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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

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ABSTRACT

PURPOSE: Mutations in the *PDE6B* gene cause recessive severe Retinitis Pigmentosa (RP). *PDE6B* encodes the β subunit of the rod-specific phosphodiesterase (βPDE), whose absence results in toxic levels of intracellular Ca^{++} and photoreceptor cell death. Ca^{++}-blockers, such as nilvadipine, as well as light restriction slow photoreceptor degeneration in animal models of βPDE deficiencies. The goal of our 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 post natal day 2 (P2). 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 histological analyses were performed at P35.

RESULTS: We observed significant morphological photoreceptor protection after subretinal administration of AAV vectors encoding EGFP. This further increased after administration of AAV2/8 or AAV2/5 encoding for βPDE and was not associated with significant functional improvement. Photoreceptor protection was higher after AAV2/8- than 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 here 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 aimed at blocking the apoptotic pathways activated by βPDE deficiency.

INTRODUCTION

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 (PR)[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 photoreceptor cells.

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 4-5% of all arRPs in the US[5] and 6% of arRPs in Spain[6]. Patients with homozygous mutations in the *PDE6B* gene present a classic severe RP, which is manifested with symptoms such as night blindness from childhood and absence of any rod response at the ERGs[1, 5]. PDE6 is a heterotetrameric complex composed of two catalytic subunits (α and β) and two inhibitory subunits (γ), which regulates cytoplasmic cGMP levels in rod photoreceptors in response to light. 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 Ca^{++}) channels; 3) the hyperpolarization of the rod plasma membrane, and, ultimately, 4) the generation of the receptor potential at the photoreceptor 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 Ca^{++}, leading to PR death through apoptosis[7]. Due to 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 have been identified, which recapitulate the human condition[8-10].

Attempts at replacing *pde6b* in the *rd1* mouse by adenoviral[11-12], adeno-associated[13] and lentiviral vectors[14] have failed to produce evidence of prolonged and sustained morphological and functional photoreceptor rescue, presumably due to the limitations of the vectors used (resulting in low levels of photoreceptor transduction) and to the severity of the *rd1* degeneration.

Calcium channel-blockers, such as cis-D-diltiazem[15-17] or nilvadipine[18], have been used to delay retinal degeneration in both murine[15, 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 *rcd1* dogs did not confirm this beneficial effect[16-18]. Recent studies have suggested the protective effect of nilvadipine, another calcium antagonist[18]. In addition, the transient inhibition of the photo-transduction cascade obtained by dark rearing appears to further delay the rate of *rd10* retinal degeneration by as much as 4 weeks [10]. Recently, Pang *et al.* have demonstrated that gene replacement combined with dark rearing results in *rd10* morphological and functional improvement[19].

The safety and efficacy of adeno-associated viral (AAV) vector-mediated retinal gene transfer has been demonstrated in several species[20], including humans[21-28]. Since the generation of the first AAV vector[29-30], AAV2/2, where the first number defines the vector genome and the second the capsid, dozens of AAV variants have been isolated, some of which have been converted in gene delivery vehicles[31]. AAV 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* photoreceptor transduction with an efficiency that is 6-fold higher than AAV2/5, considered thus far the most efficient for photoreceptor targeting[32]. Consistently with this, Tan *et al.* and Sun *et al.* have shown AAV2/8-mediated protection in a model of Leber Congenital Amaurosis due to Aipl1 deficiency[33-34], suggesting that AAV2/8 may be more efficient than AAV2/5[19] and AAV2/2[11] vectors, which were previously used as retinal gene transfer tools in the *rd10* and *rd1* models of βPDE deficiency, respectively.

Our aim here 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.

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 administered with daily intraperitoneal nilvadipine injections from postnatal day 7 (P7) to P24, or P28, or P35. The early postnatal administration (P7) was performed to prevent PR degeneration in *rd10* mice[8], and the time points of harvesting were selected according to the timing of retinal degeneration in this mouse model[8]. The number of rows of PR nuclei was counted to quantify drug efficacy. Nilvadipine treatment resulted in a significant increase of rows of PR nuclei compared to untreated animals (Fig. S1A, at P24) although the protective effect of the compound was lost by P35. Similarly, as shown by Chang *et al*., we found that dark rearing delayed *rd10* PR degeneration (Fig. S1B)[10]. These data indicate that nilvadipine and dark environment delays 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 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 AAV2/8 vectors, which we demonstrated to be the best in murine PR transduction among a series of AAV serotypes tested[32], as opposed to AAV2/5, a serotype known to efficiently transduce PR of various species[35-37].

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 photoreceptor-specific rhodopsin (RHO) promoters (Fig. 1A). We then tested whether the transduction mediated by AAV2/5 and AAV2/8 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 AAV2/8 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 AAV2/8 encoding for βPDE but not in those infected with the control vector encoding for EGFP (Fig. 1B). Lysates from wild-type retinae 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 hemagglutinin (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 ($1.2x10^9$ GC/eye) and AAV2/1-CMV-*EGFP* ($1.2x10^8$ GC/eye), while the left eyes were injected with an AAV2/1-CMV-*EGFP* as 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/halt photoreceptor 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 to obtain 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, Kolstad *et al.* and Park *et al*. have recently demonstrated that AAV intravitreal

administration resulted 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 humour[38-39]. Based on these findings, we tested if intravitreal delivery of AAV2/5 or 2/8 resulted in *rd10* PR transduction.

We injected AAV2/5- and 2/8-CMV-*EGFP* ($1x10^9$ 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 histological analysis. No significant outer retina transduction was observed, except for certain areas of the retinal pigment epithelium (RPE) and of Müller cells in the retinas injected with AAV2/8 at P21 (data not shown). We thus concluded that subretinal administrations 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 AAV2/8 vectors encoding for βPDE ($2.1\ x10^9$ GC/eye), while the left eyes were injected with the same doses of AAV2/5 or AAV2/8 vectors encoding EGFP. Electrophysiological analyses (ERGs) were performed at P35 (Fig. 2) and showed no electrical response neither in scotopic (dark) nor in photopic (light) conditions in either eye (Fig. S2A). Harvesting of the eyes for histological analyses was performed at P35. Histological analysis showed that delivery of AAV2/8 vectors encoding for βPDE results in better morphological rescue than AAV2/5 (Fig. 3 and Fig. S3A). Similarly to previous findings[40-41], we observed a significant PR protection after subretinal administration of AAV vectors encoding EGFP, thus suggesting that injury associated with retinal injection may trigger 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 AAV2/8 encoding βPDE or

EGFP (2.1 x10^9 GC/eye). Mice were administered with nilvadipine on a daily basis from P7 to P28 and/or kept in darkness from around embryonic day 14 until they were 28 days old. ERGs were performed at P35, one 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 Fig. 2 had no effect on PR electrical activity measured at P35, ERGs were measured at P35 in wild-type C57BL/6 administered with nilvadipine following the Fig. 2 schedule. Their a- and b-wave amplitudes were similar to age-matched non-injected 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 number of PR nuclei in the outer nuclear layer (ONL) was evident compared to animals treated with AAV-EGFP plus nilvadipine (Fig. 4 and Fig. S3B). However, no further significant improvement was observed when gene replacement was coupled to either dark or nilvadipine plus dark treatment (Fig. 4A). In addition, this protective effect was lost at P60 (data not shown). No significant functional rescue was observed, neither in scotopic nor photopic conditions, thus suggesting that combination of nilvadipine and/or dark rearing with AAV2/8-mediated gene replacement results in partial and transient morphological improvement in *rd10* mice (Fig. S2B, C, D). Recently, Pang *et al.* have reported consistent PR functional rescue following AAV2/5-mediated retinal *pde6b* gene transfer in dark-reared *rd10* mice. It is possible that the time of gene delivery (P14 for Pang *et al*., P2-P4 in this study) and the area of retina treated (reported to be more than 50% in the study by Pang *et al.*, while we obtain 20-30% of EGFP-transduced retina after subretinal injections in newborn mice) influence the entity of photoreceptor functional rescue obtained.

The data presented in Figures 3 and 4 were produced mainly using AAV vectors encoding the human *PDE6B* gene under the transcriptional control of the CMV promoter. Some eyes were injected with vectors containing the RHO promoter (n=4) or the murine *pde6b* cDNA (n=2) and

gave results similar to those injected with the AAV-CMV-*PDE6B* vectors suggesting that the therapeutic outcome we observe is independent of the transgene specie or of the use of a photoreceptor-specific promoter.

The severity of the *rd10* phenotype and the pathogenic mechanism of RP retinal degeneration may require higher levels of gene expression than those provided by the AAVs tested: RP initially affects the peripheral retina, resulting in the degeneration of rods, while the cones and central vision are preserved at this stage[1]. With the progression of the disease, the cones also degenerate (rod–cone degeneration) suggesting a non cell-autonomous mechanism of cell death. Therefore, widespread PR transduction (beyond the levels obtained here) may be desirable to prevent detrimental effects from non-transduced PRs. Modified AAV serotypes appear to provide higher levels of PR transduction resulting in better rescue (Pang JJ *et al*. *IOVS* 2010*;* 51:ARVO E-Abstract 2527) than what Pang *et al.* [19] and we obtained here. In addition, in *rd10* mice rod degeneration starts around P18, but ERG reveals alterations in the physiology of the inner retina as early as P18 before any obvious morphological change of inner neurons is evident (around P25)[42]. All these observations suggest that an early high and widespread βPDE expression is necessary to restore PDE6 activity and inhibit rod apoptosis in models of βPDE deficiency.

MATERIALS AND METHODS

Generation of the plasmid constructs and AAV vectors production.

For the production of AAV encoding EGFP and βPDE, the pAAV2.1-CMV-*EGFP*[43], the pAAV2.1-CMV-*PDE6B* (a kind gift of M. Hildinger, TIGEM, Naples, Italy), the pAAV2.1-CMV-*pde6b-HA* and the pAAV2.1-RHO-*PDE6B* were used[43]. To generate pAAV2.1-CMV-*pde6b-HA,* the *pde6b* gene was amplified from murine cDNA with primers NotI-HA-forward 5'-GCGGCCGCCATGTATCCGTACGACGTACCAGACTACGCAAGCCTCAGTGAGGAACAG-3' containing the influenza virus hemagglutinin (HA) tag and HindIII-reverse 5'-

AAGCTTTTATAGGATACAGCAGCAGG-3'.The PCR products were then digested with NotI and HindIII and cloned into pAAV2.1-CMV-*EGFP*. pAAV2.1-RHO-*PDE6B* was obtained exchanging the CMV promoter of pAAV2.1-CMV-*PDE6B* with the human RHO promoter derived from pAAV2.1-RHO-*EGFP* digested with NheI and NotI[32]. AAV2/8 and 2/5 vectors were produced by the TIGEM vector core by triple transfection of 293 cells followed by two rounds of $CsCl_2$ purification[43]. For each viral preparation, physical titers [genome copies (GC)/ml] were determined by dot blot analysis[44] and by PCR quantification using TaqMan (Applied Biosystems, Foster City, CA, U.S.A.)[45].

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. *Rd10* mice (obtained from The Jackson Laboratories, Bar Harbor, ME, U.S.A.), 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 2 (P2), as described[46]. Early postnatal administration was chosen in order to provide the optimal therapeutic effect with respect to disease progression. Before vector administration, pups were anesthetized by hypothermia. Mouse pups were injected subretinally with 0,75 µl (the dose of vector is specified in the results and discussion section) of AAV2/5 or AAV2/8 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 the subretinal vector administrations, the eyelids of the newborn mouse are opened artificially by an incision on the skin between the upper and the lower lid. The eye is exposed and a conjunctival peritomy is made. A 33-gauge needle is passed through the sclera and then the injection is delivered[46].

Nilvadipine (a generous gift from Astellas Pharma Inc., Tokyo, Japan) was administered by intraperitoneal injections, from postnatal day 7 in *Rd10* mice. Nilvadipine 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 physiological saline solution. The injections were performed once a day (0.05 mg/kg). To allow clearance of nilvadipine and prevent interactions with ERG measurements the final injection was given one week before recordings were made.

Dark rearing.

Late-term (around embryonic day 14) pregnant *rd10* females were moved from a normal 12-hour light/12-hour dark cyclic light environment in a continuously dark room until the newborn pups were 24, 28 or 35 days old (Fig. S1). For gene transfer experiments pups were reared in a normal 12-hour light/12-hour dark cyclic light environment from P28 to P35 (Fig. 2, 3 and 4).

Cos7 cells transfection and infection.

Cos7 cells were plated in 6-well plates at a concentration of $3x10^5$ cell/well. Fourty-four hrs later, the cells were transfected with 1 μg of the pAAV2.1-CMV-*EGFP* or the pAAV2.1-CMV-*PDE6B* by using Fugene (Roche, Basel, Switzerland) or incubated for two hrs with 10^5 GC/cell of AAV2/8- or AAV2/5-CMV-*EGFP* or AAV2/8- or AAV2/5-CMV-*PDE6B* in serum free DMEM. Forty-eight hrs later the cells were harvested by scraping for Western blot analyses (see next section).

Western blot analyses.

Western blot was performed on retinas and on Cos7 cells. Retinas were harvested, as described[47]. Samples were lysed in hypotonic buffer [10 mM Tris-Hcl ph 7.5, 10 mM NaCl, 1,5mM MgCl2, 1% CHAPS, 1mM PMSF, protease inhibitors] and separated by 10% SDS-PAGE. After blotting, specific proteins were labeled using anti-βPDE (Abcam, Inc., Cambridge, MA, U.S.A., 1:500) and anti-α tubulin (Sigma-Aldrich, Milan, Italy, 1:1000) antibodies.

Histological analyses.

Mice were sacrificed, and their eyeballs were then harvested and fixed overnight by immersion in 4% paraformaldehyde. Before harvesting the eyeballs, the nasal aspect of the sclerae is marked by cautery, in order 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 eyecup intact. Mice eyecups were infiltrated with 30% sucrose for cryopreservation, and embedded in tissue freezing medium (O.C.T. 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 DAPI (Vectashield, Vector Lab, Inc., Peterborough, UK) and retinal histology images were obtained with a Zeiss Axiocam (Carl Zeiss, Oberkochen, Germany) with 40X magnification. The sections were also stained with hematoxylin and eosin (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 were counted. A minimum of three sections/ slide, representative of the entire eyecup, were analyzed. For each section, the number of nuclei in the ONL was separately counted on the nasal, central and temporal sides. The nasal, temporal and central counts of each section were independently averaged, obtaining a number that was 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 (O.C.T., see Histological analyses section for inclusion procedures) were permeabilized for 20 minutes with 1x PBS, 0.2% Triton X-100, 1% Normal Goat Serum (NGS), blocked with 10% NGS, and than incubated for 2h with HA-antibody (1:1000, Covance, Emeryville, CA, U.S.A.). After washing, sections were incubated for 1h with secondary antibody Alexa–Fluo 594 (donkey anti-mouse IgG, Invitrogen, Gaithersburg, MD, U.S.A.). The

retinal sections were mounted with Vectashield with DAPI (Vector Lab, Inc., Peterborough, UK) before fluorescence photography was obtained with a Zeiss Axiocam (Carl Zeiss, Oberkochen, Germany) with 40X magnification.

Electrophysiological recordings.

For ERG analysis *rd10* mice were dark-adapted for 180 min, 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, Milan, Italy) at 2 ml/100 g of body weight, accommodated in a stereotaxic apparatus under dim red light, their pupils dilated with a drop of 1% tropicamide (Alcon Laboratories, Inc., Fort Worth, TX, U.S.A.) 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 through 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 4-5 min). The electrophysiological signals were recorded throughout gold plate electrodes inserted under the lower eyelids in contact with the cornea. 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 aim to dissect 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.

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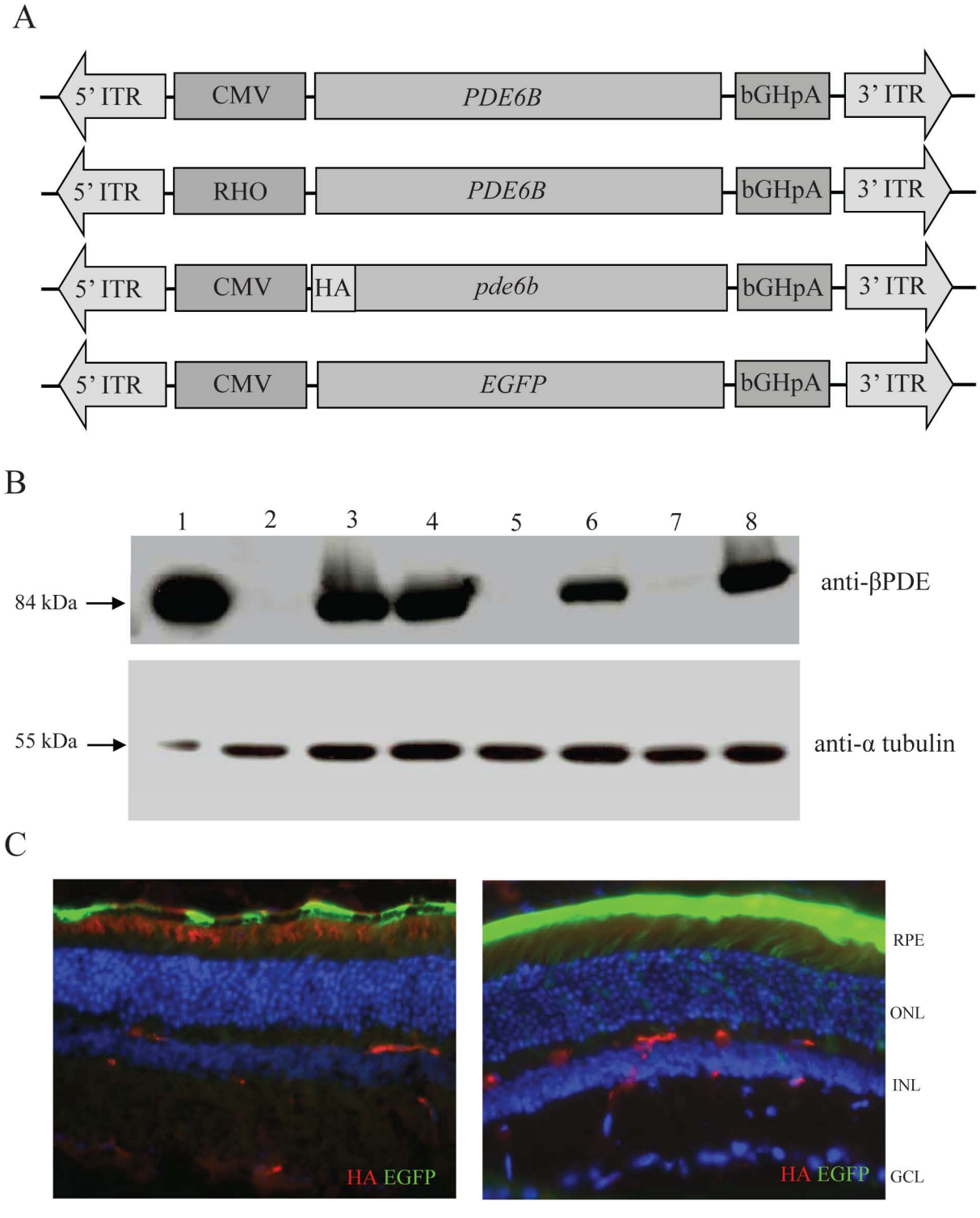
Figure Legends

Fig. 1 βPDE expression following AAV2/5 and AAV2/8 delivery in vitro and in vivo.
(A) Schematic representation of the AAV vectors used.
(B) Western blot analysis with anti-βPDE (top panel) and anti-α tubulin (bottom panel) antibodies of lysates from wild-type retina (lane 1), from Cos7 cells transfected with pAAV2.1-CMV-*EGFP* (lane 2) or pAAV2.1-CMV-*PDE6B* (lane 3 and 4), Cos7 cells transduced with AAV2/5-CMV-*EGFP* (lane 5) or AAV2/5-CMV-*PDE6B* (lane 6) and transduced with AAV2/8-CMV-*EGFP* (lane 7) or AAV2/8-CMV-*PDE6B* (lane 8). Anti-α tubulin was used as loading control.
(C) HA immunostaining (red) of retinal section from C57BL/6 eyes injected with AAV2/8-CMV-*pde6b*-HA + AAV2/1-CMV-*EGFP* (left) or AAV2/1-CMV-*EGFP* (right). The AAV2/1-CMV-*EGFP* vector was coinjected with AAV2/8-CMV-*pde6b*-HA to localize the injection area. Abbreviations: GCL, ganglion cells layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

Fig. 2 Schematic representation of the experimental plan.
Rd10 mice were injected at postnatal day 2 (P2) with AAV vectors encoding for βPDE or EGFP. Nilvadipine was administered intraperitoneally (i.p.) on a daily basis from P7 to P28. Electrophysiological (ERG) and histological analyses (Histo) were performed one week after the last drug injection (P35). Mice were kept in the dark from the embryonic day 14 (E14).

Fig. 3 Photoreceptor preservation in *rd10* mice after AAV-mediated βPDE delivery.
(A) The histograms represent the number of rows of photoreceptor nuclei in the outer nuclear layer (ONL) at postnatal day 35 of untreated wild-type (WT) or *rd10* mice, either untreated (NT) or injected with AAV2/8 or AAV2/5 encoding for human βPDE in one eye or EGFP in the controlateral.
Values shown are means ± SE. n= number of animals in each group; * p≤ 0.05; **p≤ 0.025; ***p≤ 0.0001.
(B) 4',6-diamidino-2-phenylindole (DAPI) staining of representative retinal sections analyzed in panel A. For abbreviations see legend of Fig. 1C.

Fig. 4 Photoreceptor preservation in *rd10* after AAV-mediated gene replacement in combination with dark rearing and/or nilvadipine treatment.
(A) Retinal morphological 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 photoreceptor nuclei in the outer nuclear layer (ONL) at postnatal day 35. Experimental groups are as before (see legend of Fig. 3). Values shown are means ± SE. n= number of animals in each group. *p ≤ 0.05.
(B) 4',6-diamidino-2-phenylindole (DAPI) staining of representative retinal sections analyzed in panel A. For abbreviations see legend of Fig. 1C.


A
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CMV
PDE6B
bGHpA
3' ITR
5' ITR
RHO
PDE6B
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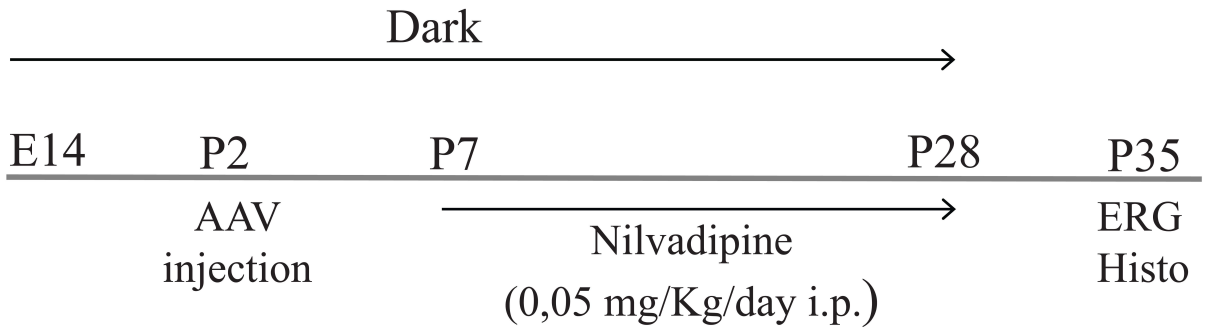
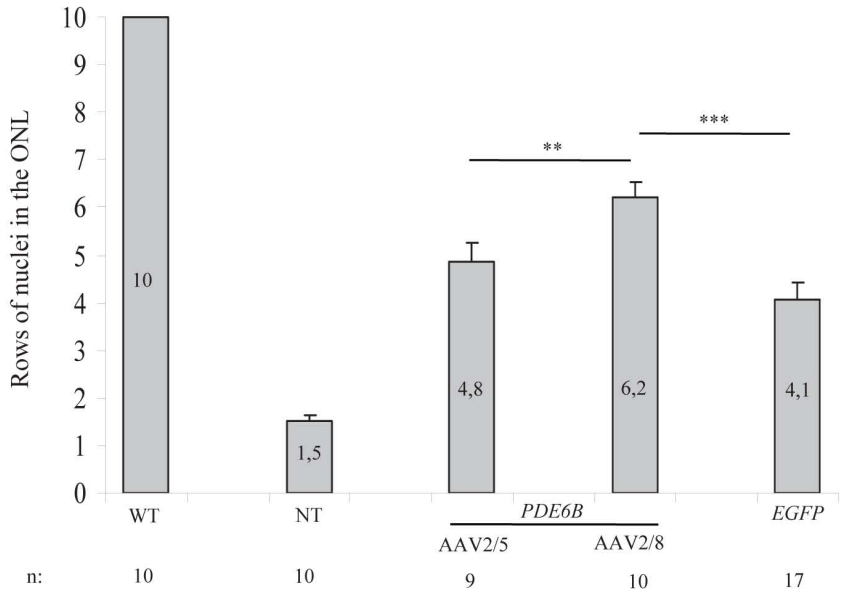
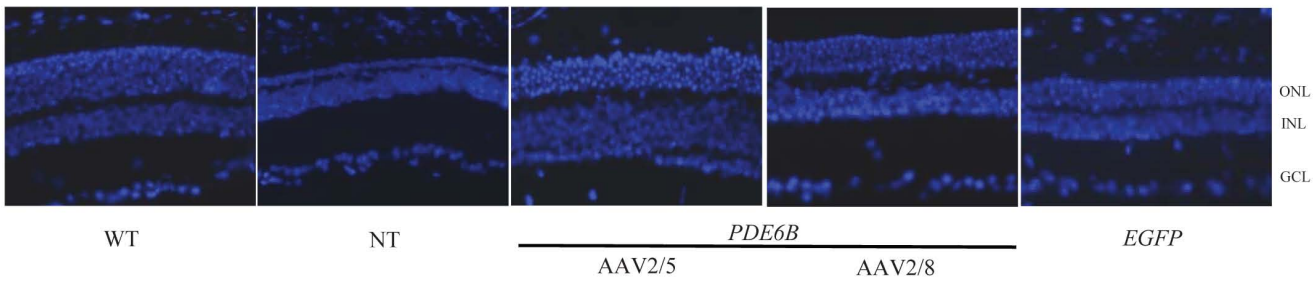
5' ITR
CMV
HA
pde6b
bGHpA
3' ITR
5' ITR
CMV
EGFP
bGHpA
3' ITR
B
1
2
3
4
5
6
7
8
84 kDa
anti-βPDE
55 kDa
anti-α tubulin
C
HA EGFP
HA EGFP
RPE
ONL
INL
GCL

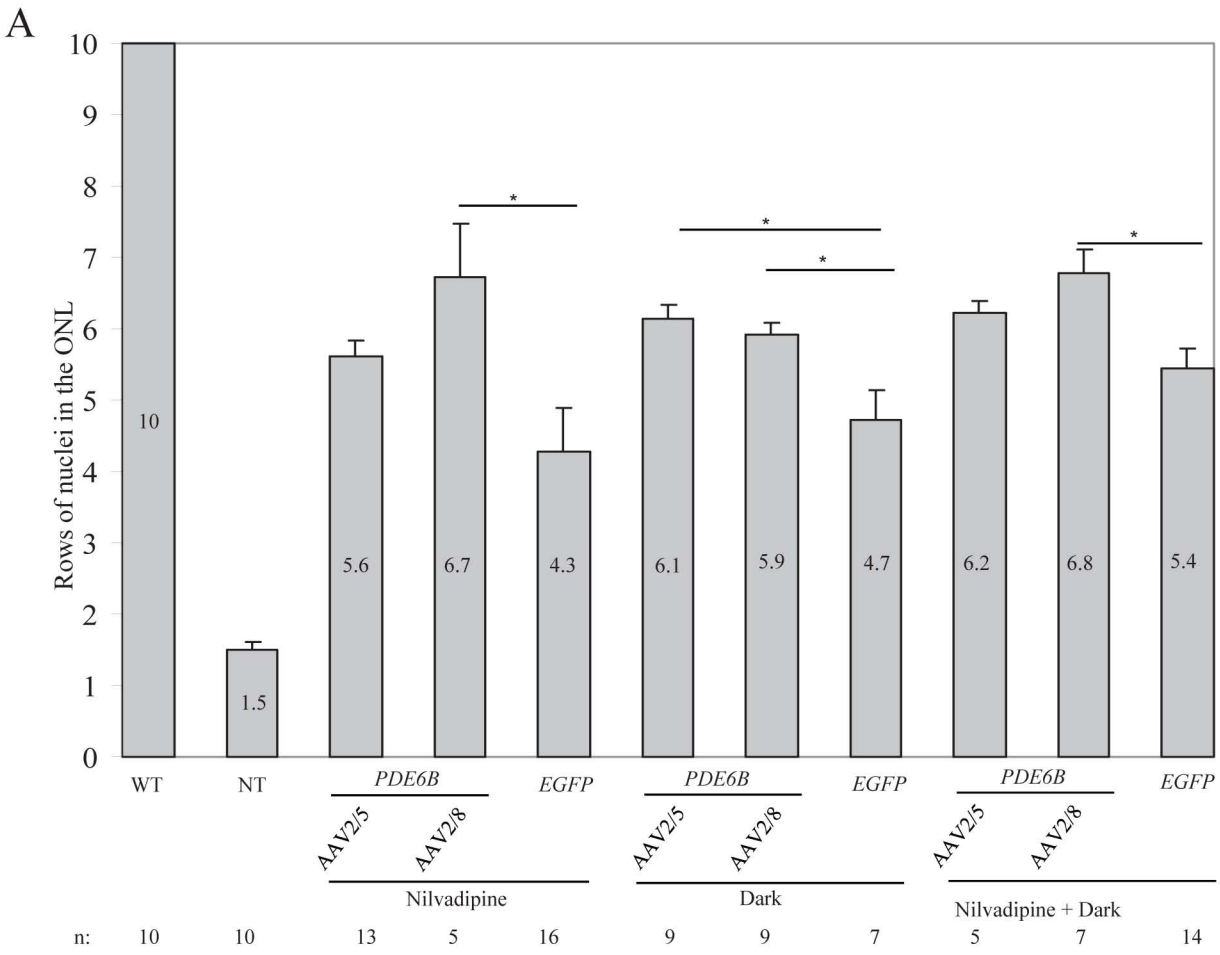

Dark
E14
P2
P7
P28
P35
AAV
injection
Nilvadipine
(0,05 mg/Kg/day i.p.)
ERG
Histo

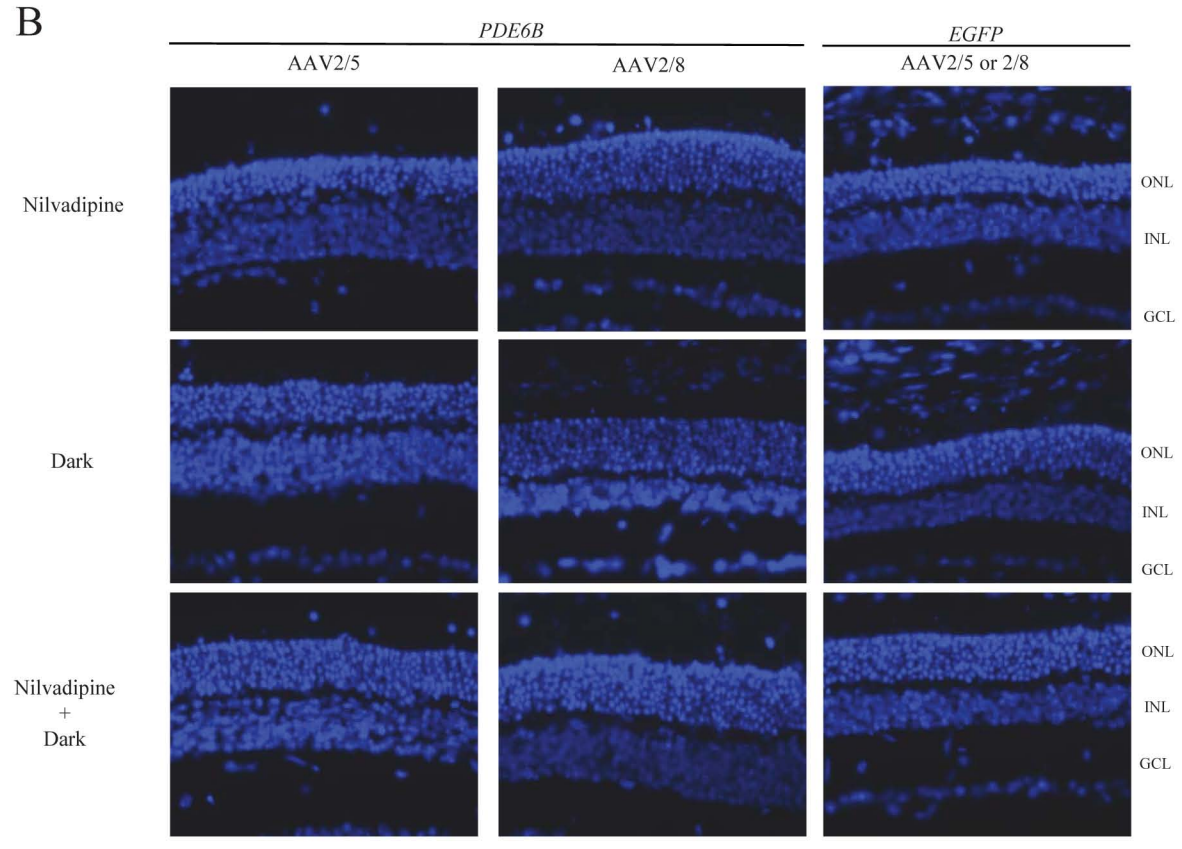
A


Rows of nuclei in the ONL
10
9
8
7
6
5
4
3
2
1
0
10
1,5
4,8
6,2
4,1
**
***
WT
NT
PDE6B
AAV2/5
AAV2/8
EGFP
n:
10
10
9
10
17

B


ONL
INL
GCL
WT
NT
PDE6B
AAV2/5
AAV2/8
EGFP


A
Rows of nuclei in the ONL
10
9
8
7
6
5
4
3
2
1
0
10
1.5
5.6
6.7
4.3
6.1
5.9
4.7
6.2
6.8
5.4
*
*
*
*
WT
NT
PDE6B
EGFP
PDE6B
EGFP
PDE6B
EGFP
AAV2/5
AAV2/8
AAV2/5
AAV2/8
AAV2/5
AAV2/8
Nilvadipine
Dark
Nilvadipine + Dark
n:
10
10
13
5
16
9
9
7
5
7
14


B
PDE6B
EGFP
AAV2/5
AAV2/8
AAV2/5 or 2/8
Nilvadipine
Dark
Nilvadipine
+
Dark
ONL
INL
GCL
ONL
INL
GCL
ONL
INL
GCL