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Longitudinal evaluation of expression of virally delivered transgenes in gerbil cone photoreceptors

MATTHEW C. MAUCK,[1] KATHERINE MANCUSO,[2] JAMES A. KUCHENBECKER,[2]
THOMAS B. CONNOR, JR.,[2] WILLIAM W. HAUSWIRTH,[3] JAY NEITZ,[2] AND MAUREEN NEITZ[2]

[1]Departments of Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, Milwaukee, Wisconsin
[2]Department of Ophthalmology, Medical College of Wisconsin, Milwaukee, Wisconsin
[3]Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, Florida

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Abstract

Delivery of foreign opsin genes to cone photoreceptors using recombinant adeno-associated virus (rAAV) is a potential tool for studying the basic mechanisms underlying cone based vision and for treating vision disorders. We used an *in vivo* retinal imaging system to monitor, over time, expression of virally-delivered genes targeted to cone photoreceptors in the Mongolian gerbil (*Meriones unguiculatus*). Gerbils have a well-developed photopic visual system, with 11–14% of their photoreceptors being cones. We used replication deficient serotype 5 rAAV to deliver a gene for green fluorescent protein (GFP). In an effort to direct expression of the gene specifically to either S or M cones, the transgene was under the control of either the human X-chromosome opsin gene regulatory elements, i.e., an enhancer termed the locus control region (LCR) and L promoter, or the human S-opsin promoter. Longitudinal fluorescence images reveal that gene expression is first detectable about 14 days post-injection, reaches a peak after about 3 months, and is observed more than a year post-injection if the initial viral concentration is sufficiently high. The regulatory elements are able to direct expression to a subpopulation of cones while excluding expression in rods and non-photoreceptor retinal cells. When the same viral constructs are used to deliver a human long-wavelength opsin gene to gerbil cones, stimulation of the introduced human photopigment with long-wavelength light produces robust cone responses.

Keywords: Cone photoreceptor, Color vision, Gene therapy, rAAV, L-opsin

Introduction

Recombinant adeno-associated virus (rAAV) holds promise as a means of delivering therapeutic genes to the retina (Acland et al., 2001, 2005; Bainbridge et al., 2006; Pang et al., 2006) and as an experimental tool for probing the neural circuitry for vision (Mancuso et al., 2007). In humans, cone photoreceptors mediate both high acuity and color vision; thus, they are an important target for expression of rAAV vectored genes from both a therapeutic and scientific perspective. Although non-human primates are the ideal model of human cone based vision (Bennett et al., 1999; LeMeur et al., 2005; Mancuso et al., 2007) there are advantages to developing a rodent model in which to explore cone vision using viral mediated gene transfer. Among rodents, gerbils have the advantage for studying cone vision in that 12–15% of their photoreceptors are cones compared to 1–3% for mice and rats, and, unlike mice, adult gerbil cone photoreceptors do not co-express UV and M cone opsins (Govardovskii et al., 1992).

Address correspondence and reprint requests to: Matthew C. Mauck, Departments of Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226. E-mail: mmauck@mcw.edu

In the experiments described here, expression of transgenes in cones is directed by cone opsin regulatory sequences. One application of this technology is to use gene transfer to increase the number of spectrally different opsins expressed in the retina. This can be used in experiments to address questions about the circuitry for color vision and its neural plasticity. Virally mediated gene transfer has some advantages over previous experiments that have expanded the cone photopigment complement in mice using targeted gene replacement of the endogenous mouse M-opsin with a human L-opsin gene (Smallwood et al., 2003; Jacobs et al., 2007). Targeted gene replacement can only be done in mice and it is expensive. Viral gene transfer can be done in any animal, it can be done at different developmental stages, and it is cost effective making it possible to design experiments which include multiple manipulations including directing a variety of transgenes to either M or S cones. A long-term goal of the experiments described here is to establish gerbils as a model system for studying cone vision, the neural circuitry for color vision, and the plasticity of the visual system by targeting expression of opsin transgenes to M cones versus UV cones.

Here we examine the duration and long-term characteristics of transgene expression in gerbil cones, the fraction of cones that are transduced, the photoreceptor subtype specificity of human cone

opsin gene regulatory sequences in gerbils, and the functionality of a human L cone photopigment in the gerbil retina. This is the first report of rAAV-vectored gene delivery and expression in the gerbil retina. Previously, reporter gene expression under the control of the same genetic regulatory sequences was examined at time points up to 6 weeks post-injection in ferrets, guinea pigs, and rat (Li et al., 2007). In the present study, we used *in vivo* fluorescence imaging to follow reporter gene expression for two years post injection. We report that rAAV-vectored gene delivery can be used to produce a long term level of expression that is highly cell-type specific and that a human L opsin transgene produces robust signals in gerbil cones. After initial expression of the transgene, there are changes in level of reporter gene expression and in the degree of cell-type specificity which stabilizes after about 5 months post injection. These results can be explained by a simple stochastic model that is useful for understanding the parameters that are important for optimizing the level of expression and cell-type specificity using viral gene delivery.

Materials and methods

Viral vectors

Three different recombinant adeno-associated virus (rAAV) constructs were used for the studies reported here: (1) rAAV.CHOPS2053.GFP carries a gene for green fluorescent protein (GFP) under control of the enhancer and promoter from the human OPN1LW gene contained on a 2053 base pair (bp) DNA segment designated CHOPS2053 for Cone Human OPSin (Wang et al., 1992). The viral construct was described previously (Li et al., 2007; Mancuso et al., 2007), and is anticipated to direct expression of GFP preferentially in gerbil M cones, (2) rAAV.HB570.RHLOPS, is identical to rAAV.CHOPS2053.GFP except that the gene for GFP was replaced with a 1.2 kb Not I restriction fragment carrying a recombinant human L opsin (RHLOPS) cDNA and the CHOPS2053 segment was replaced by the human blue cone opsin gene promoter on a 570 bp DNA fragment (HB570). This construct is anticipated to direct expression of RHLOPS preferentially to gerbil UV cones. RHLOPS was derived from plasmid hs7 (Nathans et al., 1986) and extends from hs7 nucleotide position 474 to 1689 (Fig. 7 in Nathans et al. (1986)). The full sequence of hs7 was confirmed and it was discovered that a change in codon 180 was required to make the plasmid we had match the reported sequence. The change was made using the QuickChange kit (Stratagene, LaJolla, CA) and confirmed by sequencing. The wavelength of peak sensitivity of the encoded photopigment is ~560 nm (Carroll et al., 2002). In addition, the coding sequence for the C-terminal tail was altered to replace the last 13 amino acids of human L opsin with the last 15 from human S opsin for studies outside the scope of this manuscript, (3) rAAV.HB570.GFP is identical to the construct described in (2) except that the RHLOPS was replaced with the gene for GFP. The construct was described previously (Glushakova et al., 2006).

All three viral preparations were made as serotype 2 virus packaged in the capsid from serotype 5 (rAAV-2/5). The concentrations of injected virus are as follows: rAAV.CHOPS2053.GFP was 8×10^{13} viral particles/ml, rAAV.CHOPS2053.RHLOPS was 2.0×10^{13} viral particles/ml, and rAAV.HB570.GFP was 2.4×10^{14} viral particles/ml. The following abbreviations are used throughout this article: rAAV: recombinant Adeno Associated Virus; LCR: Locus Control Region; GFP: Green Fluorescent Protein; rAAV.CHOPS2053.GFP: targeting construct directing GFP to M-cones; rAAV.HB570.GFP: targeting construct directing GFP to S-cones; rAAV.CHOPS2053.GFP: targeting construct directing L-opsin to M-cones; RHLOPS: Recombinant Human L-OPS; CHOPS2053: human L/M cone promoter; HB570: Human Blue cone promoter; mfERG: multifocal ElectroRetinoGram.

Animals

Five adult male gerbils were used for these studies: Ger27, Ger28, Ger31, Ger36, and Ger41. Ger27 received two 5 μl injections of rAAV.CHOPS2053.GFP that had been through two freeze-thaw cycles, Ger31 and Ger28 each received two 5 μl injections of a 1:1 (volume:volume) mixture of rAAV.CHOPS2053.GFP and rAAV.CHOPS2053.RHLOPS, and Ger36 received three 5 μl injections of rAAV.CHOPS2053.GFP. Mixing two viral constructs together allowed us to simultaneously monitor the location of the injection by visualizing GFP expression and to monitor function of the viral delivered human L cone photopigment with the multifocal electroretinogram, but effectively reduced the dosage of rAAV.CHOPS2053.GFP by half compared to the animal that received 10 μl of only rAAV.CHOPS2053.GFP. Ger41 received three 5 μl injections of rAAV.HB570.GFP. Ger41, Ger36, Ger27 were sacrificed 134, 538, and 138 days post-injection, respectively. At this writing Ger31 and Ger28 are still alive and showing GFP activity.

Subretinal injection procedure

Gerbils were anesthetized with an intramuscular (IM) injection of Ketamine (50 mg/kg) and Xylazine (2 mg/kg) diluted in sterile saline and supplemented as needed to sustain anesthesia throughout the procedure. Atropine sulfate (4 mg/kg, IM) was given to decrease mucus secretion. The eye was dilated by topical application of 1.0% Tropicamide and a temporary, slight proptosis of the right eye was induced using a small hole made in a piece of sterile latex and swabbed with 5% providone-iodine. For local anesthesia 0.5% proparacaine drops and subconjunctival injection of 0.03 ml injection of 1% lidocaine were administered. A limited temporal peritomy was performed, and a 27 gauge needle was used to make a lateral sclerotomy 1 ml (mm) posterior to the limbus. While viewing the eye under a surgical microscope, the injection cannula was guided through the sclerotomy, into the posterior chamber, through the retina, ultimately into the subretinal space where the virus was injected.

Virus injections were made using a straightened 30 gauge, 22 mm long, blunt anterior chamber cannula (BD Visitec 585738) connected to a 500 μl syringe (Hamilton Gastight #1750 Teflon Leur Lock with slots, Hamilton, Reno, NV) *via* 36 inches of 30 gauge Teflon tubing with adapters at both ends (Hamilton, Reno, NV). The syringe, cannula, and tubing were all sterile. The syringe was mounted on a KD Scientific model 210 syringe pump set to a syringe diameter of 3.26 mm and primed with sterile lactated ringers. Using the withdraw mode on the pump, 10–20 μl of virus was drawn into the tip of the cannula at a rate of 100 μl/min. The pump was immediately switched to infusion mode at a rate of 1060 μl/min, and an infusion volume of 5 μl. Each 5 μl bleb covers approximately 15% of the gerbil's retinal area which corresponds to 9.6 mm^2 of the gerbil retina (Wikler et al., 1989). The cannula was placed in the subretinal space, and 5 μl of virus solution was injected at a rate of 1060 μl/min. Up to three 5 μl injections were made in each gerbil retina with each injection placed at a different location. The cannula was removed from the eye, and images of

the injections taken (described below). Eye proptosis was reversed by removing the patch of latex, and a subconjunctival injection of 0.03 ml Kenalog (10 mg/ml) and 0.03 ml of Cephazolin (100 mg/ml) was given subcutaneously on the mucosal surface of the lower eyelid, followed by topical application of antibiotic ointments.

Retinal imaging

Using a RetCamII (Clarity Medical Systems, Inc., Pleasantown, CA) and a lens that allowed a 130° field of view, fundus photographs of gerbil retinas were acquired both immediately before and after performing subretinal injections of virus, and at various time points post-injection. Gerbils were anesthetized as described above for each imaging session. GFP fluorescence was visualized using the fluorescence detection configuration of the RetCamII in which a Xenon bulb served as the light source and a 510 nm cut-off filter was placed between the lens and light source. Fluorescence images were collected periodically to obtain the time course of GFP expression in individual animals.

Histological preparation and confocal microscopy

Gerbils were sacrificed and each eye was marked for orientation and enucleated. Retinas were dissected away from the RPE in ice cold Hanks Buffer and fixed for 4 h at room temperature in 4% paraformaldehyde in phosphate buffer solution (PBS) pH 7.4, then washed twice with PBS for 30 min. Photoreceptors were labeled by incubating fixed retinas overnight at 4°C in a solution of 10% normal goat serum (Vector Laboratories, Burlingame, CA) and 0.2% Triton X-100 in PBS, washing twice with PBS for 15 min, then incubating overnight at 4°C in a 1:750 dilution of rhodamine-conjugated peanut agglutinin (PNA, Vector Laboratories, Burlingame, CA) in PBS. After washing for 30 min in PBS, retinas were mounted, photoreceptor side up, using VectaShield mounting medium (Vector Laboratories). Tissue was examined with a Leica confocal microscope, and using a 100× objective and a 4× optical zoom, a z-series was obtained by averaging the signal four times for each of 19–0.2 micron thick sections. The PNA labeling that we observe is most robust in the outer segment but does weakly extend over the inner photoreceptor sheath as well as the cone pedicle. Each confocal z-series reported in the manuscript extends through the inner segment and we would detect if outer segments were sheared off during the preparation for histology. In the sections imaged, we did not observe any GFP positive cells without outer segments or any GFP positive cones that were not also PNA positive.

Color multifocal ERG

A custom multi-focal ERG (mfERG) described in this issue by Kuchenbecker et al. (2008) was constructed by modifying a RETIscan mfERG (Roland Consult, Inc., Brandenburg, Germany). The modified mfERG has 2048 paired green and red LEDs mounted on the inside surface of a full-field hemisphere subtending 150° of visual angle. This instrument allowed measurement of the amplitude of ERG waveforms from 64 distinct regions tiling the surface area of the retina.

Results

Characterization of GFP expression in M-cones

The time course of GFP expression in the gerbil retina is illustrated by the series of fluorescence fundus photographs shown in Fig. 1. The animal whose retina is shown in Fig. 1a received two 5 μl injections of rAAV.CHOPS2053.GFP and the animal in Figs. 1b and 1c received two 5 μl injections of a mixture of rAAV.CHOPS2053.GFP and rAAV.CHOPS2053.RHLOPS, effectively diluting the titer of GFP virus he received. The only observable expression observed under RetCam viewing conditions is from the GFP transgene, and therefore, our measure of expression (GFP fluorescence) is proportional to the titer (particles/ml) of GFP construct injected.

At every time point, the animal in Fig. 1a showed a more robust GFP signal than the animal in Figs. 1b and 1c as expected from the proportionally lower dose of virus carrying the GFP transgene in Figs. 1b and 1c. Consistent with other reports (Bennett et al., 1997; Rolling et al., 1999; Bainbridge et al., 2003; Alexander et al., 2007; Li et al., 2007), GFP fluorescence was reliably detected by about 2 weeks post-injection and has lasted for more than two years (Fig. 1c). Except at very early time points post-injection, there was relatively little variability in fluorescence intensity across the treated areas of individual retinas. There was an abrupt drop in GFP signal with little gradient of fluorescence at the border of the retinal detachment formed by the injection.

To investigate further the loss in GFP signal over time, we evaluated the expression dynamics quantitatively by measuring the mean pixel intensities over the area of GFP fluorescence using ImageJ (http://rsb.info.nih.gov/ij/ref). We used retinal landmarks to measure the pixel intensities from the same region of retina within an individual animal's eye over time. For three gerbils that received the rAAV.CHOPS2053.GFP virus, the mean pixel intensities are expressed as a percentage of maximum for each animal and plotted as a function of time in Fig. 2a. This procedure removes the variability among animals in Fig. 2a, and allows changes relative to the maximum pixel intensity for each animal to be compared across subjects. The GFP fluorescence rapidly increased in the weeks immediately following the injection, rising to a peak at about 110 to 120 days, then declined, until about 200 days post injection when a stable level that was maintained long term was achieved (Fig. 2a). The quantitative results for the time course of GFP fluorescence intensity reported here are similar to those reported for other species in which rAAV carrying the GFP gene under control of the CMV promoter was used (Bennett et al., 1997; Rolling et al., 1999; Bainbridge et al., 2003). The present study extends the quantitative assessment by many months to include the time at which a stable level is achieved and maintained very long term, and focuses on cone-specific expression.

The decline in GFP intensity over time appears to be due to progressively fewer cells expressing the transgene at later time points. Cell death does not account for the decline since cone photoreceptor cell densities from histology were about 50 cones/mm^2 after GFP expression levels had fallen; this value is consistent with previously published results from wild type animals (Govardovskii et al., 1992). There is variability inherent in the injection procedure that leads to variability in the amount of virus that is delivered to the subretinal space and this is usually observed as a difference in the quality and characteristics of the retinal detachment that forms. For example, Ger28 and Ger31, received the same injection volume and virus cocktail, but the injection detached a much larger area of retina in Ger31 than in Ger28. Hence, Ger28's photoreceptors were exposed to a smaller dose of virus than were Ger31's. As can be seen in Fig. 2a, both gerbils show a decline in GFP fluorescence over time, but the reduction is much more dramatic in the animal that received a lower dose of virus (Fig. 2a). Based on the observations illustrated in Figs. 1a,

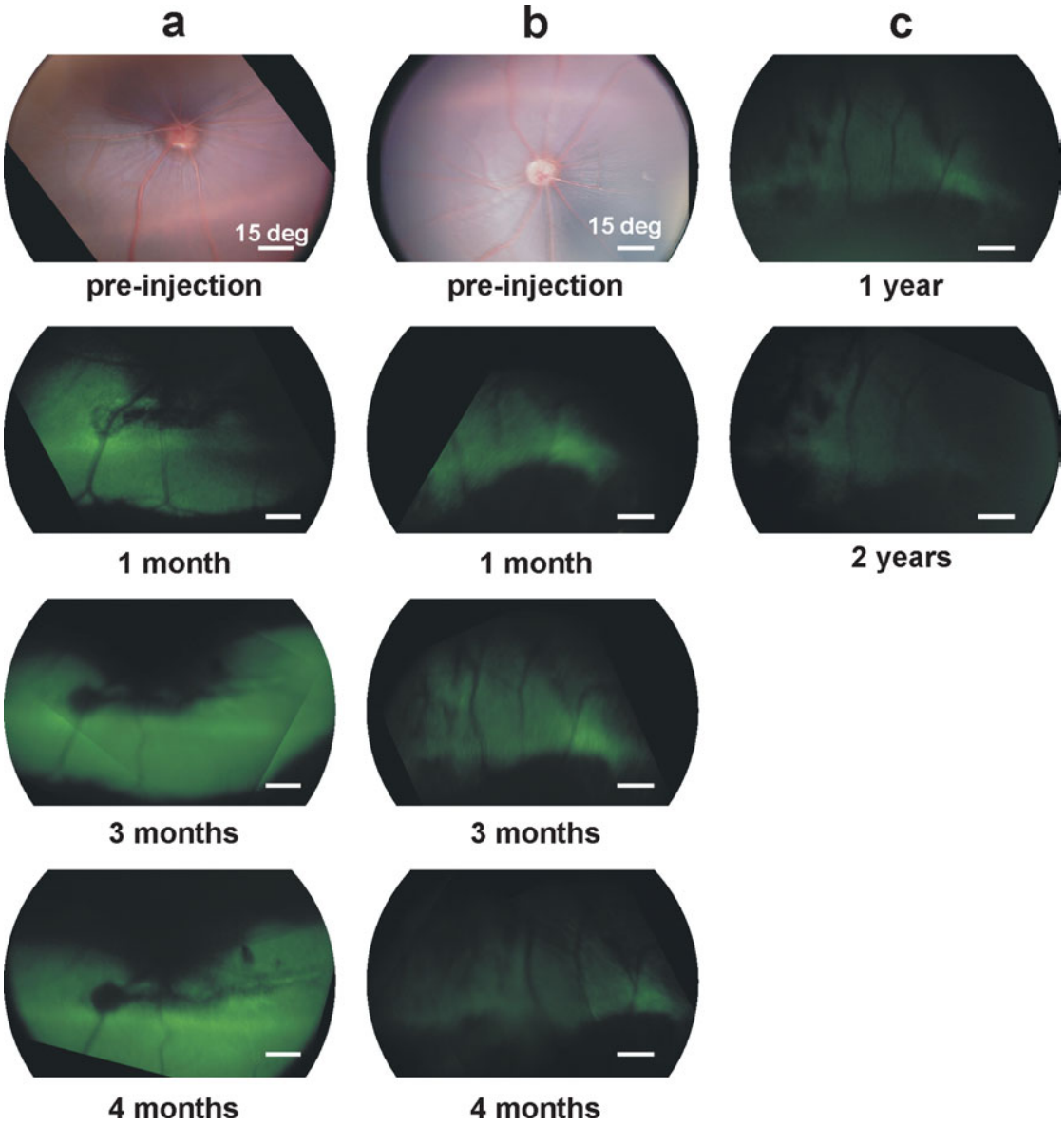

Fig. 1. Fundus images of real-time GFP expression in two gerbils. Pre-injection images were taken using white light, all other images were taken using a Xenon bulb as the light source and a 510 nm cutoff filter placed between the light source and the lens. Images were obtained at various times-post injection, as indicated on individual images. (**a**) Images from the gerbil Ger27 who received two 5 ul injections of rAAV.CHOPS2053.GFP (directed expression to M-cones). (**b**) Images from the gerbil Ger31 who received who received about half as much rAAV.CHOPS2053.GFP (directed expression to M-cones) as Ger27. (**c**) Fluorescence fundus images from gerbil Ger31 1 year and 2 years after injection.

1b, 1c, and 2a, we hypothesized that the amount of virus to which the cells are exposed determines the final, stable level of expression.

To investigate this hypothesis further, we compared the fluorescence intensity over time for two geographically well-separated locations within the area of retina detached by the same injection. The spots chosen represented the extremes in intensity of GFP fluorescence. One location received full exposure to the virus and the other was near the edge of the bleb and laid under a larger vessel; these two factors restricted exposure of the retina in that region. From the known topography of cones in the gerbil retinas, we know that the two locations are similar in cone density (Govardovskii et al., 1992). Time courses for fluorescence intensity for the two locations relative to the maximum pixel intensity across the entire fluorescent region are plotted in Fig. 2b. The region with less exposure to the virus had a lower final stable level of fluorescence. These results support the hypothesis that the long-term, stable level of transgene expression varies as a function of how much virus the cells are exposed to at the time of injection.

In order to evaluate the fraction of cones that are stably transduced by the virus, we sacrificed two gerbils one 4 months and another at 1.5 years post-injection (Figs. 3a, 3b, and 3c). Confocal microscopy was used to calculate the ratio of cones double labeled with PNA and GFP (transduced cones) versus all PNA positive cells (all cones) to estimate the percentage of cones that were transduced. In the gerbil sacrificed 4 months post-injection, we analyzed regions of the image near the injection site,

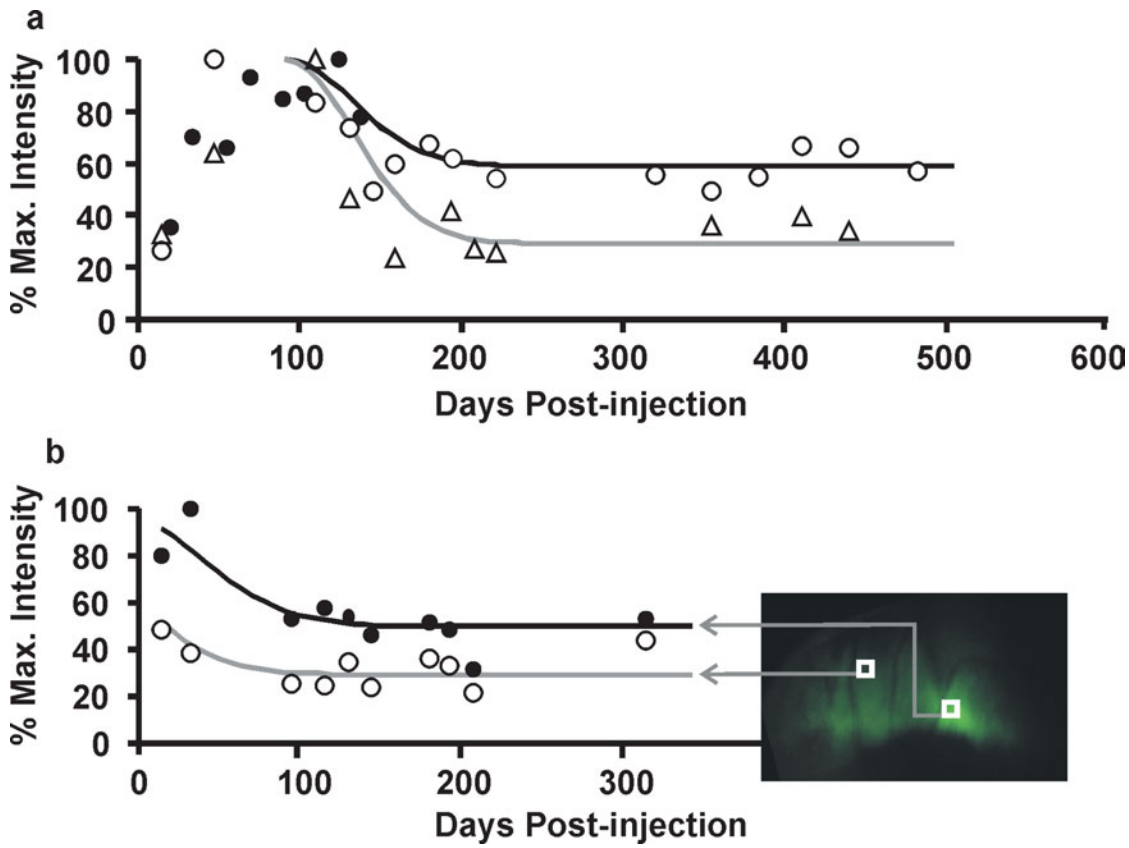

Fig. 2. Quantification of GFP fluorescence over time. (**a**) Fluorescence intensity versus the maximum pixel intensity for each animal is plotted as a function of time for three animals treated with rAAV.CHOPS2053.GFP: Ger31 (white circles), Ger27 (black circles), Ger28 (triangles). (**b**) Time course of GFP fluorescence intensity for a region of near the injection site (high fluorescence, black circles) and distal to the injection site (weaker fluorescence, white circles) for one of the injection sites for Ger31. The curves drawn in both (**a**) and (**b**) were best-fit curves generated from a model describing changes in intensity predicted over time.

distal to the injection site, at an intermediate location, and at the border of the region of retina detached by the injection. For all three locations within the detached region, about 42 ± 3% (300/724) of the cones were double labeled with PNA and GFP, with differences in fluorescence intensity across regions being mostly accounted for by decreasing density of cones with increasing eccentricity.

For the gerbil sacrificed at 1.5 years post-injection (Fig. 3a) we assessed four regions of the bleb and approximately 37 ± 3% (462/1237) of cones labeled with PNA also expressed GFP. Thus, anytime from 4 months to 1.5 years post injection, the percentage of cones that express the GFP transgene appears to stabilize around 40%.

Characterization of GFP expression in S-cones

The time course of expression of the virus, rAAV.HB570.GFP, is shown in Fig. 4. As with GFP under control of the human L opsin promoter, GFP fluorescence increased over time, reaching a maximum at about 3 months post injection, followed by a decline in intensity. The magnitude of pixel intensity for animals treated with rAAV.HB570.GFP is 3–5% of the signal observed in a time-matched image of a gerbil expressing GFP in M cones. This result is consistent with selective targeting of GFP expression to gerbil S cones. At 140 days post-injection, the gerbil was sacrificed, and the cones labeled with PNA. Confocal microscopy was used to calculate the fraction of cones double labeled with GFP and PNA versus those labeled only with PNA. Figs. 3d, 3e, and 3f show confocal micrographs of a wholemounted retina, and in this image, 3.6% (18/502) of the PNA labeled cells also contained GFP. These results further support the conclusion that the HB570 promoter targets GFP expression to S cones in gerbils.

Colocalization of fluorescence and function

The function of gene products delivered through gene therapy is crucial to the eventual treatment of retinal disease and to the success of experiments to investigate neural circuitry. We used a cone-isolating color multifocal electroretinography (mfERG) to evaluate cone photopigment function for a human L opsin delivered by gene therapy. As described above in methods, a 1:1 mixture of the rAAV.CHOPS2053.GFP and rAAV.CHOPS2053.RHLOPS was injected so that the pattern of GFP fluorescence could be compared to the pattern of activity in the mfERG, as shown in Fig. 5. In Fig. 5b, a fluorescence fundus image of the treated retina at 6 months post-injection defines the area of retina for which we would also expect to see increased sensitivity to long-wavelength light mediated by the human L cone photopigment. Areas of the retina that do not show GFP fluorescence serve as an internal control, and should not show increased sensitivity to long-wavelength light. As illustrated

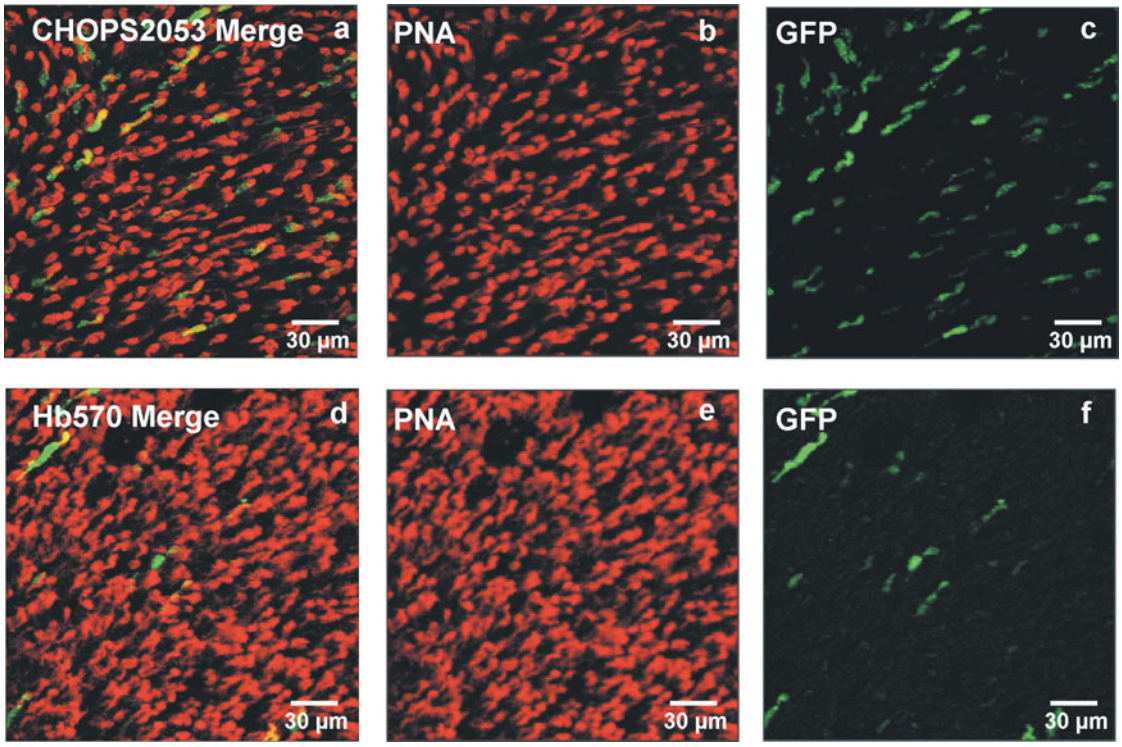

Fig. 3. (**a**) Merged 3-D reconstruction (z-series) of the outer photoreceptor layer at with PNA staining of the cone outer photoreceptor sheath (red) and GFP expression (green) targeted to gerbil M-cones via rAAV.CHOPS2053.GFP (Ger36). Yellow regions are areas of co-localization of PNA labeling and GFP fluorescence. The red (PNA). (**b**) and green (GFP) (**c**) channels are shown separately. (**d**) Merged 3-D reconstruction (z-series) of the outer photoreceptor layer with PNA staining of the cone outer photoreceptor sheath (red) and GFP expression (green) targeted to gerbil S-cones via rAAV.HB570.GFP. The red (PNA). (**e**) and green (GFP) (**f**) channels are shown separately.

in Figs. 5a and 5b, there is excellent correspondence between the regions of fluorescence and increased sensitivity to long-wavelength light (Fig. 5a). Conversely, regions that do not fluoresce also do not show increased sensitivity to long-wavelength light in the mfERG.

Discussion

Recently, the rAAV.CHOPS2053.GFP virus was shown to target transgene expression to cones of mice, ferrets, rats, and New World primates (Wang et al., 1992; Alexander et al., 2007; Li et al., 2007; Mancuso et al., 2007). As is typical for mammals, mice, ferrets, and rats, have two cone types—UV/S cones sensitive to ultraviolet (UV) or short-wavelengths (S), and M/L cones sensitive to relatively longer wavelengths. In rats and ferrets, transgene expression under the control M pigment specific sequences was selectively targeted to M cones, but in mice, it was expressed in both UV and M cones; which is not surprising as mice co-express their endogenous UV and M cone photopigments (Rohlich et al., 1994; Applebury et al., 2000). In gerbils, at early time points post injection, nearly all cones express GFP; however, after fluorescence intensity has stabilized, about 40% of cone photoreceptors express GFP. The distribution of GFP positive photoreceptors and the observation that nearly all GFP positive photoreceptors were also positive for PNA labeling indicates that expression was specific for cones. This specificity is time dependent with many more cells labeled with less specificity at earlier time points, for example, as has been reported at six weeks post-injection in the rat and ferret. Presumably, the initially high transgene copy number overwhelms repressive mechanisms in S-cones and rods that normally function to keep L/M opsin turned off in those cells. We attribute the change in specificity to a decrease in number of transgene cassettes per cell that occurs over time after the initial injection ultimately falling with the range in which repressive mechanisms operate effectively in S-cones and rods. An estimated two to five percent of gerbil cones are UV cones (Govardovskii et al., 1992); if only M cones express GFP from the rAAV.CHOPS2053.GFP virus, then 35–40% of gerbil M cones expressed GFP at 4 months and 1.5 years post-injection. Since GFP fluorescence declines between the peak at about 4 months post-injection until about 6 months post-injection when the final, stable level of GFP expression is reached (Fig. 2), the 35–40% of cones labeled at 4 months post-injection may represent a value slightly higher than the final percentage of cones that stably express GFP at 1.5 years. These results indicate that with the present technology even when undiluted virus is injected, the maximum fraction of cones that can be stably transduced is about 40%.

In a previous study using the same S-opsin promoter carrying rAAV.HB570.GFP virus used here, it was shown at 22 to 32 days post-injection to target transgene expression to all photoreceptors in rats in the proportion ~5:4:1 rods:UV/S cones:M cones (Glushakova et al., 2006). Assuming that at the 20–30 day time point almost all S-cones express the transgene, a proportion of 500 to 1000 rods for every S-cone would indicate that as many as 1:500 rods express GFP. Similarly, if M cones outnumber S cones by

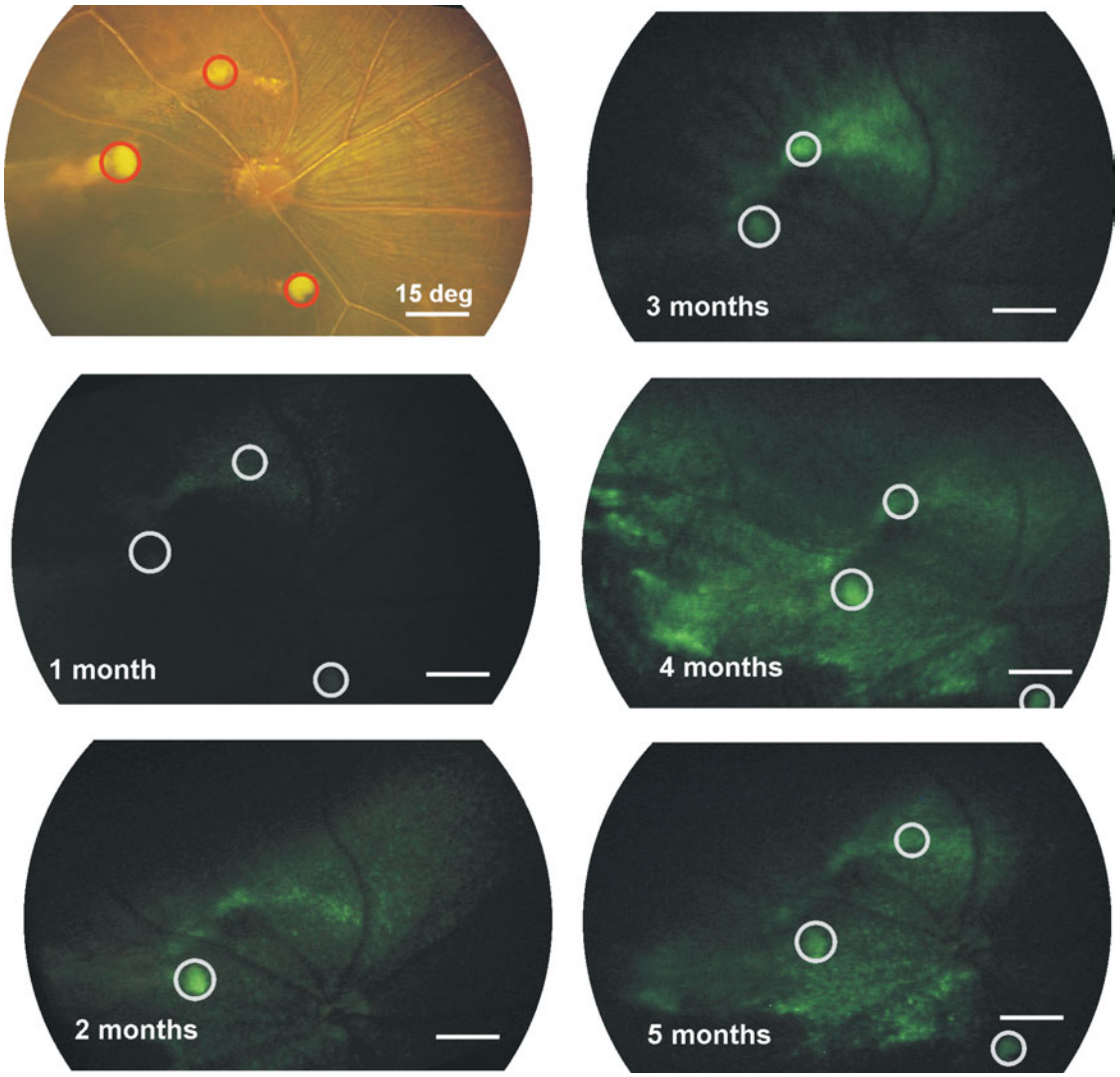

Fig. 4. (**a**) Serial fluorescence fundus images of GFP fluorescence in a gerbil retina treated with S-cone targeted GFP. The circled regions are areas of needle penetrations that appear autofluorescent. The subretinal fluid injected (15 ul) detached virtually the entire retina and thus pan-retinal fluorescence was observed.

about 20:1, about 1:400 M cones express the transgene at an early time point in the rat. As illustrated in Fig. 4b, at 4 months post-injection in the gerbil, all GFP positive cells that we identified were also PNA positive, indicating that rods did not express GFP driven by the S opsin promoter in retinal regions examined at this time point. Our results (Figs. 1, 2, and 3) indicate that expression of the rAAV delivered transgenes is in flux for 5 to 6 months after the injection before expression settles to a stable level that is maintained long-term. This change appears to be principally the result of a decrease in transgene cassettes over time (Glushakova et al., 2006). AAV2/5 enters rods and cones, and Glushakova et al. (2006) suggest that cellular machinery responsible for responsible for suppressing UV/S opsin expression from the HB570 promoter in adult rods and M cones may be overwhelmed by the high number of transgene cassettes per cell.

In the rat, it was estimated that the injection introduces 10^5 to 10^6 virus particles per photoreceptor, but by about 1 month post injection, there are only five to 10 copies of virus per photoreceptor (Glushakova et al., 2006). The results presented here allow us to estimate the number of active trangenes that are maintained per photoreceptor for the long term. A simple model can explain the changes *in vivo* fluorescence observed over time. As observed previously, after subretinal injections, we found that GFP fluorescence increases to a maximum and then declines. In retinal areas that develop only weak fluorescence, the number of active GFP transgenes decline to an endpoint below detection. When the treatment is most effective, the fluorescence intensity stabilizes at a high level and persists without diminishing. A simple model in which stable transgene expression is achieved via two probabilistic events can explain these results. First, immediately after injection, only a small fraction of virus particles in the extracellular environment enter the photoreceptor and second, only a fraction of these virus particles entering the cell and contributing to initial GFP expression are retained for the long term. Each event can be modeled as a fixed probability.

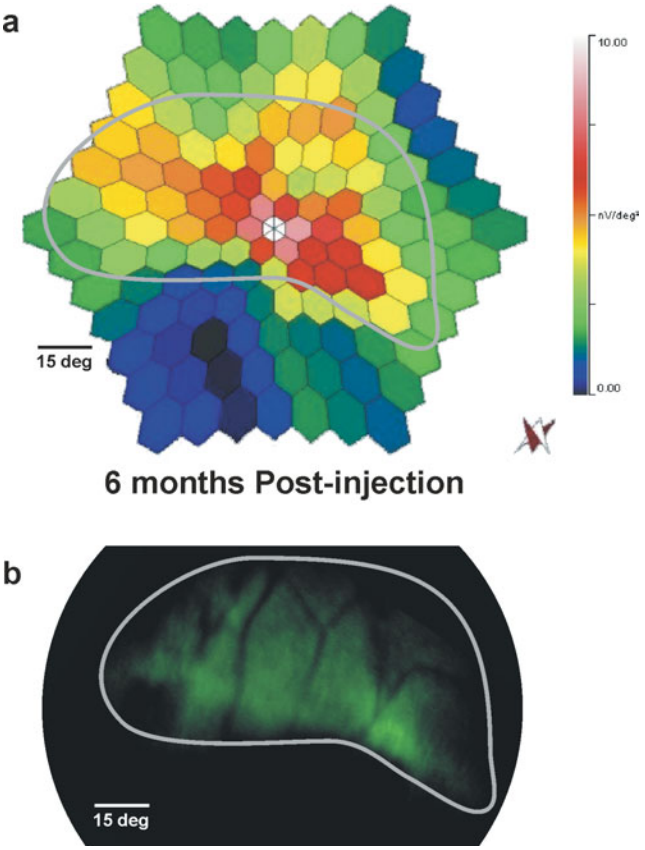

Fig. 5. A 1:1 mixture of the rAAV.CHOPS2053.GFP and rAAV.CHOPS2053.RHLOPS was injected sub-retinally in a gerbil to demonstrate co-localization of GFP fluorescence (**b**) with photoreceptor response to an L-isolating stimulus measured with a mfERG taken 6 months post-injection. (**a).** In this figure, the gray outline corresponds to the region of the GFP fluorescence that was created by the initial subretinal injection.

The number expressing transgenes per cell can estimated because of two aspects of the results presented here that simplify the problem. (1) In the long term, even under optimal conditions, 60% of cones do not express GFP. Thus we know that the number of expressing transgenes in the non-fluorescent cones is zero. (2) Since there are two possible long term outcomes for any transgene initially entering a cell, it can either be maintained or lost, the number of particles can be approximated by a binomial distribution. Given that 60% of cones have zero transgenes maintained, the number having 1, 2, 3, etc. to be approximated from the binomial distribution. Having an estimate of the probability distribution for the number of stable transgenes per cell, the distribution of particles initially entering the cell can be determined from the change in expression observed from early to late. From those values the probability that a particle will enter a cell initially can be determined from knowing the initial concentrations of viral particles per photoreceptor.

Particles per photoreceptor was determined with the knowledge that pseudotyped AAV preferentially infects photoreceptors and that each 5 μl injection covers ~9.6 mm^2 of retina which contains ~3.6 million photoreceptors. There were approximately 3.48 $\times$ 10^{10} infections particles per μl in the injection mixture which yields roughly 50,000 infectious particles per photoreceptor in each subretinal bleb. Initially, we estimate that between 90 and 99% of cones express GFP. Assuming that each viral particle has the same chance of entering the photoreceptor and that the number of particles per photoreceptor will follow a binomial distribution, the calculated probability that any one viral particle will enter a photoreceptor and drive GFP expression is about 1 in 20,000. The initial binomial distribution of the number of particles per cell is shown in Fig. 6a (dashed line) for an average injection of 50,000 per photoreceptor. Assuming a constant probability of 1/20,000, theoretical distributions are shown for lower concentrations of 35,000 per photoreceptor (black solid line) and 15,000 per photoreceptor (gray line).

Knowing the fraction of photoreceptors that stably express GFP long term allows us to determine the probability of transgene cassettes initially entering a photoreceptor, described in Fig. 6a, becoming stable. Assuming a fixed probability that produces a binomial distribution, and that 40% of photoreceptors stably express one or more GFP transgenes, we estimate that the fixed probability that any one viral particle inside a cell will become stable and express in the long term is 23%. Fig. 6b shows how theoretically, as concentrations of the initial injection are reduced, the number of stably transduced photoreceptor drops. For a starting concentration of 35,000 virus particles per photoreceptor (solid black line) the fraction of cones that stably express the transgene (containing one or more viral transgenes) would theoretically drop to 29%, and for viral concentrations 15,000 per photoreceptor (gray line) the fraction of cells that stably express GFP would theoretically drop to 12% as illustrated Fig. 6b. This shows that a relatively large concentration of virus is required to get significant long term expression. At the highest concentrations achievable in this study, nearly half of the cones stably express GFP and binomial statistics predict that most of those cones retain a single stable copy of the transgene. We predict that concentrations of the virus below about 20% of that used here would result in only a very small fraction of cones transduced and fluorescence levels that may be below those detectable with *in vivo* methods.

These statistics also speak to the issue of misexpression of GFP under the control of S-opsin regulatory sequences reported earlier in the rat. The binomial distributions shown in Fig. 6 indicate that for the highest concentration of vector, around 1/200 cells contain six or more transgene cassettes at early time points. The high number of transgene cassettes may overwhelm factors in the M cones and rods that normally suppress expression of the S-opsin in those cells and may account for the S-opsin expression levels of one in several hundred that is observed early on. However, Fig. 6b illustrates that 5 to 6 months after the injection, the number drops such that very few photoreceptors would have more than two cassettes, a level which would be within the cellular machinery's ability to suppress expression from the human S-opsin promoter.

The injection procedure generates a retinal detachment that is delimited by a border of retina adhered to the RPE. We investigated whether gene expression extended beyond the visible region of the detachment created by the injection and it is clear from confocal micrographs that there is no GFP fluorescence beyond the border. The sub-retinal injection exposes tissue within the region of the bleb to the vector, but does not come in contact with the tissue past the boundaries. Previously, it was reported that in rat, the region of the retinal detachment gave rise to a GFP fluorescence that extended over 2.5 times the area of the histological abnormality created by the detachment (Daniels et al., 2003). At least in gerbil, results reported here are consistent with the conclusion that the histological abnormalities only encompass a fraction of the entire region of the detachment.

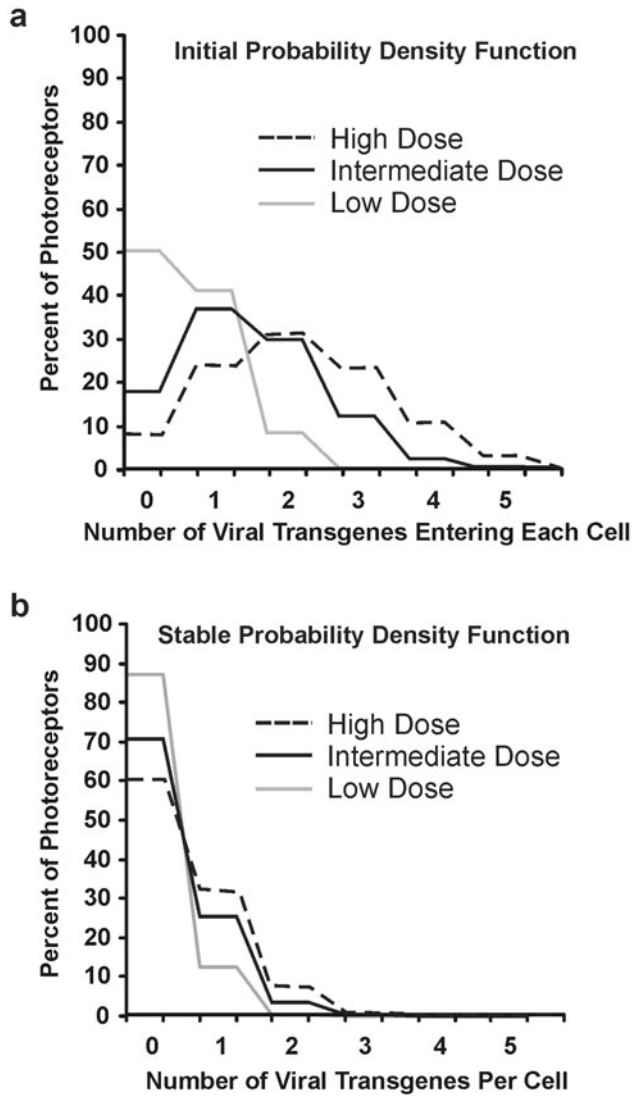

Fig. 6. Two probabilistic events explain changes in GFP fluorescence over time and the long term stability of photoreceptor transduction by rAAV-GFP. The first probability is that associated with a given virus entering a cell. The second probability is that associated with viral cassette inside a cell becoming stably expressed. The two discrete, binomial probability density functions in this figure reflect the dependence of long term transgene expression on these two probabilities. (**a**) binomial probability density function representing the percentage of photoreceptors (y-axis) containing given number of viral particles after initial transfection (x-axis) at three concentrations (high: 50,000 viral particles per photoreceptor; intermediate: 35,000 viral particles per photoreceptor; and low: 15,000 viral particles per photoreceptor). At the earliest time points following an injection of ~50,000 viral particles per photoreceptor (dashed line), >90% of cone photoreceptors show GFP fluorescence thus less than 10% have zero this increases to near, 50% of cells (grey line) for a low initial viral concentration. The high concentration curve best models the experimental data. Over time the amount of GFP fluorescence declines before reaching a stable level presumably because viral transgenes become inactive. (**b**) The binomial probability density function describing the percentage of photoreceptors expressing the viral transgene long term. The probability that a viral transgene expression will be maintained for the long term is dependent on the number of viral particles initially infecting each cell (described in (**a**)). Fitting our results to the stochastic model indicates that 23% of the viral transgenes initially entering the cell become stable for the long term. For the initial, high concentration injection (~50,000 particles per photoreceptor) approximately 40% of cells contain 1 or more stable transgenes.

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References

Acland, G.M., Aguirre, G.D., Bennett, J., Aleman, T.S., Cideciyan, A.V., Bennicelli, J., Dejneka, N.S., Pearce-Kelling, S.E., Maguire, A.M., Palczewski, K., Hauswirth, W.W. & Jacobson, S.G. (2005). Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. *Molecular Therapy* **12**, 1072–1082.

Acland, G.M., Aguirre, G.D., Ray, J., Zhang, Q., Aleman, T.S., Cideciyan, A.V., Pearce-Kelling, S.E., Anand, V., Zeng, Y., Maguire, A.M., Jacobson, S.G., Hauswirth, W.W. & Bennett, J. (2001). Gene therapy restores vision in a canine model of childhood blindness. *Nature Genetics* **28**, 92–95.

Alexander, J.J., Umino, Y., Everhart, D., Chang, B., Min, S.H., Li, Q., Timmers, A.M., Nawes, N.L., Pang, J., Barlow, R.B. & Hauswirth, W.W. (2007). Restoration of cone vision in a mouse model of achromatopsia. *Nature Medicine* **13**, 685–687.

Applebury, M.L., Antoch, M.P., Baxter, L.C., Chun, L.L.Y., Kalk, J.D., Farhangfar, F., Kage, K., Krzystolik, M.G., Lyass, L.A. & Robbins, J.T. (2000). The murine cone photoreceptor: A single cone type expresses both S and M opsins with retinal spatial patterning. *Neuron* **27**, 513–523.

Bainbridge, J.W.B., Tan, M.H. & Ali, R.R. (2006). Gene therapy progress and prospects: The eye. *Gene Therapy* **13**, 1191–1197.

Bainbridge, J.W., Mistry, A., Schlichtenbrede, F.C., Smith, A., Broderick, C., De Alwis, M., Georgiadis, A., Taylor, P.M., Squires, M., Sethi, C., Charteris, D., Thrasher, A.J., Sargan, D. & Ali, R.R. (2003). Stable rAAV-mediated transduction of rod and cone photoreceptors in the canine retina. *Gene Therapy* **10**, 1336–1344.

Bennett, J., Duan, D., Engelhardt, J.F. & Maguire, A.M. (1997). Real-time, noninvasive in vivo assessment of adeno-associated virus-mediated retinal transduction. *Investigative Ophthalmology and Vision Research* **38**, 2587–2863.

Bennett, J., Maguire, A.M., Cideciyan, V., Schnell, M., Glover, E., Anand, V., Aleman, T.S., Chirmule, N., Gupta, A.R., Huang, Y., Gao, G.-P., Nyberg, W.C., Tazelaar, J., Hughes, J., Wilson, J.M. & Jacobson, S.G. (1999). Stable transgene expression in rod photoreceptors after recombinant adeno-associated virus-mediated gene transfer to monkey retina. *Proceedings of the National Academy of Sciences USA* **96**, 9920–9925.

Carroll, J., Neitz, M. & Neitz, J. (2002). Estimates of L:M cone ratio from ERG flicker photometry and genetics. *Journal of Vision* **2**, 531–542.

Daniels, D.M., Shen, W.Y., Constable, I.J. & Rakoczy, P.E. (2003). Quantitative model demonstrating that recombinant adeno-associated virus and green fluorescent protein are non-toxic to the rat retina. *Clinical Experimental Ophthalmology* **31**, 439–444.

Glushakova, L.G., Timmers, A.M., Pang, J., Teusner, J.T. & Hauswirth, W.W. (2006). Human blue-opsin promoter preferentially targets reporter gene expression to rat s-cone photoreceptors. *Investigative Ophthalmology and Vision Research* **47**, 3505–3513.

Govardovskii, V.I., Rohlich, P., Szel, A. & Khokhlova, T.V. (1992). Cones in the retina of the mongolian gerbil, *Meriones unguiculatus*: An immunocytochemical and electrophysiological study. *Vision Research* **32**, 19–27.

Jacobs, G.H., Williams, G.A., Cahill, H. & Nathans, J. (2007). Emergence of novel color vision in mice engineered to express a human cone photopigment. *Science* **315**, 1723–1725.

Kuchenbecker, J.A., Sahay, M., Tait, D.M., Neitz, M. & Neitz, J. (2008). Topography of the long- to middle-wavelength sensitive cone ratio in the human retina assessed with a wide-field color multifocal electroretinogram. *Visual Neuroscience* **25**, 301–306.

LeMeur, G., Weber, M., Yann, P., Mendes-Madeira, A., Nivard, D., Deschamps, J., Moullier, P. & Rolling, F. (2005). Postsurgical assessment and long-term safety of recombinant adeno-associated virus-mediated gene transfer into the retinas of dogs and primates. *Archives of Ophthalmology* **123**, 500–506.

Li, Q., Timmers, A.M., Guy, J., Pang, J. & Hauswirth, W.W. (2007). Cone-specific expression using a human red opsin promoter in recombinant AAV. *Vision Research* **48**, 332–338.

Mancuso, K., Hendrickson, A.E., Connor, T.B., Jr., Mauck, M.C., Kinsella, J.J., Hauswirth, W.W., Neitz, J. & Neitz, M. (2007). Recombinant adeno-associated virus targets passenger gene expression to cones in primate retina. *Journal of the Optical Society of America A Optics, Image Science, and Vision* **24**, 1411–1416.

Nathans, J., Thomas, D. & Hogness, D.S. (1986). Molecular genetics of human color vision: The genes encoding blue, green, and red pigments. *Science* **232**, 193–202.

Pang, J.J., Chang, B., Kumar, A., Nusinowitz, S., Noorwez, S.M., Li, J., Rani, A., Foster, T.C., Chiodo, V.A., Doyle, T., Li, H., Malhotra, R., Teusner, J.T., McDowell, J.H., Min, S.H., Li, Q., Kaushal, S. & Hauswirth, W.W. (2006). Gene therapy restores vision-dependent behavior as well as retinal structure and function in a mouse model of RPE65 Leber congenital amaurosis. *Molecular Therapy* **13**, 565–572.

Rohlich, P., van Veen, T. & Szel, A. (1994). Two different visual pigments in one retinal cone cell. *Neuron* **13**, 1159–1166.

Rolling, F., Shen, W., Tabarias, H., Constable, I., Kanagasingam, Y., Barry, C.J. & Rakoczy, P.E. (1999). Evaluation of adeno-associated virus-mediated gene transfer into the rat retina by clincal fluorescence photography. *Human Gene Therapy* **10**, 641–648.

Smallwood, P.M., Olveczky, B.P., Williams, G.L., Jacobs, G.H., Reese, B.E., Meister, M. & Nathans, J. (2003). Genetically engineered mice with an additional class of cone photoreceptors: Implications for the evolution of color vision. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 11706–11711.

Wang, Y., Macke, J.P., Merbs, S.L., Zack, D.J., Klaunberg, B., Bennett, J., Gearhart, J. & Nathans, J. (1992). A locus control region adjacent to the human red and green visual pigment genes. *Neuron* **9**, 429–440.

Wikler, K.C., Perez, G. & Finlay, B.L. (1989). Duration of retinogenesis: its relationshop to retinal organization in two cricetine rodents. *The Journal of Comparative Neurology* **285**, 157–176.