Intravitreal Injection of AAV2 Transduces Macaque Inner Retina

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PURPOSE. Adeno-associated virus serotype 2 (AAV2) has been shown to be effective in transducing inner retinal neurons after intravitreal injection in several species. However, results in nonprimates may not be predictive of transduction in the human inner retina, because of differences in eye size and the specialized morphology of the high-acuity human fovea. This was a study of inner retina transduction in the macaque, a primate with ocular characteristics most similar to that of humans.

METHODS. In vivo imaging and histology were used to examine GFP expression in the macaque inner retina after intravitreal injection of AAV vectors containing five distinct promoters.

RESULTS. AAV2 produced pronounced GFP expression in inner retinal cells of the fovea, no expression in the central retina beyond the fovea, and variable expression in the peripheral retina. AAV2 vector incorporating the neuronal promoter human connexin 36 (hCx36) transduced ganglion cells within a dense annulus around the fovea center, whereas AAV2 containing the ubiquitous promoter hybrid cytomegalovirus (CMV) enhancer/chicken-β-actin (CBA) transduced both Müller and ganglion cells in a dense circular disc centered on the fovea. With three shorter promoters—human synapsin (hSYN) and the shortened CBA and hCx36 promoters (smCBA and hCx36sh)—AAV2 produced visible transduction, as seen in fundus images, only when the retina was altered by ganglion cell loss or enzymatic vitreolysis.

CONCLUSIONS. The results in the macaque suggest that intravitreal injection of AAV2 would produce high levels of gene expression at the human fovea, important in retinal gene therapy, but not in the central retina beyond the fovea. (Invest Ophthalmol Vis Sci. 2011;52:2775–2783) DOI:10.1167/iovs.10-6250
patterns of AAV2 in the macaque eye by intravitreal injection is qualitatively similar to that in the smaller eye of a foveated New World primate marmoset,20,21 yet significantly different from that in other species—in particular, rodent models.

Methods

Subjects

Eight adult macaque monkeys were used, each weighing approximately 6 kg with ages ranging from 3 to 11 years at the time of injection (Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6250/-/DCSupplemental). Eyes and retinas were normal in all the monkeys, except for one with a history of ganglion cell loss from a cortical infection and two that had been given intravitreal injections of microplasmid, which produces vitreoretinal detachment (Supplementary Materials and Methods, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6250/-/DCSupplemental). Head posts were implanted in the monkeys used for AO imaging, as previously described.29 All animal procedures were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Office of Laboratory Animal Care at the University of Rochester.

Viral Vectors

Preparation of Vectors. AAV vectors were packaged and purified by standard methods22 in the Flannery laboratory at the University of California, Berkeley. Briefly, AAV was packaged by triple transfection (Lipofectamine 2000; Invitrogen, Carlsbad, CA) of transfer and helper plasmids into AAV293 cells. After harvest, lysis, and iodixanol ultracentrifugation, the interphase between the 54% and 40% iodixanol fraction and the lower three quarters of the 40% iodixanol fraction were extracted and further purified by heparin-affinity chromatography. The eluent was concentrated and the buffer exchanged by using a centrifugal filter unit (Amicon Ultra-15; Millipore, Billerica, MA). The virus was washed three times with 15 mL of sterile phosphate-buffered saline (PBS) with 0.001% Tween. The vector was then titered by quantitative (q)PCR, relative to standards.19

Promoter and Gene Payload of AAV2 Vector. The AAV vectors produced for this study were AAV2/2 (i.e., AAV2 inverted terminal repeat containing genomes packaged inside AAV2 capsid, termed AAV2 in this article), carrying the GFP (either the enhanced [e]GFP or humanized [h]GFP transgene (Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6250/-/DCSupplemental). One of the following five promoters (Table 1) was used to drive the transgenes: chicken-β-actin (CBA) with cytomegalovirus (CMV) enhancer, human connexin 36 (hCx36), human synapsin (hSYN), and shortened versions of CBA and hCx36 (smCBA and hCx36sh, respectively). In some cases, the transduction efficiency was evaluated by injection of 5 μL of the same vector into wild-type rat eyes. For primate injections, viral vectors were shipped on ice to the University of Rochester from the University of California (UC) Berkeley. The viral vector was then stored at 4°C until use, normally within 1 to 2 weeks. All in vivo imaging and histology were performed at the University of Rochester.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Symbol</th>
<th>Size (kb)</th>
<th>Reference</th>
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<tr>
<td>Chicken-β-actin promoter with cytomegalovirus enhancer</td>
<td>CBA</td>
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<td>Boye SL, et al. IOVS 2006;47:ARVO E-Abstract 852</td>
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<td>smCBA</td>
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<td>hCx36</td>
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<td>Greenberg K, PhD dissertation, UC Berkeley, 2007</td>
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<tr>
<td>Shortened hCx36 promoter</td>
<td>hCx36sh</td>
<td>1.8</td>
<td>Greenberg K, PhD dissertation, UC Berkeley, 2007</td>
</tr>
<tr>
<td>Human synapsin 1 gene promoter</td>
<td>hSYN</td>
<td>0.5</td>
<td>Kugler et al.24,25</td>
</tr>
</tbody>
</table>

The sequences of CBA and smCBA are described elsewhere (Boye SL, et al. IOVS 2006;47:ARVO E-Abstract 852). The sequences of hCx36 and hCx36sh have not been published (Greenberg K, PhD dissertation, UC Berkeley, 2007). The 2.8-kb (hCx36) and 1.8-kb (hCx36sh) fragments of the hCx36 promoter were cloned from human genomic DNA by standard molecular biology techniques and high-fidelity PCR. The promoter fragments were then ligated into an AAV2 backbone vector containing GFP and the woodchuck post-transcriptional regulatory element (WPRE). All vectors were sequenced for PCR fidelity. The sequence of hSYN has been described by Kugler et al.24,25

Intravitreal Injection. Viral vector (40–100 μL) was injected into the vitreous of each eye through the sclera approximately 3 mm behind the limbus, using a 30-gauge needle, with precautions taken to avoid infection. The condition of the injected eyes was monitored closely for up to 2 weeks. Three eyes from two animals were pretreated by an intravitreal injection of 50 μg (in 100 μL distilled water) of recombinant microplasmid (Thrombogenics, Leuven, Belgium; Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6250/-/DCSupplemental), a recombinant serine protease that is used to degrade the vitreoretinal junction and produce posterior vitreal detachment in the human eye.26 These injections were given in identical procedures 5 days before the injection of viral vector.

In cases in which both eyes were injected with vector, the second injection was performed less than 1 month after the first one (Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6250/-/DCSupplemental), to avoid immune inactivation of the second injection by antibodies against AAV2 from the first injection.27 Serum antibody titer against AAV2 was measured in the Schaffer laboratory at UC Berkeley (Supplementary Materials and Methods, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6250/-/DCSupplemental). We excluded from analysis one monkey that had a high serum antibody titer against AAV2 before vector injection (Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6250/-/DCSupplemental).

In Vivo Transduction Evaluation

Fundus Imaging. Color and fluorescence fundus images were taken of each retina with a fundus camera (model TRC-50DX, Topcon, Paramus, NJ) that had been modified to image GFP fluorescence, by an excitation bandpass filter of 457 to 487 nm (FF01-472/50; Semrock, Rochester, NY) and a barrier filter with a bandpass of 502.5 to 537.5 nm (FF01-520/35; Semrock, Rochester, NY). Contrast and brightness of all fundus images presented in this article were adjusted (Photoshop; Adobe Systems, Waltham, MA) for better visualization of the relatively weak fluorescence signal of the GFP expressed in retinal neurons. Fundus images from the right eyes were flipped horizontally to match those from the left eyes, so that all fundus images presented herein have the same orientation. Scale bars for fundus images (Figs. 1A, 1B, 4A, 7) were calculated, assuming that the distance between the fovea center and temporal edge of the optic disc is 11.8°.28

AO Imaging. A fluorescence adaptive optics scanning laser ophthalmoscope (FAOLSO) was used to image retinal neurons that expressed GFP in vivo, as described elsewhere.19,25 During imaging sessions, the monkeys were anesthetized with isoflurane at a dosage (typically 2%) sufficient to minimize large ocular movements and eliminate microsaccades. Images (1500 frames at a field of view of...
The retina, with pigment epithelium attached, was separated from the animal immediately after euthanatization and fixed by immersion in 4% paraformaldehyde (in 0.1 M phosphate buffer).

For animals euthanatized for histologic analysis (Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6250/-/DCSupplemental), the eyes were enucleated from the animal immediately after euthanatization and fixed by immersion in 4% paraformaldehyde (in 0.1 M phosphate buffer). The retina, with pigment epithelium attached, was separated from the sclera. Retinal whole mounts were flattened on a glass slide by several radial cuts made at the periphery and covered with a coverslip with mounting medium (Vectorshied; Vector Laboratories, Burlingame, CA). Transverse sections were prepared from retinal tissue embedded in agar and sectioned parallel to the horizontal raphe at 60-μm thickness on a microtome with vibrating blade (Microm International GmbH, Walldorf, Germany). Some samples were processed with immunostaining to increase the sensitivity of detection of GFP in the tissue. After they were washed in 0.01 M PBS and pretreatment solution (0.4% Triton X-100 in 0.01 M PBS), the retinas were incubated in blocking buffer (5% normal goat serum and 0.4% Triton X-100 in 0.01 M PBS) for 4 to 6 hours at room temperature following by overnight at 4°C, to reduce nonspecific binding. We reacted the retinas with the primary antibody chicken anti-EGFP (Millipore Bioscience Research Reagents, Temecula, CA) at a dilution of 1:100 and reacted the retinas with the secondary antibody goat anti-chicken, conjugated with Alexa 488 (Invitrogen, Carlsbad, CA), to visualize the primary antibody staining (1–2 days, in dark at 4°C).

**Histology**

**Ex Vivo Preparation.** For animals euthanatized for histologic analysis (Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6250/-/DCSupplemental), the eyes were enucleated from the animal immediately after euthanatization and fixed by immersion in 4% paraformaldehyde (in 0.1 M phosphate buffer). The retina, with pigment epithelium attached, was separated from the sclera. Retinal whole mounts were flattened on a glass slide by several radial cuts made at the periphery and covered with a coverslip with mounting medium (Vectorshied; Vector Laboratories, Burlingame, CA). Transverse sections were prepared from retinal tissue embedded in agar and sectioned parallel to the horizontal raphe at 60-μm thickness on a microtome with vibrating blade (Microm International GmbH, Walldorf, Germany). Some samples were processed with immunostaining to increase the sensitivity of detection of GFP in the tissue. After they were washed in 0.01 M PBS and pretreatment solution (0.4% Triton X-100 in 0.01 M PBS), the retinas were incubated in blocking buffer (5% normal goat serum and 0.4% Triton X-100 in 0.01 M PBS) for 4 to 6 hours at room temperature following by overnight at 4°C, to reduce nonspecific binding. We reacted the retinas with the primary antibody chicken anti-EGFP (Millipore Bioscience Research Reagents, Temecula, CA) at a dilution of 1:500. The reaction was performed initially at room temperature for 2 hours, then at 4°C for 3 to 4 days. After several washes in PBS, we reacted the retinas with the secondary antibody goat anti-chicken, conjugated with Alexa 488 (Invitrogen, Carlsbad, CA), to visualize the primary antibody staining (1–2 days, in dark at 4°C).

**Ex Vivo Confocal Imaging.** Ex vivo whole mount and transverse sections were imaged with a confocal microscope (LSM 510 Meta; Carl Zeiss Meditec, Thornwood, NY). GFP fluorescence in the...
tissue or Alexa 488 signal after immunostaining were imaged with settings optimized for FITC. Image stacks were obtained across z-depths for whole mount tissue. The z-projection and transverse views of the image stacks were generated with NIH Image software (http://rsb.info.nih.gov/nih-image/). Brightness and contrast of the images presented in this article were adjusted (Photoshop; Adobe Systems, Waltham, MA) for better visualization. Montages of images from adjacent retinal regions were processed in the same program.

**Conversion between Angular and Retinal Distances.**

Since in vivo fundus and AO images were measured in angular distance (degrees of visual angle) and ex vivo confocal imaging were measured in retinal distance (in micrometers), we assumed 225 μm in retinal distance for 1° in angular distance.31 Converted values are quoted in parentheses.

**RESULTS**

AAV2 transduction in the macaque eye occurred in the fovea and peripheral retina, but not in the central retina outside the fovea. Foveal transduction was more consistent across promoters than was peripheral transduction.

**AAV2-hCx36-GFP Transduces Foveal Ganglion Cells, but Not Müller Cells**

Intravitreal injection of AAV2-hCx36-GFP resulted in a dense annulus of GFP expression around the fovea, clearly visible in vivo, as evaluated with either fundus or AO imaging (Fig. 1; four eyes were tested; Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6250/-/DCSupplemental). The inner edge of the annulus was approximately 1.5° (≈0.3 mm) from the foveal center, which closely matches the location of the outer edge of the foveal avascular zone32 (Figs. 1A, 1B). The outer edge of the annulus was approximately 2.5° (≈0.6 mm) from the fovea center. GFP-expressing axon bundles projecting from the annular ring formed an arcuate pattern converging on the temporal side of the optic disc (Fig. 1B).35

Individual GFP-expressing cell bodies were visible in the AO images (Fig. 1C). The density of transduced cells declined gradually over approximately 100 μm, from GFP expression in many retinal cells at the outer edge of the annulus to expression below the detection limit (Figs. 1C, 2C, 2D). The first appearance of the transduction illustrated in these data was rapid, with the fluorescence signal of GFP first visible by fundus camera less than 1.5 months after injection into the eye shown in Figure 1A, and the expression remained stable for more than 2.5 years after vector injection.

Through-focus AO images of the GFP-expressing annulus showed a complete lack of GFP expression in the Müller cells with the neuronal hCx36 promoter (Figs. 1D, 1E). GFP-expressing cells in the inner portion of the annulus were in sharp view at deep focus (Fig. 1D, right, arrows), but not at the superficial focus (Fig. 1E, arrows), whereas GFP-expressing cells in the outer portion of the annulus were in sharp view at the superficial focus (Fig. 1E, arrowheads), but not at the deep focus (Fig. 1D, arrowheads). The deep-focus image (Fig. 1D) showed a few scattered cells within the foveal avascular zone (left, open triangle), which may be ganglion cells, as described by Grünert et al.34

Ex vivo confocal images of retinal whole mounts confirmed AAV2-hCx36-GFP transduction in foveal cells in the ganglion cell layer (GCL), but did not involve Müller cells. Because ganglion cells comprise 95% of the neuronal cell population in the GCL in the fovea (displaced amacrine cells are 5% or less55–56; see also Curcio and Allen13 for human retina), most of the GFP-expressing cells are ganglion cells. The inner edge of the GFP-expression annulus showed no expression of GFP in Müller cells below the ganglion cell layer (Fig. 2B), confirming the in vivo observation (Figs. 1D, 1E). The outer edge of the GFP-expression annulus also showed no intervening Müller cell processes between the individual cell somas in the GCL (Figs. 2C, 2D). GFP expression was also visible in the inner plexiform layer (IPL), where the dendrites of transduced ganglion cells extended (Fig. 2B). We also observed scattered GFP-expressing cell somas in the inner nuclear layer (INL), close to the IPL/INL border, which could have been amacrine cells57 (data not shown).

While AAV2-hCx36-GFP produced strong foveal transduction in normal eyes, no retinal transduction of foveal GFP was seen in the central retina outside the annulus (data not shown). In peripheral retina, we observed scattered transduction of cells in the GCL, especially along the blood vessels (data not shown). GFP expression was also found in the peripheral retina in eyes that received enzymatic vitreolysis with microplasmin before vector injection (see the Methods section and Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6250/-/DCSupplemental). For the eye shown in Figure 3, GFP expression was visible in the Müller and retinal cells in both the GCL and INL at the distal edge of the far nasal peripheral retina, extending approximately 2 mm (≈9°) toward the central retina. This peripheral transduction was visible in fundus images as dense GFP-expressing axons entering the optic disc from the nasal retina (data not shown).

**Figure 2.** Ex vivo (histologic) evaluation of AAV2-hCx36-GFP transduction in the macaque central retina, showing dense GFP expression within the GCL in fovea. (A) Diagram of the foveal GFP expression, illustrating the location of the images in (B–D), which are from an eye transduced with AAV2-hCx36-GFP after treatment with microplasmin. Images in (B–D) show GFP expression within the ganglion cells and their axons, not amplified with immunostaining. (B) Confocal images of the inner edge of the annulus of GFP expression in whole mount view (top) and transverse view (bottom) reconstructed from the portion of the whole mount image stack between the two dashed horizontal lines. GFP expression is visible in GCL and IPL at retinal locations away from the foveal slope. NA, 1.2. (C) Confocal image of the outer edge of the annulus of GFP expression showing dense, expressing ganglion cells and their axons. NA, 0.8. (D) Higher magnification image that partially overlaps with the portion of (C) marked by the dashed rectangle. NA, 1.2.
AAV2-CBA-GFP Transduced both Ganglion Cells and Müller Cells in the Fovea and the Peripheral Retina

Intravitreal injection of AAV2-CBA-GFP resulted in a disc of GFP expression centered on the fovea and extending to more than 1.5° (~0.3 mm) eccentricity, which was clearly visible in vivo, as evaluated by fundus and AO images (Figs. 4A–C; one eye was 1.5°). The expression centered on the fovea and extending to more than 36 µm. Scale bar: (E-G) same as in (D).

Figure 3. AAV2-hCx36-GFP transduction in the far peripheral retina after treatment with microplasmin. (A, B) GFP expression in ganglion cells (arrowheads) and Müller cells (arrows) from the nasal edge of the retina near the ora serrata. In the fluorescence fundus image of this eye, axon bundles from transduced ganglion cells were visible in the nerve fiber layer entering the optic disc from the nasal side (data not shown). In (B), the transverse view (right) was reconstructed from the portion of whole mount (left) image stack between the two dashed vertical lines. Transverse view shows the processes of the Müller cell (arrow) extending toward the outer retina. GFP expression was not amplified with immunostaining. NA, 1.2.

Through-focus AO images showed that foveal Müller cells are densely transduced. The three-dimensional morphology of the GFP-expressing Müller cells and distribution of ganglion cells in the foveal region were seen through a series of AO images across retinal depths from the outer retina toward the inner retina (Figs. 4D–G). In Figure 4D, Müller cell processes at the outer retina are in focus, while in Figure 4G, more superficial ganglion cell somas are in focus. The lateral displaced processes of the Müller cells and their somas were best visualized in the intermediate focuses (Figs. 4E, 4F, respectively). These GFP-expressing Müller cells were responsible for the solid, disc-like appearance of the GFP expression in the fundus image (Fig. 4A).

Ex vivo confocal images confirmed that AAV2-CBA-GFP transduced both foveal ganglion cells and Müller cells (Fig. 5). As shown in the transverse section through the GFP-expressing
Central retina
disc centered on the fovea (Fig. 5B), GFP-expressing Müller cells extended their processes throughout the full thickness of the retina, with the scleral end of their processes terminating near the outer limiting membrane (OLM) and the vitread end of their processes terminating at the ILM.38 The lateral displacement between the scleral and vitread ends of the Müller cell processes reflect the underlying anatomy: Müller cells ensheathe cones in the fovea center that provide inputs to ganglion cells that are displaced away from the fovea center.39 GFP-expressing ganglion cells extended to slightly greater eccentricity than did the Müller cells (Fig. 5B). Most GFP-expressing cell somas in the INL appeared to be Müller cells (Fig. 5B).

Transduction of peripheral retina by AAV2-CBA-GFP was evident in the ex vivo confocal images. The density of GFP-expressing retinal cells gradually increased from an eccentricity of approximately 8 mm (∼30°) from the fovea center (data not shown) to the edge of the retina (Fig. 6A). Density of GFP-expressing retinal cells at the far peripheral retina was relatively sparse compared with that seen immediately around the fovea center (Fig. 6A compared with Fig. 5). As illustrated by the example shown in Figures 6B–D from one far peripheral location, most of the GFP-expressing cells were Müller cells, and transduction in ganglion cells was less pronounced, such that the axons of these ganglion cells could not be seen in the fundus images (data not shown).

**Shorter Promoters Were Ineffective in Normal Retinas, but Produced Transduction in Retinas with Ganglion Cell Loss, or Enzymatic Vitreolysis**

Since AAV vectors have a limited packaging capacity, we explored the use of shorter neuronal and ubiquitous promoters to drive GFP expression in the primate retina.

We examined transduction by two short neuronal promoters—hSYN34 and the shortened hCx36 promoter (hCx36sh)—in normal and diseased eyes with ganglion cell loss (one eye for each condition; Supplementary Table S1 and Supplementary Fig. S1, http://www iovs org lookup suppl doi 10.1167 iovs.10-6250 DC Supplemental) and compared both to transduction with the hCx36 promoter. As shown by the fundus images (Figs. 7A, 7C), intravitreal injection of AAV2 with neither promoter produced visible GFP expression in normal retinas over durations that exceeded the time needed for transduction by AAV2-hCx36, but in both cases (at comparable titer; Supplementary Table S1, http://www iovs org lookup suppl doi 10.1167 iovs.10-6250 DC Supplemental) produced visible GFP expression (Figs. 7B, 7D) in eyes with loss of ganglion cells (Supplementary Fig. S1A, http://www iovs org lookup suppl doi 10.1167 iovs.10-6250 DC Supplemental). However, the lower titers of the AAV2hSYN-GFP and AAV2-hCx36sh-GFP than the AAV2-hCx36-GFP precluded comparison of the transduction efficiency of the two short neuronal promoters to hCx36 promoter (Supplementary Table S1, http://www iovs org lookup suppl doi 10.1167 iovs.10-6250 DC Supplemental).

We also examined the transduction by the short CBA promoter (smCBA) in a normal eye and in an eye that received enzymatic vitreolysis with microplasmind see (the Methods section) before vector injection (one eye for each condition; Supplementary Table S1, http://www iovs org lookup suppl doi 10.1167 iovs.10-6250 DC Supplemental) and compared both to the transduction with CBA promoter (at comparable titer; Supplementary Table S1, http://www iovs org lookup suppl doi 10.1167 iovs.10-6250 DC Supplemental). As shown by fundus images (Figs. 7E, 7F), intravitreal injection of AAV2-smCBA-GFP produced no visible GFP expression in normal retina (Fig. 7E) over a duration that exceeded the time needed to produce expression with the full-length CBA promoter, but produced visible GFP expression in the eye pretreated with mi-
croplasmin (Fig. 7F). For the latter, the appearance of foveal transduction (Fig. 7F) was similar to that of the full-length CBA promoter (Fig. 4A), and we confirmed by histology that both foveal ganglion cells and Müller cells were transduced (data not shown). Besides foveal transduction, prominent transduction in far peripheral ganglion cells was visible in the fundus image (Fig. 7F) as dense GFP expression in axons entering the optic disc from the nasal retina, and we verified by histology that both Müller cells and ganglion cells at the distal edge of far peripheral retina were transduced (data not shown).

**DISCUSSION**

In this study, intravitreal injection of AAV2 into the macaque eye produced dense ganglion cell and Müller cell transduction in only a narrow region surrounding the fovea, with no transduction in the central retina beyond the fovea and little or no transduction in the peripheral retina. This result differs from the more uniform transduction reported after intravitreal injection in other species, including rodents, and raises the question of how well studies in nonprimates are predictive of gene therapy results in humans.

**What Limits Transduction of the Macaque Inner Retina?**

The limited transduction we observed may have been due to the physical barriers posed by the anatomy of the macaque retina, such as the NFL and ILM, which lie along the surface of the retina, between the GCL and vitreous.

The variation in thickness of the NFL across the retina is consistent with the transduction patterns observed in this study. There is little NFL covering the foveal retinal location where inner retinal cells were transduced (Figs. 1B, 4A). Outside the fovea, superior and inferior to the optic disc, NFL thickness can reach a maximum thickness of more than 200 μm in the macaque retina and more than 300 μm in the human retina. These are the regions where no transduction was observed. When NFL thickness was greatly reduced in retinas with ganglion cell loss, inner retina transduction was enhanced (Figs. 7B, 7D; Supplementary Fig. S1, http://www.iovs.orglookup/suppl/doi:10.1167/iovs.10-6250/-/DCSupplemental). A similar phenomenon has been reported in degenerated rodent retina. Together, these results suggest enhanced transduction in the disease-compromised retina, an advantage from the gene-delivery perspective.

Although the ILM is thin compared with theNFL, its thickness is inversely related to the inner retina transduction efficiency observed in this study. Macaque (and human) ILM is thinnest in the fovea, where transduction was best, moderately thicker in the far peripheral retina, where expression was modest, and thickest in the posterior retina near the fovea, where almost no transduction was observed. Furthermore, the ILM is thinner above the retinal vessels, where we observed focal transduction of retinal cells along blood vessels at eccentricities beyond approximately 30° (data not shown). Microplasmin, which disrupts the border between the vitreous and ILM, increased transduction by AAV2-hCx36 and AAV2-smCBA in the far peripheral retina where the ILM is relatively thin, similar to the increased transduction in rodent when the ILM was disrupted by a protease. The human and macaque ILM are very similar, and thus the ILM is a likely a barrier to AAV-mediated gene delivery in humans.

The pattern of foveal transduction could also reflect other features of the primate retina that interact with AAV2 capsids, such as the distribution of cell surface receptors, since AAV2 binds selectively to heparin sulfate proteoglycan and three co-receptors. The topographic distribution of these receptors has not been measured in macaque retina. However, the dense transduction at the fovea indicates that promoter selectivity is not the basis for a lack of transduction in other areas of retina.

**Which Animal Models Are Optimal for Developing Human Gene Therapy?**

The wide variety of animal models used in gene therapy research each fills an important role. Rodents (e.g., mice) are widely used species that are well-suited for genetic manipulations, whereas dogs and cats provide several unique models of retinal degeneration and greater similarity to human retina because of the large eye size and higher acuity area centralis. The nonhuman primate is better suited than the other species for predicting transduction in humans because of the unique morphology of the retina in species that have a fovea.

Recently, transduction of the inner retina by intravitreal injection was studied in one nonhuman primate, the New World marmoset, using an AAV2 virus with either the CAG
(hybrid CMV early enhancer/chicken β-actin) or CMV (human cytomegalovirus immediate early gene) promoter. The marmoset has a small eye, with an average axial length of 11 mm, substantially smaller than that of the macaque (18 mm) and human (24 mm).

Marmosets have a well-developed fovea, similar to that of the macaque and humans in both overall shape and the extent of ganglion cell displacement away from the foveal center.

However, the transduction of marmoset retina observed by Ivanova et al. was quite different from that observed in the macaque in our laboratory in three respects: cellular selectivity, the spatial pattern of transduction across the retina, and the depth of transduction through the retina. The CBA promoter used in the present study and the CAG promoter used in the marmoset study of Ivanova et al. are similar. Despite this similarity, the macaque showed Müller cell and ganglion cell transduction at the fovea and largely Müller cell transduction in peripheral retina, while the marmoset showed no Müller cell transduction at any retinal location, but substantial ganglion cell transduction across the retina. The spatial pattern of transduction across the retina was also different, with the macaque showing dense GFP expression in foveal ganglion cells, but little GFP expression in peripheral ganglion cells, while the marmoset showed relatively sparse foveal ganglion cell transduction, but substantial peripheral ganglion cell transduction. Finally, the depth of transduction through the retina also differed between the macaque and marmoset. Neuronal transduction in the macaque retina was confined to the GCL and inner portion of the INL, whereas the transduction in the marmoset extends to outer retina (e.g., photoreceptors).

We speculate that these differences between the macaque and marmoset could be due both to species differences in viral tropism and to a difference in the topology of a physical barrier, such as the ILM, which has not been studied in the marmoset. Given that the macaque is closer to humans in evolutionary lineage and that there is great similarity in physical attributes of the two species, such as eye size and ILM thickness, it is likely that the transduction observed in macaques in this study provides a closer prediction of transduction by intravitreal injection in humans than that observed in the marmoset. However, further research is needed, due to differences in promoter, age, and viral titer between the studies in the two species.

Choice of Vector and Promoter for Transducing Macaque Inner Retina

AAV2, with the hCx36 promoter, produced selective transduction of foveal ganglion cells, but not of Müller cells, making it a good choice for inserting gene products into ganglion cells for neurophysiology studies or ganglion cell neuroprotection in diseases such as glaucoma. However, the hCx36 promoter fragment used in this study was large (2.8 kb), and it occupies much of the 4.7-kb genomic capacity of AAV2, leaving little space for transgenes substantially larger than GFP (0.7 kb). The CBA promoter (1.7 kb) is smaller than hCx36 and produces good transduction of both foveal and peripheral ganglion cells and Müller cells. The major drawback of the CBA promoter was the extended time until expression occurred.

The short promoters—hSYN, hCx36sh, and smCBA—did not produce visible transduction in healthy retina over an extended time and thus are not effective choices for use in macaques. Although the smCBA promoter yielded similar expression to full-length CBA when injected into an eye pretreated with microplasmin, the hCx36sh and hSYN promoters remained ineffective, even when injected into eyes altered by ganglion cell loss. That the AAV2 vectors with two of these promoters (smCBA and hSYN) have produced excellent retinal transduction in other mammals (Boye SL, et al. IOVS 2006;47: ARVO E-Abstract 852) illustrates the unique difficulty in achieving gene expression in the primate retina.

The Importance of In Vivo, High-Resolution AO Imaging in Monitoring Viral Transduction in the Macaque

Although histologic verification remains the standard method for evaluating the efficiency and selectivity of transduction by viral vectors, in vivo imaging is particularly important in investigating therapeutic effects of gene therapy in primates, as it can eliminate the need to serially kill different animals for histology. Fundus imaging can reveal the time course and spatial pattern of expression in each animal. Because AO imaging has substantially greater sensitivity and spatial resolution than fundus imaging, as well as axial sectioning capacity (through-focus; Figs. 1D, 1E, 4D–G), it is able to reveal details of GFP expression that could not be determined from the fundus images. This distinction was particularly evident in this study when visualizing ganglion cell transduction by CBA, which was clear in the AO images (Figs. 4B–G), but barely observable in the fundus image (Fig. 4A).

Implications of This Study for Human Retinal Gene Therapy

A significant issue in moving viral-mediated gene therapies for human retinal disease from small animal efficacy studies to clinical applications is the potential differences between the eye and retina of the animal model and the human (e.g., retinal cell-surface properties, promoter selectivity, and anatomy of the retina). Studies in the ideal animal model, the macaque monkey, are severely limited by availability, cost, and lack of appropriate disease models, but must be performed to develop successful retinal gene therapy for humans. This study demonstrates that barriers to transduction of the inner retina in the macaque are substantial, but also shows that the use of high-resolution, in vivo adaptive optics imaging greatly facilitates such investigations.

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References

AAV2 Transduction in Macaque Inner Retina


