Proteosomal Degradation Impairs Transcytosis of AAV vectors from Suprachoroidal Space to Retina

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Abstract

Suprachoroidal injection provides a new route of delivery for AAV vectors to retinal pigmented epithelial cells and photoreceptors that can be done in an outpatient setting and is less invasive and potentially safer than subretinal injection, the most common route of delivery for ocular gene therapy. After suprachoroidal injection of AAV8 or AAV9 vectors, there is strong transduction of photoreceptors, but it is unclear how vector traverses the retinal pigmented epithelium. In this study, we found that transduction of photoreceptors was significantly increased after suprachoroidal injection of AAV2tYF-CBA-GFP versus AAV2-CBA-GFP vector. Compared with AAV2, AAV2tYF is more resistant to proteosomal degradation. Treatment with protease inhibitors significantly increased photoreceptor transduction after suprachoroidal injection of AAV5-GRK1-GFP. These data suggest that after suprachoroidal injection, AAV vectors access photoreceptors by transcytosis through retinal pigmented epithelial cells during which they are subject to proteosomal degradation, which if suppressed can enhance transduction of photoreceptors.

Introduction

There has been considerable progress utilizing ocular gene therapy to replace mutant genes in patients with an inherited retinal degenerations[1,2] or to provide sustained release of therapeutic proteins, such as anti-angiogenic proteins.[3–5] The most common route of vector administration for ocular gene therapy is subretinal injection which is done as part of a surgical procedure in which the vitreous including the posterior hyaloid is removed and a small-gauge cannula is used to inject vector through the retina into the subretinal space. This results in detachment (also referred to as a bleb) of about 20% of the retina where vector-containing fluid separates photoreceptors from the retinal pigmented epithelium (RPE). The fluid is rapidly absorbed concentrating the vector and facilitating its entry into photoreceptors and RPE throughout the area of the resolving detachment resulting in high transfection efficiency and robust transgene expression. In addition to boosting expression, confinement of vector predominantly in the subretinal space limits exposure outside the eye minimizing immune response. A disadvantage of subretinal injection is that expression is for

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the most part limited to the region of the bleb and hence about 20% of photoreceptors and RPE for a 300 μl injection volume. This can be addressed by creating multiple blebs, but even with multiple blebs, it is difficult to transfect   50% of photoreceptors and RPE which is not ideal for gene replacement since the gene defect remains uncorrected throughout a large area of the retina. The posterior retina including the macula is generally prioritized because it is responsible for vision that is most critical for functioning, but separation of the macula, and particularly the fovea, from the RPE may cause a reduction in visual acuity that does not always recover.[6] Also, there are often subretinal adhesions in the eyes of patients with inherited retinal degeneration making it more difficult to create a bleb and increasing risk. This is because greater infusion pressure may be needed and since the fovea is the thinnest and hence weakest part of the retina, fluid under pressure in the subretinal space may break through the fovea creating a macular hole which may decrease vision.[7] In addition, vitrectomy is associated with a 1% risk of retinal detachment which may require another surgical procedure and can potentially result in reduced vision.

Suprachoroidal injection provides a new route of administration for viral vectors which does not require a surgical procedure and hence can be done in an outpatient clinic. In rats, suprachoidal injection of AAV8-CBA-GFP resulted in expression of GFP in photoreceptors and RPE around the entire circumference of the eye with a gradient in level of expression; there was high expression on the side of the injection and weak, detectable expression on the opposite side.[8] In the larger eyes of primates and pigs, a single injection resulted in detectable expression as far posterior as the optic nerve. However, suprachoroidal injection of AAV2-CMV-GFP resulted in weak expression of GFP confined to a small area adjacent to the injection site. While AAV8 vectors have greater transfection efficiency than AAV2 vectors after subretinal injection,[9] the difference is not as great as that seen after suprachoroidal injection.

Phosphorylation of exposed tyrosine residues of AAV2 capsid targets the vector for proteosomal degradation and reduces transduction efficiency, and mutation of these exposed tyrosines improved transduction efficiency in murine hepatocytes.[10] AAV2 vectors with 3 surface tyrosines changed to phenylalanine (AAV2tYF) have particularly good transduction efficiency in mice after subretinal or intravitreous injection.[11] In this study, we sought to test the feasibility of delivering AAV2tYF vectors by suprachoroidal injection.

Materials and Methods

Viral Vectors

AAV2tYF-CBA-GFP, AAV2-CBA-GFP, AAV2tYF-GRK1-GFP, and AAV5-GRK1-GFP vectors were provided by Applied Genetic Technologies Corporation (Gainesville, FL).[11]

Animals

All animals were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research, and protocols were reviewed and approved by the Johns Hopkins University Animal Care and Use Committee. Norway Brown rats (male and female) were purchased from Charles River

(Frederick, MD, USA). No sample size calculations were done in this study, but instead used sample sizes similar to those in previously published studies investigating expression after suprachoroidal injection of AAV vectors. Rats were randomly selected for different treatment groups.

Suprachoroidal Injection of Vector in Rats

Rats were randomly assigned to treatment groups striving for equal numbers in each group. They were anesthetized with ketamine/xylazine followed by instillation of 0.5% proparacaine (Akorn Pharmaceuticals, Lake Forest, IL) and 5% povidone iodine in each eye. Eyes were visualized with a Zeiss Stereo Dissecting Microscope (Zeiss, Oberkochen, Germany). A 30-gauge needle on 1 ml syringe was used to generate a partial thickness (4/5 way through sclera) circumferential opening in the sclera 1 mm posterior to the limbus, and a 34-gauge needle with a blunt 45 degree bevel connected to a 5 μl Hamilton syringe (Hamilton Company, Reno, NV) containing vector was inserted into the scleral opening with the bevel facing downward and slowly advanced through the remaining scleral fibers into the suprachoroidal space. The plunger of the syringe was slowly advanced to expand the suprachoroidal space and inject 3 μl containing 7.8×10^9 GC of AAV2tYF-CBA-GFP, 6.87×10^9 GC of AAV2-CBA-GFP, 7.8×10^9 GC of AAV2tYF-GRK1-GFP, 7.8×10^9 GC of AAV5-GRK1-GFP, 3.9×10^9 GC of AAV5-GRK1-GFP+20 μM MG132(Millipore Sigma, Burlington, MA), or 3.9×10^9 GC of AAV5-GRK1-GFP+1 μM bortezomib (Millipore Sigma, Burlington, MA) and held in place for 30 seconds, after which the needle was withdrawn while holding a cotton tipped applicator over the injection site. Visualization of the fundus showed a shallow choroidal detachment on the side of the injection. Antibiotic ointment (Moore Medical LLC, Farmington, CT) was applied to the ocular surface and rats were returned to their cages.

Tissue harvesting and histology

Rats were euthanized by isoflurane inhalation followed by cervical dislocation which is approved by the Panel on Euthanasia of the American Veterinary Medical Association. Eyes were removed and fixed in 4% paraformaldehyde. Eyes used for flat mounts had the anterior segment and vitreous removed and then retina and the remaining eyecup were isolated and flat mounted separately. Only the RPE is visualized on the surface of the flat mounted eyecup, so this will be referred to as RPE flat mount. Eyes for ocular sections were frozen in optimal cutting temperature media (Fisher Scientific, Walkersville, MD) and 10 μm frozen sections were placed on slides. Sections were stained with Hoechst (Vector Laboratories, Burlingame, CA) and anti-GFP antibody (A21312, life Technologies Corporation, Eugene, OR). Both flat mount and ocular sections were examined by fluorescence microscopy. Two investigators participated in all experiments. One investigator coded slides and the other examined them and obtained images. After the images were obtained the investigators broke the code together to complete the analysis.

Measurement of GFP protein in retinal or RPE/choroid homogenates

Rat retinal and eyecup samples were isolated under a dissection microscope and put in RIPA buffer (Sigma Aldrich, Arlington, MA) containing protease inhibitor cocktail (Roche, 68298 Mannheim, Germany). Samples were sonicated for 4–5 seconds (Sonic Dismembrator

Model 300, Fisher Scientific), cooled in an ice bath for 5 minutes, centrifuged for 10 minutes at 14,000 rpm (Eppendorf, Germany), and supernatants were stored at −80C°. Total protein concentration was measured using Bradford CCB-G250 protein-binding assay. Briefly, concentrations of bovine serum albumin (Millipore Sigma, Burlington, MA) ranging from 20–140 µg/ml were used as standards. Twenty-five µl of sample or standard were added to duplicate wells of 96-well plates followed by 125 ul Protein Assay Dye (diluted 1:5, Bio-Rad, California, USA). After a 5-minute incubation on a shaker, absorption was measured at 595nm in a Spectra Max Plus 384 Microplate Reader (Molecular Devices, San Jose, California).

GFP protein levels were measured using a GFP SimpleStep ELISA kit (ab171581, Abcam, Cambridge, MA). Briefly, 50 µl of sample or GFP standard dilutions were added to duplicate wells of 96-well plates followed by 50µl of antiGFP antibody Cocktail. Plates were incubated at 25°C for 1 hour, washed 5 times with rinse buffer, and after addition of 100 µl of TMB substrate solution, they were incubated at 25°C in the dark for 10 minutes. After addition of 100 µl of stop solution, absorption was measured at 450nm with a Spectra Max Plus 384 Microplate Reader. All measurements were made by an investigator masked with regard to treatment group.

Statistical analysis

Two investigators participated in all analyses. One placed a code on samples and the other performed analyses masked with regard to treatment group. No data were excluded from analysis. GraphPad Prism version 6.0 software (La Jolla, CA) was used for assessment of data distribution and statistical analysis. All experiments were repeated once for replication. Shapiro-Wilk test showed data were normally distributed and therefore Student's t-test was used for comparison of two data sets and one-way ANOVA with Tukey's Test for multiple comparisons was used for comparison of more than two data sets. Variance was similar among the groups. A p-value 0.5 was considered statistically significant. Variance was similar between groups that were compared.

Results

Poor expression of GFP after suprachoroidal injection of AAV2-CBA-GFP

A previous study demonstrated poor expression of GFP after suprachoroidal injection of AAV2-CMV-GFP.[8] In some situations methylation can result in reduced expression by the CMV promoter.[12,13] In this study, we sought to rule out the promoter as a potential source of the poor transduction that was seen after suprachoroidal injection of AAV2-CMV-GFP by testing AAV2 vector in which a chicken beta actin (CBA) promoter was used to drive GFP expression (AAV2-CBA-GFP). Two weeks after suprachoroidal injection of 7.8×10^9 genome copies (GC) of AAV2-CBA-GFP in Brown Norway rats, retinal flat mounts (Figure 1A) and RPE flat mounts (Figure 1B) showed only focal areas of hyperfluorescence around the site of injection. An ocular section from a region near the injection site was immunohistochemically stained for GFP and examined by fluorescence and light microscopy. A merged image showed GFP in some RPE cells, but very little detectable in photoreceptor cells (Figure 1C).

Expression of GFP in the retina and RPE after suprachoroidal injection of AAV2tYF-CBA-GFP

Two weeks after suprachoroidal injection of 7.8×10^9 genome copies AAV2tYF-CBA-GFP in Brown Norway rats, retinal flat mounts showed GFP fluorescence in about ¼ of the retina (Figure 1D). RPE flat mounts showed very strong fluorescence in a relatively focal area near the injection site with weaker fluorescence throughout a much larger area (Figure 1E). High magnification of a region of a RPE flat mount that appeared to show weak fluorescence at low power, showed some hexagonal RPE cells with strong fluorescence and others with weak fluorescence (Figure 1F). An ocular section through the center of the cornea showed strong hyperfluorescence in photoreceptors extending about ¼ around the circumference of the eye (Figure 1G). Fluorescence appeared weaker and more variable in RPE cells beneath the fluorescent photoreceptors. At high magnification, strong GFP expression was seen in photoreceptor inner and outer segments and cell bodies and weak expression was seen in RPE cells, as well as some cells in the inner retina (Figure 1H and I). Four weeks after injection, GFP expression in photoreceptors was similar to that seen after two weeks, but expression appeared stronger in RPE cells and was present in more cells of the inner retina (Figure 1J).

Strong, selective expression of GFP in photoreceptors after suprachoroidal injection of AAV2tYF-GRK1-GFP

For some applications, there may be advantages to selective transgene expression in photoreceptors, avoiding expression in RPE cells. The rhodopsin kinase (GRK1) promoter is a photoreceptor-specific promoter that promotes expression in both rods and cones.[14] Two weeks after suprachoroidal injection of 7.8×10^9 genome copies (GC) of AAV2tYF-GRK1-GFP, ocular sections through the center of the cornea showed hyperfluorescence in outer nuclear layer extending about ¼ of the circumference of the eye (Figure 2A). Sections were immunohistochemically stained for GFP and high magnification merged fluorescent and light microscopic images showed strong expression of GFP in photoreceptors (Figure 2B). There was a sharp demarcation with no GFP expression seen above the outer nuclear layer. Retinal flat mounts showed hyperfluorescence throughout about ¼ of the retina (Figure 2C). Two weeks after suprachoroidal injection of 7.8×10^9 GC of AAV2tYF-CBA-GFP or AAV2tYF-GRK1-GFP the level of GFP in retinal homogenates was significantly greater in AAV2tYF-CBA-GFP-injected versus AAV2tYF-GRK1-GFP-injected eyes (Figure 2D). The level of GFP in RPE/choroid/sclera homogenates in AAV2tYF-CBA-GFP-injected eyes, which was significantly greater than that seen in AAV2tYF-GRK1-GFP-injected eyes (below limit of detection). Four weeks after injection, the level of GFP in retinal homogenates in AAV2tYF-GRK1-GFP-injected eyes had increased and was not significantly different from that in AAV2tYF-CBA-GFP-injected eyes (Figure 2E). The level of GFP in RPE/choroid/sclera homogenates remained significantly greater in AAV2tYF-CBA-GFP-injected versus AAV2tYF-GRK1-GFP-injected eyes.

Inhibition of proteosomal degradation increases GFP expression in photoreceptors after suprachoroidal injection of AAV5-GRK1-GFP

Two weeks after suprachoroidal injection of 7.8×10^9 GC of AAV5-GRK1-GFP, an ocular section through the center of the cornea showed GFP fluorescence in the outer portion of the retina extending about 1/6 around the circumference of the eye (Figure 3A). The boxed region in Figure 3A immunohistochemically stained for GFP with light microscopy images merged with the fluorescence microscopy images showed GFP expression limited to photoreceptor inner and outer segments and cell bodies (Figure 3B). Retinal flat mounts showed strong fluorescence in the quadrant that the injection was done (Figure 3C). MG132 is a peptide and bortezomib is a kinase inhibitor that each block proteosomal degradation of proteins and have been shown to increase transduction of cells directly contacted by AAV vectors.[15–17] Compared with suprachoroidal injection of AAV5-GRK1-GFP, injection of AAV5-GRK1-GFP in combination with MG132 or bortezomib resulted in significantly higher levels of GFP protein in retinal homogenates (Figure 3D). The level of GFP protein was below the limit of detection in RPE/choroid/sclera homogenates in all groups.

Discussion

Suprachoroidal injection provides a new route of delivery for ocular gene transfer that has potential advantages over subretinal injection in that it can be done in an outpatient setting without vitrectomy and without separation of photoreceptors from the RPE.[8] This suggests that delivery of vectors by suprachoroidal injection may be safer and less invasive than delivery by subretinal injection. In addition, the spread of vector in the suprachoroidal space is greater than spread in the subretinal space because reporter gene expression is limited to the region of the bleb caused by the subretinal injection. The wider spread of vector in the suprachoroidal space has the potential benefit of obtaining transgene expression over a much wider area of RPE and photoreceptors after a single injection, but there is greater dilution of vector concentration resulting in lower expression per cell. This may be overcome by increasing the volume injected or performing multiple injections, but additional studies are needed to explore these issues.

There are also other differences between subretinal and suprachoroidal injection of viral vectors that must be studied. While suprachoroidal injection of AAV8-CBA-GFP in rats resulted in widespread expression of GFP in photoreceptors and RPE, there was very limited GFP expression after suprachoroidal injection of AAV2-CMV-GFP.[8] The difference was much greater than that previously seen comparing subretinal injection of AAV8 and AAV2 vectors in primates.[9] In addition to difference in AAV serotype, the promoters were different and while the CMV promoter is generally a strong ubiquitous promoter there are instances when it can be silenced.[12,13] In this study, we performed suprachoroidal injection of AAV2-CBA-GFP and found low-level expression of GFP in both RPE and photoreceptors, similar to what was seen after suprachoroidal injection of AAV2-CMV-GFP.

Compared with suprachoroidal injection of AAV2-CBA-GFP, suprachoroidal injection of AAV2tYF-CBA-GFP resulted in far greater transduction of photoreceptors. This indicates that proteosomal degradation is a major barrier to photoreceptor transduction when AAV2 vector is delivered into the suprachoroidal space. Utilization of the photoreceptor-specific

GRK1 promoter in AAV2tYF vector resulted in selective expression in photoreceptors after suprachoroidal injection. Compared with suprachoroidal injection of AAV2tYF-CBA-GFP, suprachoroidal injection of AAV2tYF-CBA-GFP resulted in more gradual onset of GFP expression in photoreceptors, but peak expression levels were not statistically different. This indicates that the suprachoroidal route of injection can be used for selective expression of transgenes in photoreceptors.

Compared with suprachoroidal injection AAV2-GFK1-GFP, suprachoroidal injection of AAV5-GFK1-GFP resulted in greater expression of GFP in photoreceptors, but it was still quite limited. When AAV5-GFK1-GFP was injected in the presence of MG132 or bortezomib, which each suppress proteolytic degradation of proteins in the proteosome, there was a significant increase in GFP expression in photoreceptors. This confirms that proteosomal degradation of AAV vectors is a relative barrier to photoreceptor transduction after their delivery into the suprachoroidal space.

Since proteosomal degradation occurs intracellularly, these data suggest that AAV vectors injected into the suprachoroidal space access photoreceptors by passing through RPE cells. This is consistent with previous studies that have shown that AAV vectors enter cells by endocytosis and can either travel to the nucleus and mediate gene expression or pass through the cell to transduce other cell types. The major endocytic infection route for AAV vectors is through the clathrin-independent carriers/GPI-anchored-protein-enriched endosomal compartment (CLIC/GEEC) pathway, but cell entry also occurs by dynamin-dependent endocytosis which does not contribute to infection.[18] The latter route is used by wild type AAV for transcytosis to spread infection beyond epithelial barriers.[19] We hypothesize that this route is used by AAV vectors to penetrate through RPE cells and transduce photoreceptors after suprachoroidal injection. The efficiencies of these two endocytic routes are cell-type- and serotype-specific but may vary independently. Based upon the current study and a previous study,[8] it appears that transcytosis from the basal to apical surface of RPE cells is far more efficient for AAV8 and AAV9 vectors than for AAV2 or AAV5 vectors. After subretinal injection, there is direct contact of vector with both photoreceptors and RPE cells, with no need for transcytosis, and by this route of delivery, AAV2 efficiently transduce both cell types, although not as efficiently as AAV8.[9] After suprachoroidal injection of AAV2, there is poor transduction of RPE cells as well as photoreceptors indicating limited infectious endocytosis as well as transcytosis. This indicates reduced AAV2 entry through the CLIC/GEEC pathway at the basal versus the apical surface of RPE cells. The inefficient infectious endocytosis and transcytosis of AAV2 at the basal surface of RPE cells is partially overcome by capsid modifications that reduce proteosomal degradation. This suggests that AAV2 vector particles traversing each of these endocytic pathways are subject to a detour to the proteasome and degradation.

Thus, while there are likely to be other factors that contribute to the poor transduction efficiency of AAV2 vectors after suprachoroidal injection, proteosomal degradation plays a substantial role so that AAV2tYF can be considered for this route of administration. Like AAV2, AAV5 is also subject to proteosomal degradation, which decreases photoreceptor transduction after suprachoroidal injection. We hypothesize that this may be generalizable to all serotypes of AAV vectors and it will be important to test this in future studies, because it

implies that all AAV vectors access photoreceptors by transcytosis through RPE cells during which they are subject to proteosomal degradation, which can be modulated to improve photoreceptor transduction after vector injection into the suprachoroidal space.

Acknowledgements

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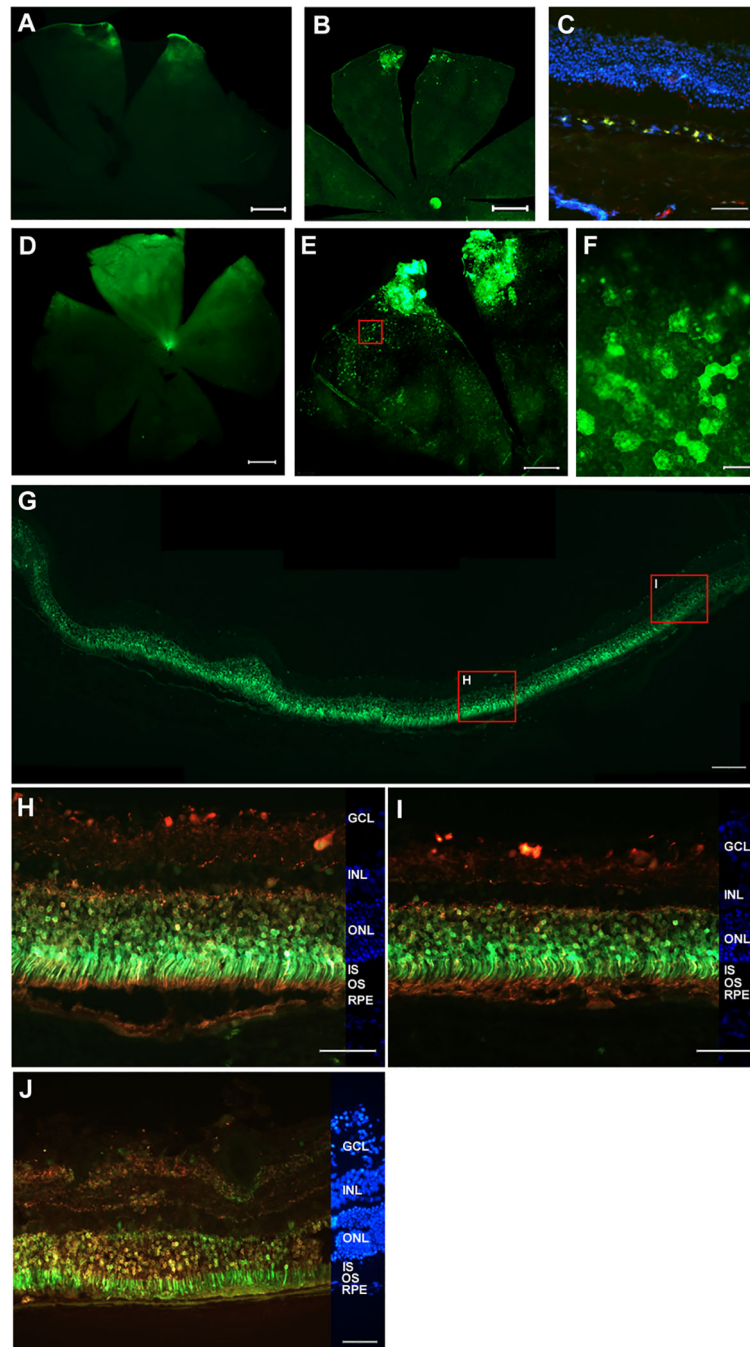
Figure 1. Good expression of GFP in retina and retinal pigmented epithelium after suprachoroidal injection of AAV2tYF-CBA-GFP but not AAV2-CBA-GFP

Brown Norway rats were given a suprachoroidal injection of 7.8×10^9 genome copies (GC) of AAV2-CBA-GFP or AAV2tYF-CBA-GFP and after two or four weeks, retinal flat mounts or ocular sections were examined by fluorescence microscopy. Two weeks after injection of AAV2-CBA-GFP, retinal flat mounts (A, scale bar = 1000μm) and RPE/choroid flat mounts (B, scale bar = 1000μm) showed a few focal areas of hyperfluorescence around the injection site, but nothing above background elsewhere in the retina or RPE. An ocular section near the injection site was examined for GFP fluorescence (green),

immunohistochemically stained for GFP (red), and nuclei were counterstained with Hoechst. A merged image showed GFP expression in a few RPE cells (C, scale bar = 50μm). Two weeks after suprachoroidal injection of AAV2tYF-CBA-GFP, retinal flat mounts showed GFP fluorescence in about ¼ of the retina (D, scale bar=1000μm) and RPE/choroid flat mounts showed very strong fluorescence in a relatively focal area surrounded by weaker fluorescence (E, scale bar = 250μm). High magnification of the boxed region in (E) showed hexagonal RPE cells with strong fluorescence and others with weak fluorescence (F scale bar = 50μm). An ocular section through the center of the cornea showed GFP fluorescence in photoreceptors and weaker more heterogeneous fluorescence in RPE cells extending about ¼ around the circumference of the eye (G, scale bar=250μm). The section was immunohistochemically stained with antiGFP (red) and high magnification merged images of the two boxed regions in G (H a bit closer to the injection site than I), showed strong expression of GFP in photoreceptor inner and outer segments and nuclei, and somewhat weaker expression in RPE cells and some cells in the inner retina (H and I, scale bars = 50μm). Four weeks after suprachoroidal injection of 7.8×10^9 GC AAV2tYF-CBA-GFP, an ocular section through the posterior part of the eye was stained with antiGFP and merged fluorescent and light microscopic images showed GFP fluorescence in the RPE, photoreceptor inner and outer segments, some photoreceptor nuclei, and some cells in the inner retina (J, scale bar= 50μm).
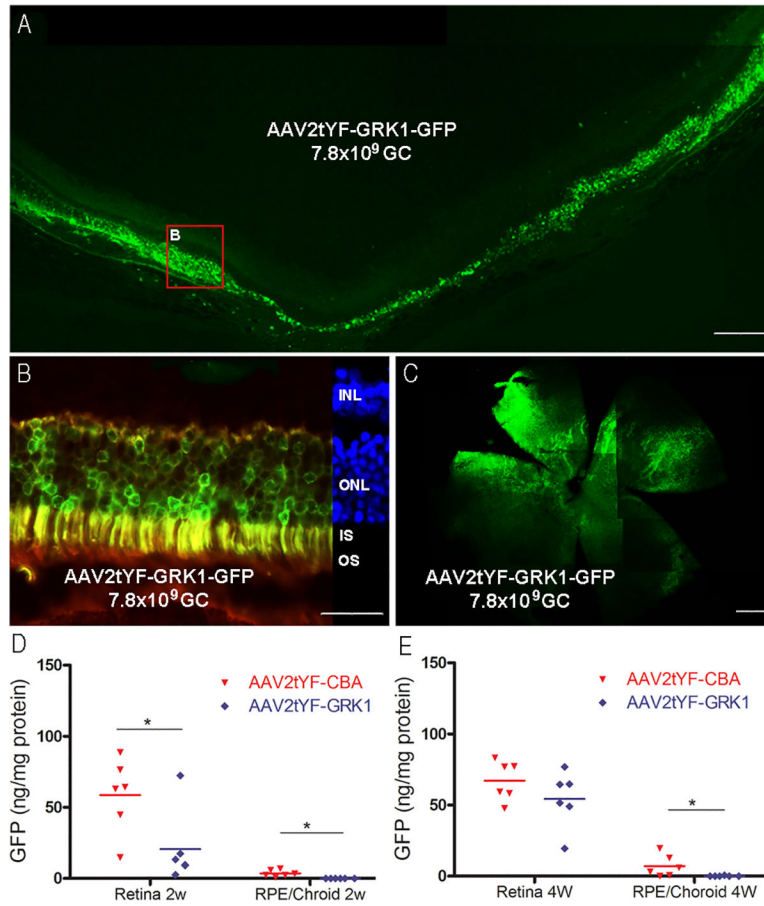
Figure 2. Strong, selective expression of GFP in photoreceptors after suprachoroidal injection of AAV2tYF-GRK1-GFP

Two weeks after suprachoroidal injection of 7.8×10^9 genome copies (GC) of AAV2tYF-GRK1-GFP in Brown Norway rats an ocular section through the center of the cornea showed GFP fluorescence extending about ¼ around the circumference of the retina (A, scale bar=250μm). The section was immunohistochemically stained with antiGFP (red) and high magnification merged images of the boxed regions marked B showed GFP expression limited to photoreceptor inner and outer segments and cell bodies (B, scale bar = 25μm). Retinal flat mounts showed GFP fluorescence in the quadrants surrounding the site of injection (C, scale bars = 1000μm). Two weeks after suprachoroidal injection of 7.8×10^9 genome copies (GC) of AAV2tYF-GRK1-GFP or AAV2tYF-CBA-GFP, mean retinal GFP protein level measured by ELISA was significantly higher in photoreceptors or RPE choroid in AAV2tYF-CBA-GFP-injected eyes (C, *p<0.05 by Student's t-test). Four weeks after injection, mean retinal GFP protein level had increased in the AAV2tYF-GRK1-GFP-injected group and was not statistically different from that in the AAV2tYF-CBA-GFP-injected group (D). Mean RPE/choroid GFP protein levels were still significantly greater in the AAV2tYF-CBA-GFP-injected group compared with the AAV2tYF-GRK1-GFP group (*p<0.05 by Student's t test).
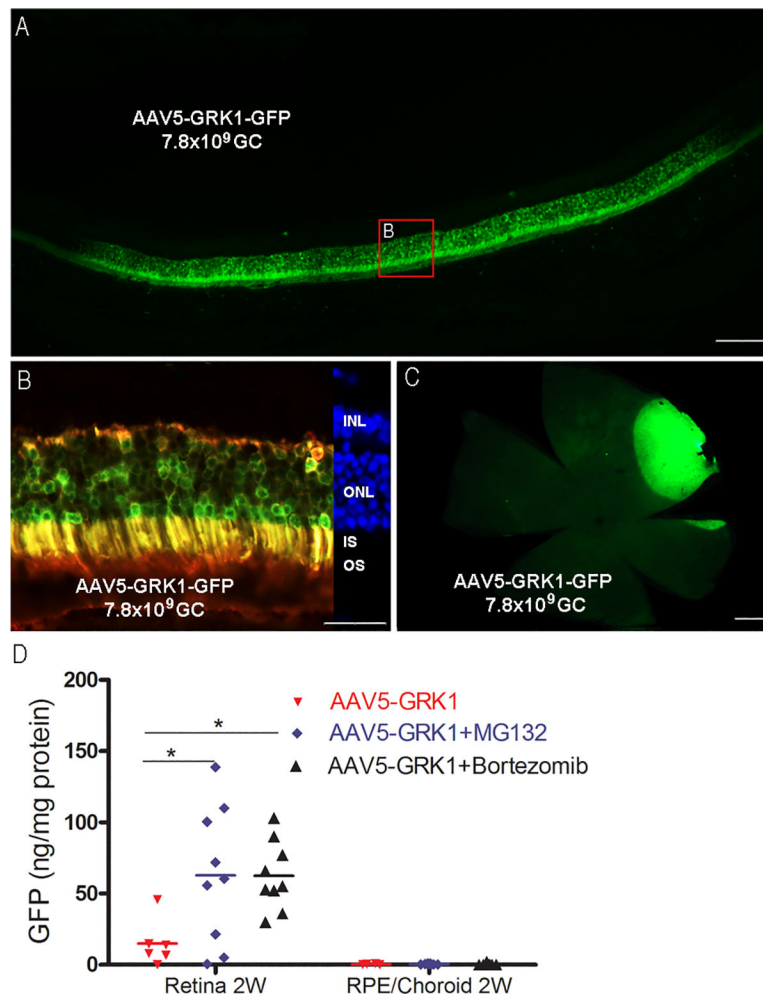
Figure 3. Protease inhibitors increase GFP expression in photoreceptors after suprachoroidal injection of AAV5-GRK1-GFP.

Two weeks after suprachoroidal injection of 7.8×10^9 GC of AAV5-GRK1-GFP, an ocular section through the center of the cornea showed GFP fluorescence extending about 1/6 around the circumference of the retina (A, scale bar=250μm). The section was immunohistochemically stained with antiGFP (red) and high magnification merged images of the boxed region in A showed GFP expression limited to photoreceptor inner and outer segments and cell bodies (B, scale bar = 25μm). Retinal flat mounts showed strong fluorescence in the quadrant that the injection was done (F, scale bars = 1000μm). Two weeks after suprachoroidal injection of 3.9×10^9 GC of AAV5-GRK1-GFP alone (n=6) or containing 20 μM MG132 (n=8) or 1 μM bortezomib (n=9), GFP protein was measured in retinal or RPE/choroid homogenates by ELISA. Mean GFP level per mg protein was significantly higher in the MG132 and bortezomib groups than the control group. (*p<0.05 by ANOVA with Tukey's Test for multiple comparisons).