A Novel Method Combining Vitreous Aspiration and Intravitreal AAV2/8 Injection Results in Retina-Wide Transduction in Adult Mice

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Citation: Da Costa R, Röger C, Segelken J, Barben M, Grimm C, Neidhardt J. A novel method combining vitreous aspiration and intravitreal AAV2/8 injection results in retina-wide transduction in adult mice. *Invest Ophthalmol Vis Sci.* 2016;57:5326-5334. DOI:10.1167/ iovs.16-19701 **PURPOSE.** Gene therapies to treat eye disorders have been extensively studied in the past 20 years. Frequently, adeno-associated viruses were applied to the subretinal or intravitreal space of the eye to transduce retinal cells with nucleotide sequences of therapeutic potential. In this study we describe a novel intravitreal injection procedure that leads to a reproducible adeno-associated virus (AAV)2/8-mediated transduction of more than 70% of the retina.

METHODS. Prior to a single intravitreal injection of a enhanced green fluorescent protien (GFP)expressing viral suspension, we performed an aspiration of vitreous tissue from wild-type C57Bl/6J mice. One and one-half microliters of AAV2/8 suspension was injected. Funduscopy, optical coherence tomography (OCT), laser scanning microscopy of retinal flat mounts, cryosections of eye cups, and ERG recordings verified the efficacy and safety of the method.

RESULTS. The combination of vitreous aspiration and intravitreal injection resulted in an almost complete transduction of the retina in approximately 60% of the eyes and showed transduced cells in all retinal layers. Photoreceptors and RPE cells were predominantly transduced. Eyes presented with well-preserved retinal morphology. Electroretinographic recordings suggested that the new combination of techniques did not cause significant alterations of the retinal physiology.

CONCLUSIONS. We show a novel application technique of AAV2/8 to the vitreous of mice that leads to widespread transduction of the retina. The results of this study have implications for virus-based gene therapies and basic science; for example, they might provide an approach to apply gene replacement strategies or clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 in vivo. It may further help to develop similar techniques for larger animal models or humans.

Keywords: AAV2/8, intravitreal injection, retinal degeneration, retinal dystrophy, viral vector

M utations in more than 200 genes are known to cause retinopathies and vitreoretinopathies.¹ Despite this enormous genetic heterogeneity, ocular gene therapy has become a reality and is intensively studied in eye research.²⁻⁴ Adenoassociated viruses (AAVs) are applied to reprogram terminally differentiated retinal cells and to ameliorate genetic conditions.^{5,6} Among the AAV capsid serotypes that have been tested to transduce the different cell types of the retina, serotypes 2, 5, and 8 were found to have a broad applicability and high transduction efficiency.⁷⁻¹⁷ In humans, AAV serotype 2 was successfully employed to treat the blinding condition of Leber's congenital amaurosis using gene augmentation therapy.¹⁸⁻²¹ To replace the function of the mutated gene in vivo, the coding region of the *RPE65* gene was transferred to retinal pigment epithelial (RPE) cells.²² Similar approaches have recently been successfully applied to the condition of choroideremia.²³

Subretinal injection is a Food and Drug Administrationapproved technique to mediate retinal transduction using AAV vectors and has been successfully applied to several animal models of retinal diseases. An alternative method to apply AAVs is injection into the vitreous. Intravitreal injections of naturally occurring AAVs frequently showed lower transduction efficacies compared to subretinal injections.²⁴⁻²⁷ Nevertheless, AAV2/2 was reported to result in a widespread transduction of the retina following intravitreal injections in mice, but predominately transduced Müller and retinal ganglion cells.²⁸⁻³² Adenovirus-associated virus 2/8 was less efficient when injected into the vitreous.^{29,33,34} Capsid mutants improved the transduction efficacies and/or changed the tropism of different AAV serotypes.³⁵⁻³⁹

Intravitreal injections would offer several advantages to the treatment of retinopathies or vitreoretinopathies. As the injected AAVs distribute via diffusion in the vitreous gel,



intravitreal injections show the potential to result in a complete and homogenous transduction of the retina. Thus, the treatment of retinal disorders would benefit from improved intravitreal injection techniques. An increase in the retinal area transduced by the AAV could result in enhanced preservation of the visual function upon treatment.

In this report, we demonstrate that aspiration of vitreous tissue prior to injecting AAV2/8 suspensions increases the probability of obtaining a widespread transduction of the retina in mice. After a single intravitreal injection, enhanced green fluorescent protein (GFP) expression was detected in all retinal layers. The method presented here provides an alternative technique to apply gene therapeutic approaches to disease models using intravitreal injection and might guide the development of improved treatment regimens for different animal models and patients suffering from inherited retinopathies or vitreoretinopathies.

MATERIALS AND METHODS

Generation of Recombinant AAV Vectors

A single-stranded AAV vector (ssAAV2/8_CMV_GFP) and a selfcomplementary AAV vector (scAAV2/8_mCMV_GFP) were tested in this study. Both recombinant AAVs featured the GFP coding sequence encompassed within the inverted terminal repeats (ITRs) of the AAV serotype 2 and were pseudotyped with the capsid of the AAV serotype 8. The expression of GFP was controlled by either the complete cytomegalovirus (CMV) promoter in ssAAV2/8_CMV_GFP or a minimal version of this promoter lacking its upregulatory domain in scAAV2/ 8_mCMV_GFP. To remove the upregulatory domain in the CMV promoter, all nucleotides more than 104 base pairs upstream from the TATA box were deleted. In addition to the GFP expression cassette, the ssAAV2/8-CMV-GFP contained the β-globin intron 1 enhancing sequence and woodchuck hepatitis virus posttranscriptional regulatory element. The production and purification procedures of AAVs were extensively described by Grieger and colleagues.⁴⁰ Briefly, both AAVs were produced by polyethylenimine transfection of HEK293T cells with the helper plasmid pHGTI_Adeno1, the plasmid pLT-RC08 encoding for Rep proteins of serotype 2 and Cap proteins of serotype 8, and the AAV genome plasmid containing GFP in either a single-stranded or self-complementary manner. Viruses were extracted from the cell nuclei 48 hours post transfection through freezing-thawing cycles and dounce homogenization of the transfected cells. Cell debris was cleared by centrifugation, DNA contaminants were degraded with Benzonase (E8263; Sigma-Aldrich, Steinheim, Germany), and supernatants were further purified by ultracentrifugation on iodixanol gradient. Finally, the iodixanol buffer was exchanged with PBS and the virus suspensions were further concentrated by ultrafiltration on filter units Amicon Ultra-15 with a cutoff at 100 kDa (UFC910008; Merck Millipore, Billerica, MA, USA). Vector genomes were determined by quantitative PCR. Briefly, viral DNA was extracted from viral suspensions using the Chemagic Magnetic Separation Module I (PerkinElmer, Baesweiler, Germany) according to the manufacturer's instruction. DNA samples were diluted to 0.02 ng/µL. Quantitative PCRs using a standard curve were performed on the diluted virus-encoding plasmids. The primers and probe were designed to anneal to the GFP gene (each 5'-3'; forward primer: tgccactcaaccgaccaat, reverse primer: gggattcccacgggtgtt, FAM/TAMRA-labeled probe: aagctggtccttac tctagactcgctagggtcacgtctct). The measurements were performed in quadruplicate on ABI Prism 7900HT and the results analyzed using SDS software version 2.2.2 (Applied Biosystems,

Life Technologies, Zug, Switzerland). Virus preparation of scAAV2/8 resulted in approximately 200 μ L viral suspension at 2.5 × 10¹¹ genome copies/mL (gc/mL). The viral suspension of ssAAV2/8 contained 2.5 × 10¹² gc/mL.

Animals

All experimental procedures were approved by the Veterinary Authorities of Zurich and Oldenburg and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All mice used in this study were on a C57/Bl6J background and were wild-type littermates. Adult mice 2 to 4 months of age were used to investigate the potential of vitreous aspiration in improving AAV transduction of the retina. Transduction efficacy was evaluated 3 to 4 months post injections. Supplementary Figure S1 provides an overview of procedures used in this study.

Vitreous Aspiration and Intravitreal Injection

Mice were first anesthetized by intraperitoneal injection of midozolam (5 mg/kg), medetomidin (0.5 mg/kg), and fentanyl (0.05 mg/kg). Pupil dilation was achieved with topical application of tropicamide 1% for 1 minute followed by application of phenylephrine 2.5% for 1 minute. Animals were kept on a heating pad at 37°C and eyes were moisturized with Lacrinorm gel (Bausch & Lomb, Zug, Switzerland). Syringes were mounted on a M3301R micromanipulator (WPI, Hertfordshire, England). To place the eye in a proper orientation and have access to the desired injection site, the eyelid was pulled toward the forehead using a surgical suture while a second one was used to attach the sclera slightly above the superior limbus and pull the eye downward in order to expose the central region of the superior sclera (Supplementary Fig. S2B). Spring scissors with a cutting edge of 2.5 mm were used to cut and remove most of the scleral tissue from the injection site (Supplementary Fig. S2C). A hole was made 2 mm posterior to the superior limbus using a sterile 30-gauge (G) needle. The needle was inserted 1.5 mm into the vitreous (Supplementary Fig. S2D). Using a surgical microscope, the position of the needles within the eye was visualized (Supplementary Fig. S2A). The needle opening was oriented toward the temporal retina facing neither the optic nerve nor the lens. Part of the vitreous was aspirated by pulling the plunger of the syringe up to a maximum of 50 µL on the graduation scale prior to removing the needle from the eye, a procedure that in the following is denoted vitreous aspiration (Supplementary Fig. S2E). Whether this procedure leads to a removal of a small portion of the vitreous or results only in a vitreous tap is a matter for further investigations. Hemorrhages on the retina were rarely observed. We excluded animals with clear retinal hemorrhages from the study, but included those that showed mild bleeding around the injection site at the sclera. Through the previously made hole, a 33-G blunt-end needle (Hamilton, Bonaduz, Switzerland) mounted onto a 5-µL glass syringe (Hamilton) was inserted 1.5 mm into the eye (Supplementary Fig. S2F). Adeno-associated virus suspension or PBS (1.5 µL) was intravitreally injected into the eye. After vitreous aspiration, 12 eyes were injected using each of the two viruses (ssAAV2/8_CMV_GFP and scAAV2/8_mCMV_GFP). In addition, 66 eyes were injected using ssAAV2/8_CMV_GFP applying two previously published techniques to inject into the vitreous but without prior vitreous aspiration. Injections were performed at 0.1 μ L/s, and after the injection, the needle was left in the eye for 2 minutes in order to prevent reflux of the viral suspension. The needle and surgical sutures were gently removed from the eye and the animal was kept warm for 20 minutes prior to administration of antagonists of the

anesthetics (a mixture of atipamezole [0.75 mg/kg], flumazenil [0.2 mg/kg], and naloxone [0.12 mg/kg]). Detailed information about the equipment used during the procedure is provided in Supplementary Table S1.

Fundus Imaging and Optical Coherence Tomography (OCT)

Fundus photographs were taken 3 to 4 months post injections with a Micron III (Phoenix Research Labs, Pleasanton, CA, USA) system equipped with an excitation filter at 482 nm and an emission filter at 536 nm. In addition, OCT scans and corresponding retinal fundus images were taken using the Phoenix Image-Guided OCT (Phoenix Research Labs) and Micron IV, respectively. Images were captured post injection at indicated time points using Micron OCT and StreamPix 5 commercial software (Phoenix Research Labs). Animals were anesthetized with an intraperitoneal injection of ketamine (85 mg/kg) and xylazine (5 mg/kg) 20 minutes prior to fundus imaging. The distribution of the GFP signals of the central retina was measured utilizing ImageJ (http://rsb.info.nih.gov/ij/ index.html; in the public domain). Briefly, photographs centered on the optic disc were used; the photographs were converted into grayscale 8-bit images, and an intensity threshold was applied to select GFP-positive areas from background signals. The number of GFP-positive pixels was counted and the percentage of positive pixels in the image was calculated. Statistical analyses were performed using Prism 7 (GraphPad Software, La Jolla, CA, USA).

Immunostaining and Morphology

After fundus imaging, animals were killed and both eyes enucleated. Eyes were fixed overnight at 4°C in a 4% paraformaldehyde in PBS solution. Retinal pigment epithelium and other retinal cell layers were prepared from the fixed eyes. The RPE cell layer was washed three times in PBS and mounted on objective trays with Fluoromount (Sigma-Aldrich). Retinae were fixed with 4% paraformaldehyde for 2 additional hours at room temperature prior to permeabilization by three consecutive incubations (10 minutes each) in a 0.5% Triton X-100 PBS solution. Unspecific antibody binding was blocked by incubation of the retinae in a 0.05% Tween 20 and 0.5% bovine serum albumin PBS solution. Samples were then incubated with primary antibodies for either 2 hours at room temperature or 16 hours at 4°C. Retinal ganglion cells (RGCs) were immunostained using a 1:100 dilution of a goat polyclonal antibody against the ganglion cell-specific transcription factor Brn3a (Sc-31984; Santa Cruz Biotechnology, Dallas, TX, USA). Blue cone outer segments were immunostained using a 1:500 dilution of a goat polyclonal antibody against S-opsin (Sc-14363, Santa Cruz Biotechnology). Alexa 568- and Alexa 647-conjugated secondary antibodies (Life Technologies) were applied for 2 hours at room temperature. Secondary antibodies were used at a working dilution of 1:200. Samples were mounted on objective trays with Fluoromount (Sigma-Aldrich). Confocal images were taken using a TCS SP8 microscope (Leica, Wetzlar, Germany). Images were processed using either the LAS AF Lite software (Leica) or Imaris v7.6.5 (Bitplane, Zurich, Switzerland).

To evaluate the retinal morphology, we performed Nissl staining. Cryosections (20 μ m) were washed three times in 0.1 M phosphate buffer (PB) for 10 minutes followed by 20-minute incubation in Nissl staining solution. Cryosections were washed in water for 3 minutes. Slides were incubated in 70% acetic ethanol for 30 seconds, followed by 95% acetic ethanol for 30 seconds and isopropanol for 3 minutes. Slides were cleared in xylol for 3 minutes. Stained cryosections were embedded in Eukitt (Sigma-Aldrich).

For further morphologic analysis, eyes were enucleated and fixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2, 0.1 M) according to the previously described procedure.⁴¹ Nasal and temporal halves were separated by cutting through the optic nerve head and embedded in epon plastic. Semithin cross sections (0.5 μ m) were counterstained with toluidine blue and analyzed by light microscopy (Axioplan; Zeiss, Jena, Germany).

Tissue Fixation and Cryosectioning

For eye cup preparations, mice were killed by cervical dislocation and eyes were enucleated and put into physiological PBS (pH 7.4) solution. Eyes were fixed in 2% paraformaldehyde (Carl Roth, Karlsruhe, Germany) for 2 to 5 minutes. Cornea, lens, and vitreous were removed and eve cups were fixed for 60 minutes in 2% paraformaldehyde in 0.1 M PB. After several washing steps in PB, eye cups were cryoprotected in PB containing 30% sucrose either overnight or for 2 hours at 4°C and embedded in cryoblock Surgipath FSC 22 Clear Compound (Leica). The central part of the eye cup was cut into 20-µm thin slices (vertical sections) on a Leica CM1860 cryotome and stored at -20°C. GFP and Enhanced green fluorescent protein and 4',6-diamidino-2-phenylindole (DAPI) fluorescence were visualized by fluorescence microscopy (Leica DM6B) or confocal microscopy (TCS-SL Confocal Microscope, Leica).

In Vivo Electroretinography

Electroretinograms (ERGs) were recorded from anesthetized noninjected (n = 6) and PBS-injected (n = 5) wild-type mice 4 and 15 weeks after injection. Animals were dark adapted for at least 12 hours, followed by procedures performed under dim red light (>620 nm), as previously described.⁴² Electroretinograms represent the recordings of averaged responses to a sequence of white full-field flashes (5 ms, band pass filtered 1-1000 Hz), ranging from 0.4 Hz (20 flashes) for low intensities to 0.066 Hz (5 flashes) for the brightest intensities (Ganzfeld ERG; Roland Consult, Brandenburg a.d. Havel, Germany). Scotopic ERGs comprised 10 light intensities, ranging from -3.5 to 1 log cds/m². Photopic ERGs were recorded in the presence of bright background light (25 cds/m²) comprising five light intensities, ranging from -1 to $1 \log cds/m^2$. Data analysis was performed using Chart v5.5 (AD Instruments, Hastings, UK). Data were evaluated for statistical differences using 2-way analysis of variance (ANOVA) in Prism 5 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Fundus Images

The transduction efficacy of AAV2/8-GFP was evaluated from 90 intravitreal injections. Twenty-four eyes were injected with prior aspiration of the vitreous, whereas 66 eyes were injected without prior vitreous aspiration. Fundus images of the central retina were taken from all eyes. The retinal area that showed GFP expression was calculated for each image. Representative fundus photographs under bright-light illumination or applying a GFP filter are presented in Figure 1. Phosphate-buffered saline injections after vitreous aspiration served as controls, and these eyes were compared to eyes injected with GFP-expressing ssAAV2/8-GFP or scAAV2/8-GFP.

Without vitreous aspiration prior to the intravitreal injection, eyes presented with only two or three smaller GFPpositive areas, including cells around the injection site and cells in close vicinity to the retinal vasculature at the optic nerve. Occasionally, this injection technique resulted in an



FIGURE 1. Fundoscopy and measurement of transduced retinal area. Fundus images of an eye that received a vitreous aspiration and intravitreal PBS injection are presented in (**A**, **E**). Representative fundus pictures of an eye intravitreally injected with ssAAV2/8_CMV_GFP without prior vitreous aspiration are shown in (**B**, **F**), with prior pressure applied around the injection site (per Chiu et al.⁴³) in (**C**, **G**), and with prior vitreous aspiration in (**D**, **H**). (**A**-**D**, *upper*) show images taken under bright-light illumination. Images in (**E**, **H**) show the GFP signal only. Graph in (**I**) compares the distribution of GFP signals in eyes injected without or with prior vitreous aspiration (vitrectomy). *Horizontal bars* indicate the distributions' medians. Graph in (**J**) shows the probability of obtaining a GFP coverage above 70%, between 30% and 70%, between 10% and 30%, and below 10%.

additional GFP-positive area at the estimated site of delivery of the viral suspension (Supplementary Figs. S3C, S3D).

We attempted to improve the transduction efficacy of the standard intravitreal injections and applied a procedure to mice that was originally described by Chiu et al.⁴³ for rats. We inserted a 30-G needle into the vitreous and removed the needle without aspirating vitreous material from the eye. Subsequently, a gentle pressure was applied to the eye and close to the injection site prior to injecting the viral suspension.⁴³ As presented in Figure 1, the mechanical stress during insertion of the 30-G needle and/or the pressure applied to the eye enlarged the area covered by GFP, especially around the injection site.

These results encouraged us to develop a novel technique that robustly improves the transduction efficacies of intravitreal injections of AAV2/8. We used a 30-G needle to perform an aspiration of the vitreous prior to intravitreal injections of AAV particles. The plunger of the attached syringe was pulled to the 50-µL mark to aspirate vitreous tissue shortly before removal of the needle. In the following, this procedure is referred to as "vitreous aspiration" in mice. To verify the integrity of the retina after vitreous aspiration, we used a high-resolution operation binocular, fundoscopy with the Micron III/IV, and OCT measurements. After 2 to 4 month post injection, we did not find indications that this procedure leads to retinal alterations or detachment. Nissl stainings of cryosections suggested a well-preserved retinal morphology (Supplementary Fig. S4). Although many retinas presented without clear retinal alterations at days 1 and 3 post injection, retinas might also show detached parts that reattached after 3 to 7 days (for a representative example see Supplementary Fig. S5). Morphologic evaluation of these eyes showed well-preserved retinal structures after 14 days (Supplementary Figs. S5A, S5B). Occasionally, mice presented with detachments of large retinal areas that seemed to result in retinal remodeling as shown in Supplementary Figures S5C and S5D. Consequently, we suggest evaluating the outcome of the vitreous aspiration and intravitreal injection by funduscopy and/or OCT at day 3 post injection and considering exclusion of animals with strong retinal detachments. A more frequent side effect of the novel injection procedure was the occurrence of cataracts in approximately one-fourth of the eyes (a representative example is shown in Supplementary Fig. S4).

The GFP-positive retinal areas in eyes that received the vitreous aspiration in combination with intravitreal injection were subjectively larger compared to those with the other techniques tested (Figs. 1A-H). We quantified this observation and compared transduced retinal areas between eyes that received a vitreous aspiration and eyes intravitreally injected without prior aspiration of the vitreous (Fig. 1I). A Mann-Whitney test (P = 0.0009, confidence level of 99%) confirmed that the mean of the retinal area expressing GFP after transductions using the novel injection technique (M =55.54%) was significantly higher than in eyes without the vitreous aspiration (M = 11.7%). Without aspiration of the vitreous, 50% of the eyes had less than 5% of the central retinal area transduced. Only 1 eye out of 66 presented with a transduction over 70%. In contrast, the majority of the eyes with vitreous aspiration (14 out of 24) showed GFP expression in more than 70% of the retinal area (Fig. 1J). The probability of obtaining a transduction over 70% of the retina is significantly increased by the novel injection technique as demonstrated by



FIGURE 2. GFP expression within retinal layers of eyes after vitreous aspiration and intravitreal injection. Pictures were taken from a representative retina. The corresponding eye was injected with 1.5 μ L ssAAV2/8_CMV_GFP at 2.5 × 10¹² gc/mL. Confocal images of the GFP expression were obtained from the retinal ganglion cell and nerve fiber layer (GCL+NF [**A**, **F**]), in the inner nuclear layer (INL [**B**, **G**]), in the outer nuclear layer (ONL [**C**, **H**]), at the photoreceptor outer and inner segments (OS+IS [**D**, **I**]), and in the retinal pigment epithelium (RPE [**E**, **J**]). A scan through the entire retinal thickness showing all retinal layers transduced within the same area is presented in (**K**). A representative fluorescence profile along the retinal thickness is presented in (**L**). GFP-positive axons leaving Brn3a-positive cells were detected (**M**). Projection of the ganglion cell axons could be documented on a retinal flat mount that was poorly transduced in the central retinal (**N**). The optic nerve of GFP-positive retinae showed prominent GFP-positive fibers (**O**). *Green*: GFP; *red*: Brn3a; *magenta*: S-opsin. Of note, the fundus of the presented eye is shown in Supplementary Figure S6, *upper*.

Fisher's exact test (P < 0.0001). Eyes with fluorescent signals exceeding 70% in the central retina were also largely transduced in the inferior, superior, nasal, and temporal retina (Supplementary Figs. 6A-E). Although not directly comparable due to different promoters and viral titers, the ssAAV2/8-GFP and scAAV2/8-GFP did not show obvious differences in the distribution of GFP signals across fundus images (Supplementary Figs. 6F-J).

GFP Expression Throughout the Retinal Layers

We asked the question whether vitreous aspiration prior to AAV2/8 injection leads to transduction of several retinal layers. Indeed, fluorescence scans documented that all retinal layers showed GFP-positive cells (Fig. 2), suggesting that the viral particles were able to penetrate throughout the retina. In the photoreceptor layer, the GFP fluorescence was approximately four times higher than in other retinal layers (Fig. 2L). Immunohistochemistry of retinal flat mounts using the RGC marker Brn3a showed costaining with GFP-positive cells, suggesting that RGCs were successfully transduced (Figs. 2A, 2F). Dendrites and axons leaving the ganglion cell body were found to be transduced (Figs. 2M, 2N) and GFP-positive axons were detected in optic nerve preparations (Fig. 2O), further

confirming that the RGCs were infected by the AAV2/8-GFP viruses. Additionally, cells in the inner nuclear layer (INL), as well as the inner plexiform layer (IPL) and outer plexiform layer (OPL), were fluorescently labeled. In areas of retinal GFP expression, RPE cells were also transduced.

Two months after applying the novel injection technique, analysis of cryosections of mouse eyes confirmed that most of the photoreceptor and RPE cells were positively labeled by GFP (Fig. 3). The preparations showed weaker GFP signals in several other cell layers of the retina. As supported by confocal microscopy (Figs. 3D, 3E), the strongest signals were seen in the photoreceptor and RPE layers. These results confirm the findings shown in Figure 2.

ERG Measurements of Eyes Following Vitreous Aspiration

We aimed to evaluate whether the combination of vitreous aspiration and intravitreal injection leads to physiological alteration in the retina. Analyses of electroretinograms of eyes that received a vitreous aspiration were followed by PBS injections and compared to ERGs from untreated wild-type littermates (Supplementary Fig. S7). Electroretinograms were recorded 4 and 15 weeks after vitreous aspiration and injection.



FIGURE 3. AAV8-mediated GFP immunofluorescence in the retina after vitreous aspiration and intravitreal injection into the mouse eye. The cryosection (**A**) and fundus image (**B**) illustrate the retina-wide GFP expression. *Scale bar*: 200 µm. An enlargement of the fluorescence signals of the area marked by an *arrowhead* is shown in (**C**). Confocal images are shown in (**D**, **E**). As expected for AAV2/8, mostly RPE and photoreceptors were transduced, whereas other cell layers show less GFP expression. *Scale bar*: 50 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

The novel injection procedure did not cause striking differences in physiological properties of the retina. Only at 4 weeks after injection, the highest-intensity flash of the scotopic a-wave showed a significant alteration, an observation that was lost after an additional 11 weeks (Supplementary Fig. S3). All other measurements showed no significant alteration at all light intensities and time points. Thus, the ERG measurements support that few or no physiological differences were induced to the retina applying the novel injection procedure described herein.

In conclusion, we developed a novel technique to apply AAVs intravitreally, which increases the probability of transducing the central and peripheral retina to above 70%. Furthermore, the technique allows the transduction of all retinal layers from the ganglion cell layer (GCL) to RPE.

DISCUSSION

Adeno-associated virus-mediated basic science applications and gene therapies to treat retinal/vitreoretinal disorders would benefit from increased transduction efficacies of viral particles across the retina. Herein, we show that intravitreal AAV2/8 administration had the potential to transduce the entire retina when a single injection was combined with vitreous aspiration.

Since previously published intravitreal injection procedures using AAV2/8 less efficiently transduced retinal cells than AAV2/2 and mainly targeted retinal ganglion and Müller glia cells, subretinal administration was more commonly applied for AAV2/8.^{29,33,34} Nevertheless, recent studies have drawn attention to disadvantages of subretinal injections. Electroretinogram measurements performed on mice 3 months post injection revealed that the amplitude of both a- and b-waves was significantly decreased compared to that in fellow control eyes. The decrease was persistent after 12 months. In contrast, ERGs of eyes injected in an intravitreal manner were not affected.³⁴ Structural changes that explain the reduced ERG amplitudes have been characterized in retinal detachment models.44 Upon detachment of retinal photoreceptors from the RPE, cone and rod outer segments were shortened. Opsins no longer specifically localized to the outer segments but were detected in the inner segments of photoreceptor cells. Most retinal cell types were shown to undergo remodeling after retinal detachment. In addition, connectivity within the retina was also affected. Neurite projections of both ON and OFF bipolar cells no longer located to their target layers.⁴⁵ Although our analysis only occasionally indicates such strong retinal alterations after the combination of vitreous aspiration and intravitreal injections, we cannot completely exclude that the new injection technique described herein leads to a peripheral retinal detachment. Future experiments will help to evaluate this possibility. The majority of eyes showed a well-preserved retinal morphology several weeks after the application of the novel technique. Electroretinogram measurements suggested that the new method does not lead to a persistent reduction of functional properties in the retina and thus supported the notion that frequent retinal remodeling due to the vitreous aspiration was unlikely (Supplementary Fig. S7). It will be a matter for future investigations to further optimize the combination of vitreous aspiration and injection to largely avoid side effects.

Intravitreal injection typically would not require retinal detachment and thus is considered to preserve retinal structures. Avoiding shear forces and increased pressure (e.g., due to subretinal injections) might be relevant to retinae affected with dystrophies and structural alterations. Nevertheless, preclinical trials for the treatment of Leber's congenital amaurosis (LCA) successfully improved visual function in animal models treated via subretinal injection.² So far, two cell types have been efficiently targeted by AAVs delivered into the vitreal space: RGCs and Müller cells.^{25,35,36} Dalkara et al.⁴⁶ identified the inner limiting membrane (ILM) as a major barrier to the diffusion of AAV into the outer retina. The ILM is rich in

polysaccharides and proteins, such as laminin, that may bind to the AAV capsids, hence preventing their diffusion toward the outer retina.^{47,48} Retinal ganglion cells and Müller cells, which are in contact with the ILM, were more efficiently transduced with intravitreal injections of AAV2/8. Using pronase as adjuvant during intravitreal injection of AAVs improved transduction efficacy of the outer retina by degrading laminins of the ILM. However, the safety of pronase applications to patients is uncertain. These studies encouraged us to try a different procedure that aimed at improving transduction efficacies via a mechanical aspiration of the vitreous during the surgical intervention.

Since the naturally occurring AAV2/8 and other serotypes showed limited capacity in transducing photoreceptors from the vitreal space in mice,46 several studies have successfully modified the AAV capsid proteins in order to change tropism and/or improve transduction efficacy.37-39 The AAV capsid variant AAV7m8 showed the capacity to perform panretinal transduction of photoreceptors from the vitreous. Thus, our study complements the development of capsid mutants toward panretinal transduction of all retinal layers with naturally occurring AAV2/8. Future studies will show whether the combination of our technique and efficient capsid mutants will further improve the transduction efficacy of AAVs. We speculate that such combinations of gene therapeutic approaches may help to correct gene defects in preclinical animal models. Furthermore, basic science approaches to modify functions of cells within the retina (e.g., clustered regularly interspaced short palindromic repeats [CRISPR]/Cas9 applications) or alterations of retinal properties (e.g., using growth factors applied to the vitreous) might benefit from the novel technique.

The reason for the increase in transduction efficacies as observed with the new injection technique is not fully understood. We hypothesize that vitreous aspiration removes three obstacles that influence transduction efficacy after intravitreal injection. First, the vitreous applies strong pressure to viral suspensions placed intravitreally and forces the suspensions to exit the eye through the injection site. Vitreous aspiration favors the interaction of viral particles with the retinal tissues and improves the diffusion of the particles toward the outer retina. Second, the ILM, previously shown to bind viral particles, may be destabilized by vitreous aspiration.^{46,47} Pores may be created in the ILM, allowing viral particles to diffuse through the retina. Third, it has been shown that the vitreous contains neutralizing antibodies acting against AAV capsids.⁴⁹ Therefore, aspiration of the vitreous might prevent or reduce antibody-mediated neutralization of AAV vectors. A schematic drawing illustrating improved diffusion of the viral particles into the retina after vitreous aspiration is shown in Figure 4.

Although vitreous aspiration performed here differs from vitrectomy in clinical settings, a combination of vitrectomy and/or ILM peeling might enhance the therapeutic effect of AAV-based gene therapies in larger animal model or even in patients. Vitrectomy is frequently performed in patients suffering from prominent vitreous floaters, vitreous hemorrhage, and diabetic retinopathy.50 Inner limiting membrane peeling in combination with vitrectomy has also been established for epiretinal membrane and macular hole surgery.⁵¹⁻⁵³ Due to anatomic differences, the combination of vitrectomy and ILM peeling as performed in humans is not applicable to the mouse eye. Studies in larger animal models are required in order to test the potential of vitrectomy/ vitreous aspiration and/or ILM peeling in improving AAV transduction in patients receiving ocular gene therapy. Interspecies differences need to be considered. Another vitrectomy technique, based on those applied to patients, has already been tested in rats and showed an improved



FIGURE 4. Schematic model of the diffusion of AAV particles in eyes after vitreous aspiration and intravitreal injection. In (**A**), AAV particles either reflux from the vitreous (*red arrow*) due to the intravitreal pressure (P1) or bind to the ILM, therefore preventing the transduction of the outer retina. The vitreous aspiration depicted in (**B**) reduces the intravitreal pressure to P2, which is now lower than the outside pressure P0, preventing the reflux of virus particles and supporting the distribution of the virus within the vitreous. In our model shown in (C), vitreous aspiration creates pores in the ILM, allowing free diffusion of AAV particles to the outer retina.

transduction of Müller cells using adenoviral vectors.²⁴ In contrast, posterior vitrectomy and posterior hyaloid membrane peeling, in combination with transductions using capsid mutant AAV2 particles, recently were applied to a canine model but failed to increase GFP signals in the retina.⁵⁴ Boyd et al.⁵⁴ observed an inflammatory response possibly against the GFP transgene and a reduced level of GFP-positive cells following vitrectomy. As our vitreous aspiration is different from vitrectomy in larger animal models and humans, it is difficult to compare these studies directly. Our procedure might be best described as a vitreous aspiration or vitreous tap that did not result in the removal of a significant portion of the vitreous.

In conclusion, we describe a novel vitreous aspiration and intravitreal injection technique that leads to widespread AAV transduction of the mouse retina. The technique is suitable for testing on AAV-based ocular gene therapies and basic science questions in mouse models and may help to develop improved intravitreal injection techniques to treat large animal models

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