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Gene delivery to the eye using adeno-associated viral vectors

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Abstract

Adeno-associated virus (AAV) vectors provide a useful way to deliver genes to the eye. They have a number of important properties which make them suitable for this purpose, not least their lack of significant pathogenicity and the potential for long-term transfection of retinal cells. The optimal methods for AAV-mediated gene delivery are determined by the location and characteristics of the target cell type. Efficient gene delivery to photoreceptors and pigment epithelial cells following subretinal injection of AAV has been achieved in various animal models. AAV-mediated gene therapy has been shown to slow photoreceptor loss in rodent models of primary photoreceptor diseases and in dogs with a naturally occurring disease similar to human Leber's congenital amaurosis (LCA). Efficient gene delivery to other cell types such as retinal ganglion cells (RGCs), however, has been more problematic. In this article, we review the potential uses of AAV-mediated gene delivery to the eye. We describe the selection of an appropriate AAV vector for ocular gene transfer studies and discuss the techniques used to deliver the virus to the eye and to assess ocular transfection. We emphasize our techniques for successful gene transfer to retinal ganglion cells, which have often proven challenging to transfect with high efficiency. Using a modified AAV incorporating a chicken β-actin (CBA) promoter and the woodchuck hepatitis posttranscriptional regulatory element, we describe how our techniques allow approximately 85% of rat retinal ganglion cells to be transfected within 2 weeks of a single intravitreal virus injection. Our techniques facilitate the study of the pathogenesis of RGC diseases such as glaucoma and the development of novel new treatments based on gene therapy.

Keywords: Adeno-associated virus; Retinal ganglion cell; Gene therapy; Glaucoma; Eye; Green fluorescent protein; Woodchuck hepatitis posttranscriptional regulatory element

1. Introduction

The potential for gene delivery to the eye using adeno-associated virus (AAV) vectors has received much recent attention. Gene transfer experiments in animal models allow vision researchers to study the mechanisms of retinal degenerative diseases and to explore possible new treatments for ocular diseases using gene therapy techniques. Recombinant AAV vectors have a number of important advantages over other vectors which make them suitable for such studies, in particular a relative lack of pathogenicity and their ability to induce long-term transgene expression in the eye [1–3]. AAV vectors can be used to transfect a variety of ocular cell types including photoreceptors [4–6], retinal pigment epithelial cells [4,7,8], Muller cells [9], retinal ganglion cells (RGCs) [2,10], trabecular meshwork cells, and corneal endothelial cells [11].

There are two main approaches by which therapeutic AAV-mediated gene transfer might be useful in the context of ocular disease. First, AAV-mediated gene therapy has the potential to correct the specific gene defect in conditions where the defect is well understood. Correction of an ocular genetic defect requires gene delivery directly to the defective cells and has been successfully used to slow photoreceptor loss in several rodent models of primary photoreceptor disease [6,12–14]. As an example, AAV-mediated transgenes have recently been shown to restore photoreceptor structure and function in retinal degeneration slow (*rds*) mice [15]. *rds* mice have a mutation in the *Prph2* gene, coding for a photoreceptor-specific membrane glycoprotein called peripherin-2, which causes development of photoreceptor disks and causes the outer segments to fail. Mutations in *Prph2* have also been demonstrated in

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retinitis pigmentosa, a human disease which causes progressive visual loss. *rds* mice injected subretinally with AAV–*Prph2* developed new outer segment structures containing rhodopsin and looking ultrastructurally similar to normal rod photoreceptor outer segments. Electroretinography of treated animals showed dramatic improvements in physiological measures of photoreceptor function in the short term [15], although follow-up studies showed that photoreceptor death continued despite transient restoration of function [14].

AAV-mediated gene replacement has also been used recently to restore visual function in a dog model of Leber's congenital amaurosis (LCA), a retinal degeneration that can cause severe childhood visual loss [16]. The gene defect in the naturally occurring dog model, a mutation in the *RPE65* gene which codes for a retinal pigment epithelium (RPE) cell membrane-associated protein involved in retinoid metabolism, also occurs in human LCA. An AAV carrying wild-type *RPE65* was able to restore vision as assessed by electroretinography, pupillometry, and psychophysical and behavioral tests.

For many ocular conditions, however, no specific genetic defect has been characterized. It is likely that many of these diseases will turn out to involve pathology more complex than a well-characterized mutation in a single gene. Glaucoma, the second leading cause of blindness in the world [17], is an example of an ocular disease which is likely to involve the interaction of multiple genetic and environmental factors and as such is unlikely to be "cured" by the replacement of a single gene. Yet, in such circumstances, a second strategy for gene therapy may be useful. This involves not replacing a defective gene, but using gene transfer to reduce loss of function by ameliorating the effect of the primary defect(s). As examples of this approach, AAV-mediated transfection of retinal cells with the gene for basic fibroblast growth factor (FGF-2), glial cell line-derived neurotrophic factor, and ciliary neurotrophic factor have been demonstrated to slow photoreceptor loss in rat models of retinitis pigmentosa [9,12,13].

Recent successes with AAV vectors in animal models mean that human clinical trials of AAV-mediated gene therapy for some severe photoreceptor degenerative diseases are already being planned. However, success with disease models involving cells other than photoreceptors and RPE cells has been much more limited. Optimization of techniques to target appropriate genes to appropriate retinal cells therefore remains an important goal for the future development of ocular gene transfer technology. Here, we describe the selection of an appropriate AAV vector for ocular gene transfer studies and discuss the techniques used to deliver the virus to the eye and to assess ocular transfection. We emphasize the techniques for successful gene transfer to RGC, which have often proven challenging to transfect with high efficiency.

2. Factors influencing ocular transfection by AAV

The efficiency of transfection of particular cell types in the eye is determined by a number of variables including the site of injection, the AAV serotype and titer, the amount of passenger DNA, and the specific gene promoters and enhancing elements used.

2.1. Injection site

Transduction of RPE cells and photoreceptors is most efficiently achieved by subretinal injection of AAV. Indeed, subretinal injection provides an almost ideal route for delivery of AAV to these cells. The subretinal space has a relatively high degree of immunoprivilege [18] and typically very little evidence of inflammation is seen in the vicinity of the injection site. Subretinal injection induces a bleb of concentrated virus in intimate contact with photoreceptors and RPE cells without the need for perforation of the neuroretina. The injected subretinal fluid is gradually and completely resorbed by the pumping mechanisms of the RPE, restoring the normal anatomical relationship between the photoreceptors and the RPE. The exact technique for successful subretinal injection varies for different species, but in all cases the goal is to minimize damage to ocular structures such as the lens and vasculature while avoiding leakage from the injection site.

Intravitreal delivery of AAV can be used to transfect Muller cells and RGC, as described in detail below, in addition to anterior segment cells. Theoretically, the potential for a host immune response to AAV is greater following intravitreal injection compared to subretinal virus injection. Despite the lack of a significant local inflammatory response, both subretinal and intravitreal injection of AAV have been shown to induce a small but significant increase in AAV-specific antibody responses systemically. A strong systemic antibody response to green fluorescent protein (GFP) has also been reported in dogs following intravitreal administration of AAV–GFP [1]. However, additional transduction events do occur following repeated subretinal injections of AAV despite the systemic immune response [18]; so multiple intraocular deliveries of AAV may still convey additional benefits.

2.2. Virus subtypes

Although AAV serotype 2 (AAV2) has been most extensively studied as a potential vector, there are currently six known serotypes of AAV [19]. Different AAV serotypes have different virion shell proteins and, as a consequence, vary in their ability to bind to and transfect different host cell types. Since optimized promoters and regulatory elements are worthless unless an AAV binds to a target cell and is internalized, it is becoming

apparent that viral capsid differences may be crucial determinants of AAV expression efficiency and tropism. Recently, the influence of AAV1 to 5 virion shells on AAV2 transgene transduction has been studied by cross-packaging of a single AAV2 vector genome into multiple AAV serotypes [19]. This work has demonstrated that AAV capsid protein seroype has a dramatic effect on the transduction of different cell types. In the retina, expression from AAV4 and AAV5 was shown to be far more efficient than AAV serotypes 1–3. AAV1 appeared to be a particularly efficient transducer of nonneuronal cells. It is therefore apparent that optimal transfection of particular retinal cell types should involve careful selection of AAV capsid serotype, the route of delivery, and the design of the transfecting plamid.

2.3. Virus titer

Virus titer is an important determinant of efficiency of transfection. The relationship between AAV titer and transgene expression in cerebral neurons has been clearly established, with expression demonstrated to increase in a dose-dependendent manner over a 3.3-log range of viral concentration [20]. Following subretinal injections of AAV in mice, there is a close relationship between the AAV titer and the efficiency and speed of onset of photoreceptor transduction [21]. Using an AAV carrying a gene encoding enhanced GFP driven by a cytomegalovirus (CMV) promoter at three different titers, Sarra and co-workers [22] have shown substantial differences in transgene expression over time. Both the lower titers used in their study (2×10^6 and 2×10^8 infective U/ml) reached a maximum transduction rate of 15% of photoreceptors compared to 85% transduction by the highest-titer virus preparation (2×10^{10} infective U/ml). The onset of photoreceptor transduction was also much faster (10% at 7 days, 35% at 14 days, 75% at 28 days, and 85% at 84 days for the highest titer compared to a slow increase to 15% transduction over 84 days for the lowest titer). It seems likely that high viral titers will also aid transfection of other cell types within the retina and it is therefore important to maximize the viral titer to improve the efficiency of ocular gene transfer. Novel plasmids incorporating all necessary AAV packaging functions and adenovirus helper functions in a single plasmid facilitate the production of consistent titers of rAAV without wild-type contamination from either adenovirus or AAV [22,23]. Techniques to purify and concentrate recominant AAV continue to evolve and improve, with the result that very high titers can now be achieved.

2.4. Packaging size limitations

One of the limitations of AAV as a vector is the relatively small amount of passenger DNA that can be incorporated. Although genes up to 6.0 kb have been packaged into AAV, these oversized viruses were not infectious [24]. The usual packaging limit for AAV appears to be 5.1–5.3 kb [24,25], although this limit may not be absolute as some genes seem to package more easily than others. Constructs of up to 5.7 kb which still showed acceptable packaging and transduction efficiency have been made [26].

2.5. Promoter sequences

Selection of appropriate transgene promoter sequences is important in determining the efficiency and cell specificity of retinal transduction by AAV. Several promoter sequences have shown efficacy for ocular transduction. The CMV promoter can drive expression in multiple retinal cell types including Muller cells, vascular endothelial cells, and retinal neurons [2,8,12,27,28]. Transduction of RGC does occur with the CMV promoter, but in many studies, the proportion of RGC transfected is relatively low or unquantified. Several cell-specific promoters can be used in the eye, with the opsin promoter particularly effective at driving transgene expression in photoreceptors [5]. Another commonly used ocular gene promoter is chicken β-actin (CBA) [16,29]. We have found that AAV vectors incorporating hybrid CMV/CBA promoters give more efficient transduction of retinal neurons, particularly RGC, compared to AAV with CMV alone (K.R.G. Martin, 2001, unpublished observations). Other neuron-specific promoters include platelet-derived growth factor (PDGF) and neuron-specific enolase (NSE), although studies in the brain have found neuronal transduction with AAV–NSE to be less efficient than with AAV–CBA [20]. Neither NSE nor PDGF has been extensively investigated in the retina.

Given the cell-specific nature of the pathological defects in many retinal diseases, controlling the cell specificity of retinal transfection is an important goal for the future. For effective virus targeting, AAV capsid properties and promoter sequences are both important. Yet, as an example, there are currently no known promoters that can drive transgene expression preferentially in RPE cells, compared to photoreceptors. Development of optimal promoters for each cell type of interest should help improve transgene targeting.

2.6. Posttranscriptional regulation

Successful retinal transduction requires not just effective gene delivery to target cells, but also efficient translation of transfecting genes into functional protein within the cells. It is known that the presence of introns associated with a gene of interest can increase translation [30]. Some wild-type viruses exploit a similar effect to increase production of viral protein in host cells. An

example is the woodchuck hepatitis virus which uses a posttranscriptional regulatory element (WPRE) to increase viral protein translation [31]. WPRE is known to be important for high-level expression of native mRNA transcripts, acting to enhance mRNA processing and gene transport [32]. Incorporation of WPRE into AAV has been shown to improve GFP expression in cerebral neurons [33], and we have recently found that the expression of GFP by RGC can also be improved dramatically by WPRE [34].

3. Description of the method

3.1. Intravitreal injection technique

In our studies, we have used intravitreal injections of AAV vectors to transfect rat RGCs. All animals are treated in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research using protocols approved and monitored by the Animal Care Committee of the Johns Hopkins University School of Medicine. Adult Wistar rats (375–425 g) are anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg) and topical 1% proparacaine eyedrops. Pupillary dilatation is acheved with 1% tropicamide and 2.5% phenylephrine eyedrops. Using an operating microscope, a superotemporal conjunctival incision large enough to expose the sclera posterior to the lens is performed. A partial-thickness scleral pilot hole is made with a 30-G needle to facilitate penetration of the underlying sclera, choroid, and retina by a fine glass micropipette with a tip diameter of 30 μm and a tip length of 2.5 mm. We have found glass micropipettes to be preferable to metal needles because the tip diameter is smaller, the depth of injection is easier to standardize, and the tapered pipette tip effectively seals the injection site during injection, reducing leakage of the injected fluid from the eye. The micropipette is connected to a 5-μl glass syringe (Hamilton, Reno, NV) by polyethylene tubing prefilled with light mineral oil (Sigma, St. Louis, MO) prior to drawing up the virus stock. The rat lens occupies a large proportion of the intraocular volume and care must be taken to avoid penetration of the lens during intravitreal injection. It is also important to avoid damage to the vortex vein which is located lateral to the superior rectus muscle. The injection site that we find most effective is located about 3 mm posterior to the superotemporal limbus. We retract the superior margin of the lateral rectus muscle so that the muscle will help to tamponade the injection site at the end of the procedure, although we have found that an oblique and posteriorly directed intravitreal injection with a fine glass micropipette has little tendency to leak. Injections are given slowly over 1 min to allow diffusion of the virus stock. After each injection, the injection site is visualized with a standard indirect ophthalmoscope using a 90-diopter condensing lens (Volk Optical, Mentor, OH). The presence of significant hemorrhage, retinal detachment, or lens touch are noted, although these complications are rare. The whole procedure takes about 2 min per animal.

3.2. Subretinal injection technique

The technique for subretinal injection in the rat is similar to that described above for intravitreal injection. However, we have found that a shorter glass micropipette (about 1.5 mm) works more effectively. After formation of the partial-thickness scleral pilot hole, the glass micropipette is directed more obliquely than for intravitreal injection, into the space between the RPE and the neuroretina. Correct cannula placement can be verified by observation of a bullous retinal detachment following subretinal injection without evidence of a retinal hole or tear. Injection of a volume of 2–3 μl is usually sufficient to cause a subretinal bleb involving 30–40% of the fundus.

3.3. In vivo visualization of transduction

In animals transduced with GFP, particularly when photoreceptors and RPE cells are transduced, it is possible to visualize green fluoresence in vivo as a measure of ocular transduction efficiency in a number of species including rat [35,36], mouse [37], and monkey [27]. As GFP has absorption–emission characteristics similar to those of sodium fluorescein, efficiency and duration of GFP gene expression can be followed using the same photographic equipment as is widely used for fluorescein angiography in a clinical setting [36]. Using such techniques, AAV-mediated GFP expression was visible by 2 weeks after subretinal virus injection in the rat, increased between 2 and 8 weeks, and fell slightly between 8 and 16 weeks [36].

3.4. Preparation of tissues and quantification of retinal ganglion cell transduction

For AAV incorporating GFP, we visualize transfected RGC using fluorescent microscopy on flat retinal whole mounts or histological sections of the optic cup. Good retinal transduction is achieved by 2 weeks after intravitreal AAV injection. We find that preservation of retinal integrity is improved by intracardiac perfusion of animals with 4% paraformaldehyde, although this is not essential. GFP fluorescence appears to be unaffected by paraformaldehyde and glutaraldehyde fixation, but methanol fixation and regimens that involve reducing conditions should be avoided [38]. Retinal whole mounts are prepared after enucleation of the globe by removing the anterior segment with a blade and care-

fully transferring the whole retina to a microscope slide. Four relieving incisions are made to allow the retina to be flattened. The retinas are coverslipped with Vectashield (Vector Laboratories, Burlingame, CA) to reduce the quenching of GFP fluorescence and sealed with rubber cement. GFP fluorescence persists for at least 4 weeks if the slides are stored in the dark at 4 °C. If free-floating tissue is stored in antifreeze solution at −20 °C, GFP fluorescence and antigen detection can be preserved almost indefinitely. GFP fluorescence can be visualized using a specific GFP filter set (Omega Optical, Burlington, VA) fitted to a Zeiss Axioskop (Zeiss, Thornwood, NY). A standard fluorecein isothiocyanate (FITC) or FITC long-pass filter set may also be used. Indeed, for the form of GFP that we use [39], we actually find that a FITC long-pass filter gives a better signal-to-noise ratio, critical for detection of low levels of expression. This is because autofluorescence appears golden yellow under FITC which is easier to distinguish from GFP than the greener autofluorescence seen under a specific GFP filter set. However, FITC filters differ between different manufacturers, so the filter characteristics need to be carefully checked.

We calculate the density of GFP positive cells in digital images of 10 randomly sampled 40× whole mount fields under fluorescence microscopy, representing a 2.5% sample of the total retinal area. Measurement of retinal whole mount area allows estimation of the total number of transfected cells per retina. We count the number of axons in optic nerve cross sections using a standardized method that we have reported in detail elsewhere [40] to give an estimate of the total number of RGC. Hence, we can estimate the proportion of RGC transfected. The number of RGC transfected can also be calculated as a proportion of the number of cells retrogradely labeled following fluorogold injection to the superior colliculus—this technique consistently produces a slightly higher estimate of the total number of RGC. We have found that the efficiency of transfection of RGC following intravitreal AAV injection is dramatically improved by incorporation of the woodchuck hepatitis posttranscriptional regulatory element in the viral construct [31,33]. Using the above technique with an AAV containing GFP under the control of a CBA promoter and incorporating WPRE (AAV–CBA–GFP–WPRE), we have been able to achieve transfection densities of 1828 ± 299 cells/mm^2 ($72{,}273 \pm 11{,}814$ cells/retina). This represents transfection of approximately $84.5 \pm 13.8\%$ of the total number of RGC as estimated by axon counting in optic nerve cross sections or $74.7 \pm 12.2\%$ of the number of back-labeled RGC counted after fluorogold injection to the superior colliculus (mean ± standard deviation, $n = 13$). This highly efficient transduction of rat RGC is achieved 2 weeks after a single intravitreal injection of 2 μl viral stock at a concentration of 2×10^{12} particles/ml [34] (Figs. 1A and B). The axons of large numbers of GFP-labeled cells can be followed continuously from the retinal periphery to the optic nerve in retinal whole mounts, confirming that transfected cells are RGC (Fig. 2A). In addition, the entire dendritic tree structure of a significant minority of RGC is clearly revealed by GFP labeling (Fig. 2B), allowing us to study the morphological responses of RGC to insults such as elevated intraocular pressure.

We use retinal cross sections for histological localization of GFP positive cells within the retina. Following intravitreal injection of AAV–CBA–GFP–WPRE, we find that transfected cells lie almost exclusively in the RGC layer of the retina (Fig. 2C), except in the immediate vicinity of the injection site (Fig. 2D) where cells deeper in the retina may also be transfected. We find that GFP fluorescence survives standard ocular cryopreservation techniques well. Prior to sectioning, we isolated optic cups by immersion fixation in 4% paraformaldehyde for 45 min followed by serial exposure to 2% paraformaldehyde/5% sucrose, 5% sucrose, 10% sucrose, 12.5% sucrose, and 15% sucrose for 30 min each and then 20% sucrose overnight (all sucrose solutions are 0.2 M phosphate buffered, pH 7.2). Optic cups are embedded in OCT (Sakura Finetek USA, Torrance, CA) and sectioned to 8 μm. This technique allows excellent preservation of the histological structure of the retina, and GFP fluorescence can be directly visualized using the GFP filter set. We capture digital images of all sections (Zeiss Axiocam; Zeiss). Immunohistochemistry for GFP or other transgenes of interest can also by carried out on the cryopreserved histological sections.

4. Suggestions for troubleshooting

When the transfection efficiency for the retinal cell type of interest appears poor, a number of explanations should be considered.

4.1. Virus titer and viability

It should be confirmed that the virus stock contains a high titer of viable virus. Repeated freeze–thaw cycles can dramatically reduce the transducing ability of AAV and should be avoided. We find that AAV can remain stable and capable of high-efficiency transfection when stored at 4 °C for over 6 months.

4.2. Injection technique

For intraocular injection, the micropipette or needle used should be as fine as possible to reduce leakage. Injections should be given slowly over 1–2 min to allow intraocular diffusion of virus. If backflow of the injected

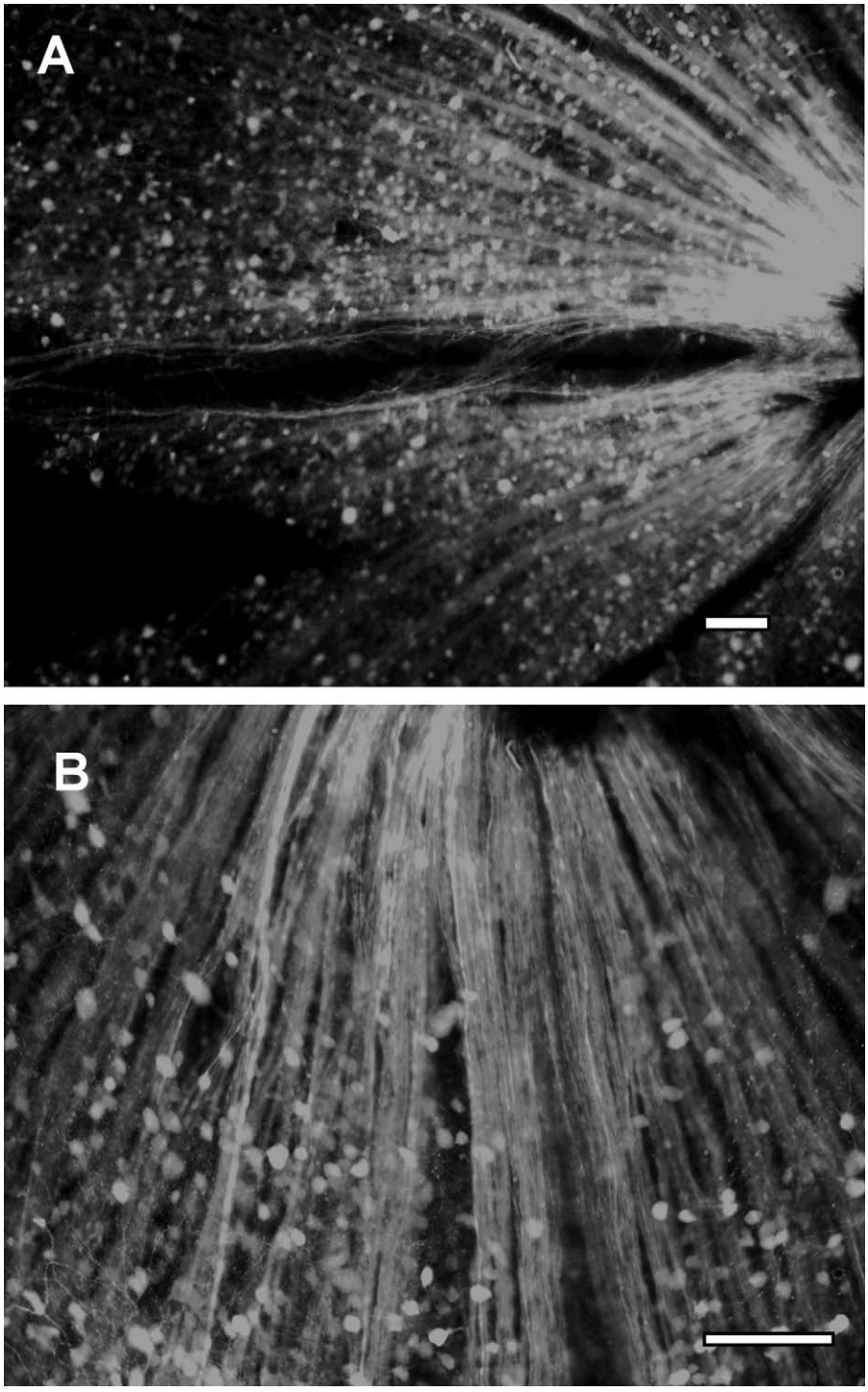

Fig. 1. Highly efficient transduction of rat retinal ganglion cells with GFP 2 weeks after intravitreal administration of 2 μl AAV–CBA–GFP–WPRE at a concentration of 2×10^{12} particles/ml (see Section 3). Representative retinal whole mounts as visualized by fluorescence microscopy using a GFP filter set are shown. GFP expression was extensive and almost confluent throughout the whole retina (A) in all replicate retinas examined ($n = 13$) with numerous RGC axons clearly visible (B). Bars, 100 μm.

fluid at the injection site is noticed, an anterior chamber paracentesis can be performed and a small volume of aqueous humor removed to create space. The intraocular injection site should be visualized, either during or immediately after injection to ensure correct placement of virus and to exclude complications. Planar ophthalmoscopy using a coverglass in contact with the cornea is a common method to visualize the rodent fundus, but we prefer noncontact ophthalmoscopy to reduce the risk of pressure-induced leakage at the injection site.

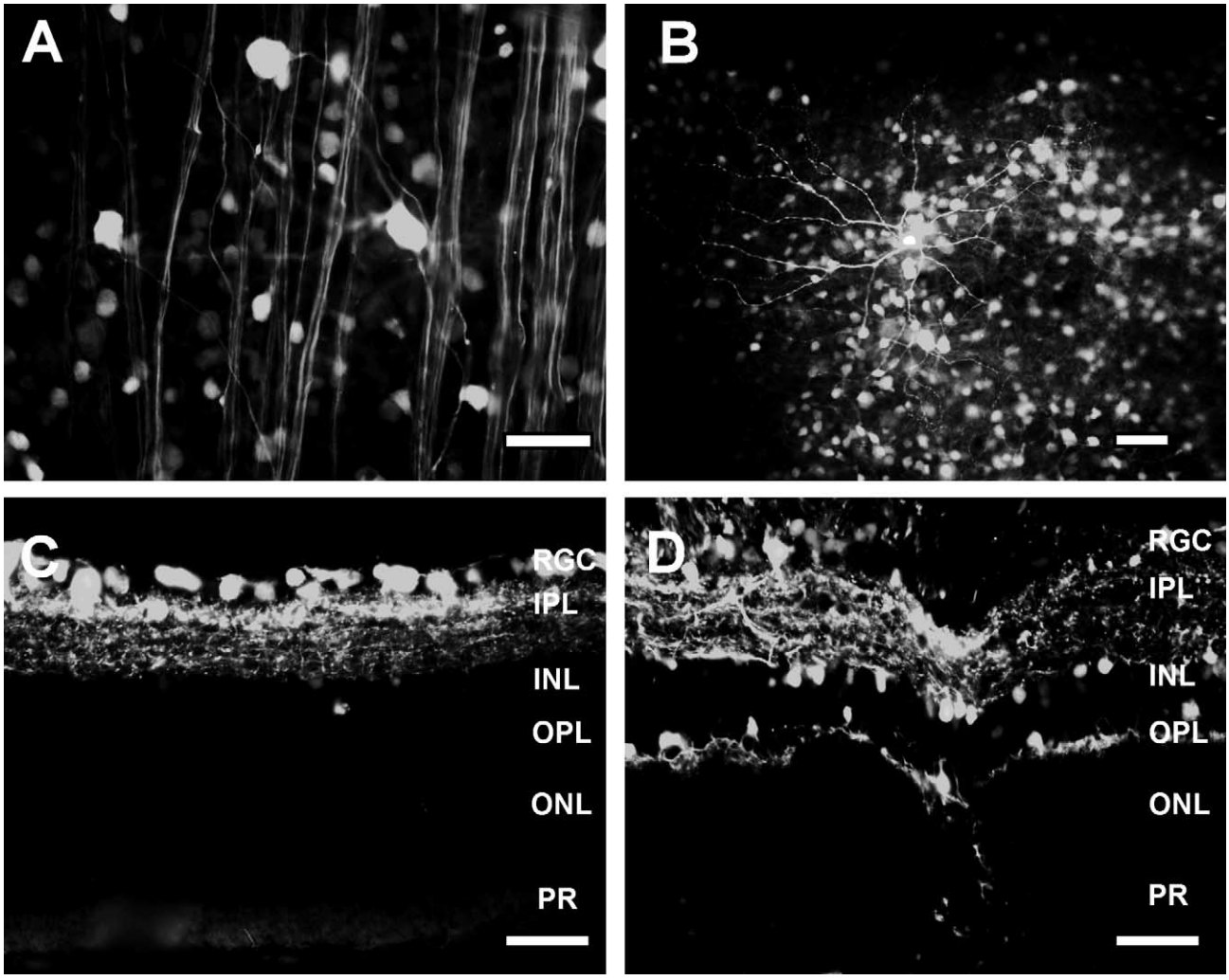

Fig. 2. Transfection of the rat retina with AAV–CBA–GFP–WPRE allowed individual RGC and their axons to be seen clearly (A). The entire dendritic tree of a subset of RGC was also visualized (B). In retinal cross sections ($n = 7$ eyes), GFP-labeled cells were localized almost exclusively to the RGC layer (C) except in the immediate vicinity of the injection site, where cells deeper in the retina were also transfected (D). Bars, 50 μm.

4.3. GFP visualization

AAV incorporating GFP allows rapid and straighforward assessment of transduction, but poor visualization of GFP fluorescence does not necessarily mean poor transfection. Although relatively resistant to standard paraformaldehyde- and glutaraldehyde-based fixation techniques, fluorescence from the oxidized GFP chromophore can be diminished by exposure to reducing conditions or to acetone-based sealants [38]. Mounting media which protect against photobleaching (e.g., Vectashield) can be extremely useful, particularly when the fluoresence signal is weak. If no fluorescence signal is seen, it should be verified that the correct filter set is being used—not all FITC-type filters are suitable. It is also essential that a suitable form of GFP is used; wild-type GFP molecules become misfolded at temperatures above 30 °C and a GFP modified for use in mammals is therefore crucial. Background autofluorescence within the eye does occur and tends to be most prominent in the RPE layer. Careful examination of control retinas is therefore an essential part of the assessment of retinal transduction with GFP. When viewed with a FITC filter set, autofluorescence usually appears yellowish-green compared to the distinctive bright green of GFP. This distinction can sometimes be less obvious under a dedicated GFP filter set.

4.4. Low-efficiency transfection in target cell type

If poor tranduction persists despite delivery of viable virus to the correct anatomical location in the eye, it may be necessary to change the promoter sequences used or to try a posttranscriptional regulatory element to increase transgene protein translation.

5. Concluding remarks

AAV-mediated gene transfer is a powerful technology with which to explore the pathology of ocular diseases and to investigate potential new therapeutic approaches. The eye is an excellent candidate for gene therapy, given its small size, its relative anatomical isolation, its numerous well-characterized genetic defects, and the ease with which vectors can be delivered to the immediate vicinity of cells involved in a particular disease. Successful gene transfer to specific retinal cell populations requires a clear understanding of the cellular specificities of the viral serotype and promoter sequences used, together with repeatable and reliable techniques for delivering the virus to the eye. Posttranscriptional regulatory elements can be useful in improving the efficiency of transduction, and we have found WPRE to be particularly impressive in this regard. As an example of what is possible with AAV-based technology in the eye, we are currently using AAV-mediated gene therapy to explore the possible neuroprotective effects of tranducing RGC with neurotrophic factor genes. Such techniques, if successful, could be an important future adjunct to the treatment of humans with glaucoma and other neurodegenerative disorders of the optic nerve. If the current success of ocular gene therapy research is maintained, it is our belief that the eye may be perhaps the the first organ for which gene therapy is used routinely in a clinical setting.

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