Targeted Ablation of the Pde6h Gene in Mice Reveals Cross-species Differences in Cone and Rod Phototransduction Protein Isoform Inventory*

Received for publication, September 18, 2014, and in revised form, March 2, 2015. Published, JBC Papers in Press, March 4, 2015, DOI 10.1074/jbc.M114.611921

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Background: Phosphodiesterase-6 (PDE6) is a multisubunit enzyme essential for visual signal processing. Rare mutations in the human PDE6H gene result in incomplete color blindness.

Results: Pde6h-deficient mice exhibit no signs of photoreceptor dysfunction.

Conclusion: PDE6 configurations differ between species and are more interchangeable than previously thought.

Significance: Presence of related isoforms in the retina may allow adjustments of the phototransduction components thereby preventing the occurrence of pathological conditions.

Phosphodiesterase-6 (PDE6) is a multisubunit enzyme that plays a key role in the visual transduction cascade in rod and cone photoreceptors. Each type of photoreceptor utilizes discrete catalytic and inhibitory PDE6 subunits to fulfill its physiological tasks, i.e. the degradation of cyclic guanosine-3′,5′-monophosphate at specifically tuned rates and kinetics. Recently, the human PDE6H gene was identified as a novel locus for autosomal recessive (incomplete) color blindness. However, the three different classes of cones were not affected to the same extent. Short wave cone function was more preserved than mid- and long wave cone function indicating that some basic regulation of the PDE6 multisubunit enzyme was maintained albeit by an unknown mechanism. To study normal and disease-related functions of cone Pde6h in vivo, we generated Pde6h knock-out (Pde6h−/−) mice. Expression of PDE6H in murine eyes was restricted to both outer segments and synaptic terminals of short and long/middle cone photoreceptors, whereas Pde6h−/− retinas remained PDE6H-negative. Combined in vivo assessment of retinal morphology with histomorphological analyses revealed a normal overall integrity of the retinal organization and an unaltered distribution of the different cone photoreceptor subtypes upon Pde6h ablation. In contrast to human patients, our electroretinographic examinations of Pde6h−/− mice suggest no defects in cone/rod-driven retinal signaling and therefore preserved visual functions. To this end, we were able to demonstrate the presence of rod PDE6G in cones indicating functional substitution of PDE6. The disparities between human and murine phenotypes caused by mutant Pde6h/ PDE6H suggest species-to-species differences in the vulnerability of biochemical and neurosensory pathways of the visual signal transduction system.

Transgenic and knock-out mouse models are widely used to study the physiology of phototransduction and the cellular mechanisms of vision impairment. In general, light detection in the murine and human retina follows the same principles, which involve two types of photoreceptors. At low light intensities visual cues are mainly detected by rods, whereas cones are important for color vision and vision at high light intensities (1, 2). Despite these differences in light sensitivities, phototo- transduction in rods and cones involves related biochemical signaling pathways and a set of highly homologous but unique factors, including the different types of opsins, G-proteins, catalytic and regulatory subunits of phosphodiesterase-6 (PDE6), the various pore-forming and auxiliary subunits of the cGMP-gated cation channels (CNGs), as well as proteins involved in phototransduction recovery such as opsin kinases, arrestins, membrane guanylate cyclases, i.e. GC-E and GC-F and GC-activating proteins (3–7).

The multisubunit enzyme PDE6 plays an essential role for the visual signal transduction process in both rods and cones; however, each type of photoreceptor utilizes discrete PDE6 subunits to fulfill its physiological tasks, i.e. the degradation of cyclic guanosine-3′,5′-monophosphate (cGMP) at specifically tuned rates and kinetics (8). Rod PDE6 is composed of two

*This work was supported by Deutsche Forschungsgemeinschaft Grants KFO134 and Ru573/5 (to P. R. and B. W.) and KFO134-Se837/5-2 and Se837/6-2 (to M. W. S.).

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5 The abbreviations used are: CNG, cGMP-gated cation channel; ACHM, achromatopsia; cd, candelis; PNA, peanut agglutinin; GlyPhos, glycogen phosphorylase; OS, outer segment; IS, inner segment; SLO, scanning laser ophthalmoscopy; OCT, optical coherence tomography; ERG, electroretinogram; IF, immunofluorescence; L, long wavelength-sensitive; S, short wavelength-sensitive; M, medium wavelength-sensitive.
distinct PDE6α and PDE6β subunits that form a catalytic core encoded by the Pde6α and Pde6β gene, respectively. In the dark, the rod PDE6α/PDE6β heterodimer is inhibited by two identical γ-subunits (PDE6G) that are encoded by the Pde6g gene (9). In cone photoreceptors, PDE6 is formed by two identical PDE6α’ subunits produced by the Pde6c gene and by two inhibitory PDE6γ’ subunits (PDE6H), which derive from the Pde6h gene (10, 11).

Activation of PDE6 in rods and cones requires the Gα-subunits from the G-protein transducin (Gtα), which upon light-induced release of Gtβγ binds to PDE6 and thereby displaces the respective inhibitory γ-subunits (12). Light-induced activation of the different PDE6 multisubunit enzymes in photoreceptors results in a rapid drop in the intracellular concentration of cGMP and subsequently in closure of heterotetrameric CNG channels (3). In addition to many neuronal and cardiovascular functions of cGMP (13–15), cGMP binds to and activates CNG channels producing a stimulus-induced receptor potential. Ligand-dependent activation of photoreceptor CNG channels results in the so-called “dark current,” a depolarizing current carried by a sustained entry of Na+ and Ca2+ ions, which trigger neurotransmitter, i.e. glutamate release from photoreceptors as major synaptic input to bipolar cells.

Mutations in genes that code for proteins of the visual transduction system are a major cause of visual impairments (16). In this line, several studies report primary loss of proper PDE6 functions in photoreceptor pathology. Both the gene-targeted mouse model and humans revealed that defects in all so far identified genes encoding Pde6 subunits cause hereditary retinal diseases. For example, a mutation in the Pde6b gene produced an early onset retinal degeneration in the rd1 mouse model (17). In humans, mutations in PDE6B cause autosomal dominant night blindness (18) or recessive retinitis pigmentosa (19) characterized by night blindness, initial rod photoreceptor degeneration, and gradual loss of vision leading to blindness (20). Similarly, mutations in the Pde6a and in the PDE6G genes were identified in several families with segregating autosomal recessive retinitis pigmentosa (21, 22). Accordingly, an ablation of the rod γ-subunit in gene-targeted Pde6g mice resulted in rapid retinal degeneration resembling human retinitis pigmentosa (23). We and others previously reported mutations in the human PDE6C gene encoding for the cone-specific catalytic PDE6α’ subunits in patients that develop autosomal recessive inherited achromatopsia (ACHM) with early-onset cone photoreceptor dysfunction (24). Consistently, the cpfl1 mutant mouse strain, which carries a spontaneous mutation in the Pde6c gene, develops pronounced cone dysfunction and photoreceptor degeneration (25). Likewise, mutations in the human genes for cone transducin α-subunit (GNAT2) (26), cone CNG channel (CNGA3 and CNGB3) subunits (27), and PDE6C (24) as well as PDE6H (28) lead to autosomal recessive (incomplete) ACHM. Yet, important unresolved issues remained in case of the PDE6H mutation. In the three affected patients reported by Kohl and co-workers (28), a nonsense mutation within exon 2 of PDE6H was predicted to result in a truncated protein of only 11 amino acid residues lacking all conserved domains relevant for transducin binding (29, 30) and inhibition of the catalytic activity of PDE6. Because cone PDE6H includes 83 amino acid residues (11, 31), trichromatic color vision should be completely lost. However, S-cone function was more preserved than L- and M-cone function indicating that basic regulatory properties of the PDE6 multisubunit enzyme were maintained in S-cones by an unknown mechanism.

To elucidate the time course and mechanism(s) of cone degeneration triggered by loss of PDE6H function, we generated a Pde6h−/− mouse model that lacks the entire Pde6h locus. However, Pde6h−/− mice showed a normal retinal morphology, regular cone distribution, and unaltered retinal function in electroretinographic recordings despite the confirmed absence of cone PDE6H in Pde6h−/− mutant retinae. Evidence for the expression of the Pde6g-derived rod inhibitory subunit PDE6G in cones served as explanation of this unexpected phenotype. In contrast to humans, these results indicate that loss of Pde6h in dichromatic mouse retina can functionally be compensated by rod PDE6G. Together, the available evidence from previous reports (see Table 1) and this study reveal important species-to-species differences in their capabilities to respond to pathophysiological conditions of the visual transduction system.

Experimental Procedures

Animal Welfare and Genetic Background—Experimental mice were bred and maintained at the animal facility of the Institute of Pharmacy, Department of Pharmacology, Toxicology and Clinical Pharmacy, University of Tübingen. All procedures concerning animals were performed with permission of local authorities and conducted in accordance with the German legislation on the protection of animals. The mice were kept in temperature- and humidity-controlled cabinets, in a standard 12-h light-dark cycle with ad libitum access to food and water. For the experiments, global Pde6h knock-out mice (genotype Pde6h−/−) were compared with age or age- and litter-matched wild-type mice (genotype Pde6h+/+) on a hybrid SV129/C57BL/6 genetic background. Animals were used irrespective of their gender at an age of 12–52 weeks.

Generation of the Pde6h-deficient Mouse Model—The Pde6h homology arm fragments for the assembly of the targeting construct were taken from a bacterial artificial chromosome isolated from a 129/Sv mouse bacterial artificial chromosome library (RZPD, Berlin, Germany). The targeting vector contained a Neo/Tk selection cassette flanked by two loxP sites located downstream of exon 4 and a single loxP site together with a new SpeI restriction site (for subsequent identification of the homologous recombination by Southern blot analysis) in intron 2. Therefore, the entire protein coding exons 2–4 of the Pde6h gene were flanked by two loxP sites (Fig. 1A). R1 embryonic stem (ES) cells were electroporated with the linearized targeting construct and screened for G418-resistant clones. Homologous recombination was confirmed by Southern blot analysis upon HindIII- and SpeI-digested ES cell DNA. One correctly targeted clone (L3+/+) was injected into C57BL/6 blastocysts. The resulting chimeras were mated with C57BL/6 mice to obtain germ line transmission, resulting in heterozygous Pde6hL3+/− offspring. Pde6hL3+/− mice were crossed to Cre deleter animals (genotype CMV-Cre(LoxP))(32) to excise the coding exons of Pde6h and the Neo/Tk cassette. This produced double
transgenic animals with the desired Pde6h−/−; CMV-CreTG/0 genotype. Pde6h−/−; CMV-CreTG/0 mice were then backcrossed to C57BL/6 mice to eliminate the Cre transgene. Heterozygous offspring (genotype Pde6h+/−) were intercrossed to establish homozygous Pde6h-deficient mice (Pde6h h−/−) and littermate control animals for the experiments. Animals were genotyped by PCR using mouse tail genomic DNA with three primers (F1, 5′-TCCATGTGAGGGAAGCAG-3′; F2, 5′-CGATGT-GAGCTTAGGGCTCTG-3′; and R, 5′-GCTTCCAACATC-CCATCTCTCC-3′) that amplified either the WT (446 bp) or the Pde6h knock-out allele (346 bp) (Fig. 1D).

Immunoblotting of Proteins from Mouse Retina and of Recombinant PDE6H Protein—For protein extraction from pooled mouse retinas (n = 4–6 per genotype), enucleated eyes were homogenized mechanically (Ultra-Turrax) in homogenization buffer (20 mM Tris-HCl (pH 8.3), 0.67% SDS, 238 mM KCl) and suspended again in cell culture medium, and 150–200 μl of the suspension were plated on glass slides coated with poly-D-lysine/PBS overnight (1:100; Sigma) equipped with 0.3% Triton X-100/PBS, as described previously (36).

For immunofluorescence (IF) staining, retinal sections or cultured retina-derived single cell cultures were washed with PBS, blocked with 5% normal goat serum in PBS for 1 h, and incubated overnight at 4 °C in primary antibody dilutions. The primary antibodies used for the IF were specific for PDE6H (1:1000 dilution; as described previously (28)), PDE6G/H (1:1000 dilution; Santa Cruz Biotechnology), glycogen phosphorylase (1:1000 dilution (37–39)), and peanut agglutinin (PNA) conjugated to FITC or Alexa 568 (1:100 dilution, Sigma). Detection of the primary antibody-antigen complexes was carried out by appropriate Cy2- or Cy3-conjugated secondary antibodies (1:200 dilution, Dianova, Hamburg, Germany) and mounted in PermaFluor (Thermo Fisher Scientific, Waltham, MA) containing 1 μg/ml Hoechst (Sigma) for the nuclear counterstain. Axio-imager Z1 or ApoTome fluorescence microscopes (Zeiss, Oberkochen, Germany) were used to examine the IF.

Digital quantification of the PDE6H or PDE6G/H fluorescence in Pde6h−/− and WT retina was performed in optical slices double-stained for PNA-FITC and PDE6H or PNA-FITC and PDE6G/H. Quantitative analyses of fluorescence intensities at defined coordinates were measured in grayscales using the Axiovert software for a 200 μm high field of view. All data were normalized against the corresponding background fluorescence.

Histology of Retinal Fine Structure and Whole Mount Immunohistochemistry—For the histological assessment, 8-μm sections of paraffin-embedded retina were used. Hematoxylin and eosiin staining (H&E) was performed according to a previously published protocol. Digital images were obtained by the use of an AxioCam MRC attached to an Axiovert 200 M microscope (Zeiss).

For retinal whole mount staining, eye cups were dissected, and the orientation of the retina was marked by a small incision. Isolated retinae were flat-mounted on slides, fixed for 40 min with 2% paraformaldehyde, transferred onto a filter paper with the photoreceptor layer up, and kept in 0.1 M phosphate buffer. Whole mounts were washed in 0.1 M phosphate buffer and blocked with 5% ChemiBLOCKER (EMD Millipore Billerica) containing 0.5% Triton X-100 and 0.05% NaCl for 1 h. Primary antibodies were applied overnight at 4 °C. After several washing steps, whole mounts were sealed in Vectashield to prevent photobleaching.

In Vivo Electroretinography—Full-field electroretinograms (ERGs) were recorded from Pde6h−/− and WT littermate mice at an age of 4–5 months according to the procedures described.
previously (40). In brief, mice were dark-adapted overnight before the experiments and anesthetized with a subcutaneous injection of a mixture of ketamine (66.7 mg/kg body weight) and xylazine (11.7 mg/kg body weight). The pupils were dilated, and single-flash ERGs were obtained under dark-adapted (no background illumination, 0 cd/m²) and light-adapted (with a background illumination of 30 cd/m² starting 10 min before recording) conditions. Single white-flash stimuli ranged from −4.0 to 1.5 log cd s/m² under dark-adapted and from −2.0 to 1.5 log cd s/m² under light-adapted conditions. Ten responses were averaged with inter-stimulus intervals of 5 s (for −4 to −0.5 log cd s/m²) or 17 s (for −0.5 to −1.5 log cd s/m²). Responses to trains of brief flashes (flicker) for a fixed intensity (0.5 log cd s/m²) with 12 frequencies (0.5, 1–3, 5, 7, 10, 12, 15, 18, 20 and 30 Hz) were obtained without any background illumination (0 cd/m²), which were averaged either 20 times (for 0.5 to 3 Hz) or 30 times (for 5 Hz and above). Bandpass filter cutoff frequencies were 0.3 and 300 Hz for all ERG recordings. ERG data were recorded from four WT and four Pde6h−/− animals.

Scanning Laser Ophthalmoscopy and Optical Coherence Tomography—Scanning laser ophthalmoscopy (SLO) and spectral domain optical coherence tomography (OCT) imaging were done in the same session as the ERGs in Pde6h−/− and WT. SLOs and OCTs were obtained as reported previously (41, 42).

Scanning laser ophthalmoscopy was performed with a Heidelberg Retina Angiograph (HRA I, Heidelberg Engineering, Germany). The HRA features two argon wavelengths (488 and 514 nm) in the short wavelength range and two infrared diode lasers (795 and 830 nm) in the long wavelength range. Laser wavelengths used for fundus visualization were as follows: 830 nm (infrared channel) and 514 nm (red-free channel). Additionally, the 488- and 795-nm lasers were used for fluorescein and indocyanine green angiography, respectively. Fluorescein angiography was performed using an s.c. injection of 75 mg/kg body weight sodium fluorescein (University Pharmacy, University of Tübingen, Germany) and indocyanine green angiography following an s.c. injection of 50 mg/kg body weight indocyanine green (ICG-Pulsion, Pulsion Medical Systems AG, Munich, Germany). SD-OCT imaging was performed with a Spectralis HRA + OCT device from Heidelberg Engineering featuring a broadband superluminescent diode as low coherent light source (43). Each two-dimensional B-scan recorded with the equipment set to 308 fields of view consists of 1536 A-scans acquired at a speed of 40,000 scans/s. Optical depth resolution is 7 μm with digital resolution reaching 3.5 mm. Image data were analyzed using the proprietary software package Eye Explorer from Heidelberg Engineering.

RESULTS

Generation of Pde6h−/− Mice—To define the role of the inhibitory subunit of cone phosphodiesterase-6 for photoreceptor function in vivo, we generated mice lacking a functional Pde6h. Exons 2–4, which include the entire protein coding sequences of murine Pde6h, were flanked with two loxP sites and a Neo/TK selection cassette by homologous recombination (Fig. 1A). ES cells were screened for correct targeting by Southern blot analysis (Fig. 1B). The integrity of the loxP sites was tested in vitro using L3/+ ES cells that were subjected to a transient expression of the Cre recombinase. As expected, this second targeting resulted in ES cells that were either heterozygous for the conditional (L2) or null (L1 or −) allele of Pde6h (Fig. 1, A and C). To establish a conditional and constitutive knock-out mouse line, we used one correctly targeted L3/+ clone for the blastocyst injection and generated four chimeric mice, two of which showed germ line transmission of the L3/+ allele. We obtained heterozygous Pde6h-deficient (Pde6h+/−) progenies by mating the germ line founders, which carried the targeted Pde6h allele (Pde6hL1/+L2) with a transgenic Cre-deleter mouse line (Fig. 1D) (32). Offspring heterozygous for the targeted Pde6h allele were subsequently backcrossed to C57BL/6 mice to obtain heterozygous Pde6h+/− animals that lost the Cre recombinase transgene. Intercrossing of Pde6h+/− mice yielded age- and litter-matched Pde6h−/− knock-out and WT (genotype Pde6h+/+) animals at the expected Mendelian ratios (Fig. 1D). Pde6h−/− and Pde6h+/− mice were viable and fertile and did not show any gross abnormalities confirming that the Pde6h gene is not essential for a normal pre- and postnatal development.

Lack of the PDE6H Protein from Retinal Cone Photoreceptor Cells in Pde6h−/− Mice—To verify the retinal distribution of PDE6H on a cellular level and to confirm the absence of the PDE6H protein in the Pde6h−/− model, we first investigated retinal cryosections and primary retinal cell cultures obtained from WT and Pde6h−/− mice. Immunofluorescence staining was performed by using in-house-generated antibodies that were either specific for PDE6H or glycogen phosphorylase (GlyPhos), a widely used cone-specific marker. As in previous studies (28), the co-localization experiments in WT retina showed that the PDE6H protein was exclusively located in cone photoreceptors and not present in any other retinal cell type. The highest PDE6H intensities were detected in cone outer segments (OS), whereas inner segments (IS) and synaptic terminals of cone photoreceptors showed a weaker (but consistent) staining. Retinal sections of Pde6h null mice remained PDE6H-negative confirming efficient deletion of the Pde6h gene in the KOs (Fig. 2A). Interestingly, we observed a normal cellular morphology and layering of Pde6h-deficient retinе that were obtained from young and old mice, i.e. about 52-week-old mice indicating that the lack of PDE6H protein does not cause a loss of photoreceptor cells neither during development nor as a result of a postnatal progressive degenerative process.

To discriminate exactly between rod and cone expression of PDE6H, we analyzed enzymatically dissociated murine retina. In these cultures, the different types of photoreceptors can be well discriminated from any other retinal cell types. Primary cells derived from Pde6h−/− and WT retinе were plated and analyzed by immunofluorescence staining using the above-mentioned antibodies against PDE6H and GlyPhos. These co-localization experiments revealed that PDE6H was present in all GlyPhos-positive cells from dispersed WT retinas, whereas GlyPhos-positive cells from Pde6h−/− retinas could not be labeled for PDE6H (Fig. 2B). Importantly, the primary cells confirmed that the PDE6H protein was restricted to cone photoreceptors because we never observed PDE6H in GlyPhos-nega-
tive cells (data not shown). Together, our present findings are in good agreement with previous reports (11) implying that the retinal expression of PDE6H in the eye is indeed restricted to cone photoreceptors.

We next examined the distribution of the PDE6H protein in rat and bovine retina (Fig. 2). In rat retina, highest PDE6H intensities were observed in the cone photoreceptor IS. Similarly, PDE6H protein in bovine retina was more concentrated to the IS than the OS. Immunoreactivity for PDE6H appeared to be high in the base region of the bovine cone OS, whereas in the distal portion of the OS much less PDE6H was detectable.

Normal Retinal Morphology, Cone Subtype Distributions, and Photoreceptor Densities in Pde6h−/− Mice—Because the genetic ablation of the catalytic Pde6 subunits in both cones or rods and of the rod-specific γ-subunit of PDE6 in mice caused prominent histological changes, including photoreceptor degeneration, and result in severe functional defects of the retina (21–25, 28), we performed a combined histomorphological and cone distribution study in our Pde6h−/− mouse model.

Pde6g null mice that carry a deletion of the inhibitory γ-subunit of rod PDE6 exhibit a severe and progressive loss of photoreceptor cells leading to early retinal degeneration (23). In contrast, hematoxylin and eosin staining of retinal paraffin sections from young (data not shown) and old Pde6h−/− animals did not show any obvious morphological abnormalities, i.e. neither the overall structure of the retina nor its organization into the typical layers were altered by the lack of the PDE6H protein (Fig. 3A). Next, we performed an IF analysis on whole mount staining obtained from Pde6h−/− and WT retina using two different antibodies directed against M- or S-opsins, respectively.

**FIGURE 1. Targeted deletion of the murine Pde6h gene using the Cre/loxP system.** A, targeting strategy. Top, murine WT Pde6h gene locus and targeting vector containing a Neo/Tk cassette flanked by two loxP sites (black triangles) downstream of the coding exons 2–4 and a single loxP site upstream of exon 2. Middle, targeted allele (L3) after neomycin (Neo) selection. Bottom, treatment of a targeted allele with Cre recombinase and subsequent negative selection using gancyclovir, which is toxic in the presence of thymidine kinase (Tk) yielded a modified Pde6h allele where the coding exons are flanked by two loxP sites (L2) or a null allele with a deletion of all coding exons (L1). Diagnostic restriction sites, i.e. HindIII, SpeI, and BamHI as well as the position of all probes for the Southern analysis (gray bars), are indicated. Black arrows indicate the position of the PCR primers used to identify the different Pde6h alleles. B, first targeting, representative ES cell Southern blots. To verify the correct integration of the 3′-homology arm, genomic ES cell DNA was digested with HindIII and hybridized with the 3′-probe yielding an 8.4- and a 6.2-kb fragment representing the WT and the targeted L3 allele (left). To analyze the correct integration of the 5′-homology arm, genomic ES cell DNA was digested with SpeI and hybridized with the 5′-probe yielding a 14.7- and a 4.7-kb fragment representing the WT and the targeted L3 allele (right). C, Southern blot of L3 ES cells after transfection with Cre recombinase. DNA was digested with BamHI and hybridized with the 3′-probe yielding 18.8-, 12.6-, and 5.8-kb fragments representing the WT, null (L1), and conditional knock-out allele (L2), respectively. D, PCR genotyping of murine tail tip DNA from WT, heterozygous (Pde6h−/+), and Pde6h−/− animals using specific primers F1, F2, and R (arrows in A). The size of the expected amplicons is 446 bp for WT and 346 bp for the knock-out allele, respectively.
Surprisingly, we did not observe any differences in the distribution of M-opsin- (Fig. 3B) and S-opsin-expressing cones (Fig. 3C) in ventral and dorsal parts of the retina between both genotypes. The nonhomogeneous distribution of S-opsin expression in Pde6h−/− and in WT retinas, i.e. a high number of S-opsin-expressing cones in the ventral part and a low abundance in the dorsal part of the retina represent the physiological distribution in mice and correlate well with previous reports (44, 45). From our recent analysis of three human patients that carry a mutation in PDE6H (28), we expected that M-cone (and M-/S-cone) functions and therefore their numbers would be affected in the Pde6h null retina. However, on the histomorphological level, we did not find any signs for M- or S-cone degeneration (Fig. 3B). These histological investigations were corroborated by an in vivo assessment of the retinal morphology using SLO imaging and OCT.

In vivo SLO en face fundus imaging (Fig. 3D) as well as angiography (data not shown) demonstrated normal fundus appearance and normal retinal vasculature, respectively. Analysis of retinal morphology with in vivo OCT imaging demonstrated regular retinal thickness and normal retinal layering (Fig. 3E), suggesting, together with the histomorphological examinations (Fig. 3, A–C), that Pde6h is dispensable for retinal integrity and the proper organization of cone photoreceptors.

Ablation of Pde6h Does Not Alter Mouse Cone and Rod Photoreceptor Functions—To assess whether the lack of Pde6h has consequences for photoreceptor functions, full-field single-flash ERGs were recorded from Pde6h−/− and WT littermates.
under both dark-adapted and light-adapted conditions (Fig. 4, A and B). The initial negative-deflecting a-wave that appears at middle and high intensities is initiated by photoreceptors. The so-called b-wave, a large positive deflection that follows the a-wave, mainly includes ON-bipolar cell activity and hence represents signaling downstream of the photoreceptors. Because the input to bipolar cells is derived from the preceding photoreceptor cells, the b-wave indirectly also reflects the activity of rods and cones and is a practical indicator for photoreceptor-ON-bipolar cell functionality. In contrast to the ERG phenotype of the three human patients that carry a stop mutation in the PDE6G homolog (28), dark-adapted and light-adapted single-flash ERGs of WT and Pde6h−/− mice appeared similar in size and shape (Fig. 4, A, B and D), and we could not detect any difference in b-wave amplitudes (Fig. 4C). These results suggest that the deletion of Pde6h in mice does not affect rod- or cone-dependent ON-bipolar cell signaling. By recording ERGs during a flicker stimulation (Fig. 4, E and F), we further obtained some additional information about cone ON- and OFF-pathway signaling in Pde6h−/− mice. In this flicker frequency series, the stimulus intensity remains the same at 0.5 log cd s/m², whereas stimulus frequency is increased stepwise from 0.5 to 30 Hz without any adapting background illumination. Under the given conditions, the flicker responses are dominated by activity in the rod system below 5 Hz, cone ON-pathway between 5 and 15 Hz, and cone OFF-pathway above 15 Hz (46). At all applied stimulus frequencies, there was no remarkable difference in flicker responses between WT and Pde6h−/− mice (Fig. 4, E and F). Together, the ERG measurements suggest no defects in cone-driven or rod-driven retinal signaling and that overall retinal function is not impaired in Pde6h−/− mice.

Cone Photoreceptor Cells in Pde6h−/− Mice Express the Rod Pde6g Homolog—In contrast to the three patients with homozygous nonsense mutations in PDE6H, our in vivo ERG analysis revealed that Pde6h−/− mice neither exhibit reduced cone functions nor absent flicker responses indicative for a homologous phenotype (28). We therefore reasoned that an ablation of the entire Pde6h gene in dichromatic mouse retina would be compensated by a mechanism that maintains a regular light-evoked signal processing from cones to bipolar cells. We first tested whether an unexpected presence of the PDE6G protein (the rod isoform of PDEγ) in Pde6h−/− cones was responsible for a preserved regulation of the catalytic subunits of PDE6. FITC-linked PNA was used as cone marker on mouse WT and Pde6h−/− retinal sections for co-labeling experiments with antibodies specifically detecting either the PDE6H protein (Fig. 5A) or both homologs i.e. PDE6G and PDE6H (Fig. 5, C and D).

In agreement with our previous results obtained from co-labeling of cone photoreceptors with GlyPhos antibodies (Fig. 2, A and B), we observed high expression of PDE6H in the outer segments of PNA-FITC-labeled cones of the WT retina, whereas photoreceptors from Pde6h null mice remained PDE6H-negative (Fig. 5A). As expected, the PDE6G/H common antibody homogeneously labeled the entire photoreceptor layer, including the PNA-FITC-positive cones of WT retina (Fig. 5C). By using the same PDE6G/H common antibody, we observed a highly analogous staining pattern in Pde6h−/− retina (Fig. 5C), i.e. co-staining of PNA-FITC-tagged cones with the PDE6G/H common antibody. Because we repeatedly confirmed full ablation of the PDE6H protein in the Pde6h−/− mouse model, this finding points to the presence of PDE6G in Pde6h−/− cones.

To avoid superimposing fluorescence signals from rod to cones, we digitally measured and quantified relative fluorescence intensities in optical sections devoid of scattered light and upon background subtraction in WT and Pde6h−/− (Fig. 5, B, D, and E). Again, PNA-FITC fluorescence was used to detect cone outer segments (peak of green fluorescence; arrows in Fig. 5D). Subsequently, corresponding PDE6H or PDE6G fluorescence intensities at the same coordinates, obtained by labeling of the retina with PDE6H or PDE6G/H antibodies, were deter-
mined. As expected, the quantification demonstrated that the PDE6H fluorescence signal was absent from the cone outer segments of Pde6h−/−/H11002/−/H11002 upon labeling the retina with PDE6H-specific antibody (Fig. 5, A and B). In contrast, labeling of the Pde6h null retina with a common PDE6G/H antibody resulted in significant fluorescence signals in the cone photoreceptors (Fig. 5, D and E). Indeed, the relative amount of cone fluorescence in the Pde6h−/− and WT retina was similar upon labeling the photoreceptors with PDE6G/H antibody. We conclude from this finding that the usually “rod-specific” PDE6G protein is present in cones, thereby preventing catalytic deregulation of PDE6, which would otherwise cause cone dysfunction and/or degeneration. This finding is further supported by immunoblotting analysis of retina lysates derived from WT and Pde6h−/−/H11002/−/H11002 mice showing no differences in the total PDE6G plus PDE6H protein levels (Fig. 5F) and control samples of recombinant PDE6H protein to determine the specificity of the antibodies (Fig. 5, F and G).

DISCUSSION

To study the molecular pathomechanisms of visual dysfunction, in particular cone dystrophies and ACHM in vivo, we generated mice in which the inhibitory subunit of cone photoreceptor Pde6h was genetically deleted. Based on previous findings from different gene-targeted mouse models (17, 23, 25, 43) and human patients, we expected the loss of PDE6H func-
tion to result in deregulated PDE6 activity and thereby impaired cone signal transduction and photoreceptor loss. However, neither our immunohistochemical labeling of photoreceptors using different cone and rod markers (Figs. 2 and 5) nor in vivo analyses by SLO and OCT imaging (Fig. 3, D and E) revealed any degenerative processes or morphological alterations in *Pde6h<sup>−/−</sup>* retinas. Importantly, M- and S-opsin expression was distributed normally throughout the *Pde6h*-deficient
photoreceptor cell layer (Fig. 3, B and C), although we confirmed the absence of PDE6H in these cells. To assess the retinal function of Pde6h+/− mice in vivo, we performed a set of ERG analyses (Fig. 4). Light- and dark-adapted measurements and different stimulus conditions were tested to investigate discrete rod or cone functions and as well as mixed rod and cone responses. In contrast to the patients with a nonsense mutation in PDE6H that suffer from incomplete ACHM due to severely reduced cone functions (28), we did not find any signs of distorted photoreceptor function in Pde6h−/− ERGs at all applied stimuli (Fig. 4). Importantly, retinal signaling after single-flash stimulation in dark-adapted and light-adapted flash ERGs as well as the dynamic properties by repetitively stimulating the retina in flicker ERGs were not different between Pde6h+/− and WT mice. Together, these findings identify important species-specific differences for the roles of mouse Pde6h and human PDE6H. Murine Pde6h is either dispensable for visual signal transduction in cones or the chronic lack of PDE6H may be efficiently substituted by PDE6G in vivo in mice.

The presence of rod- and cone-specific isoforms for the various components of the phototransduction signaling cascade is fairly conserved in vertebrate species. The exclusivity of their expression in either rods or cones and thus the primary impairment of one of the two types of photoreceptors upon gene ablation have been demonstrated in numerous animal models. However, there are several important exceptions in which species-specific differences in gene expression or the phenotypic presentation of mutants has been reported. For instance, GRK1 and GRK7 are considered to encode the G-protein receptor kinases that phosphorylate the photopigment during the photoreceptor signal off in rods and cones, respectively. Immunohistological analysis revealed prominent differences in the expression of GRK1 and GRK7 in rods and cones between species (47, 48). Moreover, mutations in GRK1 in humans cause Oguchi disease, a hereditary form of night blindness with a marked decrease in rod response and recovery kinetics (49, 50), whereas ablation of Grk1 in the mouse results in equally impaired rod light response and recovery kinetics in both rods and cones (51, 52). Similarly, differences between man and mouse were reported for retinal photoreceptor guanylate cyclases (GC-E and GC-F) and the interacting guanylate cyclase activator proteins 1–3 (5, 6, 53). Indeed, the distribution and expression levels of these important factors vary a lot between species; hence, rearrangements made upon disruption of a single guanylate cyclase or guanylate cyclase activator proteins may result in rather unique phenotypes. For example, GCAP1 and GCAP2 expression levels in rod and cone photoreceptors differ between a number of species, and GCAP3 is expressed exclusively in human and zebrafish cones but is not detectable in mice (54–57). In the rod-dominated rat and mouse retinae, the outer segment layer shows uniform GC-E immunoreactivity (58, 59), whereas in monkey and human retina, GC-E labeling of cones seems to be more intense than in rod outer segments (58, 60, 61). In contrast to human GC-F that is present in photoreceptor cell bodies and inner segments (54, 62), murine GC-F is largely restricted to cone OS (53). Distinct expression patterns for GC-E and GC-F imply cross-species differences in the development, function, and/or survival of photoreceptors when one or the other isoform is functionally impaired or eliminated. Indeed, recessive mutations in the human GC-E gene (GUCY2D) are known to cause Leber’s congenital amaurosis type 1 (Table 1), an early onset rod/cone dystrophy with severe early onset visual impairment (63). GC-E-deficient mice, however, develop cone dystrophy with rods remaining morphologically intact maintaining light responsiveness (64). Along the same lines, mutations in the GCAP1 gene have been associated with an autosomal dominant form of cone dystrophy in human patients (65–67), whereas expression of GCAP1/Gcap2-deficient mouse rescues rod photoreceptor response in this mutant (68).

We find it very unlikely that deregulated cGMP degrading activity of the catalytic PDE6 subunits in cones can be tolerated by the cone photoreceptor. Indeed, the available data from mutant zebrafish, the cpfl1 mutant mouse model that carries a spontaneous mutation in the catalytic subunit of cone phosphodiesterase (i.e. Pde6c), and from humans with mutations within the PDE6C gene (25, 69) point toward the importance of a tightly regulated PDE6 in cones. Reduced PDE6C activity interferes with the visual signal transduction process thereby causing cone dysfunction, photoreceptor degeneration, and ACHM (25). Because our genetic approach to inactivate Pde6h revealed no retinal phenotype, we tested for potential compensatory mechanisms and found strong evidence for expression of the rod-specific PDE6G protein in cones of Pde6h−/− mice (Fig. 5). For dichromatic mice, this finding suggests that PDE6G may effectively participate in cone PDE6 regulation to allow accurate visual signal transduction even in the absence of functional PDE6H.

Our data are in good agreement with several reports that found a reciprocal substitution of the catalytic PDE6 subunits in rod and cone photoreceptors. Kolaandaiavelu et al. (70) gener-

FIGURE 5. Analysis of rod PDE6G expression in Pde6h−/− cones by double immunofluorescence labeling. A co-labeling of mouse WT and Pde6h−/− (KO) retina sections with a PDE6H-specific antibody (red, 1:1000) and peanut hemagglutinin (PNA-FITC, green, 1:100) as a cone marker. Co-localization of PDE6H and PNA was detectable in WT but not in Pde6h−/− retina. B, measurement of relative fluorescence intensities was performed for 20 cone OS of each genotype for PDE6H and PNA. Only coordinates that were positive for PNA (green, cones) were considered for the quantification. As expected, Pde6h−/− outer segments revealed no PDE6H fluorescence, PNA immunoreactivity of cones, however, was similar between genotypes. C and D, co-labeling of mouse WT and Pde6h−/− retina sections with PNA-FITC (green, 1:100) as cone marker and an antibody staining both PDE6G and PDE6H protein (red, 1:1000) in lower (C) and at a higher magnification (D). DAPI was used as a nucleus-staining fluorochrome. C, single channel (left, middle) and multichannel (right) panels are displayed. D, peaks of PNA-FITC fluorescence intensity in histograms (boxes) were used to identify the localization of cones. At these coordinates (arrows), relative fluorescence intensities for PDE6G/H (red) and PNA (green) were digitally measured in grayscale and quantified as shown in E. Only coordinates that are positive for PNA (green, cones) were analyzed for PDE6G/H (and PDE6H) fluorescence intensities (red). Data were normalized to background fluorescence. E, measurement of relative fluorescence intensities was performed for 20 cone OS. Pde6h−/− outer segments revealed PDE6G/H immunoreactivity, suggesting that rod PDE6G is present in Pde6h−/− cones. F, Western blot analysis of PDE6G/H protein of WT and Pde6h−/− retinas. Heterologously expressed recombinant PDE6H protein (rPDE6H) serves as a positive control. Pde6h−/− retina showed no reduction in total PDE6G/H protein levels compared with WT retina. G, specificity of the PDE6H antibody was validated with recombinant PDE6H (rPDE6H) by immunoblotting. B and E, n.s. indicates non-significant difference between groups; ***, p < 0.001.
Nomenclature—We thank Stephen Tsang for discussions; Brigitte Pfeiffer-Guglielmi for providing the GlyPhos antibody; Johannes Wilbertz from the Karolinska Center for Transgene Technologies (Stockholm) for performing blastocyst injections; and Marc Freichel from the Institute of Pharmacology in Heidelberg for providing Cre deleter mice.

TABLE 1

Exemplary gene-targeted mouse models failing to produce a phenotype like in humans

| Gene (human/mouse ortholog) | Human disease | Phenotype of gene-targeted mouse model | Ref. |
|-----------------------------|--------------|----------------------------------------|------|
| Neuro-sensory system        |              |                                        |      |
| CLRN1/Clrn1                 | Usher syndrome type3 (USH3) | Mutations in the murine orthologous gene lead to cochlear and vestibular dysfunction but not to retinal defects | 75   |
| CRB1/Crb1                   | Retinitis pigmentosa (RP12), Leber congenital amaurosis (LCA8) | Cone responses in Gucy2e−/− mice are undetectable, but morphologically normal rods show paradoxical behavior in their responses to light, whereas human patients present with rod/cone dystrophy | 31, 64 |
| MYO7A/Myo7a                 | Usher syndrome type1 (USH1B) | Mutations in the murine orthologous gene lead to cochlear and vestibular dysfunction but not to retinal defects | 78   |
| GUCY2D/Gucy2e               | Leber congenital amaurosis type1 (LCA1) | Mutations in the murine orthologous gene lead to cochlear and vestibular dysfunction but not to retinal defects | 79, 80 |
| RDH12/Rdh12                 | Leber congenital amaurosis (LCA13) | Retinal homogenates from Rdh12-deficient mice exhibit markedly decreased capacity to reduce exogenous retinaldehydees in vitro. Furthermore, the bisretinoid compound directinoid-pyridinium-ethanolamine (A2E) is increased in Rdh12-deficient mice of various genetic backgrounds. However, mutant mice do not show a retinal degeneration phenotype | 81   |
| ZNS                         | TGF1/Tgf1     | In contrast to human patients, homozygous null mice present no congenital malformations and are viable and fertile | 82   |
| OCRL1/Oct11                 | Ocularcerebrorenal syndrome of Lowe | Murine null mutants show no apparent phenotype possibly due to a functional compensation by Inpp5b | 83   |

Acknowledgments—We thank Stephen Tsang for discussions; Brigitte Pfeiffer-Guglielmi for providing the GlyPhos antibody; Johannes Wilbertz from the Karolinska Center for Transgene Technologies (Stockholm) for performing blastocyst injections; and Marc Freichel from the Institute of Pharmacology in Heidelberg for providing Cre deleter mice.
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