AAV-Mediated Gene Replacement, Either Alone or in Combination with Physical and Pharmacological Agents, Results in Partial and Transient Protection from Photoreceptor Degeneration Associated with βPDE Deficiency

Mariacarmela Allocca, Anna Manfredi, Carolina Iodice, Umberto Di Vicino, and Alberto Auricchio

PURPOSE. Mutations in the PDE6B gene cause recessive, severe retinitis pigmentosa (RP). PDE6B encodes the β subunit of the rod-specific phosphodiesterase (βPDE), which, when absent, results in toxic levels of intracellular Ca²⁺ and photoreceptor cell death. Ca²⁺ blockers, such as nilvadipine, as well as light restriction, slow photoreceptor degeneration in animal models of βPDE deficiencies. The goal of the study was to evaluate the efficacy of AAV2/5- or AAV2/8-mediated gene replacement in combination with nilvadipine and/or with light restriction in the rd10 mouse bearing homozygous pde6b mutations.

METHODS. AAV vectors encoding either βPDE or EGFP were subretinally administered at postnatal day (P)2. Nilvadipine was administered from P7 to P28. For light restriction, pregnant rd10 mice were kept in a dark environment until their pups were 28 days old. All functional and histologic analyses were performed at P35.

RESULTS. Significant morphologic photoreceptor protection was observed after subretinal administration of AAV vectors encoding EGFP. This protection further increased after administration of AAV2/8- or -2/5 encoding for βPDE and was not associated with significant functional improvement. Photoreceptor protection was higher after AAV2/8- than after AAV2/5-mediated delivery and was not significantly augmented by additional drug therapy and/or light restriction. The protective effect was lost after P35.

CONCLUSIONS. In conclusion, more efficient gene transfer tools than those used in this study, as well as a better understanding of the disease pathogenesis, should be explored to increase the effect of gene replacement and to design gene-based strategies that block the apoptotic pathways activated by βPDE deficiency. (Invest Ophthmol Vis Sci. 2011;52:5713–5719) DOI:10.1167/ iovs.10-6269

From the 1Telethon Institute of Genetics and Medicine (TIGEM) and 2Medical Genetics, Department of Pediatrics, Federico II University, Naples, Italy.

Supported by the European Commission under the FP7 AAVEYE Project, Grant HEALTH-2007-B-223445, and the FP7 TREATRUSH Project, Grant 242013.

Submitted for publication July 23, 2010; revised December 1, 2010; accepted December 20, 2010.

Disclosure: M. Allocca, None; A. Manfredi, None; C. Iodice, None; U. Di Vicino, None; A. Auricchio, None

Corresponding author: Alberto Auricchio, Telethon Institute of Genetics and Medicine. Via P. Castellino, 111, 80131 Naples, Italy; auricchio@tigem.it.

Investigative Ophthalmology & Visual Science, July 2011, Vol. 52, No. 8
Copyright 2011 The Association for Research in Vision and Ophthalmology, Inc.

Reprint requests to: Alberto Auricchio, 1,2

Retinitis pigmentosa (RP) is the term given to a set of genetically and clinically heterogeneous retinal diseases affecting 1.5 million people.1,2 Symptoms include night blindness, progressive loss of the peripheral visual field, and eventually loss of central vision caused by degeneration of photoreceptor cells (PRs).1,2 RP may be inherited as an autosomal dominant (ad), autosomal recessive (ar), X-linked, or simplex/multiplex disease.3,4 An increasing number of genes responsible for RP have been identified, most of which are expressed specifically in PRs.

Mutations in the PDE6B gene encoding for the β subunit (βPDE) of the rod cGMP phosphodiesterase 6 (PDE6) are a cause of arRP, accounting for 4% to 5% of all arRPs in the United States and 6% of arRPs in Spain.5 Patients with homozygous mutations in the PDE6B gene present with classic, severe RP, which manifests with symptoms such as night blindness from childhood and the absence of any rod response at the ERGs.6,7 PDE6, which regulates cytoplasmic cGMP levels in rod PRs in response to light, is a heterotetrameric complex composed of two catalytic subunits (α and β) and two inhibitory subunits (γ). On light stimulation, PDE6 activation leads in turn to (1) the reduction of cytoplasmic cGMP levels, (2) the closure of cGMP-gated cation (Na⁺ and Ca²⁺) channels, (3) the hyperpolarization of the rod plasma membrane, and, ultimately, (4) the generation of the receptor potential at the PR synapse. The absence of PDE6 activity due to mutations in PDE6B results in the disruption of the phototransduction cascade and to high levels of intracellular cGMP and therefore of Ca²⁺, leading to PR death by apoptosis.7 Because of the crucial role of PDE6 in the rod phototransduction cascade, mutations in the PDE6B gene result in severe RP, for which no cure is currently available.

Two spontaneous murine models (rd1 and rd10 mice) and a canine model (rcd1 dogs) of arRP with mutations in the pde6b gene that replicate the human condition have been identified.8–10 Attempts at replacing pde6b in the rd1 mouse by adenoviral,11,12 adenoassociated,13 and lentiviral14 vectors have failed to produce evidence of prolonged, sustained morphologic and functional PR rescue, presumably because of the limitations of the vectors used (resulting in low levels of PR transduction) and the severity of the rd1 degeneration.

Calcium channel blockers, such as β-cis-diltiazem15–17 or nilvadipine,18 have been used to delay retinal degeneration in both murine15,16,18 and dog models of βPDE deficiency.17 In an initial study testing the efficiency of diltiazem, researchers reported a beneficial effect in the rd1 mouse model.15 However, additional studies in the same murine model, as well as in
Recent studies have suggested the protective effect of nilvadipine, another calcium antagonist. In addition, the transient inhibition of the phototransduction cascade obtained by dark rearing appears to further delay the rate of rd10 retinal degeneration by as much as 4 weeks. Recently, Pang et al demonstrated that gene replacement combined with dark rearing results in rd10 morphologic and functional improvement.

The safety and efficacy of adeno-associated viral (AAV) vector-mediated retinal gene transfer has been demonstrated in several species, including humans. Since the generation of the first AAV vector, many serotypes differ in the composition of the capsid surface proteins, which affect their tropism and transduction characteristics. In particular, we have recently shown that AAV2/8 mediates in vivo PR transduction with an efficiency that is six times higher than AAV2/5, regarded thus far as the most efficient for PR targeting. Consistent with this finding, Tan et al. have shown AAV2/8-mediated protection in a model of Leber congenital amaurosis due to Aip1 deficiency, suggesting that the AAV2/8 vector may be more efficient than the AAV2/5 and AAV2/2/11 vectors, which have been used as retinal gene transfer tools in the rd10 and rd1 models of βPDE deficiency, respectively.

Our purpose was to compare the efficiency of AAV2/5- and AAV2/8-mediated gene replacement in rd10 mice in combination, or not, with nilvadipine and/or dark rearing.

**Materials and Methods**

**Generation of the Plasmid Constructs and AAV Vector Production**

For the production of AAV encoding EGFP and βPDE, pAAV2.1-CMV-EGFP, pAAV2.1-CMV-PDE6B (a kind gift of Markus Hildinger, Telethon Institute of Genetics and Medicine [TIGEM], Naples, Italy), pAAV2.1-CMV-pde6b-HA, and pAAV2.1-RHO-PDE6B were used. To generate pAAV2.1-CMV-pde6b-HA, we amplified the pde6b gene from murine cDNA with primers NotI-Ha/forward 5'-GCCGAAGGATCCCCATGTAATCGATCGAGTACGACTAGCAAGGCTAGGAGAAGG-3' containing the influenza virus hemagglutinin (HA) tag and HindIII-reverse 5'-AAGCTTATATAGATCAGCAGCGG-3'. The PCR products were then digested with NotI and HindIII and cloned into pAAV2.1-CMV-EGFP. pAAV2.1-RHO-PDE6B was obtained by exchanging the CMV promoter of pAAV2.1-CMV-PDE6B with the human RHO promoter derived from pAAV2.1-RHO-EGFP digested with Nhel and NotI. The AAV2/8 and 2/5 vectors were produced by the TIGEM Vector Core by triple transfection of 293 cells followed by two rounds of CsCl2 purification. For each viral preparation, physical titers (geometric mean) were determined via genetic analysis (TaqMan; Applied Biosystems, Foster City, CA).

**Animal Models, Vector, and Drug Administration**

All procedures on animals were performed in accordance with the institutional guidelines for animal research and with the ARVO Statement for the Use of Animal in Ophthalmic and Vision Research. R	extit{d}10 mice (obtained from The Jackson Laboratory, Bar Harbor, ME) and wild-type C57BL/6 mice (Harlan, S. Pietro al Natisone, Italy) were used in our experimental setting. Subretinal vector administration was performed at postnatal day (P)2, as described. Early postnatal administration was chosen to provide the optimal therapeutic effect with respect to disease progression. Before vector administration, the mouse pups were anesthetized by hypothermia. They were injected subretinally with 0.75 µL (the dose of vector is specified in the Results and Discussion section) of AAV2/5 or -2/8 encoding βPDE in the right eye. The same dose of AAV2/5- or AAV2/8-CMV-EGFP in 0.75 µL was injected as a control in the left eye. For subretinal vector administration, the eyelids of the newborn mouse were opened artificially by an incision on the skin between the upper and lower lids. The eye was exposed and a conjunctival peritomy made. A 33-gauge needle was passed through the sclera, and the injection was delivered.

Nilvadipine (a generous gift from Astellas Pharma Inc., Tokyo, Japan) was administered to the rd10 mice by intraperitoneal injections from postnatal day 7. The drug was dissolved in a mixture of ethanol:polyethylene glycol 400:distilled water (2:1:7) at a concentration of 0.1 mg/mL and diluted twice with a physiologic saline solution. The injections were performed once a day (0.05 mg/kg). The final injection was given 1 week before the recordings were made, to allow clearance of nilvadipine and prevent interactions with HERG measurements.

**Dark Rearing**

Late-term (at approximately embryonic day [E] 14) pregnant rd10 females were moved from a normal 12-hour light/12-hour dark cyclic light environment into a continuously dark room until the newborn pups were 24, 28, or 35 days old (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.106209-DCSupplemental). For gene transfer experiments, the pups were reared in a normal 12-hour light/12-hour dark cyclic light environment from P28 to P35 (see Figs. 2, 3, 4).

**Cos7 Cell Transfection and Infection**

Cos7 cells were plated in six-well plates at a concentration of 3 × 10⁵ cell/well. Forty-four hours later, the cells were transfected with 1 µg of pAAV2.1-CMV-EGFP or pAAV2.1-CMV-PDE6B with a proprietary transfection reagent (Fugene; Roche, Basel, Switzerland) or incubated for 2 hours with 10³ GC/cell of AAV2/8- or AAV2/5-CMV-EGFP, or AAV2/8- or AAV2/5-CMV-PDE6B in serum-free DMEM. Forty-eight hours later, the cells were harvested by scraping for Western blot analyses.

**Western Blot Analyses**

Western blot was performed on retinas and on Cos7 cells. The retinas were harvested, as described. Samples were lysed in hypotonic buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 1.5 mM MgCl₂, 1% CHAPS, 1 mM PMSF, and protease inhibitors) and separated by 10% SDS-PAGE. After the blots were obtained, specific proteins were labeled with anti-βPDE (1:500; Abcam, Inc., Cambridge, MA) and anti-α tubulin (1:1000; Sigma-Aldrich, Milan, Italy) antibodies.

**Histologic Analyses**

Mice were killed, and their eyeballs were harvested and fixed overnight by immersion in 4% paraformaldehyde. Before the eyeballs were harvested, the temporal aspect of the sclera was marked by cautery, to orient the eyes with respect to the injection site at the moment of the inclusion. The eyeballs were cut so that the lens and vitreous could be removed, leaving the eye cup intact. Mouse eye cups were infiltrated with 30% sucrose for cryopreservation and embedded in tissue freezing medium (OCT matrix; Kaltek, Padua, Italy). For each eye, 150 to 200 serial sections (10-µm-thick) were cut along the horizontal plane, the sections were progressively distributed on 10 slides so that each slide contained 15 to 20 sections, each representative of the whole eye at different levels. The sections were stained with 4',6-diamidino-2-phenylindole (DAPI; Vectorshields, Inc., Peterborough, UK) and retinal histology images were obtained with a microscope (Axioject; Carl Zeiss Meditec, Oberkochen, Germany) with 40× magnification. The sections were also stained with hematoxylin and cosin (Sigma-Aldrich, Milan, Italy), according to standard procedures, and retinal histology was analyzed by light microscopy. To quantify PR rescue, the number of nuclei in the outer nuclear layer (ONL) of each eye was counted. A minimum of three sections per slide, representative of the entire eye cup, were analyzed. For each section, the number of nuclei in the ONL was separately counted on
the nasal, central, and temporal sides. The counts of each section were independently averaged, obtaining the average of the three sides for each eye. The counts from each group were then averaged, and standard errors were calculated.

**Immunofluorescence**

For HA staining, the tissue sections (OCT; see the Histologic Analyses section for inclusion procedures) were permeabilized for 20 minutes with 1× PBS, 0.2% Triton X-100, and 1% normal goat serum (NGS); blocked with 10% NGS; and then incubated for 2 hours with HA antibody (1:1000; Covance, Emeryville, CA). The sections were washed, incubated for 1 hour with the secondary antibody AlexaFluor 594 (donkey anti-mouse IgG; Invitrogen, Gaithersburg, MD), and mounted with antifade medium (Vectashield with DAPI; Vector Laboratories, Inc., Peterborough, UK). Fluorescence photographs were obtained (Axioskop; Carl Zeiss Meditec) at 40× magnification.

**Electrophysiological Recordings**

For ERG analysis, rd10 mice were dark adapted for 180 minutes. They were anesthetized with an intraperitoneal injection of Avertin (1.25% wt/vol of 2,2,2-tribromoethanol and 2.5% vol/vol of 2-methyl-2-butanol; Sigma-Aldrich) at 2 mL/100 g of body weight and positioned in a stereotaxic apparatus under dim red light. Their pupils were dilated with a drop of 1% tropicamide (Alcon Laboratories, Inc., Fort Worth, TX) and the body temperature maintained at 37.5°C. ERGs were evoked by 10-ms flashes of different light intensities ranging from 10⁻⁴ to 20 cd m⁻² s⁻¹ generated by a Ganzfeld stimulator (CSO, Florence, Italy). To minimize the noise, three different responses evoked by light were averaged for each luminance step (the time interval between light stimuli was 4 to 5 minutes). The electrophysiological signals were recorded through gold-plate electrodes inserted under the lower eyelids in contact with the cornea. The electrodes in each eye were referred to a needle electrode inserted subcutaneously at the level of the corresponding frontal region. The different electrodes were connected to a two-channel amplifier. Amplitudes of a- and b-waves were plotted as a function of increasing light intensities. After completion of responses obtained in dark-adapted conditions (scotopic) the recording session continued with the purpose of dissecting the cone pathway mediating the light response (photopic). To this end, the ERG in response to light of 20 cd m⁻² was recorded in the presence of a continuous background light (background light set at 50 cd m⁻²). For each group, the mean b-wave amplitude was plotted as a function of luminance (transfer curve) under scotopic and photopic conditions.

**RESULTS AND DISCUSSION**

**Effect of Nilvadipine and Dark Rearing on rd10 Retinal Degeneration**

We initially tested the efficacy of nilvadipine on rd10 PR degeneration in our experimental setting. Rd10 mice were given daily intraperitoneal nilvadipine injections from postnatal day (P7) to P24, P28, or P35. The early postnatal administration (P7) was performed to prevent PR degeneration in the rd10 mice,9 and the time points of harvesting were selected according to the timing of retinal degeneration in this mouse model.8 The rows of PR nuclei were counted, to quantify drug efficacy. Nilvadipine treatment resulted in a significant increase in rows of PR nuclei compared with the number in untreated animals (Supplementary Fig. S1A, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6269/-/DCSupplemental, at P24), although the protective effect of the compound was lost by P35. Similarly, as shown by Chang et al.,10 we found that dark rearing delayed rd10 PR degeneration (Supplementary Fig. S1B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6269/-/DCSupplemental). These data indicate that nilvadipine and a dark environment delay PR loss in the rd10 mouse model.

Based on these findings, we hypothesized that these treatments could expand the therapeutic window to allow AAV-mediated transduction, and we decided to test AAV-mediated gene replacement, with and without nilvadipine treatment and/or dark rearing in the rd10 model. In addition, we planned to compare the efficacy of the AAV2/8 vectors, which we demonstrated to be the best in murine PR transduction among the series of AAV serotypes tested,32 as opposed to AAV2/5, a serotype known to efficiently transduce PRs of various species.40–42

<table>
<thead>
<tr>
<th>Dark</th>
<th>E14</th>
<th>P2</th>
<th>P7</th>
<th>P28</th>
<th>P35</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV injection</td>
<td>Nilvadipine (0.05 mg/Kg/day i.p.)</td>
<td>ERG Histo</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 2.** The experimental plan. Rd10 mice were injected at P2 with AAV vectors encoding for βPDE or EGFP. Nilvadipine was administered IP daily from P7 to P28. ERG and histologic analyses (Histo) were performed 1 week after the last drug injection (P35). The mice were kept in the dark from E14.
Assessment of βPDE Expression In Vitro and In Vivo

We produced AAV2/5 and -2/8 vectors encoding human or murine βPDE or EGFP under the control of the ubiquitous cytomegalovirus (CMV) or the PR-specific rhodopsin (RHO) promoters (Fig. 1A). We then tested whether the transduction mediated by AAV2/5 and -2/8 encoding for βPDE resulted in the expression of the expected protein in vitro and in vivo. To this end, Cos7 cells were infected with AAV2/5 or -2/8 encoding for EGFP or human βPDE. Western blot analysis of cellular lysates with anti-βPDE antibody showed a band corresponding to βPDE in the samples infected with AAV2/5 or -2/8 encoding for βPDE, but not in those infected with the control vector encoding for EGFP (Fig. 1B). Lysates from wild-type retinas and lysates from Cos7 cells transfected with the pAAV2.1-CMV-PDE6B were used as positive controls. For the in vivo expression experiments, we used a vector expressing murine βPDE with the influenza virus HA tag because rd10 mice express a mutant βPDE recognized by anti-βPDE antibodies. Four-week-old C57BL/6 mice were injected subretinally at P28 in the right eye with a mixture of AAV2/8-CMV-pde6b-HA (1.2 × 10^9 GC/eye) and AAV2/1-CMV-EGFP (1.2 × 10^8 GC/eye), and the left eyes were injected with an AAV2/1-CMV-EGFP as the control. Recombinant βPDE-HA expression was detected by immunofluorescence with anti-HA antibodies on retinal sections and was found to properly localize to the PR outer segments (Fig. 1C).

Thus, the AAV vectors we have produced efficiently express βPDE in vitro and in vivo and could be further tested for their ability to slow or halt PR degeneration in the rd10 mouse model.

Intravitreal Administration of AAV2/5 and -2/8 to the rd10 Retina

Subretinal delivery of viral vectors is preferred to intravitreal delivery for obtaining outer retinal transduction. However, subretinal injections are more complex than intravitreal injections, and the transduction of the retinal region is generally restricted to the area surrounding the injection site. Moreover, Park et al.43 and Kolstad et al.44 have recently demonstrated that AAV intravitreal administration results in the transduction of the outer retina in models of retinal disease, in which the retinal architecture is mainly altered by the potential disruption of the inner limiting membrane, which divides the retina from the vitreous hu-

![A](image1.png)

![B](image2.png)

**Figure 3.** PR preservation in rd10 mice after AAV-mediated βPDE delivery. (A) The histograms represent the number of rows of PR nuclei in the ONL at P35 of untreated wild-type (WT) or rd10 mice, either untreated (NT) or injected with AAV2/8 or -2/5 encoding for human βPDE in one eye and EGFP in the contralateral one. Data are the mean ± SE; n, number of animals in each group; *P ≤ 0.05; **P ≤ 0.025; ***P ≤ 0.0001. (B) DAPI staining of representative retinal sections analyzed in (A). For abbreviations, see the legend to Figure 1C.
Based on these findings, we tested whether intravitreal delivery of AAV2/5 or -2/8 would result in rd10 PR transduction. We injected AAV2/5- and -2/8-CMV-EGFP (1 × 10^9 GC/eye) intravitreally in rd10 mice at P8, P15, or P21. One week after the injection, the eyes were harvested and the retinas were processed for histologic analysis. No significant outer retina transduction was observed, except in certain areas of the retinal pigment epithelium (RPE) and in the Müller cells in the retinas injected with AAV2/8 at P21 (data not shown). We thus concluded that subretinal administration of AAV vectors should be used for gene delivery to the rd10 retina.

Assessment of Rescue after AAV-Mediated Gene Replacement in the rd10 Animal Model

Rd10 animals were injected subretinally at P2 in the right eye with AAV2/5 or -2/8 vectors encoding for βPDE (2.1 × 10^9 GC/eye), and the left eyes were injected with the same doses of AAV2/5 or -2/8 vectors encoding EGFP. Electrophysiological analyses (ERGs) were performed at P35 (Fig. 2) and showed no electrical response in scotopic (dark) or in photopic (light) conditions in either eye (Supplementary Fig. S2A, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6269/-/DCSupplemental). Harvesting of the eyes for histologic analyses was performed at P35.

**Figure 4.** PR preservation in rd10 after AAV-mediated gene replacement in combination with dark rearing and/or nilvadipine treatment. (A) Retinal morphologic rescue in rd10 mice after AAV-mediated PDE6B gene delivery in combination with dark rearing and/or nilvadipine treatment. The histograms represent the number of rows of PR nuclei in the ONL at P35. Experimental groups are as described in Figure 3. Data are the mean ± SE; n, number of animals in each group. *P ≤ 0.05. (B) DAPI staining of representative retinal sections analyzed in (A). For abbreviations, see the legend to Figure 1C.
Histologic analysis showed that delivery of AAV2/8 vectors encoding for βPDE resulted in better morphologic rescue than did the AAV2/5 vectors (Fig. 3 and Supplementary Fig. S5A, http://www iovs org/lookup/suppl do10.1167/iovs.106269/-/DCSupplemental). Similar to previous findings,15,46 we observed a significant PR protection after subretinal administration of AAV vectors encoding EGFP, thus suggesting that injury associated with retinal injection triggers a neurotrophic response in the rd10 retina.

Given the limited improvement obtained by the administration of vectors encoding for βPDE, the next step was to test the effect of gene replacement combined with dark rearing and/or nilvadipine treatment. rd10 mice were injected subretinally at P2 with AAV2/5 or -2/8 encoding βPDE or EGFP (2.1 × 109 GC/eye). The mice were received nilvadipine daily from P7 to P28 and/or kept in darkness from ∼E14, until they were 28 days old. ERGs were performed at P35, 1 week after the last nilvadipine injection, to allow drug clearance and to avoid any interference with PR function (Fig. 2).

To confirm that the experimental plan of nilvadipine administration depicted in Figure 2 had no effect on PR electrical activity measured at P35, ERGs were measured at P35 in wild-type C57BL/6 mice given nilvadipine according to the schedule in Figure 2. Their a- and b-wave amplitudes were similar to those of the age-matched noninjected mice used as controls (data not shown).

When delivery of AAV2/8 vectors encoding for βPDE was coupled with nilvadipine treatment, a significant improvement in the number of PR nuclei in the ONL was evident, compared with those of the age-matched noninjected mice used as controls (data not shown).

The data presented in Figures 3 and 4 were produced with null vectors, and the method of gene delivery (P14 for Pang et al., P2–P4 in this study) and the area of retina treated (reported to be ∼50% of EGFP-transduced retina after subretinal injections in newborn mice) influence the extent of PR functional rescue obtained. The authors thank Graciana Diez-Roux and Luciana Borrelli for a critical reading of the manuscript, Maurizio Di Tommaso for technical help with the animal work, and the TGEM Vector Core for producing the AAV vectors.

References
17. Pearce-Kelling SE, Aleman TS, Nickle A, et al. Calcium channel blocker D-cis-diltiazem does not slow retinal degeneration in the...


