was similar to the light avoidance of wt mice (n = 4) (Fig. 5B).

After establishing that we can restore light avoidance behavior, we asked how long the restoration of the light response would last following a single intravitreal injection of MAG0 as the receptor protein on the cell surface turns over. Following a single intravitreal injection of MAG0 in rd1 mice expressing LiGluR in RGCs, the light-induced firing of RGCs in isolated retinas was found to decline with a time constant of ~9 d (τ = 8.8 d) (Fig. 5C). The behavioral preference for the dark compartment declined over a similar time course following a single intravitreal injection of MAG0 (Fig. 5D).

Because LiGluR-MAG0 targeted to either RGCs or ON-BCs can restore the ability of blind mice to distinguish light from dark, we wanted to test whether it would also enable animals to distinguish temporal patterns of light and use this information in the context of a learned behavior. Based on our finding that LiGluR-MAG0 can follow moderate frequencies of intensity modulation (Fig. 3 E and F), we created two visual stimuli of identical intensity, with the cue stimulus flashing at a rate of 2 Hz and the decoy stimulus emitting light of constant intensity. These visual stimuli were presented in a radial arm water maze that was identical in size and the decoy stimulus emitting light of constant intensity. These visual stimuli were presented in a radial arm water maze that was identical in size and was designed to encourage exploration and discovery of the correct platform (38). (A) Schematic showing the light/dark box for the open field test. (B) Percentage of time spent in the dark compartment plotted before (black) and after (gray) administration of MAG0 for rd1 RGC-LiGluR (n = 18), rd1 ON-BC-LiGluR (n = 13), and wt mice (n = 4). Data are mean ± SEM. ***P < 0.005, ****P < 0.0005; paired Student t test (light intensity: 7 mW/cm² or 1.6 × 10¹⁶ photons per cm²–s⁻¹). (C) Biological τ½ of intravitreally injected MAG0, RGC-LiGluR-expressing rd1 mice were injected with a single dose of MAG0 in vivo 24 h before day 1. Subsequently, on days 2–14, retinal explants were prepared and responses to 3-s flashes were plotted. The decay of LiGluR-MAG0-induced light responses was fit with an exponential curve decay constant τ = 8.75 ± 0.79 d. (D) Efficiency of MAG over time in vivo. RGC-LiGluR-expressing rd1 mice (n = 7) from B were tested over the course of 10 d. The percentage of time spent in the dark compartment was plotted for rd1 RGC-LiGluR mice before MAG0 (n = 18) and for rd1 RGC-LiGluR mice after receiving a single intravitreal dose of MAG0 at day 2 (n = 18), day 4 (n = 7), day 6 (n = 7), day 8 (n = 7), and day 10 (n = 7). Data are mean ± SEM. *P < 0.05, **P < 0.01; multiple t tests with Bonferroni correction. (E and F) Forced two-choice associative learning task with a modified radial arm maze. (E) Schematic of the maze with dimensions given in centimeters. (F) Performance of the four groups of mice on day 1 (black) vs. day 8 (gray). Percentage of correct choices is plotted for sham (PBS)-treated rd1 mice (−LiGluR, −MAG0) (n = 8); rd1 RGC-LiGluR + MAG0 (n = 9); rd1 ON-BC-LiGluR + MAG0 (n = 6); and wt mice (n = 6). Data are mean ± SEM. *P < 0.05, **P < 0.005, paired Student t test (light intensity at the divider 5 mW/cm² or 1.1 × 10¹⁶ photons per cm²–s⁻¹).
LiGluR in RGCs Restores the Retinal Light Responses in a Canine Model of Retinal Blindness. Next, we wanted to confirm that the LiGluR-MAG0 system could be virally delivered and drive functional light responses in a larger animal model. To assess whether LiGluR-MAG0 is effective in a human-sized eye, we expanded our study to the canine model, which provides both anatomical and pathological similarities that are clinically relevant for testing retinal therapies (39). The rod-cone dystrophy (rd1) model has a nonsense mutation in PDE6B, the same gene that is defective in the rd1 and rd10 mice. We used an AAV2/2(4YF) vector in combination with the ubiquitous cytomegalovirus CMV promoter variant CAG to deliver the LiGluR transgene to RGCs (Fig. 6 A and B). Intravitreal injection of AAV2/2(4YF)-CAG-LiGluR in the area centralis, a region of high RGC density in the canine retina (40), resulted in potent expression in RGCs by 8 wk post-injection (Fig. 6 C and D).

MEA recordings were performed in three degenerated retinas from two mutant rd1 dogs that had been intravitreally injected 8–11 wk earlier with AAV2/2(4YF)-CAG-LiGluR. Stimulation with high-intensity (75 mW/cm² or 1.7 × 10¹⁰ photons per cm⁻² s⁻¹) blue light in the absence of MAG0 did not alter the baseline RGC firing activity (Fig. 6E). However, following 20 min of incubation in 100 μM MAG0 and thorough washout, strong and repeated periods of light-induced firing were seen that peaked shortly after the onset of light (Fig. 6 F and G). To explore the sensitivity of LiGluR-MAG0 to photopic light levels typically encountered within the brightly illuminated working/ living environment of humans, we performed MEA recordings in the rd1 canine retina at different levels of blue light ranging from 6.8 to 0.43 mW/cm² or 1.5 × 10⁸ photons per cm⁻² s⁻¹ to 9.7 × 10¹⁴ photons per cm⁻² s⁻¹, respectively. Light stimulations (1-s duration) induced RGC firing that was still detectable at the lowest (0.43 mW/cm² or 9.7 × 10⁷ photons per cm⁻² s⁻¹) irradiance and increased with higher intensities (Fig. 6H). Responses to all five intensities had a transient component and a sustained component, and showed similar kinetics with rapid inactivation time from steady-state to baseline levels (40 ms) upon offset of light (Fig. 6I).

To develop future psychophysical tests to be used in dogs and human patients for the assessment of visual function recovery after intervention with LiGluR-MAG0, we examined the responses to retina stimuli at combinations of higher frequency (4 Hz) and shorter duration (50 ms), and low light intensity (0.85 mW/cm² or 1.9 × 10¹³ photons per cm⁻² s⁻¹) stimulations. Whereas peak firing rate was reduced and a slight delay was introduced in comparison to responses achieved with longer stimulations at higher intensities, a distinct light-induced ON response of RGCs that peaked at the end of the 50-ms stimulation period could be detected (Fig. 6J). Rapid relaxation of the photoswitch (τ_on ≈ 28 ms) occurred upon returning to darkness (Fig. 6J, Inset).

Discussion

In this study, we show the translational potential of the LiGluR-MAG0 system for retinal gene therapy to cure human blindness. In a mouse model of the human retinal degenerative disease RP, we observed restoration of a useful retinal output in response to light when LiGluR was expressed either in the most upstream or the most downstream cell types that survive after photoreceptor degeneration. In vivo, these retinal responses restored an innate light-guided behavior and enabled light-associated learning based on cues with distinct temporal patterns.

Earlier work validated the use of LiGluR in conjunction with a first-generation MAG0 photoswitch in RGCs for vision restoration (23). However, the therapeutic utility of the first-generation MAG0 was limited by two properties. First, the spectral sensitivity of the original MAG0 chromophore was outside of the visible range, peaking in the UV light at 380 nm (41), which penetrates the lens poorly and is damaging to corneal, lens epithelial, and retinal cells. Second, LiGluR-MAG0 was bistable, requiring a second longer wavelength pulse of light to reset the system after each activating event (19, 41), which would necessitate additional hardware for potential clinical applications. We solved these problems with our photoswitch, MAG0-MAG0.
which is activated by blue light similar to blue cone photoreceptors, responds well to broad-spectrum visible light, and rapidly and spontaneously turns off in the dark (24). LiGluR-MAG0<sub>460</sub> responds dynamically to incremental changes in light intensity and supports reliable retinal output with intensity modulations at moderate frequencies of 4–10 Hz.

We compared two gene therapy target cell types, RGCs and ON-BCs, for LiGluR-MAG0<sub>460</sub> expression in the degenerating mouse retina. RGCs are well-suited therapy targets for late-stage disease because they have been shown to stay morphologically intact (4) with minimal remodeling following photoreceptor degeneration compared with the other retinal cells (27). Furthermore, RGCs are easily targeted due to their proximity to the vitreous, enabling strong, uniform, and widespread expression after intravitreal injection of AAV vectors. ON-BCs, however, are promising therapeutic targets for early-stage disease because they are located upstream in the retinal circuit and provide an opportunity to preserve aspects of retinal processing (32, 33).

Robust light responses were restored to the RGCs of retinas isolated from blind mice when LiGluR-MAG0<sub>460</sub> was installed in either the RGCs themselves or in ON-BCs. The sensitivity, ability to follow frequency-modulated light, and on/off kinetics in response to full-field illumination were similar for the two cell types. The similarity is striking considering that expression was much sparser after viral delivery to the ON-BCs (compare Fig. S2A and B). Comparable sensitivity at lower expression suggests that the signal amplification due to convergence from BCs to RGCs (42) makes the ON-BC population particularly effective for vision restoration and highlights the value of further improvement for gene delivery to these cells.

There was an important distinction between the properties of the restored retinal output activity when LiGluR-MAG0<sub>460</sub> was installed in the two cell types. In rd1 mouse ON-BCs, LiGluR-MAG0<sub>460</sub> drove RGC activity that was temporally diverse and excited a subset of RGCs, whereas other RGCs were inhibited by light. In contrast, LiGluR-MAG0<sub>460</sub> in rd1 mouse RGCs led to uniform light responses, as one would expect from direct excitation of the RGCs. The signal diversity that emerges from the retinal circuit when LiGluR-MAG0<sub>460</sub> is installed in ON-BCs could be beneficial for restoring critical aspects of visual processing, such as contrast and edge detection (43). Single-unit RGC recordings in ON-BC LiGluR-MAG0<sub>460</sub> in both rd1 and rd10 resembled the RGC responses seen in wt retina more closely than RGC LiGluR-MAG0<sub>460</sub>. ON-BCs might therefore be the target of choice in early stages of retinal degeneration, before substantial synaptic remodeling has occurred (27). In late stages of retinal degeneration with severe circuit degeneration, it may be preferable to target the least affected cells, the RGCs, to generate a strong and synchronized output signal.

We tested the function of LiGluR-MAG0<sub>460</sub> in vivo and found that the restored retinal activity supported normal light-avoidance behavior in blind treated mice using the open field test. Having established that our treatment supported light-guided behavior, we next asked whether the LiGluR-MAG0<sub>460</sub> system would enable blind mice to learn to distinguish different visual cues in the context of a forced two-choice variant of the Morris water maze task. In this experiment, mice were challenged to associate a temporally patterned cue stimulus from a non-patterned intensity-matched decoy stimulus. The wt mice were able to learn to associate the patterned stimulus with the reward, whereas untreated rd1 mice were not and did not improve their performance over the 8 d of the experiment. Rd1 mice treated with LiGluR-MAG0<sub>460</sub> in either the RGCs or ON-BCs learned to perform as well as wt animals, indicating that the LiGluR-MAG0<sub>460</sub> system operates as more than just a mere light meter and can inform mice about qualitative aspects of the visual world. In this study, we did not attempt to record visually evoked potentials, record electroretinograms, or test for a pupillary light reflex as we did for our previous study (23). Instead, we focused our efforts on learned associations and demonstrated the ability of mice to recognize temporal patterns in the water maze task. We have not yet tested these mice for spatial pattern recognition or temporal and spatial pattern resolution.

Our results in the mouse model encouraged us to test if our treatment could be translated to a larger animal model. Specifically, we set out to test virus-mediated expression of the LiGluR receptor and the ability of LiGluR-MAG0<sub>460</sub> to drive light responses in retinal explants. To this end, we selected the rd11 dog, which, like the rd1 mouse, has severe and early-onset rod and cone degeneration (44, 45). We developed a virus for canine RGC transduction and tested patches of retina collected from the area centralis region for LiGluR-MAG0<sub>460</sub>-induced light responses in vitro. LiGluR-MAG0<sub>460</sub> in the RGCs rendered the blind dog retina light-sensitive, with characteristics closely matching the characteristics observed in the rd1 mouse. Our results show that the LiGluR-MAG0<sub>460</sub> system in excised rd11 canine retina responds well to stimulation at a light intensity found under natural environmental conditions [0.43 mW/cm<sup>2</sup>, which approximates outdoor conditions (Fig. S8)] and moderate frequencies (up to 4 Hz). These findings pave the way for future efforts in the canine model to determine toxicity and a therapeutic index for the MAG0<sub>460</sub> photoswitch and to perform behavioral testing in advanced retinal degeneration.

A recent study restored the ability to distinguish light from dark using just a chemical photoswitch that acts on native ion channels, including those native ion channels that are up-regulated in the RGCs of the degenerating mouse retina (17). Compared with this optochemical therapy, which has advantages of not requiring gene transfer and being more sensitive to light (comparison of threshold light intensities in Fig. S8), our two-component optochemical-genetic therapy approaches, a bolus supply of the photochemical restores light-guided behavior temporarily for a period of days. In the optochemical case, the limited efficacy is presumably due to washout of the molecule, whereas in our optochemical-genetic case, it is most likely due to turnover of the photoswitch-conjugated receptor. As a result, both of these approaches would benefit from sustained-release drug delivery technology.

An alternative purely optogenetic approach has proven successful for restoration of light responses in retinal cells, light responses in visual cortex, light aversion, and learned association tasks using unpatterned light. For this approach, microbial opsins are expressed in specific cell types of the degenerated retina from cone cell bodies that have lost their outer segment to ON-BCs and RGCs (10, 13, 14, 16). The simplicity of this genetic therapy is appealing, as is the ability of the microbial opsins to use the retina’s supply of 11-cis-retinal as the natural photoswitch. One concern about this approach is the possibility of an immune response to the foreign protein, which, in the worst case scenario, could spread into the brain via the optic nerve. In addition, once expressed, these opsins cannot be turned off in case of an adverse reaction. Our success with a mammalian light-gated protein that is identical in amino acid sequence to the native protein, except for a single amino acid substitution that creates the photoswitch anchoring site, reduces the risk of immune reaction. In addition, the dependence on chronic delivery of the synthetic photoswitch should make it possible to discontinue treatment in case of an adverse reaction as well as to replace the synthetic photoswitch with improved photoswitches as they become available.

In summary, we have shown that the LiGluR-MAG0<sub>460</sub> system operates successfully in either ON-BCs, at the upstream end of the degenerating retina, or at the output end of the retina, in RGCs, to restore retinal light responses and enable innate and
learned light-guided behavior in blind mice. Installation in the ON-BCs, which is probably most appropriate for early-stage degeneration, provides more diverse retinal output characteristics and may support higher quality vision, a notion that will require future testing. Importantly, the system is equally effective in the rd1 dog in vitro, paving the way for extensive testing of high-resolution vision in a preclinical setting and for clinical development. Our approach should allow for the use of a receptor protein based on the patient’s own receptor, reducing the chance of an immune response. Because the functional properties of the restored light response depend on the externally provided photoswitch, it can be tailored to the patient and improved as new photoswitches become available; equally importantly, the function of the system can be aborted in case of adverse effects by curtailing photoswitch delivery.

Methods

Animals. All mouse experiments were performed with approval of the University of California Animal Care and Use Committee. The wt mice (C57BL/6) and rd1 mice (C3H) were purchased from the Jackson Laboratory and housed on a 12:12-h reversed light:dark cycle. The animals were kept on a diet ad libitum. The ages of the treated rd1 mice ranged from p30–p60 for rAAV injections and from p90–p160 for in vivo and in vitro experiments. All experiments on dogs were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH (46) and with the US Department of Agriculture’s Animal Welfare Act and Animal Welfare Regulations, and complied with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Three rd1 dogs (PEDSB mutation) (47) with late-stage retinal degeneration (0.4, 1.8, and 3.7 y of age) and one normal dog (2.4 y of age) were used to assess viral vector tropism and for MEA experiments (details are provided below).

Injection of rAAV and MAG Photoswitch. AAVs were produced via standard methods (48). We selected rAAV2/2 carrying the LiGluR transgene under the control of the hsyn-1 promoter for RGC targeting. For ON-BC targeting, we selected rAAV22/2(4YF) carrying the LiGluR transgene under the control of the 4x repeat of the mGlur6 promoter (dkgrm6), a kind gift from Botond Roska (Friedrich Miescher Institute, Basel) and Connie Cepko (Harvard Medical School, Boston). The titer of AAV was determined via quantitative PCR relative to the inverted terminal repeat domains standard. Titers for these viruses ranged between 1 × 1012 viral genomes (vg/mL) and 1 × 1013 vg/mL. Mice were anesthetized with i.p. ketamine (72 mg/kg) and xylazine (11 mg/kg). Eyes were additionally anesthetized with proparacaine (0.5%), whereas the contralateral (left) eye received a 10-fold higher viral titer (1 × 1014 vg/mL). At 12 wk postinjection, HEK 293T cells were transfected with an LiGluR expression vector, and the eyes were enucleated and fixed in 4% paraformaldehyde (Ted Pella) for 1 h. Whole-mount retinas were prepared by making a complete circular incision around the ora serrata using scissors, removing the cornea while leaving the lens attached, gently tearing the eye apart using two forceps, and finally removing the lens. Radial cuts were made to flatten the retina, resulting in the typical cloverleaf shape. For retinal sections, whole mounts were embedded in agarose (Sigma) and sectioned transverse using a vibratome (Leica Microsystems) at medium speed, maximum vibration, and 150-μm thickness. Whole mounts and vibratome sections were incubated in blocking buffer (10% normal goat serum, 1% BSA, 0.5% Triton X-100 in PBS (pH 7.4)) for 2 h at room temperature (RT). Monoclonal antibody against Gluk2/3 (Millipore) was applied at a 1:1,500 dilution in blocking buffer overnight at 4 °C. Secondary antibody-Rabbit Alexa 488 or Alexa 594 antibody (Invitrogen) was applied at a 1:1,000 dilution in blocking buffer for 2 h at RT. Tissue was washed three times for 10 min with PBS and mounted on slides using Vectashield (Vector Laboratories) mounting medium with DAPI to stain cell nuclei. Whole mounts and sections were imaged via confocal microscopy (LSM7, Carl Zeiss). To identify off-target cell expression outside of the expected layer, we prepared a total of 44 vibratome sections from four treated retinas from three mice (previously injected with AA2V2/2-hsyn-LiGluR or AA2V2/2(4YF)-4xgrm6-LiGluR) and counted the number of labeled cell bodies outside of the RGC layer or the IPL, respectively. In dogs,ocular tissues were collected following i.v. injection of a euthanasia solution (Euthasol; Virbac). Retinal tissues used for immunohistochemistry on retinal cryosections or whole mounts were processed as previously reported (40) and examined by confocal microscopy (Leica TCS SP2; Leica Microsystems).

HEK Cell Recordings. HEK 293T cells were transfected with an LiGluR expression vector [pcDNA-GluK2(Q) L439C] using Lipofectamine 2000 (Invitrogen) and YFP as a transfection marker (24, 49) Cells were labeled after expression for 24–48 h at 37 °C. Cells were washed with external solution, incubated for 2 min with 0.3 mg/mL Con A to block ligand-induced desensitization, and labeled with ~25 μM MAG600 or regular MAG0 (19) for 40 min in extracellular solution at RT in the dark. After labeling, any unreacted MAG was removed by thorough washing with external solution. Whole-cell HEK cell recordings were performed in voltage-clamp configuration, typically at ~ -75 mV, on an inverted microscope (Olympus IX) using an Axopatch 200B head stage/ampifier (Molecular Devices) at 22–24 °C. Patch pipettes were pulled from borosilicate glass to give 3–7 MΩ resistance. Cells were labeled with internal solution [135 mM K-glucurate, 10 mM NaCl, 10 mM Hepes, 2 mM MgCl2, 2 mM MgATP, 1 mM EGTA (pH 7.4)]. The extracellular solution was 138 mM NaCl, 1.5 mM KCl, 1.2 mM MgCl2, 2.5 mM CaCl2, 10 mM glucose, and 10 mM Hepes (pH 7.3). Photoswitching (Fig. 1 C and D and Fig. S1 A and B) was achieved with a xenon-lamp light source (DG4; Sutter Instruments) in combination with excitation filters [445/20 nm, 379/34 nm, and 500/24 nm (“center”/“full width > 90%”) and a set of neural density filters (XND; Omega Optical). The DG4 light source was coupled via liquid light guide to the back-port of the microscope to give homogeneous illumination through a LUCPlanFLN (Olympus) 0.60–N.A., 22-field number objective with a magnification of 40×, yielding an irradiance up to 500 W/cm2 (445 nm) at the sample stage. Dynamic intensity modulation (Fig. S1 C and D) was performed using a collimated light-emitting diode (LED) light source (470 nm; Thorlab) coupled to the back-port of the microscope. The intensity was modulated with an analog signal from a Digidata AD-converter (Molecular Devices).

MEA Recordings. MEA recordings were performed on wt (C57BL/6) mice, untreated control mice, and treated rd1 mice (fast retinal degeneration model), as well as rd10 mice (slow degeneration model). Control mice and wt mice were used at an age >p90. Experimental mice were used 6–10 wk following rAAV injection. MEAs were used in two versions of recording. Elastomer-based MEA recordings, the retina was placed ganglion cell side down (50) in the recording chamber (pMEA 100/300r-Tpr; Multi Channel Systems) of a 60-channel MEA system with a constant vacuum pump (perforated MEA1060).
system with CVP; Multi Channel Systems). A custom-made dialysis membrane weight was placed on the retina, adding positive pressure from above. Additional weight was applied to the tissue via an anesthesia pump, adding negative pressure and improving electrode-to-tissue contact and the signal-to-noise ratio. During recording, constant perfusion of oxygenated Ames media (34 °C) was provided to the recording chamber. For rd10 mouse retina, we supplied LAP-4 (Sigma), a group III metabotropic glutamate receptor agonist, to block any residual photoreceptor-mediated response. Comparative analysis of responses before and after drug administration was used to ensure complete blockade of photoreceptor activity at a working concentration of 20 μM. Recordings lasted between 1 and 3 h. Illumination was provided by two different light sources that were both coupled to an objective with a magnification of 4x. Light intensities were measured with a handheld power meter (Thorlabs, Inc.). A 300-W mercury arc lamp (DG-4; Sutter Instruments) with a blue bandpass filter (445/50 nm; Thorlabs, Inc.) was used for initial recordings in Fig. 2 and Fig. S2. For later recordings, an LED light source (470 nm, 24.7 mW/cm² or 6.3 × 10¹⁴ photons per cm²-s⁻¹; Thorlabs, Inc.) with a collimator lens (Thorlabs, Inc.) was used for high-frequency stimulation (Fig. 3 and Fig. S5) and for all MEA recordings in Fig. 4. Data were sampled at 25 KHz filtered between 300 and 2,000 Hz and recorded using MCS rack software (Multi Channel Systems) for offline analysis. Voltage traces were converted to spike trains offline by collecting responses above a threshold. Spikes were isolated with a low-pass filter. Individual spikes were sorted into single units, which we defined as “cells,” via principal components analysis using Offline Sorter (Plexon). Single-unit spike clusters were exported to MATLAB (MathWorks) and were analyzed and graphed with custom software. For extracting firing rates in the dark, we averaged all bins over 3 s preceding the flash to minimize fluctuations. To extract firing rates in the light, the maximum response was taken, typically the 1 s duration before the flash duration. The firing rates in the dark and light were compared using the protocol described by Wong and Brown (38) with minor modifications. A radial arm maze was modified to include a forced-choice task by blocking two of the five arms of the maze (Fig. 5E) and adding a divider (dimensions: 25 cm × 25 cm) to separate the two potential “escape arms.” A similar setup was used by Wong and Brown (38) except that the divider was centered over the maze. The light intensities at the release site (2 mW/cm² or 4.4 × 10¹⁵ photons per cm²-s⁻¹) and at the divider (5 mW/cm² or 1.1 × 10¹⁶ photons per cm²-s⁻¹) were measured at the level of a handheld power meter (Thorlabs, Inc.). The LED array that cued the escape platform was triggered with square pulses to flash at 2 Hz using a stimulus generator (Stanford Research Systems).

Forced Two-Choice Water Maze Task. The water maze task was performed using the protocol described by Wong and Brown (38) with minor modifications. A radial arm maze was modified to include a forced-choice task by blocking two of the five arms of the maze (Fig. 5E) and adding a divider (dimensions: 25 cm × 25 cm) to separate the two potential “escape arms.” A similar setup was used by Wong and Brown (38) except that the divider was centered over the maze. The light intensities at the release site (2 mW/cm² or 4.4 × 10¹⁵ photons per cm²-s⁻¹) and at the divider (5 mW/cm² or 1.1 × 10¹⁶ photons per cm²-s⁻¹) were measured at the level of a handheld power meter (Thorlabs, Inc.). The LED array that cued the escape platform was triggered with square pulses to flash at 2 Hz using a stimulus generator (Stanford Research Systems).

The room was kept dark for the entire time of the experiment. Permanent records were made using a video camera (GoPro Hero3).
subsequent days. The platform and the flashing LED were moved between trials according to the following pattern: LRLRLLRLRL and RLRLLRRRLRL on alternating days. Mice that received a MAGOG100 or sham (PBS) injection were allowed to rest for >24 h afterward to avoid masking effects from anesthesia.

Statistical Analysis. The Students’ t test was used for statistical analysis of in vivo mouse physiology. Paired t tests were used for comparisons within the same group of mice before and after MAGOG treatment (Fig. 5 C and F and Fig. S7). Multiple t tests were corrected for type I errors using the Bonferroni correction.

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