

Restoration of Vision in the *pde6 β* -deficient Dog, a Large Animal Model of Rod-cone Dystrophy

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Defects in the β subunit of rod cGMP phosphodiesterase 6 (PDE6 β) are associated with autosomal recessive retinitis pigmentosa (RP), a childhood blinding disease with early retinal degeneration and vision loss. To date, there is no treatment for this pathology. The aim of this pre-clinical study was to test recombinant adeno-associated virus (AAV)-mediated gene addition therapy in the rod-cone dysplasia type 1 (*rcd1*) dog, a large animal model of naturally occurring PDE6 β deficiency that strongly resembles the human pathology. A total of eight *rcd1* dogs were injected subretinally with AAV2/5RK.cpde6 β ($n = 4$) or AAV2/8RK.cpde6 β ($n = 4$). *In vivo* and *post-mortem* morphological analysis showed a significant preservation of the retinal structure in transduced areas of both AAV2/5RK.cpde6 β - and AAV2/8RK.cpde6 β -treated retinas. Moreover, substantial rod-derived electroretinography (ERG) signals were recorded as soon as 1 month postinjection (35% of normal eyes) and remained stable for at least 18 months (the duration of the study) in treated eyes. Rod-responses were undetectable in untreated contralateral eyes. Most importantly, dim-light vision was restored in all treated *rcd1* dogs. These results demonstrate for the first time that gene therapy effectively restores long-term retinal function and vision in a large animal model of autosomal recessive rod-cone dystrophy, and provide great promise for human treatment.

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INTRODUCTION

Retinitis pigmentosa (RP), or rod-cone dystrophy, comprises a wide spectrum of incurable hereditary retinal dystrophies characterized by sequential degeneration of rod and cone photoreceptors.¹ Early symptoms include night blindness and loss of peripheral vision due to progressive rod photoreceptors degeneration. This phase is followed by cone death and concomitant loss

of central day vision. RP may be inherited as an autosomal recessive (50–60%), autosomal dominant (30–40%), X-linked (5–15%), or simplex/multiplex disease.² To date, about 45 genes associated with RP etiology have been identified; most of them are primarily expressed in photoreceptor cells.³

Mutations in the gene encoding the β subunit of rod cGMP-phosphodiesterase 6 (PDE6 β) are associated with one of the most prevalent forms of autosomal recessive RP, accounting for ~1–2% of all human RP cases.^{4–12} Rod PDE6 is localized on the disc membrane of rod outer segments where it plays a key role in the rod phototransduction cascade by controlling the level of cGMP and Ca²⁺ in the rod outer segments. Rod PDE6 is a heterotetrameric complex composed of two homologous catalytic subunits (PDE6 α and PDE6 β) and two copies of an inhibitory subunit (PDE6 γ).¹³ As both PDE6 α and PDE6 β subunits are required for rod PDE6 activity, loss-of-function mutations in the *pde6 β* gene result in an improper function of PDE6 holoenzyme and an accumulation of cGMP and Ca²⁺ in the rod outer segments. This ultimately leads to rod then cone photoreceptor death through apoptosis by non-well defined mechanisms.^{14–16}

There is currently no cure for RP caused by PDE6 β deficiency. Addition gene therapy is an attractive approach for PDE6 β -RP.

Gene therapy approaches to delay photoreceptors degeneration have already been employed in three murine models of PDE6 β -RP, the rodless 1 (*rd1*),¹⁷ the retinal degeneration 10 (*rd10*)^{18,19} and the PDE6 β H620Q.²⁰ Various vectors encoding murine or human *pde6 β* gene have been tested, including adenovirus,²¹ adeno-associated virus (AAV),^{22–25} simian immunodeficiency virus,²⁶ herpes simplex virus,²⁷ and lentivirus.^{20,28} In most studies functional rescue was limited, despite significant improvement of retinal morphology with an increased number of outer nuclear layer (ONL) nuclei and outer segment length. One of the best functional results (37% preservation of rod electroretinography (ERG) responses and prolonged photoreceptor survival up to postnatal day (P) 35) was obtained in dark-reared *rd10* mice, a model with a slower rate of degeneration than *rd1* mice, treated at P14 using an AAV2/5smCBA.mpde6 β vector.²³ This suggests that difficulties in achieving sustained long-term functional

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rescue in murine models of PDE6 β -deficiency may not be related to specific characteristics of the PDE6 β subunit but to (i) excessively rapid loss of photoreceptors in these models and mainly to (ii) difficulties in achieving sufficient level of transgene expression in mutant photoreceptors before their irreversible degeneration. This hypothesis was reinforced by a recent successful gene addition therapy in the same mouse model after subretinal injection of a fast-acting AAV2/8(Y733F)smCBA.mpde6 β vector.²⁴ In this recent “proof-of-concept” study, the authors reported on a subsequent 58% preservation of rod ERG responses, rod-mediated vision-guided behavior and rod survival for up to 6 months.

Preclinical evaluation of recombinant AAV (rAAV)-mediated gene transfer in large animal models (*i.e.*, dogs, cats, pigs, and non-human primates) is a key step for future clinical development.²⁹ Indeed, the size and anatomy of the eye in large animals provide a more relevant model than rodents in terms of pathobiology and surgical approaches. Moreover, their longevity enables long-term evaluation of the treatment, which is crucial for pathologies such as PDE6 β deficiency that typically progress over decades in humans.

The *Rcd1* dog is a natural large animal model of progressive retinal degeneration. The *Rcd1* dog carries a (2420G>A) nonsense mutation in the C-terminus of the PDE6 β subunit³⁰ leading to truncation and destabilization of the gene product and a nonfunctional PDE6 holoenzyme.^{31,32} Retinal development is normal in affected dogs until 13 days of age, at which point photoreceptor development is arrested. Rod degeneration then occurs gradually from 1 to 5 months of age, followed by cone loss within 1 to 2 years.^{33,34} This clinical course of generalized retinal atrophy, which strongly mimics retinal degeneration in humans, makes this PDE6 β deficient dog a valuable large animal model for the evaluation of gene therapy.

In the present study, we investigated the long-term efficacy of early rAAV-mediated *pde6β* gene transfer in the *rcd1* dog. Our findings demonstrate, for the first time, that gene transfer prevents long-term photoreceptor death and restores electrophysiological function and vision in this large animal model.

RESULTS

Recombinant AAV2/5 and AAV2/8 vector design and subretinal administration to *rcd1* dogs

We generated rAAV2/5 or rAAV2/8 vectors carrying the canine *pde6β* (*cpde6β*) gene under the control of the photoreceptor-specific human rhodopsin kinase (RK) promoter^{35,36} to drive rapid and strong transgene expression in photoreceptors (Figure 1).

Rcd1 dogs display an early and severe rod-cone photoreceptor degeneration that starts in the central retina at P25, during postnatal differentiation of photoreceptor cells.^{33,34} This rapid onset of retinal degeneration in *rcd1* dogs suggests that early therapeutic intervention is critical to prevent rod photoreceptor cell death in this model. To this end, we chose to treat *rcd1* dogs at P20, before extensive rod photoreceptor loss.

A total of eight *rcd1* dogs (A2–A9) were injected subretinally at the age of 20 days. Dogs A2 to A5 were treated with AAV2/5RK.*cpde6β*, whereas dogs A6 to A9 were injected with AAV2/8RK.*cpde6β* (Table 1). All injections were performed unilaterally in the right retina, except for dog A9 which received AAV2/8RK.

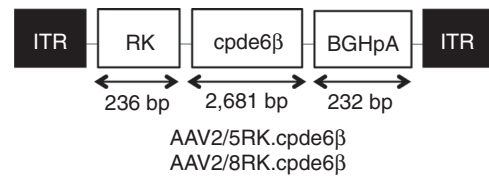


Figure 1 Schematic structure of recombinant adeno-associated virus (AAV) vectors. AAV2/5RK.*cpde6β* and AAV2/8RK.*cpde6β* vectors encode the canine *pde6β* cDNA under the control of a human RK promoter (–112 bp to +87 bp region of the proximal promoter³⁶). BGHpA, bovine growth hormone polyadenylation signal; *cpde6β*, canine *pde6β* cDNA; ITR, inverted terminal repeats from AAV2; RK, human rhodopsin kinase promoter.

Table 1 List of dogs included in the study and ERG amplitudes

Dog	Vector	Rod ERG (μ V)			30Hz Flicker (μ V)		
		1 mpi	9 mpi	18 mpi	1 mpi	9 mpi	18 mpi
		T/U	T/U	T/U	T/U	T/U	T/U
NA1		169/166	215/182	140/128	45/46	67/65	43/42
NA2	None	141/191	a	a	33/39	a	a
A1		0/0	11/15	0/0	44/38	43/56	6/9
A2		47/0	43/10	53/0	45/53	54/53	40/14
A3	AAV2/5RK. <i>cpde6β</i>	34/0	34/0	42/0	39/35	17/15	25/13
A4	(10 ¹¹ vg/ml)	40/0	52/0	nd	51/27	41/39	nd
A5		nd	a	a	nd	a	a
A6		37/0	87/0	63/0	21/46	38/15	30/11
A7	AAV2/8RK. <i>cpde6β</i>	45/0	37/0	28/0	42/29	23/17	19/9
A8	(10 ¹² vg/ml)	41/0	41/0	nd	36/28	27/21	nd
A9		19 ^b /64 ^b	a	a	37 ^b /30 ^b	a	a

Abbreviations: 30 Hz Flicker, photopic 30 Hz Flicker amplitude; A, affected; mpi, month(s) postinjection; NA, nonaffected; nd, not done; rod ERG, scotopic rod-mediated b-wave amplitude; T, treated eye; U, untreated eye; vg, vector genome.

^aSacrificed for RT-PCR and/or histology. ^bDog A9 received AAV2/8RK.*cpde6β* in both right and left eyes.

cpde6β in both eyes due to the observation of significant vector reflux into the vitreous during the subretinal injection of the right eye. Subretinal blebs were restricted to the nasal superior retina and covered ~25% of the entire retinal surface, except in the right eye of dog A9 in which the bleb was much smaller.

In all treated eyes, no apparent surgically induced retinal damage was noted by fundus photography or optical coherence tomography (OCT) at 2 months postinjection (mpi), despite the small size of the eye and the immaturity of the retina at the time of the subretinal injection (data not shown).

Assessment of transgene expression in *rcd1*-treated retinas

To evaluate *pde6β* transgene expression after subretinal injection of AAV2/5RK.*cpde6β* and AAV2/8RK.*cpde6β* vectors, presence of *pde6β* transcripts was assessed by reverse transcription (RT)-PCR in treated retinas of dogs A4 and A8, at 4 mpi (Figure 2).

As our preliminary RT-PCR analysis indicated that the *rcd1* dogs expressed mutant (2420G>A) *pde6β* transcripts at the

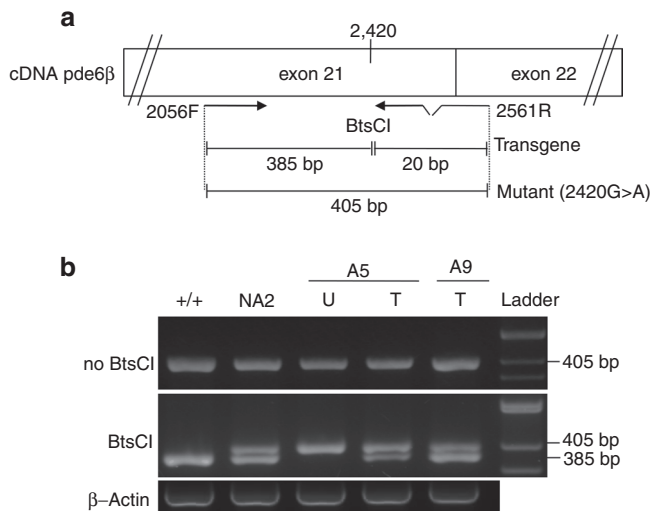


Figure 2 Detection of transgene (wild-type) and endogenous (mutant) *pde6β* transcripts in A5- and A9-treated retinas at 4 mpi. **(a)** Reverse transcription-PCR (RT-PCR) amplification of retinal cDNA using 2056F- and 2561R-specific primers generates a 405 bp product encompassing a portion of exons 21 and 22 of transgene and native *pde6β* transcripts. Mismatched 2561R specifically creates a unique *BtsCI* restriction site in PCR products arising from *pde6β* transgene transcripts, dividing the 405 bp product into 385 and 20 bp fragments. This *BtsCI* restriction site is not present in PCR products derived from mutant RNA templates. Arrows indicate the positions of the 2056F and 2561R primers used in RT-PCR. **(b)** Agarose gel electrophoresis of RT-PCR products after complete *BtsCI* digestion. Lines 1 and 2 show products from *Pde6β*^{+/+} and *Pde6β*^{-/-} (NA2) untreated control retinas, respectively. Lines 3 and 4 show products from untreated and AAV2/5RK.*cpde6β*-treated retinas of dog A5 at 4 mpi, respectively. Line 5 shows the AAV2/8RK.*cpde6β*-treated right retina of dog A9. +/+, *Pde6β*^{+/+} retina; AAV, adeno-associated virus; bp, base pairs; ladder, 1 kb molecular size ladder; T, treated retina; U, untreated retina.

time of injection (P20) (data not shown), we used allele-specific RT-PCR to ensure discrimination between wild-type (transgene) and mutant 2420G>A (endogenous) *pde6β* transcripts in treated retinas.

Total retinal RNA was reverse-transcribed and subjected to PCR amplification designed to generate a 405 bp product encompassing a portion of exons 21 and 22 of both wild-type and mutant 2420G>A *pde6β* transcripts. A mismatched reverse primer specifically created a unique *BtsCI* restriction site in the PCR products arising from wild-type *pde6β* RNA templates, dividing the 405 bp product into 385 and 20 bp fragments (Figure 2a). This *BtsCI* restriction site was not created in the PCR products derived from mutant RNA templates. Thus, one could expect the complete digestion of RT-PCR products by *BtsCI* endonuclease to result in a 385-bp digestion product in RNA templates arising from *Pde6β*^{+/+} retinas, a 405 bp product in RNA templates arising from untreated *Pde6β*^{-/-} retinas and both 385 and 405 bp products in RNA templates arising from *Pde6β*^{+/+} and rAAV-treated *Pde6β*^{-/-} retinas.

The results were as expected (Figure 2b). Transgene-derived 385 bp product was detected in both AAV2/5RK.*cpde6β*- and AAV2/8RK.*cpde6β*-treated retinas. On the contrary, this 385 bp product was never detected in the untreated *rcd1* retina. These results clearly show that vector-encoded *pde6β* was expressed in both AAV2/5RK.*cpde6β*- and AAV2/8RK.*cpde6β*-treated retinas.

AAV2/5RK.*cpde6β* and AAV2/8RK.*cpde6β* preserve retinal structure

Fundus appearance and retinal thickness were evaluated in all dogs using color fundus photography and OCT. These noninvasive examinations were performed from 2 to 18 mpi for dogs A2, A3, A6, and A7, from 2 to 8 mpi for dogs A4 and A8, and from 2 to 3 mpi for dogs A5 and A9. Recordings on age-matched untreated *Pde6β*^{+/+} (NA1) and *Pde6β*^{-/-} (A1) dogs were performed as controls. All examinations were performed bilaterally to optimize the comparison between treated and untreated eyes.

Figure 3 shows the ophthalmoscopy results obtained for rAAV2/5-treated (A3), rAAV2/8-treated (A7) and age-matched *Pde6β*^{+/+} control (NA1) dogs, at 4 and 18 mpi.

Fundus photography revealed a significant preservation of the retinal vasculature in both rAAV2/5- and rAAV2/8-treated eyes compared with an age-matched *Pde6β*^{+/+} control. In contrast, severe retinal degeneration, hyper-reflective fundi and attenuated retinal vessels were observed in the untreated left eye of dogs A3 (Figure 3b, left) and A7 (Figure 3c, left) as early as 4 mpi. By 18 mpi, only few central retinal vessels were still visible in the untreated affected eyes and pallor of the optic nerve was observed (Figure 3b,c, right).

In all rAAV-treated eyes, this preservation of fundus appearance was associated with a preservation of central retinal thickness, as assessed by long-term OCT monitoring. At 4 and 18 mpi, the treated retina of dog A3 (224 and 207 μm) was 22 to 24% thicker than the contralateral untreated retina (174 and 157 μm) (Figure 3b). Meanwhile, the treated retina of dog A7 (240 μm) was 17–21% higher than the contralateral untreated retina (199 and 190 μm) (Figure 3c). At 18 mpi, the retinal thickness of A3 AAV2/5RK.*cpde6β*- and A7 AAV2/8RK.*cpde6β*-treated retinas was 207- and 240-μm thick, respectively, representing 83–97% of that observed in age-matched noninjected *Pde6β*^{+/+} eyes (248 μm) (Figure 3a).

To more accurately define the therapeutic effect of rAAV-mediated gene transfer on retinal degeneration observed *in vivo* by OCT, we serially cryosectioned the entire treated retinas of dogs A5 and A9. The untreated left retinas of dogs A5 and NA2 were processed similarly and examined as controls.

The expression of the PDE6β subunit in treated *rcd1* eyes was not evaluated as all commercially available antibodies against the mouse or human PDE6β subunit were tested and found to be nonspecific in dogs due to crossreactivity with the canine PDE6α subunit (data not shown). Rod photoreceptors were thus identified by staining for the entire rod PDE6 holoenzyme or GNAT1.

Figure 4 shows photographs of hematoxylin and eosin stained sections and images of rod PDE6 and GNAT1 immunofluorescence obtained from nasal superior retinas of dogs A5 and NA2.

Low magnifications revealed a dramatic difference in retinal morphology between the vector-exposed and unexposed areas of the treated retina of dog A5 (Figure 4a,b). The vector-exposed region of the treated A5 retina retained a nearly normal morphology compared with the *Pde6β*^{+/+} age-matched control retina (Figure 4c, top). The ONL from the treated A5 retina was 16–17 rows thick (39 ± 3 μm, *n* = 10), accounting for ~90% of the ONL thickness of the control retina (18–19 rows, 42 ± 2 μm, *n* = 10) (Figure 4c, top). Most importantly, the mean combined inner and

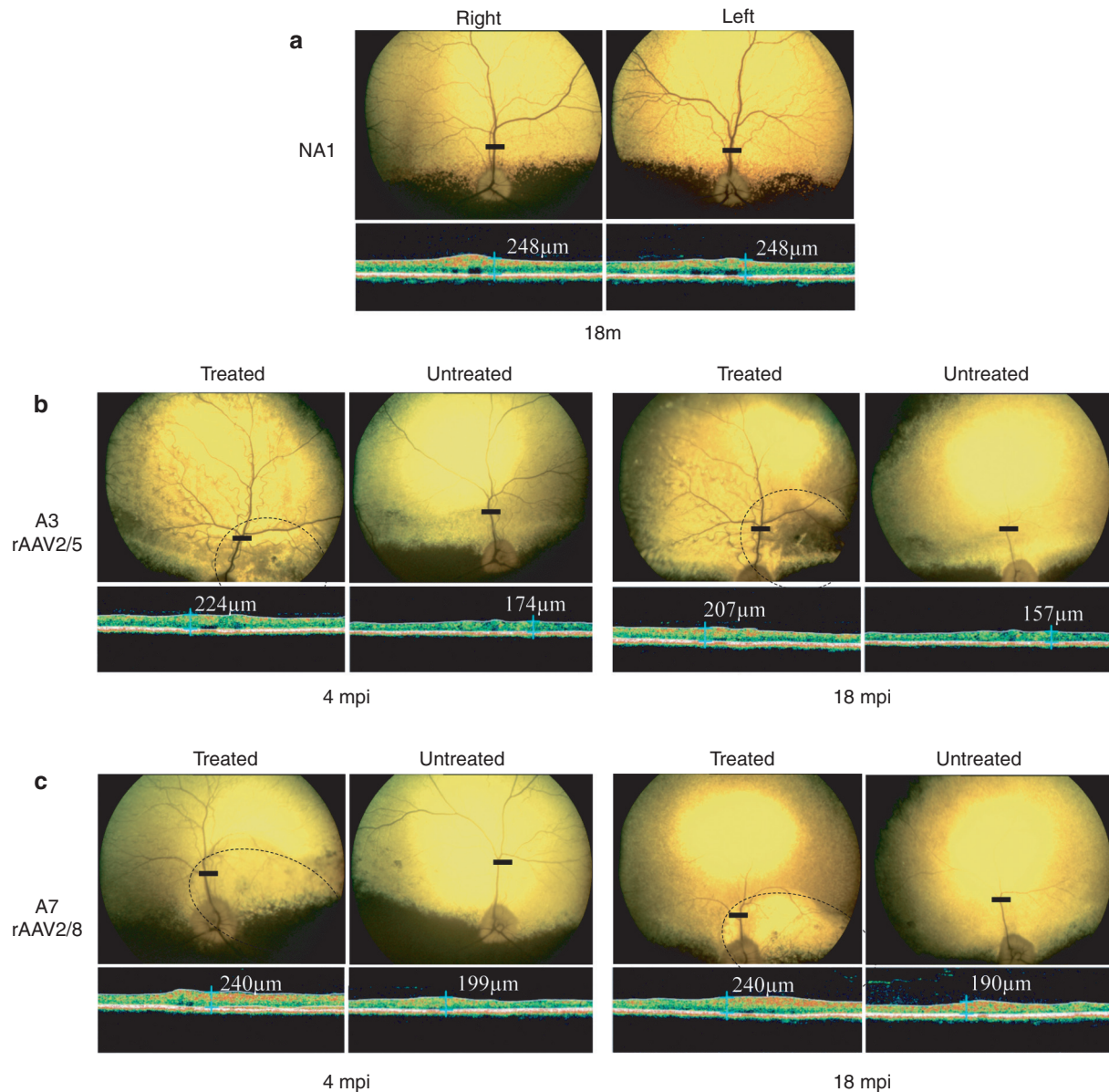


Figure 3 *In vivo* assessment of retinal morphology in dogs A3 and A7 at 4 and 18 mpi. **(a)** Fundus photographs and retinal cross-sectional images obtained from control nonaffected, untreated dog NA1 at 18 months of age. **(b)** Fundus photographs and retinal cross-sectional images obtained from dog A3 treated with AAV2/5RK.pde6 β at 4 and 18 mpi. **(c)** Fundus photographs and retinal cross-sectional images obtained from A7 treated with AAV2/8RK.pde6 β at 4 and 18 mpi. Dark circles on fundus photographs schematically represent areas of the treated retinas exposed to recombinant adeno-associated virus (rAAV) vectors. Optical coherence tomography (OCT) scans were acquired on a horizontal line shown on the fundus images (dark-line). The localization and the size of the dark-lines represent the localization and the size of the OCT scans. Retinal thicknesses at the same location were measured using calibrated calipers and indicated on the OCT scan. mpi, months postinjection; μm , micrometers.

outer segment thickness ($22 \pm 3 \mu\text{m}$, $n = 10$) was indistinguishable from that of the Pde6 $\beta^{+/-}$ control retina ($22 \pm 1 \mu\text{m}$, $n = 10$).

In the vector-unexposed region of the A5 treated retina, however, a massive loss of photoreceptor cell nuclei was observed (Figure 4c, top) similarly to that seen in the noninjected contralateral retina. The ONL was reduced to 6–7 rows ($20 \pm 2 \mu\text{m}$, $n = 10$) which represented only one-third to one-half of the number of rows observed in normal or in the vector-exposed retina respectively, and ~50% of normal or vector-exposed ONL thickness (Figure 4c, top). Moreover, photoreceptor inner and outer segments appeared to be shorter ($16 \pm 2 \mu\text{m}$, $n = 10$), less dense

and disorganized compared with the vector-exposed region and the nonaffected control retina.

Strong PDE6 labeling was found in outer segments in the vector-exposed region of the A5 treated retina (Figure 4c, middle), confirming the preservation of rod photoreceptor morphology after rAAV-mediated gene transfer. In contrast, only mild or undetectable PDE6 immunofluorescence was detected in the vector-unexposed region of the A5 treated retina and in the A5 untreated retina (Figure 4c, middle). A similar pattern of immunofluorescence was obtained using an antibody directed against GNAT1 (Figure 4c, bottom).

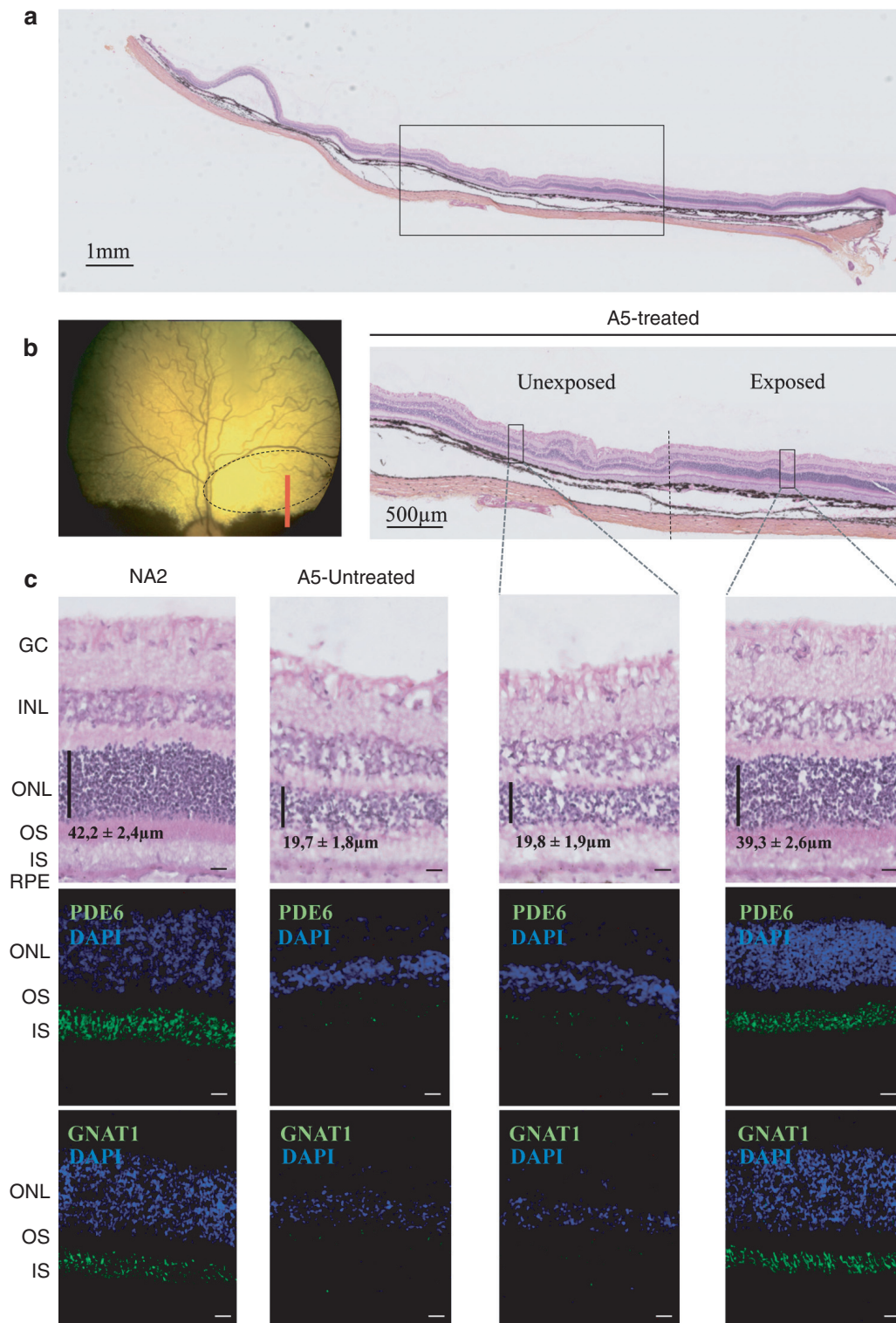


Figure 4 Post-mortem assessment of retinal morphology in dog A5 at 4 months postinjection. **(a–c)** Nasal retinal cryosections from the treated and untreated eyes of dog A5 at 4 months post subretinal delivery of rAAV2/5RK.cpde6 β in the nasal superior retina. **(a)** Wide A5 retinal section displaying vector exposed and unexposed-areas. Retinal layers remained intact while the choroid has been partially detached from the retina during the embedding process. **(b)** Fundus photograph representing the A5 retina exposed to the rAAV2/5 vector (dark circle) and the localization of the wide retinal section (red line). **(c)** Nasal retinal cryosections from unaffected, untreated dog NA2 at 5 months of age and from the untreated and treated eyes of dog A5. Serial retinal cryosections were processed for hematoxylin and eosin coloration (top) and for immunohistochemistry using antibodies against rod PDE6 (middle) or GNAT1 (bottom). Primary antibodies were detected with Alexa 488-conjugated goat anti-rabbit IgG (green). Cell nuclei were counterstained with DAPI (blue). Bar = 10 μ m. Vertical dark lines indicate ONL thickness (mean \pm SEM, $n = 10$). GC, ganglion cells; INL, inner nuclear layer; IS, inner segments; ONL, outer nuclear layer; OS, outer segments; rAAV, recombinant adeno-associated virus; RPE, outer retinal pigment.

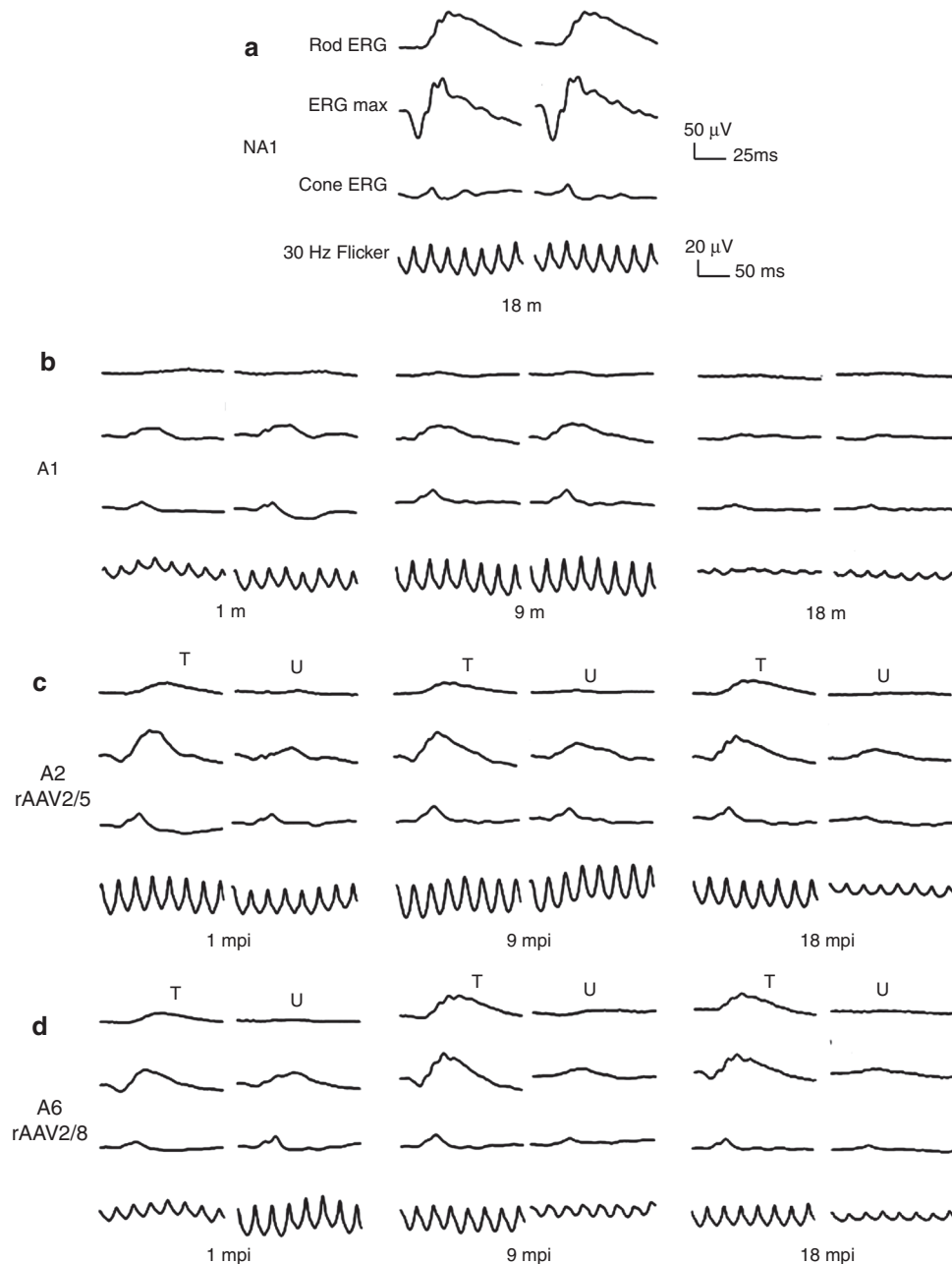


Figure 5 Bilateral full-field electroretinographic traces from dogs NA1, A1, A2, and A6 at 1, 9, and 18 months following subretinal injection. **(a)** Electroretinographic trace from control nonaffected, untreated dog NA1 at 18 months of age. **(b)** Electroretinographic traces from control affected, untreated dog A1 at 1 and 18 months of age. **(c)** Electroretinographic traces from dog A2 treated with AAV2/5RK.cpde6 β . **(d)** Electroretinographic traces from dog A6 treated with AAV2/8RK.cpde6 β . The top two recordings are low- and high-intensity dark-adapted responses, whereas the bottom two recordings show light-adapted responses (responses to single flash and 30 Hz flicker stimuli, respectively). AAV, adeno-associated virus; mpi, month(s) postinjection; T, treated eye; U, untreated eye.

The analysis of the entire retina showed that the preserved morphology covered ~25% of the total retinal surface (data not shown) and was mostly restricted to the region exposed to the vector.

AAV2/5RK.cpde6 β and AAV2/8RK.cpde6 β rescue retinal function

Retinal function was evaluated by the same investigator (L.L.) in all dogs using simultaneous bilateral full-field flash ERG. ERGs were performed on all treated dogs at different timepoints

following vector delivery, starting at 1 mpi, except for dog A5 for which retinal function was first assessed at 3 mpi. Follow-up was 18 mpi for dogs A2, A3, A6 and A7, 9 mpi for dogs A4 and A8, and 4 mpi for dogs A5 and A9. Recordings on age-matched untreated *Pde6 β ^{+/-}* (NA1) and *Pde6 β ^{-/-}* (A1) dogs were performed as controls ([Table 1](#)).

Representative ERG waveforms for dogs A1, A2, and A6 at 1, 9, and 18 mpi are shown in [Figure 5](#). The kinetics of functional recovery in dogs A2 and A6 are presented in [Figure 6](#).

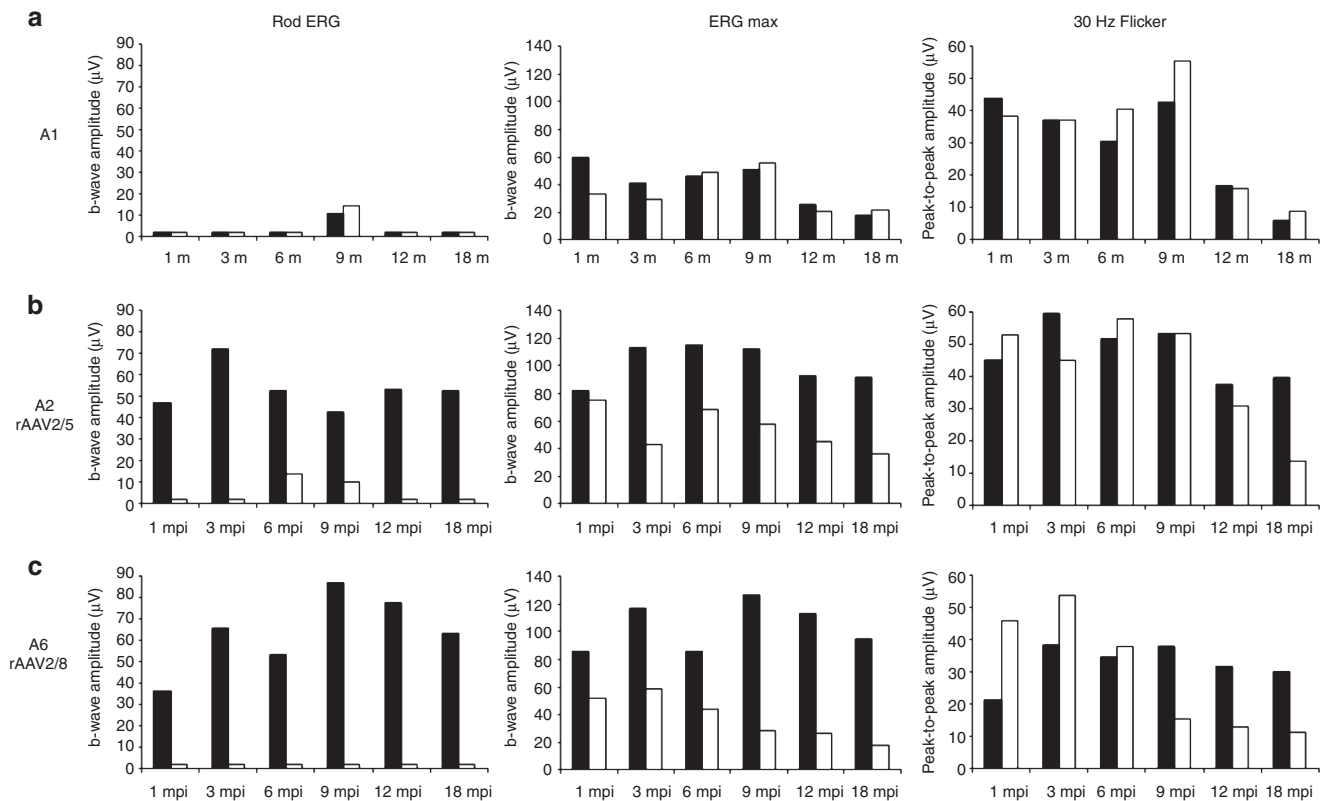


Figure 6 Kinetics of retinal function recovery in treated dogs A2 and A6. **(a)** Amplitudes of electroretinography (ERG) responses for control affected, untreated dog A1 from 1 to 18 months of age. **(b)** Amplitudes of ERG responses for dog A2 treated with AAV2/5RK.cpde6 β from 1 to 18 mpi. **(c)** Amplitudes of ERG responses for dog A6 treated with AAV2/8RK.cpde6 β from 1 to 18 mpi. The left and middle panels show scotopic rod and mixed cone-rod-mediated b-wave amplitudes, respectively. The right panel shows photopic 30 Hz flicker amplitude. Right eyes are shown in dark, left eyes in white. AAV, adeno-associated virus; mpi, month(s) postinjection.

We found that both AAV2/5RK.cpde6 β and AAV2/8RK.cpde6 β -subretinal delivery led to substantial restoration of rod function in all treated eyes as soon as 1 mpi (Table 1 and Figures 5c,d and 6b,c). In contrast, the rod responses were undetectable in the contralateral untreated eyes (Figures 5c,d and 6b,c) and in untreated control *Pde6β*^{-/-} eyes (Table 1 and Figures 5b and 6a) at this timepoint.

Although the general trend of rod function recovery was similar in all rAAV2/5- or rAAV2/8-treated eyes, interindividual's variations were observed (Table 1) and might reflect small variations on the extent and/or localization of the vector bleb.

In all rAAV2/5- and rAAV2/8-treated dogs, the amplitude of rod function recovery increased slightly between 1 and 3 mpi and remained stable thereafter, up to 18 mpi, the longest period of observation (Figure 6b,c, left). In all treated dogs, restored rod function exhibited typical a- and b-wave components (Figure 5c,d).

At 18 mpi, the median b-wave amplitude recorded on AAV2/5RK.cpde6 β - ($47 \pm 8 \mu\text{V}$, $n = 2$) and AAV2/8RK.cpde6 β - ($46 \pm 25 \mu\text{V}$, $n = 2$) treated eyes represent 35% of that recorded on nonaffected eyes ($134 \pm 8 \mu\text{V}$, $n = 2$) (Supplementary Figure S1). At 18 mpi, the maximal b-wave amplitudes were recorded on A2 AAV2/5RK.cpde6 β - ($53 \mu\text{V}$, 38% of the normal rod function) and A6 AAV2/8RK.cpde6 β - ($63 \mu\text{V}$, 45% of the normal rod function) treated retinas (Table 1 and Figure 6b,c, left).

The cone-mediated ERG (30 Hz Flicker) responses in treated and untreated eyes were similar in all AAV2/5RK.cpde6 β - and AAV2/8RK.cpde6 β -injected dogs over the first 8–12 month period postvector delivery (Figure 6b,c).

Interestingly, ERG max and 30 Hz flicker amplitudes remained stable in all treated eyes over the 12–18 mpi period while a progressive and consistent reduction of these responses was observed in all contralateral untreated eyes during this period (Table 1 and Figures 5c,d, and 6b,c).

At 18 mpi, the median amplitude of the 30 Hz flicker of AAV2/5RK.cpde6 β - ($33 \pm 11 \mu\text{V}$, $n = 2$) and AAV2/8RK.cpde6 β - ($25 \pm 8 \mu\text{V}$, $n = 2$) treated eyes represent 76 and 56% of that recorded on age-matched *Pde6β*^{+/-} control eyes ($43 \pm 1 \mu\text{V}$, $n = 2$), respectively (Supplementary Figure S1). At 18 mpi, the maximal amplitudes of the 30 Hz flicker were recorded on A2 AAV2/5RK.cpde6 β - ($40 \mu\text{V}$, 93% of the normal cone function) and A6 AAV2/8RK.cpde6 β - ($30 \mu\text{V}$, 70% of the normal cone function) treated retinas (Table 1 and Figure 6b,c, left). They were approximately threefold higher than responses recovered for their contralateral untreated eyes (14 and $11 \mu\text{V}$).

Interestingly, the level of rod function restoration and cone function preservation obtained following delivery of rAAV2/5 was not significantly different from that achieved with the rAAV2/8 (Supplementary Figure S1).

AAV2/5RK.*cpde6β* and AAV2/8RK.*cpde6β* restore vision

Vision tests were performed on A2, A3, A6, and A7 treated *Pde6β*^{-/-} dogs at 4, 8, 12, and 18 months postvector delivery (Table 2). Dim-light conditions were used to assess transmission of rescued rod activity to higher visual pathways and improvement of visually guided behavior. Bright light conditions were used to assess preservation of day vision in treated *rcd1* dogs.

Under dim-light conditions at 4 and 8 mpi, none of the treated dogs showed behavioral signs of blindness in both eyes (Table 2). However, at the latest timepoints (12 and 18 mpi), all treated dogs avoided obstacles when their untreated eye was occluded while they showed difficulty to navigate around the obstacle panels when their treated eye was occluded. They moved very cautiously with their nose to the ground and tried to feel the panels with their forelegs. Several collisions with obstacles were also noted. These results were confirmed by a significant increase in transit time. For example, dog A6 completed the obstacle course in 7 seconds with the untreated eye covered and in 30 seconds with the treated eye covered (Supplementary Video S1, part I). Dog A6 took a comparable time to complete the obstacle course with the untreated eye covered than a control nonaffected *Pde6β*^{+/-} dog with one eye covered (9 seconds, data not shown).

In bright light conditions, none of the treated dogs showed behavioral signs of blindness from 4 to 18 mpi regardless of which eye was covered (Table 2 and Supplementary Video S1, part II).

DISCUSSION

In this study, a total of eight *rcd1* dogs were subretinally injected with rAAV2/5 and rAAV2/8 vectors carrying the *cpde6β* cDNA under the control of the photoreceptor-specific RK promoter. The results demonstrated that rAAV-mediated *cpde6β* expression restored rod function and consequently, prevented photoreceptor death and concomitant vision loss in treated dogs for at least 18 mpi (the duration of the study).

Interestingly, rod-mediated ERG responses were undetectable in the *rcd1* canine model from the earliest age measured³⁷ (1 month of age, Table 1). At this age, the *PDE6β* subunit was undetectable by immunoblot and rod *PDE6* function was absent, suggesting that the *rcd1* dog carries a *null* mutation in the *pde6β* gene.³⁰ In this matter, this canine model of *PDE6β* deficiency is similar to the *rd1* murine model in which *PDE6* function is also completely lacking,¹⁷ but different from the hypomorphic *rd10* and *Pde6β*H620Q murine models in which photoreceptors develop and function almost normally before their degeneration.^{18–20}

Here, we show that both AAV2/5RK.*cpde6β* and AAV2/8RK.*cpde6β*-gene transfer restored sustained and stable rod-mediated ERG responses in all treated *rcd1* eyes as early as 1 month postinjection (Figure 5). From 1 to 18 mpi, median amplitudes of dark-adapted b-waves in rAAV-treated eyes account for 28–35% of those recorded in normal eyes (Supplementary Figure S1), consistent with the estimated area of retina directly exposed to the vectors (~25% of the total retinal surface).

This is the first demonstration that gene therapy can restore a rod function in a *null* animal model of *PDE6β* deficiency as all attempts to treat the *rd1* mice by gene addition therapy have failed to provide functional rescue.^{21,26,27} In contrast, in the *rd10*

Table 2 Evolution of dim- and bright-light vision in *PDE6β*^{-/-} treated dogs

Dog	Vector	Behavioral test					
		Dim-light			Bright-light		
		4 mpi	12 mpi	18 mpi	4 mpi	12 mpi	18 mpi
		T/U	T/U	T/U	T/U	T/U	T/U
A2	AAV2/5RK. <i>cpde6β</i>	+/+	+/-	+/-	+/+	+/+	+/+
A3		+/+	+/-	+/-	+/+	+/+	+/+
A6	AAV2/8RK. <i>cpde6β</i>	+/+	+/-	+/-	+/+	+/+	+/+
A7		+/+	+/-	+/-	+/+	+/+	+/+

Abbreviations: -, impaired vision; +, correct vision; mpi, months postinjection; T, treated eye; U, untreated eye.

Vision tests were performed in dim (1.5 ± 0.8 lux) or bright (260 ± 13 lux) light at different timepoints postinjection. An opaque lens was used to alternatively cover treated (right) and untreated (left) eyes.

mouse, previous studies demonstrated that subretinal injection of AAV2/5smCBA.*mpde6β* led to the preservation of 37% of rod ERG responses up to P35,²³ and that subretinal injection of AAV2/8(Y733F)smCBA.*mpde6β* led to a preservation of 58% of rod ERG responses for up to 6 months after treatment.²⁴

This finding has obvious clinical significance because among the 21 causative-mutations already identified in patients with *PDE6β*-recessive RP,^{4–12} 8 (38%) are nonsense mutations predicted to lead to a complete loss of *PDE6* function.^{5–8,10} The most sustained rescue of rod function in *null* animal models of progressive photoreceptor defects reported to date was obtained in the *Aipl1*^{-/-} mouse, in which both rod and cone functions are absent at birth. In this model, subretinal injection of AAV2/8RK.*haip1* at P10 led to a 50% restoration of wild-type scotopic ERG responses for at least 4 weeks postinjection.³⁸ When scAAV2/8(Y733F)RK.*haip1* was used,³⁹ this rescued rod function was more sustained persisting until P60 (the duration of the reported study).

In addition to this sustained restoration of rod function in treated *rcd1* retinas, long-term preservation of cone-mediated ERG responses were demonstrated up to 18 mpi (Table 1 and Figures 5 and 6). This result strongly suggests that rAAV-mediated gene therapy inhibited or delayed the initiation of secondary cone death in treated *rcd1* retinas. A similar beneficial effect of the primary rod treatment on the cone function has been previously obtained in *rd10* mice treated with AAV2/8(Y733F)smCBA.*mpde6β* for at least 6 weeks postinjection²⁴ (the duration of the reported study).

Several mechanisms have been proposed to explain the non-autonomous death of cones in rod-cone dystrophies: the loss of a rod-derived trophic support, the release of a toxic factor by dying rods, an increase in oxidative damage to cones once the rods have died or an imbalance in the metabolism of cones due to changes in retinal architecture.^{14–16} In all these models, primary loss of rods is responsible for secondary cone death, indicating that protection of cones after rAAV-mediated gene transfer might be correlated with a preservation of rod cells in treated *rcd1* retinas.

Long-term preservation of central retinal thickness in treated *rcd1* eyes was observed by OCT from 4 to 18 mpi (Figure 3). Our histology data at 4 mpi suggested that this reflects the retention of rod photoreceptor cells in treated retinas (Figure 4).

Interestingly, this preservation of rod photoreceptors was not widespread but mainly restricted to nasal vector-exposed areas of treated retinas. Similar results have been recently shown in RPGR canine models of X-linked RP following subretinal injection of AAV2/5IRBP.hrpgr or AAV2/5RK.hrpgr.⁴⁰ This observation may have two notable clinical implications. First, it demonstrates that degenerating nontransduced photoreceptors have no major negative impact on transduced photoreceptor survival as previously suggested.⁴¹ Second, it surprisingly indicates that localized preservation of rod photoreceptor cells in the nasal superior retina (that did not comprise the cone-rich *area centralis*⁴²) is sufficient to maintain the nearly total cone function and subsequently delay initiation of cone loss. We do not have yet a clear explanation of the apparent complete retention of cone function observed in the treated *rcd1* retinas. It might be related to the physic and/or trophic interactions of preserved rods with other retinal cell populations.^{14–16} Further monitoring of the cone function for months or years in treated *rcd1* dogs will be essential to confirm its stability overtime and injection of additional dogs will be required to identify the underlying molecular mechanism for this long-term preservation of cone function.

The effect of the rAAV-mediated gene therapy on the vision of treated *rcd1* dogs was assessed by behavioral tests under dim (1.5 ± 0.8 lux) and bright (260 ± 13 lux) light conditions. It is important to note that, in these two conditions, it was impossible to discriminate rod- from cone-mediated vision because both types of photoreceptors were stimulated.

In dim light, all treated dogs displayed normal vision behavior using either their untreated and treated eyes up to 8 mpi (Table 2), probably due to the preservation of normal cone function in both eyes (Table 1 and Figures 5 and 6). Over the 8 to 18 mpi period, none of the treated dogs were still able to successfully negotiate the obstacle course using their untreated eye (Supplementary Video S1, part I) consistent with the progressive loss of cone function observed by ERG analysis at these late timepoints (Table 1 and Figures 5 and 6). In contrast, they maintained normal vision-elicited behavior using their treated eyes (Supplementary Video S1, part I), demonstrating that rAAV-mediated restoration of rod function and/or preservation of cone function preserve long-term night vision in treated *rcd1* dogs.

In bright light, untreated dogs displayed vision over the 18-month period (Supplementary Video S1, part II) certainly due to the residual cone function in both eyes (Table 1 and Figures 5 and 6). It will be necessary to further monitor the vision of treated *rcd1* dogs over several years to determine whether the preservation of cone function observed by ERG analysis will also lead to long-term preservation of day vision over time.

In this study, the efficacy of rAAV2/5 and rAAV2/8 vectors was comparable with both vectors providing similar rod-mediated ERG responses in treated *rcd1* eyes (Table 1). This result was surprising as rAAV2/8 (10^{12} vg/ml) was injected at a tenfold higher titer than rAAV2/5 (10^{11} vg/ml) vector. One possible explanation might be the broader dispersion of rAAV2/8 in the canine retina after subretinal delivery.⁴³ The higher potential of rAAV2/8 for widespread diffusion across synapses may reduce the global number of rAAV-delivered vector genomes in photoreceptors cells and subsequently the level of *pde6β* expression. Alternatively

or in addition, it is possible that in both rAAV2/5- and rAAV2/8-treated eyes, the saturation threshold of *pde6β* expression level was reached.

Interestingly, both rAAV2/5 and rAAV2/8 vectors provided long-term therapy in the *rcd1* dog. This result contradicts previous reports in mouse models of *Pde6β*,²⁴ *Aipl1*,³⁸ and *Rpgrip1*⁴⁴ deficiencies that have shown a strong functional advantage of rAAV2/8 and/or rAAV2/8(Y733F) vectors compared with the rAAV2/5 vector. This significant discrepancy in long-term rAAV2/5 efficiency between the *rcd1* dog and these murine models of retinal dystrophies probably reflects the slower rate of retinal degeneration in the *rcd1* dog.^{33,34} This result should be considered in the context of PDE6β-patients as they typically describe impaired or absent night vision in childhood, followed by a reduction of their visual field from young adulthood, which slowly progresses over decades.^{4–6}

In this study, we used rAAV vectors carrying the *cpde6β* under the control of the human RK promoter which has been demonstrated to drive transgene expression in both rods and cones after subretinal delivery,^{35,36,40} whereas the β subunit of PDE6 is specifically expressed in rod photoreceptors.¹³ Recombinant AAV-mediated gene therapy in *rcd1* retinas may thus led to undesirable ectopic expression of *pde6β* in transduced cones. It was unfortunately not possible to assess this *pde6β* expression in cones due to the limitations of *pde6β* immunostaining. However, based on the finding that rAAV-treated eyes exhibited no appreciable reduction of cone function over the 18-mpi period (Table 1), it is likely that this potential RK-mediated ectopic expression of *pde6β* has no toxic effects on the cone function, at least at this stage. These results have high clinical relevance because they suggest the potential safety of the human RK promoter for future treatment of *pde6β* defects.

In conclusion, this study demonstrated, for the first time, that rAAV-mediated gene transfer efficiently corrects the *pde6β* defect in a large animal model of *pde6β* deficiency, leading to (i) robust and stable restoration of rod function and (ii) concomitant long-term preservation of cone function and vision. As mutations in the *pde6β* gene are one of the leading causes of recessive RP^{4–12} and as retinal degeneration in the *rcd1* dog is highly similar to the human disease, this preclinical study represent a major step towards the future development of this therapy in patients with PDE6β-deficiencies. In a larger sense, these PDE6β results offer great promise for the treatment of many other rapid recessive rod-cone dystrophies due to a rod-specific defect.

MATERIALS AND METHODS

Plasmid construction and production of rAAV vectors. Recombinant AAV2/5.RK.*cpde6β* and AAV2/8.RK.*cpde6β* vectors were produced by triple transfection of 293 cells according to previously reported methods,⁴⁵ using the SSV9RK.*cpde6β* vector plasmid. This construct carried the *cpde6β* cDNA (2,681 bp) directly under the control of the short human RK promoter (–112 bp to +87 bp region of the proximal promoter³⁶) and the bovine growth hormone polyadenylation signal (BGHPA), flanked by two AAV2 inverted terminal repeat sequences.

For the SSV9RK.*cpde6β* construction, full-length *cpde6β* cDNA (NCBI RefSeq NM_001002934.1) was amplified by PCR from *Pde6β*^{+/+} canine retinal cDNA. Two primers designed to cover the entire sequence of the *pde6β* gene were used for this purpose. The forward

primer encoded a *Hind*III end and the first 15 bp of the *cpde6β* gene (5'-AATTAAGCTTTAGACAGCCGGACAC-3'). The reverse primer (5'-CTTTATTCATAGTTGAGTTT-3') encoded a 20 bp sequence of the *cpde6β* gene located 121 bp downstream of the stop codon. An endogenous *Eco*RV restriction site was present in the sequence of *cpde6β* 82 bp downstream of the stop codon. The PCR product (2,728 bp) was purified, digested by *Hind*III and *Eco*RV and cloned into a parental plasmid SSV9RK.crprip1, between the RK promoter and the bovine growth hormone polyadenylation site, after removal of the eGFP sequence (by *Hind*III and *Eco*RV digestion). The identity of the resulting SSV9RK.c*pdde6β* construct was verified by sequencing.

Viral vector titers were determined by dot-blot and by quantitative real-time PCR and expressed as vector genomes (vg) per milliliter (vg/ml). The final vector titers of AAV2/5RK.c*pdde6β* and AAV2/8RK.c*pdde6β* were 1.10^{11} and 1.10^{12} vg/ml, respectively.

Animals. A total of nine affected *Pde6β*^{-/-} and 1 *Pde6β*^{+/-} control Irish Setter dogs were used in this study (Table 1). The first three *Pde6β*^{-/-} individuals were kindly provided D.J. Maskell (University of Cambridge, Cambridge, UK). Animals were maintained at the Boisbonne Center (ONIRIS, Nantes-Atlantic College of Veterinary Medicine, Food Science and Engineering, Nantes, France) under a 12/12 hour light/dark cycle. All experiments involving animals were conducted in accordance with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

Subretinal administration of rAAV vectors. Subretinal injections of AAV2/5RK.c*pdde6β* and AAV2/8RK.c*pdde6β* vectors were performed on 8 affected *Pde6β*^{-/-} dogs at P20. For all dogs except A9, vector delivery was unilateral, leaving the contralateral eyes without injection as internal controls (Table 1). All subretinal injections were performed under general anesthesia induced by intramuscular injection of a mixture of diazepam (Hoffmann-La Roche, Basel, Switzerland) and ketamine (Rhone Merieux, Lyon, France) and maintained by inhalation of isoflurane gas. Pupils were fully dilated by topical administration of 0.3% atropine (Alcon Cusi SA, Barcelona, Spain), tropicamide (Novartis, Annonay, France) and phenylephrine hypochloride (Novartis).

Surgery was conducted using a transvitreal approach as previously described,⁴⁶ without vitrectomy. Under microscopic control, 80–120 μl of vector solution were injected into the subretinal space. Immediately after the injection, the localization and the extent of the retinal surface directly exposed to the vector were recorded on schematic fundus drafts as it was not possible to obtain clear fundus photography of the dog retina until 2 months of age. The precise orientation of the vector bleb was further determined by fundus photography at 2 mpi, by the observation of brighter areas that delineate the border of the treated area (data not shown).

Post-surgical care included one topical administration of 0.3% atropine (Alcon Cusi SA) and two topical administrations of Ocryl lotion (Laboratoire TVM, Lempdes, France) and gentamicin dexamethasonum (Virbac France S.A., Carros, France) daily for 10 days post-surgery.

Histology and immunohistochemistry. At 4 mpi, A5 and A9 dogs were euthanized by intravenous injection of pentobarbital sodium (Vétoquinol, Lure, France). Eyes were enucleated and fixed for 2 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS) solution before removal of the anterior chamber and the lens. Eyecups were embedded in optimal cutting temperature compound (OCT Cryomount; Microm Microtech, Francheville, France), and flash-frozen in a dry ice isopentane bath. Ten to fifteen micrometer cryosections were prepared.

For morphological examinations, sections were stained with hematoxylin and eosin before imaging by transmitter light microscopy (Nikon, Champigny sur Marne, France). Photoreceptor survival was assessed by counting the number of photoreceptor nuclei rows in one ONL column. Mean combined outer and inner segment thickness values

of vector-exposed and unexposed areas of the retina were determined by averaging ten counts, performed on ten different slices.

For immunohistochemical studies, cryosections were air-dried, washed in PBS solution and incubated in blocking solution [20% normal goat serum (Invitrogen Life Technologies, Saint Aubin, France), 0.05% Triton X-100 in PBS] for 1 hour at room temperature. The bovine rod PDE6 (1:500) (Cytosignal, Irvine, CA) or GNAT1 (1:250) (Santa-Cruz Technology, Heidelberg, Germany) antibodies were incubated overnight at 4 °C in blocking solution. After three washes in 0.05% Triton X-100 in PBS solution, slides were incubated with the secondary antibody Alexa 488 goat anti-rabbit IgG conjugate (Life Technologies, Grand Island, NY) at 1:250 for 2 hours at room temperature. Slides were washed three times with 0.05% Triton X-100 in PBS solution and once with PBS solution before counterstaining the nuclei with DAPI diluted at 1:500. Slides were mounted in Prolong Gold anti-fade reagent (Life Technologies), observed with a fluorescence microscope (Nikon) and images captured with a digital camera (Nikon).

Specificity of the primary bovine rod PDE6 antibody for canine retina was demonstrated in the *Pde6β*^{+/-} retina by absence of co-labeling with peanut lectin agglutinin conjugated with fluorescein isothiocyanate (1:250; Vector Laboratories, Burlingame, CA) (data not shown).

RT-PCR

RNA extraction and retro-transcription. Total RNA was isolated from individual flash-frozen retinas or retinal cryosections of *Pde6β*^{+/-}, *Pde6β*^{+/-}, or *Pde6β*^{-/-} dogs using TRIzol Reagent (Invitrogen Life Technologies). Rnase-free Dnase I (Ambion DNA-free kit; Invitrogen Life Technologies) was used according to the manufacturer's instructions to remove contaminating DNA before generation of cDNA by reverse-transcription. Five hundred nanograms of total RNA were reverse-transcribed using oligodT primers and M-MLV reverse transcriptase (Invitrogen Life Technologies) as per the manufacturer's instructions. Control assays without addition of reverse transcriptase were included and the products were used in the subsequent RT-PCR as negative controls.

Primers. Integrity of cDNA was determined by amplification of a 650 bp sequence of the β-actin gene using β-actinF (5'-TGACGGGGTACACCCACACTGTGCCCATCTA-3') and β-actinR (5'-CTAGAAGCATTTCGGTGGACGATGGAGGG-3') primers.

For *pde6β* amplification, 2056F (5'-GAAGATCGTGGATGAGTCTAAGA-3') and 2561R (5'-CTACTTATCATCAGTCAAGGCCA TC-3') primers were designed to specifically create a *Bst*CI restriction site in RT-PCR products arising from wild-type RNA templates. The reverse primer was designed to neighbouring exon sequences to avoid amplification of genomic DNA (Figure 2). The forward 2056F primer was designed in a *pde6β*-specific sequence to avoid amplification of canine *pde6α* RNA.

To verify the specificity of the 2056F and 2561R primer set against *pde6β*, full-length canine *pde6α* cDNA (NCBI RefSeq NM_001003073.1) was amplified by PCR from *Pde6α*^{+/-} canine retinal cDNA. The forward primer encoded a *Bam*HI end and an 18 bp sequence located 72 bp upstream of the start codon of the *cpde6α* gene (5'-ATTAAGGATCCTGACTCTGTCTTGC-3'). The reverse primer encoded an *Eco*RV end and a 15 bp sequence located 353 bp downstream of the stop codon of the *cpde6α* gene (5'-TATTGATATCTGGGTGATGAGGAGG-3'). The PCR product (3,061 bp) was purified, digested by *Bam*HI and *Eco*RV and cloned into the plasmid SSV9RK.c*pdde6β* previously constructed, between the RK promoter and the bovine growth hormone polyadenylation site after removal of the *cpde6β* cDNA (by *Bam*HI and *Eco*RV digestion). The identity of the resulting SSV9RK.c*pdde6α* construct was verified by sequencing.

Amplification and *Bst*CI digestion. The amount of cDNA used in β-actin and *pde6β* allele-specific PCR was 40 ng. Both PCR were performed using GoTaq DNA polymerase (Promega France, Charbonnières, France) and a Veriti Applied Biosystems thermocycler.

The β -actin reaction profile was as follows: an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and a final incubation step at 72°C for 7 minutes. For pde6 β , conditions of amplification were: an initial denaturation step at 95°C for 5 minutes, followed by 10 cycles at 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, 30 cycles at 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds and a final incubation step at 72°C for 7 minutes.

Actin PCR products were resolved on a 1.5% agarose gel electrophoresis. Pde6 β PCR products were purified by Nucleospin Extract II (Macherey-Nagel, Hoerdt, France) before digestion by BtsCI (New England Biolabs, Ipswich, MA) for 2 hours at 50°C according to the manufacturer's instructions and analyzed by 3.5% agarose gel electrophoresis.

Fundus photography and OCT. Fundus photography and OCT were performed bilaterally on all treated dogs every 2 months after treatment. Before clinical examinations, the pupils were topically dilated as described above. Dogs were anesthetized by intravenous injection of xylazine (Bayer Health Care, Shawnee Mission, KS) and ketamine (Rhone Merieux).

Fundus photographs were taken with a Canon UVI retinal camera connected to a digital imaging system (Lhedioh Win Software; Lheritier, Saint-Ouen-l'Aumône, France).

Optical coherence tomography was performed by doing a 3-mm horizontal line scan in vector-exposed or vector unexposed areas of the retina (Stratus 3000; Carl Zeiss S.A.S; Le Pecq, France).

Electroretinography. Retinal function of treated Pde6 $\beta^{-/-}$ dogs and age-matched untreated Pde6 $\beta^{-/-}$ and Pde6 $\beta^{+/+}$ controls was evaluated using bilateral full-field flash ERG. Initial ERG measurements were recorded at 4 weeks postinjection and every subsequent 2 months up to 4 (dogs A5 and A9), 9 (dogs A4 and A8), or 18 mpi (dogs A2, A3, A6, and A7), the latest timepoints evaluated in this study. For all dogs, each contralateral eye served as intra-individual noninjected control (except for dog A9 who received injection in both eyes).

Both pupils of animals were topically dilated as described above. Dogs were dark-adapted for 20 minutes and anesthetized following premedication by intravenous injection of thiopental sodium (Specia Laboratories, Paris, France) followed by isoflurane gas inhalation. Hydroxypropylmethylcellulose (Laboratoires Théa, Clermont-Ferrand, France) was applied to each eye to prevent corneal dehydration during recordings.

A computer-based system MonColor (Metrovision, Pérenchies, France) and contact lens electrodes (ERG-jet; Microcomponents SA, Grenchen, Switzerland) were used.

ERGs were recorded in a standardized fashion according to the International Society for Clinical Electrophysiology of Vision (ISCEV) recommendations,⁴⁷ using a protocol described previously.⁴⁸

Vision testing. Light and dim-light vision of selected treated Pde6 $\beta^{-/-}$ dogs were evaluated at regular intervals from 7 to 18 mpi by recording ambulation of dogs through an obstacle avoidance course under 260 \pm 13 lux and 1.5 \pm 0.8 lux ambient light intensities, respectively. Details of the apparatus have been described previously.⁴⁸ Animals were adapted to each light condition for 1 hour before beginning the test. An opaque lens was used to alternatively cover the treated or the untreated eye. Obstacle panel combinations were randomly determined for each test to prevent the dogs memorizing the positions of the obstacle panels. A Nightshot camera recorder (Sony DCR-PC110E PAL; Sony, New York, NY) was used for filming the dimmest light condition. All dim-light films were cleared before mounting with commercial Final Cut Pro 7 software (Apple, Cork, Ireland) using a standardized protocol.

SUPPLEMENTARY MATERIAL

Figure S1. Median ERG responses of rAAV2/5- and rAAV2/8-treated eyes overtime.

Video S1. Assessment of dim and bright light vision in dog A6 at 18 mpi.

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