AAV2-Mediated Transduction of the Mouse Retina After Optic Nerve Injury

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PURPOSE. Gene therapy of retinal ganglion cells (RGCs) has promise as a powerful therapeutic for the rescue and regeneration of these cells after optic nerve damage. However, early after damage, RGCs undergo atrophic changes, including gene silencing. It is not known if these changes will deleteriously affect transduction and transgene expression, or if the therapeutic protein can influence reactivation of the endogenous genome.

METHODS. Double-transgenic mice carrying a Rosa26-(LoxP)-tdTomato reporter, and a mutant allele for the proapoptotic Bax gene were reared. The Bax mutant blocks apoptosis, but RGCs still exhibit nuclear atrophy and gene silencing. At times ranging from 1 hour to 4 weeks after optic nerve crush (ONC), eyes received an intravitreal injection of AAV2 virus carrying the Cre recombinase. Successful transduction was monitored by expression of the tdTomato reporter. Immunostaining was used to localize tdTomato expression in select cell types.

RESULTS. Successful transduction of RGCs was achieved at all time points after ONC using AAV2 expressing Cre from the phosphoglycerate kinase (Pgk) promoter, but not the CMV promoter. ONC promoted an increase in the transduction of cell types in the inner nuclear layer, including Müller cells and rod bipolar neurons. There was minimal evidence of transduction of amacrine cells and astrocytes in the inner retina or optic nerve.

CONCLUSIONS. Damaged RGCs can be transduced and at least some endogenous genes can be subsequently activated. Optic nerve damage may change retinal architecture to allow greater penetration of an AAV2 virus to transduce several additional cell types in the inner nuclear layer.

Keywords: gene therapy, retinal ganglion cells, optic nerve damage

A deno-associated virus, serotype 2 (AAV2) has become the principal vector for transducing retinal ganglion cells (RGCs) with recombinant genes. Native AAV2 efficiently infects RGCs, transducing upward of 85% of the population of these cells¹,² after intravitreal injection of adult rodent eyes. Nearly all of the investigations on AAV2-mediated gene transfer into RGCs have been done on naïve tissues (i.e., before optic nerve damage). Nearly all of the investigations on AAV2-mediated gene transfer into RGCs have been done on naïve tissues (i.e., before optic nerve damage), in an effort to validate gene therapeutic treatment of optic neuropathies, such as glaucoma. It is likely, however, that future therapies will be conducted on RGCs in a tissue that has already experienced some level of damage. This may be problematic to achieving a successful outcome. In response to damage, RGCs undergo changes in chromatin structure that precipitate the condensation of euchromatin to heterochromatin, and the early and rapid silencing of normal gene expression.³-⁶ These changes are largely controlled by an increase in histone deacetylase (HDAC) activity, including the translocation of HDAC3 from the cytoplasm to the nucleus shortly after damage.⁷ It is not clear if these changes would present a hostile or repressive transcriptional environment to a newly introduced transgene. Recombinant AAVs introduce their genome as single-stranded molecules containing inverted terminal repeats. In the nucleus, AAV genomes are converted to double-stranded molecules, which concatenate into circular episomes,⁷ which can become chromatinized.⁸ Thus, the AAV cargo DNA may be subject to the same rules of heterochromatin formation that govern the endogenous genome. Similarly, even if the exogenous transgene was expressed, the endogenous genome may be in a state that is refractory to the actions of therapeutic transgene products such as transcription factors. Because these nuclear changes occur early in the apoptotic program of RGCs, it is possible that this could present a significant barrier to the successful outcome of any neuroprotective strategy.

Even before consideration of whether or not a transgene could be expressed, the question of how well damaged RGCs can be transduced has not been addressed. AAV2 uses heparin sulfate as the primary receptor for attachment and internalization into cells,⁷ but it also binds to the 150kDa glycoprotein AAV-receptor,¹⁰,¹¹ various growth factor receptors,¹²,¹₅ αVβ5 integrin,¹⁴ and the 37/67 kDa laminin receptor.¹⁵ It is well-established that damaging insults that result in RGC death are correlated with widespread proteolytic changes in the retinal extracellular matrix,¹⁶-¹⁸ but how this extends to the biochemistry of the RGC plasma membrane is not known. Damaged RGC somas undergo dramatic morphological changes, typified by retraction of the dendritic arbor¹⁹-²³ with evidence of nuclear and soma shrinkage.²⁴,²⁵ This spectrum of changes
makes it uncertain if the necessary cell surface cues required for viral interaction are retained in damaged cells.

To address these issues, we examined the ability of gene therapy using AAV2 to deliver the Cre recombinase to RGCs after optic nerve damage. Rosa26-\((\text{LoxP})\)-\(\text{tdTomato}\) mice were used as the reporter strain to document successful activation of an endogenous gene in response to the activity of the exogenous Cre transgene. To help preserve a normal complement of RGCs, and to mimic a neuroprotective intervention, we crossed these mice to \(\text{Bax}^{+/−}\) mice (also on the C57BL/6J background) to generate animals carrying both the reporter and one or two mutant \(\text{Bax}\) alleles. Previous studies have shown that all of the early changes in chromatin remodeling and gene silencing are duplicated in \(\text{Bax}\)-deficient mice,\(^{22}\) even though the RGCs are highly resistant to completion of the apoptotic program.\(^{20}\) Activation of the \(\text{tdTomato}\) reporter gene in damaged RGCs of these mice would simultaneously verify that AAV2 can interact with and transduce these cells; that the transgene (\(\text{Cre}\) recombinase) can be expressed; and that the transgene product can interact with the endogenous RGC genome to facilitate the expression of a normally silenced gene \((\text{Rosa26-}(\text{LoxP})\text{-}\text{tdTomato})\). Here we show that all three objectives could be met, even in mice transduced with AAV2-\(\text{Cre}\) up to 1 month after acute optic nerve injury. In addition to successful RGC transduction, optic nerve injury dramatically increased the transduction efficiency of multiple other cell types, primarily in the retinal inner nuclear layer. This increase in promiscuity of retinal cell transduction may affect future therapeutic strategies and/or outcomes using gene therapy.

**MATERIALS AND METHODS**

**Animals**

Adult mice (4–6 months of age) were used for these studies and were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental protocols were approved by the Animal Care and Use Committee and the Office of Biosafety at the University of Wisconsin-Madison. Mice were housed in microisolator cages and kept on a 12-hour light/dark cycle and maintained on a 4% sucrose diet (8604 M/R, Harland Tekland, Madison, WI, USA). \(\text{Rosa26-}(\text{LoxP})\text{-}\text{tdTomato}\) mice (B6.Cg-Gt(Rosa)26Sor8\text{tm1CAGtdTomato}\text{J}\text{Hy}, Stock 007909; Jackson Laboratories, Bar Harbor, ME, USA) were crossed with C57BL/6J animals heterozygous for a targeted deficiency of the \(\text{Bax}\) gene (gift from Stanley Korsmeyer and made homozygous \((\text{Bax}^{−/−})\) by the C58BL/6J–deficient mice.\(^{25}\) even though the RGCs are highly resistant to completion of the apoptotic program.\(^{20}\) Activation of the \(\text{tdTomato}\) reporter gene in damaged RGCs of these mice would simultaneously verify that AAV2 can interact with and transduce these cells; that the transgene (\(\text{Cre}\) recombinase) can be expressed; and that the transgene product can interact with the endogenous RGC genome to facilitate the expression of a normally silenced gene \((\text{Rosa26-}(\text{LoxP})\text{-}\text{tdTomato})\). Here we show that all three objectives could be met, even in mice transduced with AAV2-\(\text{Cre}\) up to 1 month after acute optic nerve injury. In addition to successful RGC transduction, optic nerve injury dramatically increased the transduction efficiency of multiple other cell types, primarily in the retinal inner nuclear layer. This increase in promiscuity of retinal cell transduction may affect future therapeutic strategies and/or outcomes using gene therapy.

**ONC and Viral Injections**

Mice were subjected to ONC surgery of the left eye as described previously\(^{30,31}\) and allowed to recover for different periods of time ranging from 1 hour to 4 weeks. The mice then received a single intravitreal injection of either AAV2-CMV-Cre/ GFP (Vector Biolabs, Philadelphia, PA, USA) or AAV2-Pgp-Cre (University of North Carolina Vector CORE, Chapel Hill, NC, USA) (1 \(\mu\)L containing \(2–5 \times 10^{8}\) viral genomes). Briefly, mice were anesthetized with ketamine/xylazine and the experimental eye numbed with a drop of proparacaine. The globe was first punctured using the bevel of a 30-G needle, and intravitreal injections were made using a Nanofil syringe and 35G beveled needle (World Precision Instruments, Sarasota, FL, USA), which was inserted through the puncture site. Virus was injected slowly over 60 seconds. The needle was then withdrawn over another 60 seconds, and the puncture site was covered with triple antibiotic ointment. Mice were inspected daily for ocular infections or lens opacities that may have resulted from needle damage during injection, but none of the mice used in this study exhibited any adverse effects. Four weeks after injection, the mice were euthanized and the retinas processed for analysis. All times given in reference to experimental results refer to the time between ONC surgery and intravitreal injection of virus, and do not reflect the additional 4 weeks of incubation necessary to allow for second-strand synthesis and expression of AAV2-delivered DNA content.

**Image Analysis and Immunostaining**

All procedures, where possible, were conducted in low-level lighting so as not to bleach the fluorescent signal of the \(\text{tdTomato}\) reporter protein. After euthanasia, whole globes were enucleated and emersion fixed in 4% paraformaldehyde in PBS (50 mM phosphate buffer, pH 7.4, 150 mM NaCl) at 22°C between 30 and 60 minutes. Globes were then dissected to remove the anterior chamber and lens, and the resulting eyecup was equilibrated in 30% sucrose in PBS and embedded in optimum cutting temperature compound (Tissue-Tek; Sakura Finetek USA, Inc., Torrance, CA, USA). Sections were cut at 10 \(\mu\)m and affixed to Superfrost Plus microscope slides (ThermoFisher Scientific, Waltham, MA, USA). Sections were chosen for analysis that contained substantial portions of the optic nerve. Sections were blocked in PBS containing 2% BSA and 0.1% Triton X-100 overnight at 4°C in humidified chambers. Primary antibodies were then applied in the same buffer for 48 hours at 4°C. The primary antibodies included anti-BRN3A (1:100 dilution, MAB1585; EMD Millipore, Billerica, MA, USA), anti-SOX9 (1:500 dilution, AB5535; EMD Millipore), anti-PKC\(\alpha\) (1:100 dilution, sc-208; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Calretinin (1:50 dilution, AF5065; R&D Systems, Minneapolis, MN, USA), and anti-heparin sulfate (1:100 dilution, MAB2040; EMD Millipore). After incubation, sections were washed thoroughly in PBS and then incubated in the appropriate secondary antibody conjugated to either Alexa-488 or FITC. All secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA) and used at a dilution of 1:1000 in PBS, 2% BSA, and 0.1% Triton X-100 buffer. Secondary antibodies were applied for 2 hours at 22°C, or overnight at 4°C, followed by washing in PBS. Sections were then incubated in water containing 300 ng/mL 4,6-diamidine-2'-phenylinole dihydrochloride (DAPI; ThermoFisher Scientific) for 5 minutes at 22°C, followed by washing and mounting with Immu-Mount (ThermoFisher Scientific). Sections were imaged and digitally photographed on a Nikon Eclipse Ti inverted confocal microscope (Nikon, Melville, NY, USA) configured with an Andor Laser Combiner (Andor, Belfast, Northern Ireland). Emission band pass filters used for imaging were \(\text{tdTomato}\) (580–625 nm) and Alexa-488/FITC (510–540 nm). Individual fields were imaged as Nyquist Z-stacks of 10 to 15 steps (0.22 \(\mu\)m each) and then used to construct three-dimensional images.
Transduction of Damaged RGCs

using IMARIS 7.7 image analysis software (Bitplane, Concord, MA, USA).

Transmission Electron Microscopy
Smaller regions of eyecups processed as described above, which included the retina, were emersion fixed in Karnovsky’s fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 200 mM cacodylate buffer, pH 7.4) overnight at 4°C. The tissue was then post-fixed in 1% osmium tetroxide in phosphate buffer, dehydrated, and embedded in Epon epoxy. Sections (60-90 nm) were cut and stained with 50% ethanoic uranyl acetate and Reynolds’s lead citrate and viewed using a Phillips CM120 transmission electron microscope (FEI Company, Hillsboro, OR, USA).

Counting Protocol and Statistical Analysis
The number of tdTomato-expressing cells in retinal sections was quantified as follows. Five sections each of three retinas were counted from virus-injected eyes that did not receive ONC or virus-injected eyes, 2 weeks after ONC. All counting experiments were conducted on Bax+/− mice. Counts were obtained across the entire retinal section and reported as the number of positive cells along a 100-μm distance. From the images, it was evident that positive cells in the inner nuclear layer resided generally in the outermost and innermost portions of this layer. Therefore, the inner nuclear layer (INL) was divided into two leaflets, and counts were acquired in each separately. Counts were then reported as the mean and SD for the ganglion cell layer (GCL) and the two halves of the INL, which resided generally in the outermost and innermost portions of this layer. Therefore, the inner nuclear layer (INL) was divided into two leaflets, and counts were acquired in each separately. Counts were then reported as the mean and SD for the ganglion cell layer (GCL) and the two halves of the INL. These means were compared between undamaged and optic nerve-damaged retinas using a 2-tailed Student’s t-test with a significance level of P = 0.05.

RESULTS

Comparison of Cytomegalovirus (CMV) and Phosphoglycerate Kinase (Pgk) Promoter Activity to Drive Cre Expression in Damaged RGCs
In initial experiments, Bax-deficient mice were subjected to ONC and injected with virus within 1 hour to 2 days after damage. This time window was chosen because it was expected to be before the completion of heterochromatin formation in damaged RGCs.5,25 Figure 1 shows lobes from representative retinas from this study. Retinas in eyes exposed to the AAV2-CMV-Cre virus exhibited few labeled cells, which were mostly clustered in the periphery. Previously, in injections of naïve eyes of Rosa26-(LoxP)-tdTomato mice, we have achieved widespread transduction of cells in the GCL using this virus.5 Mice injected with the AAV2-Pgk-Cre virus, however, exhibited dramatically transduction and subsequent expression of the tdTomato reporter gene in the GCL at all time points. Both Bax−/− and Bax+/− mice exhibited similar widespread transduction. All future experiments were conducted using AAV2-Pgk-Cre.

Transduction of Multiple Retinal Cell Types by AAV2-Pgk-Cre After ONC Injury
Figure 2 shows more detailed microscopic examination of the transduction of retinas in Bax−/− mice injected with AAV2-Pgk-Cre 2 weeks after optic nerve injury. Confocal imaging of the GCL showed extensive cell labeling (en fosc image, Fig. 2A), and also revealed that a proportion of tdTomato-expressing cells were present in the outer retina, including cells with long processes extending toward the outer limiting membrane. A common feature of these cells was that they were grouped into distinct clusters (Fig. 2B), whereas cells in the GCL were distributed more uniformly. Sections of retina showed extensive tdTomato-expressing cells in the GCL and INL, including cells that extended long processes to the nerve fiber layer and outer limiting membrane, suggestive of Müller cells. Additionally, there was strong labeling in the proximal optic nerve up to the point of injury (Figs. 2C, 2D). Higher-magnification imaging of the optic nerve revealed that the bulk of the reporter protein was present in presumptive RGC axons, and not in astrocytes, which were positive for SOX9 immunoreactivity (Fig. 2E).

A comparison of retinas from undamaged eyes and retinas from eyes after ONC showed that more cells in the INL appeared to be expressing the tdTomato reporter in retinas after ONC surgery (Figs. 3A, 3B). Quantification of the number of cells per 100 μm of sectioned retina showed that there was no difference in labeled cells between the two conditions in the GCL, but there were significant increases in the numbers of cells in both the innermost portion (2.4-fold increase) and outermost portion (3.4-fold increase) of the INL (Fig. 3C).

We then examined if optic nerve damage affected either the structural integrity of the inner limiting membrane or increased the overall pattern of expression of heparin sulfate throughout the retina, two of the most likely mechanisms that would lead to increased transduction of cells in the inner retina. Transmission electron microscopy of sections of the naïve mouse retina, or retinas taken from the contralateral control eye of experimental animals (n = 4) all showed a well-structured inner limiting membrane (ILM) with a flocculent material lining the vitreous face of the membrane (Supplementary Fig. S1). Retinas from eyes 5 days after optic nerve damage (n = 4) also exhibited an ILM, but also showed regions where the integrity of the membrane transitioned to a disorganized fuzzy material. This was consistent for eyes from both wild-type and Bax-deficient mice. At 14 days after optic nerve damage, some retinas exhibited no evidence of an ILM in regions. These results were consistent with regional breakdown of the ILM leading to the clusters of transduced cells in the inner retina shown in Figure 2B. Conversely, sections stained for heparin sulfate showed no distinct difference in the pattern of immunolabeling for this moiety between contralateral control and damaged retinas (Supplementary Figs. S1D, S1E). The damaged retinas were processed between 6 and 8 weeks after optic nerve damage, however, and may not reflect a more immediate response in changes in the proteoglycan distribution after damage.

Discovery of Cell Types Transduced by AAV2-Pgk-Cre
The presence of the tdTomato reporter protein in axons of the optic nerves of eyes provides compelling evidence that damaged RGCs were successfully transduced by the virus and yielded subsequent transgene expression. To validate this, however, sections of Bax−/− eyes, injected with virus 4 weeks after optic nerve damage, were counterstained with an antibody against BRN3A to identify RGC somas in the GCL. Figure 4 shows colocalization of tdTomato with a majority of BRN3A-positive cells. There were also patchy areas of reporter gene expression that were not associated with a nucleus located at the interface between the GCL and the nerve fiber layer. These may have been Müller end feet (see below). In some regions of these retinas, BRN3A labeling appeared to be redistributed to the nuclear envelop or completely extruded from the nucleus. These cells were also often positive for tdTomato expression (Fig. 5). The proportion of cells with
nuclear and extruded BRN3A labeling combined was 46.5% ± 15.5% of the nuclei in the GCL of Bax
+/−/C0/C0 mice, which compares to the 44.8% ± 9.1% of cells reported in an earlier study.32 This suggests that cells exhibiting extruded BRN3A are RGCs and that redistribution of BRN3A may be a unique phenomenon warranting further investigation. Quantitatively, we measured the proportion of cells in the GCL that exhibited either expression of one or both markers (BRN3A or tdTomato). An average of 69.7% of these cells were positive for both markers, whereas 19.8% and 10.5% were positive for...
Figure 2. Multiple retinal cell types are transduced by AA2-Pgl-Cre. (A) En fosse confocal image of the GCL of a Bax+/− mouse injected 2 weeks pONC. A large number of cells are expressing the tdTomato reporter, suggesting efficient transduction of RGCs in this layer. (B) A 45° tilt of the Z-stack of this image shows that clusters of transduced/expressing cells extend into the outer retina, with prominent cell bodies in the INL. Scale bar: 10 μm. (C) A section through a Bax+/− mouse retina and optic nerve, transduced 2 weeks pONC. Note extensive labeling of the optic nerve. Scale
either BRN3A or tdTomato alone, respectively (Fig. 6). Thus, most transduced cells in the GCL were ganglion cells. It is notable that in naïve retinas, fewer than 2% of tdTomato-expressing cells did not also express an RGC marker such as BRN3A or βIII tubulin (data not shown). Cells that exclusively expressed the tdTomato reporter may have represented RGCs that were not BRN3A positive,32,33 or could have been cells that had experienced damage-induced silencing of this gene.34 It is also possible that some of these tdTomato-positive cells were not RGCs. Rare positive cells, for example, had the appearance of astrocytes (Fig. 5), although these cells did not colocalize with a marker for macroglia (see below).

The principal cell type showing increased tdTomato expression in the INL was expected to be Müller cells, based on morphological criteria of positively labeled extensions to the inner and outer limiting membranes, and ellipsoid-shaped nuclei in the outermost region of the INL. To verify that these were Müller cells, we counterstained sections with an antibody against the transcription factor SOX9, which selectively stains the nuclei of macroglia, including astrocytes,35 and Müller cells in the retina.36 Figure 7 shows that many, but not all of the Müller cells labeled with anti-SOX9 were also strongly positive for tdTomato expression. Notably, SOX9 positive cells in the GCL, identified as astrocytes, did not show tdTomato expression, similar to the observations made in the optic nerve (Fig. 2E).

Müller cells accounted for many of the reporter-positive nuclei present in the outermost region of the INL, but did not identify a subpopulation of cells with more rounded nuclei, which were also positive. These cells were in a region of the INL known to be populated by the nuclei of rod bipolar cells. To test if these cells were transduced, sections of retinas were counterstained with an antibody against PKCδ, which selectively stains rod bipolar cells.37 Figure 8 shows that most rod bipolar cells were also expressing tdTomato. Interestingly, although the reporter protein was prevalent throughout the cytoplasm of both RGCs and Müller cells, in the bipolar cells fluorescent signal appeared to be restricted to the nuclei.

To evaluate the possibility that amacrine cells made up most cells in the innermost half of the INL, we stained sections with an antibody against Calretinin, which selectively stains AII amacrine cells in the neural retina.38 There were few if any AII amacrine cells that showed colocalization of both proteins (Fig. 9). All amacrine cells that did appear to be expressing tdTomato proved to be situated above a second tdTomato-expressing cell when the Z-stack of images was rotated. Lack of tdTomato expression was evident in all amacrine cells both in the INL, and displaced cells in the GCL (Fig. 9).

dotted line indicates the stratification of the INL into innermost and outermost sections for cell counting. (B) A retina transduced 2 weeks after optic nerve damage. A dramatic increase in the numbers of tdTomato-expressing cells is evident in the INL. No labeling of photoreceptors was evident. Scale bar: 50 μm. DAPI counterstain. (C) Quantification of labeled cells in the different regions of retinas (n = 3 retinas for each condition). Positive cells were counted along 100-μm distances of retinal sections. The means ± SDs are graphed (minimum of 44 microscopic fields per condition counted). There was no significant change in the numbers of GCL cells that were transduced (t-test, P = 0.165). In the innermost region of the INL, there was a 2.1-fold increase in the numbers of cells (*P < 0.001). In the outermost region there was a 3.4-fold increase in the numbers of cells transduced (**P < 0.0001).
DISCUSSION

We tested the ability of AAV2 to introduce a gene of interest into RGCs after optic nerve damage. This study was precipitated by the need to understand the dynamics of AAV2-mediated gene transduction in RGCs that were in the early stages of the apoptotic pathway, because we predict that this would be the condition of RGCs for future application of...
gene therapy in diseases affecting the optic nerve, even including cells that were “rescued” by a neuroprotective treatment. Perhaps the greatest concern was that RGCs undergo early atrophy of the nucleus and condensation of the genome, even under conditions in which the apoptotic pathway had been blocked. Thus, it was uncertain if an exogenous transgene would even be expressed, or if so, would the product be able to influence expression of silenced endogenous genes. The results in this study indicate that not only can damaged RGCs be transduced, but also that expression of the therapeutic gene, and subsequently an endogenous gene, can be achieved. It is important to note that, by design, successful transduction is monitored by the amplified signal from the expression of the Rosa26-(LoxP)-tdTomato reporter gene. Thus, we cannot assess the quantitative level of successful viral transgene expression, because small and large amounts of Cre expression would result in the same activation of the reporter gene, which is then independently sustained (whereas Cre expression could be transient). The amplification step, however, does dramatize the difference in overall retinal transduction between uninjured and injured retinas (see below). Additionally, it is important to note that the Rosa26 locus is well-characterized for its ability to stay transcriptionally active during transition periods when the genome of a cell is being reset to a new transcriptome, such as during development. These transitions involve nuclear remodeling of heterochromatic and euchromatic regions, similar to what we observe in apoptotic RGCs. Thus, the Rosa26 locus may have an extended transcriptional potential that is not representative of other endogenous genes.

**Promoter Differences in Transduction of Damaged RGCs**

An important observation from these experiments is the relatively poor success achieved with the CMV promoter. We interpret this result as being indicative of poor expression from the promoter in cells of the affected retina. It is well known that the CMV promoter is only transiently expressed in vivo, but this is unlikely to explain the low levels of transduced cells in our experiments. The outcome measure of

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**Figure 5.** BRN3A becomes redistributed in some RGCs after damage. (A–C) Detail of tdTomato-positive cells colocalized with BRN3A. Of the four cells shown, three exhibit BRN3A localization that is in various stages of distribution between the nuclear and cytosolic compartments (asterisks). The arrow marks a cell with typical nuclear BRN3A localization. (D–E) A tdTomato-positive cell that is negative for BRN3A labeling. The flattened morphology is similar to the normal appearance of astrocytes. The BRN3A-positive cells exhibit both nuclear (arrow) or cytosolic (asterisks) distribution of BRN3A. Scale bar: 8 μm.

**Figure 6.** Quantification of BRN3A and tdTomato-expressing cells in the ganglion cell layer of optic nerve-damaged eyes. Data were collected from both Bax+/− and Bax−/− mice (n = 2 each) transduced between 2 and 4 weeks after ONC. The number of cells expressing BRN3A, tdTomato, or both, were scored and the percentage of each category of cells from that total was plotted for each of 31 different sections. An average of 69.7% of the cells were positive for both markers, whereas 19.8% and 10.5% were positive for only BRN3A or tdTomato, respectively.
Figure 7. tdTomato reporter expression in Müller cells. Bax<sup>−/−</sup> retinas, transduced 4 weeks pONC were counterstained with an antibody against SOX9 to identify Müller cells. (A) Survey view of a retinal section showing the GCL, INL, and ONL. Scale bar: 50 μm. (B–D) Nuclei, principally in the outermost region of the INL are positive for the SOX9 antigen. Many of them also express the tdTomato reporter (arrows), indicating transduction of these cells with the AAV2-Pgk-Cre vector. Several tdTomato-expressing cells, also in this half of the INL, but with more rounded nuclei, are also present, but do not express the SOX9 antigen. Scale bar: 5 μm. DAPI counterstain.
our experiments was the activation of the Rosa26-(LoxP)-tdTomato gene, and we would assume even transient expression from the CMV promoter would have generated sufficient Cre recombinase to cause activation, as is the case in transduced naïve retinas. Reduced expression from the CMV-driven promoter may be a by-product of the increased HDAC activity in RGCs, which has been shown in other cell types to reduce the activity of this promoter.\textsuperscript{43} It is important to note

Figure 8. tdTomato reporter expression in rod bipolar cells. Bax\textsuperscript{-/-} retinas, transduced 4 weeks pONC were counterstained with an antibody against PKC\textalpha. (A) Survey view of a retinal section showing the GCL, INL, and ONL. Scale bar: 50 µm. (B–D) Rounded nuclei in the outermost region of the INL exhibit tdTomato reporter gene expression, which colocalizes with PKC\textalpha immunoreactivity (arrows). Scale bar: 5 µm. DAPI counterstain.
Figure 9. All amacrine cells do not express the tdTomato reporter gene. Bax<sup>−/−</sup> retinas, transduced 4 weeks pONC were counterstained with an antibody against Calretinin. (A) Survey view of a retinal section showing the GCL, INL, and ONL. Scale bar: 50 μm. (B–D) Calretinin expressing AII amacrine cells are present in the innermost layer of the INL and in the GCL. There was no apparent colocalization with any tdTomato-expressing cells in either layer with the Calretinin stain. Some cells, which do show overlap of stain (arrowheads), were found to be distinct overlapping cells in the section when the confocal Z-stack was rotated. Scale bar: 5 μm. DAPI counterstain.
that our initial experiments using AAV2-CMV-Cre/GFP were limited to evaluation of cells in the GCL. Sectioning of a few naive retinas transduced with this virus did reveal a few cells in similar locations of the INL were also transduced, but not to the extent of the AAV2-Pgk-Cre virus (data not shown). Therefore, the enhanced level of expression in the INL after optic nerve damage also may be a function of promoter choice. Others have shown that promoter choice improves expression of AAV2-delivered transgenes in the retina.\textsuperscript{45} We selected the Pgk promoter on the basis of phosphoglycerate kinase being an enzyme involved in glycolysis, and therefore expected to be essential to cell homeostasis under a variety of conditions, including after damage. This promoter has been used to drive transgene expression in RGCs for at least 7 months.\textsuperscript{45} Additionally, we predict that genes involved in the glycolytic pathway to continue to be expressed in Bax-deficient RGCs, because these cells remain viable for up to 18 months after optic nerve damage in mice.\textsuperscript{50}

**Retinal Cell Types Transduced After Optic Nerve Damage**

In addition to RGCs, the AAV2-Pgk-Cre virus was able to transduce (and activate tdTomato expression) in a variety of other cell types. The level of transduction of these other cells was significantly increased after optic nerve injury. It is already well known that AAV2 can infect multiple different retinal cell types. The apparent increased tropism for RGCs after intravitreal injection is most likely a function of tissue-derived barriers, such as the nerve fiber layer and the ILM, which have been shown to impede viral access to other retinal cell types.\textsuperscript{46} Enzymatic digestion of the ILM is able to increase AAV2 penetration to the outer retina.\textsuperscript{46} Similarly, alterations in the viral capsid,\textsuperscript{47,48} or mixing the virus with exosomes,\textsuperscript{49} dramatically improves retinal penetration of AAV2, allowing for the transduction of a variety of other retinal cell types, including photoreceptors and RPE.

We predict that damage to the optic nerve leads to tissue modifications in the retina that increases permeability of the AAV2-Pgk-Cre virus to access other retinal cell types. Others have shown that retinal damage resulting from photoreceptor degeneration can increase the penetration, and possibly transduction efficiency, of several AAV serotypes.\textsuperscript{50} In addition, enzymatic treatment of the ILM and vitreous results in similar increased penetration of AAV serotypes.\textsuperscript{44,46} In a primate eye, transduction of the inner cells of the retinal fovea is increased in eyes that had experienced RGC loss due to cortical infection.\textsuperscript{41} We predict that optic nerve damage produces a similar breakdown of the ILM, which appears less well organized, or absent, in retinas after crush surgery at the ultrastructural level. In the case of acute optic nerve damage, the remodeling of the ILM may not be uniform, and seems to occur in select regions allowing for viral transduction of patches or groupings of cells such as what we observed for the Müller cells. At this point, however, we cannot rule out that certain retinal cell types change their surface chemistry in response to damage resulting in a more favorable binding environment for the virus. Immunostaining for heparan sulfate, the principal cell surface ligand for AAV2 binding, did not provide conclusive evidence of a dramatic change in expression pattern in damaged retinas.

Transduced cells in the INL appeared to be positioned generally in two distinct regions, the outermost edge and the innermost edge. In the outermost edge, most of these cells are likely a combination of Müller cell somas and rod bipolar cells. Nuclear staining, which is prominent with the tdTomato reporter, shows that Müller cells have elongated nuclei, whereas the nuclei of bipolar cells are more rounded. Several rounded tdTomato-positive nuclei in this region, however, are not colabeled with the antibody to PKCa, and may represent cone bipolar cells. These results are in agreement with other studies in which AAV2 penetration into the retina was increased. Wassmer and colleagues,\textsuperscript{49} for example, showed AAV2-mediated transduction of multiple CaBPs-expressing cells, which is indicative of both rod and cone bipolars. The cell type(s) that express the tdTomato reporter in the innermost region of the INL are, as yet, not identified. Although this is the region where amacrine cells are anatomically located, neither Calretinin-positive amacrine cells (this study) or Chat-positive amacrine cells\textsuperscript{49} appear to be transduced. This is also evident in the displaced amacrine cells in the GCL. Given the position of these unknown transduced cells, however, it is still likely that they are one of the 40 different subtypes of amacrine cells in the mammalian retina.\textsuperscript{51} Last, astrocytes identified by SOX9 staining did not appear to be transduced in either the GCL or the optic nerve, although rare tdTomato-positive cells with astrocyte-like morphology were detected in the GCL.

**Practical Considerations for Using AAV2-Mediated Gene Therapy in Retinas After Optic Nerve Damage**

Gene therapy holds promise as a powerful way to treat RGCs and prevent their death and/or stimulate their regeneration. This study fills a gap in our knowledge base by showing that partially damaged RGCs can be successfully transduced, express a transgene, and that the endogenous, silenced, genome can be activated to some degree. Although successful transduction of RGCs is encouraging, the increase in transduction of multiple other retinal cell types has both potential positive and potential negative consequences. Increased transduction of cells such as Müller cells may provide a broader platform for the synthesis and secretion of therapeutic molecules such as trophic factors.\textsuperscript{52} Alternatively, molecules specifically targeting RGCs may subsequently damage, alter, or kill other retinal cell types. Under these circumstances, promoter choice will be a critical consideration.

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**References**


