Delayed dark adaptation in 11-cis-retinol dehydrogenase deficient mice:  
A role of RDH11 in visual processes in vivo

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ABSTRACT

The oxidation of 11-cis-retinol to 11-cis-retinal in the retinal pigment epithelium (RPE) represents the final step in a metabolic cycle that culminates in visual pigment regeneration. Retinol dehydrogenase 5 (RDH5) is responsible for a majority of the 11-cis-RDH activity in the RPE, but the formation of 11-cis-retinal in rdh5-/- mice suggests another enzyme(s) is present. We have previously shown that RDH11 is also highly expressed in RPE cells and has dual specificity for both cis- and trans-retinoid substrates. To investigate the role of RDH11 in the retinoid cycle, we generated rdh11-/- and rdh5-/-rdh11-/- mice and examined their electrophysiological responses to various intensities of illumination and during dark adaptation. Retinoid profiles of dark-adapted rdh11-/- mice did not show significant differences compared with WT mice, whereas an accumulation of cis-esters was detected in rdh5-/- and rdh5-/-rdh11-/- mice. Following light stimulation, 73% more cis-retinyl esters were stored in rdh5-/-rdh11-/- mice compared with rdh5-/- mice. Single-flash ERGs of rdh11-/- showed normal responses under dark- and light-adapted conditions, but exhibited delayed dark adaptation following high bleaching levels. Double knockout mice also had normal ERG responses in dark- and light-adapted conditions, but had a further delay in dark adaptation relative to either rdh11-/- or rdh5-/- mice. Taken together, these results suggest that RDH11 has a measurable role in regenerating the visual pigment by complementing RDH5 as an 11-cis-RDH in RPE cells, and indicate that an additional unidentified enzyme(s) oxidizes 11-cis-retinol or that an alternative pathway contributes to the retinoid cycle.

Abbreviations: EM, electron microscopy; ERG, electroretinogram; ES, embryonic stem; (P)SDR, (prostate) short-chain dehydrogenase reductase; RDH, retinol dehydrogenase; ROS, rod outer segment(s); RPE, retinal pigment epithelium; WT, wildtype.
INTRODUCTION

In retinal photoreceptors, a photon of light isomerizes 11-cis-retinal to all-trans-retinal, a reaction that initiates a signal transduction cascade culminating in a visual sensation (1-3). Both rod and cone opsin visual pigments contain the light-absorbing chromophore 11-cis-retinal (4). Vision is sustained through biochemical reactions involving the regeneration of isomerized chromophores in rod and cone photoreceptors in a multi-step retinoid cycling pathway (reviewed in (5,6)). The mechanism to regain light sensitivity is not completely understood, but it has been proposed to involve two distinct pathways, one for cone and the other for rod photoreceptors (7). Both pathways ultimately regenerate 11-cis-retinal conjugated to opsins by a retinylidene bond. However, cone visual pigments are regenerated much faster than rhodopsin in rods (reviewed by McBee et al. (5)). To complete the visual cycle, photoisomerized all-trans-retinal is first reduced to all-trans-retinol within the photoreceptors. This reaction is followed by the movement of all-trans-retinol to the retinal pigment epithelium (RPE) for storage in retinosomes as all-trans-retinyl esters (8,9), which are available for isomerization to 11-cis-retinol. The final reaction in the pathway involves oxidation of 11-cis-retinol to 11-cis-retinal and movement of the chromophore back to the photoreceptors (Fig. 1).

The importance of the retinoid cycle for the development and maintenance of normal vision has prompted efforts to identify the critical enzymes and cofactors responsible for regulating each reaction of the cycle. Hereditary mutations in key regulatory enzymes involved in this pathway underlie a range of disorders from mild visual acuity problems to severe retinal dystrophies (see Retnet at www.sph.uth.tmc.edu). Although the enzymatic components of many retinoid cycle reactions are well characterized, the key mediator(s) for 11-cis-retinol oxidation to
11-cis-retinal remains to be identified. Leading candidates include one or more of the retinol dehydrogenase (RDH) enzymes, a subfamily of the short-chain dehydrogenase/reductase (SDR) superfamily. RDH5 is expressed in RPE cells and has been shown to oxidize 11-cis-retinol in vitro. Mutations in the RDH5 gene have been linked to the clinical diagnosis of fundus albipunctatus and are associated with delayed dark adaptation (10). However, rdh5−/− mice display no retinal degeneration and have normal dark adaptation kinetics at bleaching levels that cause a delay in patients with fundus albipunctatus (11,12). This finding indicates that there are other RDH enzymes which facilitate retinol oxidation in the RPE.

We have recently identified additional members of the RDH family that are expressed in the eye and exhibit the ability to catalyze reduction/oxidation reactions involving retinoids (13). One of these enzymes, RDH11, was initially designated as prostate short-chain dehydrogenase reductase 1 (PSDR1) based on its hallmark SDR protein motifs and its high transcript expression level in the human prostate (14). Subsequently RDH11 was identified as a gene regulated by sterol regulatory element-binding protein (SREBP), a transcription factor that functions to coordinately regulate the expression of enzymes involved in cholesterol and fatty acid synthesis (15). SDR enzymes also utilize steroids including RDH5. RDH11, however, lacks reactivity with steroid substrates but reduces other short-chain aldehydes such as nonanal and 4-hydroxy-2-nonenal (15). RDH5 has NADH cofactor specificity and is more efficient in oxidizing retinols rather than reducing retinals in vitro (16). In contrast, RDH11 has NADPH specificity and catalyzes the reduction of retinals ~50-fold more efficiently than it does the oxidation of retinol in vitro (17). However, the in vivo substrate of RDHs might depend upon the relative concentration of substrates in the immediate environment, and several lines of evidence indicate that RDH11 can have 11-cis-RDH
activity in the RPE. First, RDH11 is expressed in RPE cells. Second, the remaining enzymatic activity in \textit{rdh5}^{-/-} RPE exhibited NADPH cofactor specificity and reduced all-\textit{trans}-, 9-\textit{cis}-, and 11-\textit{cis}-retinal (11,18). Third, the residual 11-\textit{cis}-RDH activity in the \textit{rdh5}^{-/-} RPE was membrane-associated (18), a characteristic consistent with the known subcellular localization of RDH11 (19).

In this study, we examine the physiological role of RDH11 and in particular its role in the visual retinoid cycle by generating and characterizing mice with a targeted deletion of the \textit{rdh11} gene (\textit{rdh11}^{-/-}) and combined deletions of RDH5 and RDH11 (\textit{rdh5}^{-/-}\textit{rdh11}^{-/-}). Electrophysiological and biochemical measurements demonstrate that RDH11 plays a minor but complementary role to RDH5 in the flow of retinoids and thus in dark adaptation. Analyses of the \textit{rdh5}^{-/-}\textit{rdh11}^{-/-} mice reveal the existence of additional enzymes in the retina with 11-\textit{cis}-RDH(s) activity, or the presence of an alternative pathway capable of generating the visual pigment.
MATERIALS AND METHODS

Animals—All animal experiments employed procedures approved by the Fred Hutchinson Cancer Research Center and the University of Washington Animal Care Committees, and conformed to recommendations of the American Veterinary Medical Association Panel on Euthanasia and recommendations of the Association of Research for Vision and Ophthalmology. Animals were maintained in complete darkness, and all manipulations were done under dim red light employing a Kodak No. 1 Safelight filter (transmittance >560 nm).

Construction of Targeting Vector—A probe containing nucleotides 913 to 1213 of the mouse rdh11 cDNA was used to isolate the rdh11 genomic clone containing exons 2 to 7 from a 129S4 mouse library (P. Soriano, Fred Hutchinson Cancer Research Center, Seattle, WA). The genomic region between exon 1 and 2 was PCR amplified using primers O1, 5'-GTTTTCCCAGTCACGACGAACCGGGGTGTGTCTAGGAT-3' and O2, 5'-AGGAAACAGCTATGACCATCCGGGAAGCTGAACATTAGA-3' (Fig. 2) and the KOD XL polymerase (Novagen, Madison, WI). The targeting vector was generated by PCR using KOD HiFi polymerase (Novagen). The 5' arm was generated by amplifying a 1.8 kb fragment using primers O3, 5'-AGAAGTCCCGCGCACGTGGGTCTCAAAGGATGTTTC-3' and O4, 5'-AAGTGCCGCGCATGCTTTGCTCTAAAGCTGTTG-3', and an equivalent length 3' arm was amplified using primers O5, 5'-AAGTGTCGACTACAATAGGGCTTTGG-3' and O6, 5'-AGTAAGCTTTGTAGGGCGACGTGAAGCA-3'. The 5’ arm was sub-cloned into the Not I and Sac II restriction sites of pPGKneo0x2 DTA.2 (P. Soriano) whereas the 3’ arm was sub-cloned into the Hind III and Sal I sites. Neomycin (neo) was used for positive selection and
diphtheria toxin A-fragment (DTA) was used for negative selection.

Homologous Recombination and Genotyping — Targeting vector was linearized with Sac II and electroporated into R1_129 embryonic stem (ES) cells (20), and colonies were selected in 300 µg/ml G418 for 9 days. Homologous recombination events were screened by Southern blotting and PCR using primers O7, 5'-AGGAGGGTTGCACCTTTTGCTCTCT-3' and O8, 5'-GCCCAAGTCATAGCCGAATAGCCTCTCT-3' (Fig. 2A) that hybridized to the genomic sequence outside of the targeting arm construct and within the neo gene, respectively. Positive clones were microinjected into C57BL6 blastocysts and the resulting male chimeric mice were screened for germline transmission. Genotyping by PCR analysis was carried out using primers O9, 5'-AC-TATGGCCGTGCATGTGAAGT -3' and O10, 5'-TCTCCTTCCCAATGCCTGTG-3' for wildtype (WT) (389 bp) and O9 and O11, 5'-GCTAAAGCGCATGCTCCAGA-3' for targeted deletion (300 bp). The PCR product with primers O9 and O11 was cloned and sequenced to verify proper targeting of rdh11. A second PCR analysis with primers O9 and O12, 5'-GGACCCTACCCCTTCTGCAACTG-3' were used to determine WT (1.6 kb), heterozygous (1.6 and 2.4 kb) and homozygous (2.4 kb) genotypes.

RDH5 mice were previously generated and characterized by Drs. Carola Driessen and Jacques J. M. Janssen (18,21). Genotypes of RDH5 mice were determined by PCR using primers, KORDH-s1, 5'-GGGCGAGCTGCAGTCTGCACCATC-3', and KORDH-a1 5'-GGCAAGACCTGACCTTGACAG-3' which produced a 2.9-kb fragment for the WT allele and a 1.5-kb fragment for the disrupted allele.
**Southern Blotting** — Genomic tail or ES cell DNA was digested overnight with Eco RV and electrophoresed on a 0.7% agarose gel. DNA samples were transferred onto a nylon membrane by capillary method. The membrane was incubated with a 700 bp DNA probe amplified with primers P13, 5’-ATAAGTCCCGCTGTCCTCTT-3’ and P14, 5’-TCTCCTGGCTCCTAGTAATCTAA–3’ and was subsequently labeled with [α-32P]dCTP using the random primer labeling kit (Stratagene, La Jolla, CA).

**Real time quantitative PCR**—RNA was Trizol extracted from liver and brain according to manufacturer’s protocol. Five µg of RNA was used to generate the cDNA using 0.14 mM oligo-dT (22 mer) primer and 0.2 mM of each dNTP and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real time PCR was carried out in triplicate for each sample using SYBR green PCR master mix (Applied Biosystems, San Francisco, CA), 0.2 µM of each primer, and 8 ng of cDNA. The PCR protocol used is 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. PCR was performed and analyzed using an ABI Prism 7700 sequence detector. Mouse RDH11-14 gene expression was normalized to S16 expression. PCR without cDNA templates did not produce significant amplification products. Specificity of the primers was verified by the amplification of a single PCR product, which was determined by observing a single dissociation curve from each tissue. Primers for the real-time PCR are the following:

mRDH11_F 5'ACCAAGAGCACATGGGTAGC-3', mRDH11_R 5'CTCATCAGTCTCGGTGCTT-3';

mRDH12_F 5'CCAGGAACTCCTACCTAGCTG-3', mRDH12_R 5'ACCCACATCCTTTGCTGTC-3';
mRDH13_F 5'GAGGAGCGAGTAGACATTCTGG-3', mRDH13_R
5'CCAGGGACGAGAGATTGATG-3';
mRDH14_F 5'TGGTCAGGAATGGCATGTG-3', mRDH14_R
5'GCATGATTGCGGCTAGACTG-3';

mS16_F 5'AGGAGCGATTTGCTGGTGGA, mS16_R
5'GCTACCAGGCTTTGAGATGGA-3'.

**Immunoblotting**—Mouse testes were solubilized in 6 volumes (wt/ vol) of T-PER tissue protein extraction buffer (Pierce Biotechnology, Inc. Rockford, IL). Thirty µg of protein extract was separated on a 10% SDS polyacrylamide gel and electrophoretically transferred onto 0.2 µm PVDF membranes (Invitrogen). For immunoblotting, membranes were blocked in PBS containing 0.1% Tween (PBST) and 5% milk (PBSTM) and labeled with an anti-RDH11 polyclonal antibody (2479) diluted to 1/5000 in PBSTM. After washing membranes with PBST, blots were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce) at 1/10000 dilution and antibody labeling was detected by chemiluminescence (Pierce). Anti-RDH11 polyclonal antibody was generated against the C-terminal peptide LWDVSCDLLGLPVDW conjugated to keyhole limpet hemocyanin by Genemed Synthesis Inc. (San Francisco, CA). Protein concentration was determined by the Bradford assay (BioRad, Hercules, CA) using bovine serum albumin (Pierce) as the standard.

**Analyses of Retinoids**—All experimental procedures related to extraction, derivatization, and separation of retinoids from dissected mouse eyes were carried out as described previously.
(22-24). All reactions involving retinoids were carried out under dim red light. Retinoids were separated by normal phase HPLC (Beckman, Ultrasphere-Si, 4.6 μ 250 mm) with 10% ethyl acetate and 90% hexane at a flow rate of 1.4 ml/min with detection at 325 nm, using an HP1100 HPLC with a diode array detector and HP Chemstation A.03.03 software.

Electroretinograms (ERGs)—Prior to recording, mice were dark-adapted overnight. Under safety light, mice were anesthetized by intraperitoneal injection using 20 μl/g body weight of 6 mg/ml ketamine and 0.44 mg/ml xylazine diluted with 10 mM sodium phosphate (pH 7.2) containing 100 mM NaCl. The pupils were dilated with 1% tropicamide. A contact lens electrode was placed on the eye, and a reference electrode and ground electrode were placed in the ear and on the tail. ERGs were recorded with the universal testing and electrophysiologic system UTAS E-3000 (LKC Technologies, Inc. Gaithersburg, MD). The light intensity was calibrated by the manufacturer and computer-controlled. The mice were placed in a Ganzfeld chamber, and scotopic and photopic responses to flash stimuli were each obtained from both eyes simultaneously.

Single-flash recording—Flash stimuli had a range of intensities (-3.7–2.8 log cd·s·m⁻²), and white light flash duration was adjusted according to intensity (from 20 μs to 1 ms). Three to five recordings were made with >10 s intervals, and for higher intensity intervals, intervals were 10 min or as indicated. There were no significant differences between the first and the fifth flash. Light-adapted responses were examined after bleaching at 1.4 log cd·m⁻² for 15 min. Typically, four to eight animals were used for the recording of each point in all conditions.
Double-flash recording—The protocol was followed as previously published with some modifications (25). A test flash was delivered to suppress the circulating current of the rod photoreceptors. The recovery of this current was monitored by delivering a second flash, termed the probe flash. The interval time between two flashes varied from 200 to 2000 ms. The intensity of the test flash and probe flash was 0.4 and 1.6 log cd·s·m⁻², respectively. Each trial was performed separately with 120 s interval time for dark adaptation. The amplitude of the probe flash alone was confirmed throughout the experiment to ensure that this time was sufficient. These probe flashes were also used to normalize the response of probe flashes following a test flash. The normalized amplitude of the probe flash a-wave versus the time between two flashes was plotted and fit by the linear regression algorithm in the SigmaPlot 2002 ver. 8.02 program.

Dark adaptation after intense constant illumination—Mice were dark-adapted overnight and then bleached with the background light of a Ganzfeld chamber (500 cd·m⁻²) for 3 min. After the light was turned off, a single-flash ERG at -0.2 cd·s·m⁻² was used to monitor recovery of a-wave amplitude every 5 min for 60 min. The recovery ratio was calculated by normalizing single flash a-wave amplitude responses at various times following bleaching to the WT dark-adapted a-wave response at the identical flash intensity of -0.2 cd·s·m⁻². The recovery ratio versus time after bleaching was plotted and fit by the linear regression algorithm in the Sigma Plot 2002 ver. 8.02 program. Leading edges of the ERG responses were fitted with a model of rod photoreceptor activation as previously described (23). Statistical analysis was carried out using the one-way ANOVA test.
Transmission Electron Microscopy (EM) — For transmission EM, mouse eyecups were fixed primarily in 2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.08 M PIPES, pH 7.4, containing 2% sucrose, initially at room temperature for ~1 h then at 4 °C for 24 h. The eyecups were then washed with 0.13 M sodium phosphate, pH 7.3, and post-fixed with 1% osmium tetroxide in 0.1 M sodium phosphate, pH 7.3, for 1 h at room temperature. The eyecups were dehydrated through a methanol series and transitioned to the epoxy embedding medium with propylene oxide. The eyecups were embedded for sectioning in Eponate 812. Ultrathin sections (60–70 nm) were stained with aqueous saturated uranium acetate and Reynold’s formula lead citrate prior to viewing with a Philips CM10 EM.
RESULTS

*Generation of rdh11 knockout mice*—To determine the physiological role of RDH11, we generated *rdh11*\(^{-/-}\) null mice by deleting exons 2 and 3 through homologous recombination (Fig. 2A). Exon 2 contains the NADP(H)-binding site, which is essential for enzyme activity. If the short transcript for exon 1 is stably expressed, it will produce a 22 amino acid-long peptide. Alternative splicing of exon 1 to exons 4, 5, 6, or 7 creates a frameshift resulting in a premature stop codon. Southern blotting and PCR analysis identified embryonic stem (ES) cell clones with the proper homologous recombination event for germline transmission. Southern blotting probed with the 5’-fragment derived from the *rdh11* gene labeled the anticipated DNA fragment sizes of 5.9 kb for the WT allele and 6.5 kb for the targeted allele (Fig. 2B). PCR screening with a primer outside the targeting vector arm and another within the *neo* gene verified the Southern results (Fig. 2C). Of three male chimeras with 80-90% agouti coat color, one had germline transmission of the mutant *rdh11* allele. All the experiments in this report used mice originating from this ES cell clone. Crosses of heterozygous *rdh11*\(^{+/-}\) mice produced offspring with genotypes in accordance with expected Mendelian ratios (Fig. 2D and 2E). Breeding of homozygous *rdh11*\(^{-/-}\) mice produced average litter sizes (~7-10 pups) and the offspring had normal survival and growth, indicating that *rdh11*\(^{-/-}\) mice are fertile and healthy. Besides the retina, RDH11 is highly expressed in the liver and testes. At 2 months of age, tissues of *rdh11*\(^{-/-}\) mice exhibited no histological differences grossly or upon microscopic analysis of hematoxylin- and eosin-stained tissues in comparison with organs from WT mice (data not shown).

Relative to WT mice, immunoblotting analysis demonstrated the loss or reduction of RDH11 protein in testes from *rdh11*\(^{-/-}\) or *rdh11*\(^{+/-}\) mice, respectively (Fig. 3A). Quantitative PCR
results of rdh11 mRNA expression were concordant with the protein level for RDH11 (Fig. 3B). Rdh11 transcripts were not detected in rdh11−/− liver but were measured in liver tissue from rdh11+/− and rdh11+/+ mice. Expression levels of rdh11 homologues, rdh12, 13, and 14, were analyzed to ensure that rdh11 on chromosome 12 was targeted and not the homologues. Rdh12, which is located ~16 kb from rdh11 on chromosome 12, has the same intron-exon genomic organization and shares 43% nucleotide identity with rdh11. Transcripts of rdh11 homologues exhibited similar expression levels in the livers of different rdh11 genotypes (Fig. 3B), indicating the correct targeted disruption of rdh11 and a lack of transcriptionally-based compensation for the loss of rdh11.

Retinoid Analysis—To investigate the in vivo role of RDH11 in vertebrate retinoid cycling between photoreceptors and RPE cells, retinoid levels in the eyes of WT, rdh11−/−, rdh5−/−, and rdh5−/−rdh11−/− mice were measured 48 h after dark adaptation, or 15 min after a probe flash. Typical HPLC separation profiles of retinoids are illustrated in Fig. 4. In dark-adapted conditions rdh11−/− eyes had similar levels of retinoids relative to those in WT mice. Relative to WT mice, rdh5−/− mice had elevated levels of 11/13-cis-retinyl esters, which further increased 15 min after a single light flash that photoactivated ~35% of rhodopsin (24) (Fig. 4, peaks 1, 2; Table 1), in agreement with previously published data (11,18). The separation of 13-cis- from 11-cis-retinyl esters was particularly challenging, and only partial separation of these esters was accomplished (identified by characteristic UV/visible spectra). Interestingly, after a flash probe, rdh5−/−rdh11−/− mice had even greater amounts of 11/13-cis-retinyl esters relative to those in rdh5−/− mice (Fig. 4, peaks 1, 2). Conversely, after the first critical 15 min of dark adaptation, the 11-cis-retinal chromophore regenerated with similar kinetics in all the mice. The ratios for all-trans-retinal/11-cis-retinal

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(pmol/eye) are WT 31.3 ± 3.3 %, \textit{rdh11}^-^/- 28.3 ± 3.2 %, \textit{rdh5}^-^/- 29.2 ± 3.1 %, and \textit{rdh5}^-^/-\textit{rdh11}^-^/- 32.7 ± 3.8 %.

Retinal Morphology—Since we have previously shown that RDH11 is expressed in the RPE and the Müller cells of the nerve fiber layer (13), the retinal morphology of 2-month-old \textit{rdh11}^-^/- mice was examined by light microscopy and EM. Histologically, all cell layers of the retina were comparable between the WT and \textit{rdh11}^-^/- mice (data not shown). EM micrographs revealed no obvious abnormalities in the outer nuclear layer, photoreceptors, or RPE cells of \textit{rdh11}^-^/-, \textit{rdh5}^-^/- or \textit{rdh5}^-^/-\textit{rdh11}^-^/- mice (Fig. 5). Higher magnification of RPE cells and photoreceptor outer segments demonstrated no discernible differences between mice with single or combined gene deletions.

ERG analysis—ERG response was examined under dark-adapted or light-adapted conditions using a single flash at different levels of intensity. The series of averaged waveforms obtained at different light stimuli for each genotype are shown in Fig. 6A and C, and a- and b-wave amplitude responses are plotted in Fig. 6B and D. Under dark-adapted conditions, \textit{rdh11}^-^/- and \textit{rdh5}^-^/- a- and b- wave amplitudes remained similar to those observed in WT mice (Fig. 6B). \textit{Rdh5}^-^/-\textit{rdh11}^-^/- mice, however, displayed slightly reduced a- and b- wave amplitude responses. Under light-adapted conditions, the genetically altered mice showed no significant changes in either a- or b-wave amplitudes (Fig. 6D). The leading edges of the a-wave of the ERG responses in dark-adapted conditions were fitted with a rod phototransduction model. The maximum amplitude and sensitivity of the photoresponses were reduced from maximal responses in dark-adapted conditions, and both parameters were compared with the results in WT mice as described previously (23). The
maximal amplitude and sensitivity of the \(rdh11^{-/-}, \ rdh5^{-/-},\) and \(rdh5^{-/-}rdh11^{-/-}\) mouse photoresponses remained unchanged relative to WT mice (Table 1).

Recovery function of rod photoreceptors was further studied using paired flash responses. A test flash was used to desensitize rod photoreceptors, and then a second probe flash at various times following the initial flash was used to examine the recovery of dark adaptation by monitoring a-wave amplitude responses (Fig. 7, left panel). Recovery of the a-wave in \(rdh11^{-/-}, \ rdh5^{-/-},\) and \(rdh5^{-/-}rdh11^{-/-}\) mice was normalized to a-wave amplitude responses in WT mice. Dark adaptation rates in \(rdh11^{-/-}\) mice and \(rdh5^{-/-}\) mice showed no significant difference, whereas photoreceptors in \(rdh5^{-/-}rdh11^{-/-}\) mice resensitized significantly more slowly compared with WT (\(P < 0.02; \ rdh5^{-/-}rdh11^{-/-}, 1230.3 \pm 121.3\) ms; WT, 893.3 \pm 47.8 ms) after the test flash (Fig. 7, right panel; Table 2). As mentioned above, other parameters of rod photoreceptor function (Table 2) showed no significant differences between mice with different genotypes. Taken together these results suggest that the photoexcitation function of rod photoreceptors is not affected by the loss of either \(rdh11\) or \(rdh5\) alone but is slightly reduced when both genes are disrupted, and that both genes must be functional in order to maintain normal dark adaptation kinetics following illumination. These differences might be due to a slower replacement of the chromophore once the flash is applied. To further evaluate the mild delay in dark adaptation, we determined if a more dramatic phenotype could be elicited by stressing the mice with an intense bleaching condition (500 cd·m\(^{-2}\) for 3 min) prior to monitoring the recovery of a-wave amplitude. Typical a-wave traces in the recovery phase following the bleach for WT, \(rdh11^{-/-}, \ rdh5^{-/-}\) and \(rdh5^{-/-}rdh11^{-/-}\) mice are shown in Fig. 8A and the recovery ratios are plotted in Fig. 8B. Recovery in a-wave amplitude was slightly attenuated in \(rdh11^{-/-}\) mice (\(p<0.01\)), even slower in \(rdh5^{-/-}\) mice (\(p<0.0001\)), and further delayed in
rdh5⁻/⁻rdh11⁻/⁻ mice (p<0.0001) compared with WT. To ascertain the reason for the mild but significant delay in dark adaptation kinetics, retinoid analysis was carried out immediately after the 3 min bleach and after 30 min in the dark. At time zero, photoexcitation caused the expected increase in all-trans-retinal and a concurrent decrease in 11-cis-retinal levels relative to the dark-adapted condition. No significant differences in the retinal levels were observed between the mice of different genotypes. In agreement with single-flash ERGs, the loss of RDH11 or RDH5 does not cause a defect in the photoexcitation process even under sustained visual excitation conditions. Eyes of rdh5⁻/⁻ and rdh5⁻/⁻rdh11⁻/⁻ mice had elevated levels of 11/13-cis-retinyl esters (data not shown), which is consistent with data following a single flash. However, unlike the single flash experiment, mice of different genetic backgrounds had reduced levels of 11-cis-retinal and increased levels of all-trans-retinal relative to WT mice (Fig. 9). Mice with both rdh11 and rdh5 disrupted exhibited the lowest concentration of 11-cis-retinal and the most severe delay in dark adaptation, whereas rdh11⁻/⁻ mice had higher levels of 11-cis-retinal and a milder delay. Concentrations of 11-cis-retinal appeared to correlate with dark adaptation kinetics, in agreement with the current retinoid cycle model. These results demonstrate that the subtle difference in ERG response produced by a double flash experiment can be accentuated to a more severe phenotype, provided a higher percent of rhodopsin molecules is photoactivated.
DISCUSSION

Retinoids play an essential role in vertebrate development, differentiation, and reproduction. *In vitro* studies have demonstrated the ability of RDH11 to catalyze the reduction/oxidation of *cis*- and *trans*-retinals/retinols (13,17), thereby suggesting that this enzyme can play a role in retinoid homeostasis. *Rdh11* is expressed during murine embryonic development, and levels gradually increase from embryonic day 7 to day 17 (26). Although RDH11 utilizes retinoid substrates and is expressed during embryogenesis, the studies reported here analyzing *rdh11*−/− mice did not identify any consistent abnormalities in development, post-natal survival, or fertility. The lack of a developmental phenotype in *rdh11*−/− mice can be due to the overlapping expression of RDH11 homologues (13) or other enzymes compensating for the loss of *rdh11*. In addition, RDH 11 was hypothesized to play a protective role in cells by converting highly reactive and toxic short chain aldehyde byproducts of unsaturated fatty acid oxidation to non-reactive alcohols. Since SREBP regulates fatty acid and cholesterol synthesis as well as *rdh11* expression, we examined the liver for a possible phenotype. Up to 6 months of age, no histological difference in the liver of *rdh11*−/− compared to that of the *rdh11*+/+ mice was observed, suggesting that either a liver phenotype can have a late onset, RDH11 might not play a role in preventing oxidative damage, or the mice need to be put on a special diet in order to manifest a liver phenotype. Another dehydrogenase, RDH5, is also expressed during embryogenesis (27) and catalyzes the oxidation of *cis*-retinol. Interestingly, mice with both *rdh11* and *rdh5* genes disrupted also appear healthy with no defects in development and fertility, a finding that further supports the functional redundancy of the retinoid metabolic pathway.

Maintaining the retinoid cycle in the retina is also essential for vision. In dark-adapted
conditions the retinoid levels in eyes of rdh11^−/− mice were similar to measurements in eyes from WT mice, whereas rdh5^−/−rdh11^−/− mice had elevated levels of cis-retinol and cis-retinyl esters that were greater than in rdh5^−/− eyes (Table 1). The reason for elevated cis-retinol levels in dark-adapted conditions is unclear, because the rate of 11-cis-retinal formation remained unchanged. A possible explanation is that the slower rate of 11-cis-retinol oxidation did not disrupt the 11-cis-retinal supply because the oxidation reaction rate was still comparable to the rate-limiting step in the retinoid cycling process, which is the reduction of all-trans-retinal [28,29].

One hypothesis could be that RDH5 might act as a modulator of RPE65-mediated isomerase activity such that the complex of RDH5 and RPE65 with a yet unidentified enzyme has lower isomerase activity compared with RPE65 free of RDH5 [16]. Thus, the conversion of all-trans-retinyl ester to cis-retinol by the RPE65-mediated process (Fig. 1, d) would accelerate in the absence of RDH5, whereas the subsequent oxidation of cis-retinol (Fig. 1, e) by the remaining enzymes would not be affected by the loss of RDH5. This would result in increased levels of 11-cis-retinol, and thus cis-retinyl esters due to lecithin:retinol acyl transferase (LRAT; Fig. 1 [30]).

After a continuous 3 min bleach, cis-retinal levels decreased in rdh5^−/− and rdh5^−/−rdh11^−/− mice, which can explain the delayed dark adaptation kinetics. After prolonged illumination, enzymes responsible for steps b, c, and d in Fig. 1 are capable of handling the increase in substrate concentrations because of photoisomerization of 11-cis-retinal to all-trans-retinal, but a bottleneck effect occurs at step e because of the slower oxidation rate of the compensating enzymes. Thus, 11-cis-retinal demand following mild perturbations of ~30% bleach can be met by the remaining dehydrogenase activity, but these enzymes are insufficient to rapidly replenish large depletions of
11-cis-retinal chromophore following higher bleaches. Changes in retinoid levels in rdh5+/rdh11−/− mice compared with rdh5−/− mice also provide evidence that RDH11 plays a role in the normal flow of retinoids.

Both rdh11−/− and rdh5−/−rdh11−/− mice have normal photopic and scotopic ERG kinetics (Fig. 6, Table 2), indicating that these mice are capable of regenerating the visual chromophore and have normal rod and cone photoreceptor phototransduction signaling. However, after prolonged intense illumination conditions delayed dark adaptation kinetics were observed in the single and double knockout mice. The severity of the attenuated dark adaptation (Fig. 8) appeared to correlate with decreasing 11-cis-retinal concentrations observed in rdh11−/−, rdh5−/− and rdh5−/−rdh11−/− mice relative to WT (Fig. 9). Photoreceptors in both rdh11−/− and rdh5−/− mice displayed biphasic recovery kinetics: a rapid initial phase followed by a slower period (Fig. 8). This biphasic recovery has been observed in patients exhibiting clinical fundus albipunctatus due to an Arg257Trp mutation in RDH5 (31). After a 0.5% bleach, patients have normal rod recovery, but following intermediate bleaches in the 2-12% range, a rapid partial recovery is followed by a transitory plateau. Both 11-cis-RDH-deficient mice and humans with RDH5 mutations are able to maintain normal visual recovery kinetics using the alternative pathway(s) to generate 11-cis-retinal, but mice are able to recover faster from stronger bleaching conditions than are humans. Part of the reason for these variations in the rate of dark adaptation can result from anatomical differences between the mouse and human retina. Mice are nocturnal animals with a rod-dominant photoreceptor layer essential for scotopic vision, whereas humans have evolved for both day and night vision, resulting in a cone-dominant macula and a rod-dominant periphery. Hence, clinical retinopathies affecting dark adaptation often present as a less severe phenotype in mice, simply due to the nocturnal
evolution of the rodent visual system compared with the diurnal primate one.

A mild visual phenotype in \textit{rdh5/rdh11/-} mice suggests that alternative enzymes can oxidize 11-cis-retinol to 11-cis-retinal in RPE cells. Likely candidates might be Dhrs9 (retsdr8), an oxidoreductase, which has dual substrate specificity for both 11-cis and all-trans-retinol with NADPH specificity (21) or RDH10, a SDR that catalyzes retinol oxidation with NADP specificity (32). Rather than a microsomal enzyme, perhaps the compensating enzyme can be the plasma membrane-associated 11-cis-RDH found in the RPE (33). Another possibility is that the remaining 11-cis- RDH activity observed in \textit{rdh5/rdh11/-} RPE cells results from a NAD$^+$-dependent enzyme (18). Candidate NADH-dependent enzymes might be amongst the eight SDR genes present as a cluster on mouse chromosome 10D3. \textit{Rdh5} is also located on chromosome 10D3 but it is separated from the other SDRs by ~1 Mb. RDH1, RDH5, RDH6 (CRAD1), RDH7 (CRAD2), and RDH9 (CRAD3) (34-37) catalyze retinol oxidation with NAD$^+$ specificity, suggesting that these SDRs most likely originated through local intra-chromosomal duplication events. If these enzymes are expressed in the RPE as demonstrated for RDH6 and RDH7, and if indeed RDH1, RDH7, and RDH9 lack a human orthologue, one or more of these enzymes can catalyze 11-cis-retinol oxidation and contribute to the more efficient dark adaptation mechanism in mice.

Biochemical and genetic studies indicate that alcohol dehydrogenase (ADH) metabolizes retinol to retinal (38). Database searches have identified \textit{adh1} expressed sequence tags (EST) in mouse RPE and choroid libraries and \textit{adh4} ESTs in mouse and human RPE and choroid libraries. Recently, ADH4 has been immunolocalized to the RPE and shown to have significant enzymatic activity toward \textit{cis}-retinoids (39). Although soluble ADHs catalyze retinal/retinol redox reactions, the soluble protein fraction of bovine RPE cells contained low amounts of retinol oxidative activity,
which is insufficient to explain the remaining alternative enzyme(s) activity in rdh5/- RPE. This indicates that soluble enzymes do not play a major role in the visual retinoid cycle (18). Thus, ADH1 and ADH4, although expressed in the RPE, most likely contribute a minor amount to redox activity toward cis-retinoids in RPE cells.

Factors that determine whether an enzyme catalyzes the reduction or oxidation of a substrate include the equilibrium constant, the reduced/oxidized cofactor, and substrate/product ratios. The pH independent equilibrium constant is 3.3 x10^9 for the oxidation of retinol (40). It was reported that RDH11 displayed ~50-fold more efficient activity for the NADPH-dependent reduction of all-trans-retinal than for the oxidation of all-trans-retinol (17). However, in the retina, the ratio of NADP to NAPDH is between 4 to 1 or 1.5 to 1 (41), suggesting that NADPH-dependent enzymes such as RDH11 and RDH5 have the ability to catalyze both oxidative and reductive reactions depending on the substrate concentrations. Rdh-/-, rdh11-/- and Rdh-/-rdh11-/- mice have elevated levels of cis-retinol, which is stored in the form of cis-retinyl esters, suggesting that RDH11 and RDH5 catalyze the oxidation of retinol to retinal. It should be considered that the oxidation reaction is preferred by the means of sequestering the 11-cis-retinal product by CRALBP. In other tissues where RDH11 is expressed at different substrate/product concentrations, it is conceivable that RDH11 might catalyze the reductive reaction.

EM results revealed no retinal degeneration in rdh11-/-, rdh5 -/- or rdh5-/-rdh11-/- retina, which is concordant with normal ERG kinetics. Contact between photoreceptor outer segments and RPE cells remained intact in all mice with different genotypes, suggesting that normal phagocytosis was occurring. Not surprisingly, disrupting both rdh11 and rdh5 genes did not elicit the white punctata in the fundus that is observed in patients with RDH5 mutations (data not shown).
A number of mice models are providing evidence that disruption of genes known to play a role in the retinoid cycle exhibit a more severe degenerative retinal pathology in humans compared with mice, such as aber (42,43), cralbp (44,45), and rgr (24,46) mutations. These mouse models exhibit delayed dark adaptation kinetics at higher bleaching intensities but normal ERG kinetics under dark-adapted conditions. In contrast, mice models of other genes involved in the retinoid cycle such as lrat (30) and rpe65 (47) more closely mimic human retinal dystrophies. Mutations in LRAT cause early childhood onset retinal dystrophy (CSRД), and RPE65 has been identified as one of the disease-causing genes for Leber’s Congenital Amaurosis (LCA), also an early onset form of retinal dystrophy. Thus, genes associated with later onset of retinal degeneration in humans produce a mild phenotype in mice, whereas genes causing early childhood onset retinal dystrophies produce a severe phenotype.

A total of ten LCA genes have been mapped, 8 of which have been identified: retGC1, RPE65, CRX, AIPL1, RPGRIP1, CRB1, TULP1, and most recently RDH12 (reviewed in (48,49)). Mutations in RDH12 were linked with progressive rod-cone dystrophy in a subset of LCA patients (50,51). In humans, RDH12 is located on chromosome 14, ~27 kb apart from its homologue RDH11. Both RDH11 and RDH12 have the same substrate specificity and similar enzymatic properties, but RDH11 is localized to the RPE and Müller cells, whereas RDH12 is localized to the photoreceptor cell layer. Both groups screened their cohort of patients for polymorphisms in the RDH11 gene, but neither group found an association between the disease and RDH11. A lack of a severe phenotype in rdh11−/− mice provides additional evidence that loss of RDH11 function might not manifest in LCA. If this is indeed true, a defect in the rate-limiting step of all-trans-retinal reduction in photoreceptors appears to be the critical step in the retinoid cycle. RDH12 must be the
dominant enzyme, given the redundancy of all-trans-retinal reducing enzymes such as retSDR1 (52) and prRDH (53) in photoreceptors. It will be interesting to examine whether rdh12−/− mice display a similar retinal electrophysiological and pathological phenotype as seen in LCA patients.

The fact that some fundus albipunctatus patients with RDH5 gene mutations develop cone dystrophy after the age of 40 (54) suggests that the RDH5 gene is important for maintenance of normal cone function, contrary to the proposed visual cycle for cone opsin regeneration (7). Seven genes have been identified to cause cone dystrophy, CRX (55), retGC1 (56), GUCA1A (57), GCAP1(58), ABCR (59), RDH12 (51), and RDH5 (54), but only RDH5 is expressed in the RPE. In this study no cone dysfunctions were detected in rdh11−/−, rdh5−/− and rdh5−/−rdh11−/− mice less than 6 months old.

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FIGURE LEGENDS

Figure 1: Chemistry of the retinoid cycle reactions in the vertebrate retina. The retinoid cycle reactions were reviewed recently (5). In the rod outer segment (ROS), light causes the isomerization (reaction a) of the rhodopsin chromophore, 11-cis-retinylidene (1), to all-trans-retinylidene. All-trans-retinal (2) is hydrolyzed and then reduced (reaction b) in the reaction catalyzed by all-trans-retinal-specific RDH(s). All-trans-retinol (3) diffuses to RPE where it is esterified by LRAT (reaction c) to all-trans-retinyl esters (4). All-trans-retinyl esters can be hydrolyzed by a yet unidentified retinyl hydrolase (reaction d) generating all-trans-retinol. Next, the pathway branches and all-trans-retinol, or its derivative, is isomerized to 11-cis-retinol (5) in a reaction that involves an abundant RPE protein, termed RPE65 (poorly defined reactions d). 11-cis-retinol is then oxidized by 11-cis-RDH (RDH5, RDH11) and other dehydrogenases (13) to 11-cis-retinal (6) (reaction e) to complete the cycle. In the dark, under conditions of low 11-cis-retinal utilization, 11-cis-retinol can also be esterified by LRAT to form 11-cis-retinyl esters (7) (reaction g). With chromophore depletion, hydrolysis of 11-cis-retinyl esters by retinyl ester hydrolase (REH) occurs (reaction h) (60). 11-cis-retinal diffuses across the extracellular space (reaction f), is taken up by the outer segment, and recombines with apo-rhodopsin to regenerate rhodopsin.

Figure 2: Generation of the rdh11 knockout mouse. A) Schematic diagram of rdh11 genomic organization of exons (black rectangles) 1 to 5, the targeting vector with neomycin (neo) and diphtheria toxin A-fragment (DTA) genes, and the targeted allele resulting from a homologous recombination event that replaces exons 2 and 3 with neomycin. Eco RV (E) restriction enzyme digested genomic fragments are indicated for the WT (5.9 kb) and targeted (6.5 kb) alleles, and the
5’ Southern probe flanking the targeting vector arm is shown as a gray box. Arrows O1 to O12 indicate PCR primers. B) Southern blot analysis with a 5’ probe (gray) or C) PCR analysis of targeted embryonic stem cell (ES) DNA and WT (+/+) or heterozygous (+/-) mouse tail DNA. Primers O7/ O8 amplify a 2.8 kb fragment from the targeted allele but not from the WT mice. D) and E) PCR genotyping of WT (+/+), heterozygous (+/-), and homozygous null (-/-) tail DNA with the indicated primer sets. Primers O9/ O10 produce a 389 bp fragment from the WT allele and O9/ O11 produce a 300 bp fragment from the targeted allele. Primers O9/ O12 generate fragments of 1.6 kb for (+/+), 1.6 kb and 2.4 kb for (+/-), and a 2.4 kb fragment for (-/-).

**Figure 3: Expression of RDH11 and homologues.** A) Immunoblotting analysis of testes protein extract from WT (+/+), heterozygous (+/-), and homozygous (-/-) mice using an anti-RDH11 polyclonal antibody. B) Real time quantitative PCR analysis of rdh11, 12, 13, and 14 transcript expression, using total RNA from liver of WT (+/+), heterozygous (+/-), and homozygous (-/-) mice. Each gene was analyzed separately and the transcript expression level was relative to that in the WT, which was set at 100%. *-crossreactive protein with the antibody in the mouse extracts.

**Figure 4: Chromatographic separation of non-polar retinoids from mice of different genetic backgrounds used in this study.** Retinoids were extracted from the eye and separated on normal-phase HPLC. The peaks correspond to the following retinoids: 1, 13-cis-retinyl esters; 2, 11-cis-retinyl esters; 3, all-trans-retinyl esters; 4, 4’, syn- and anti-11-cis-retinal oximes; 5, 5’, syn- and anti-all-trans-retinal oximes; 6, 11-cis-retinol; 7, all-trans-retinol. *Artifact related to a change in the solvent composition.
Figure 5: Montage of cross-section of the retinas of 2-month-old mice analyzed by transmission EM. Upper panels show the cross-section of the RPE and the photoreceptor cells. Lower panels show a higher magnification of the RPE and ROS. The preparation of sections is described in Materials and Methods.

Figure 6: Single flash ERG responses of increasing intensity for WT, rdh11+/-, rdh5+/- and rdh5+/rdh11+/- mice. Serial responses to increasing flash stimuli were obtained for WT, rdh11+/-, rdh5+/- and rdh5+/rdh11+/- mice for selected intensities under dark-adapted conditions (A) and light-adapted conditions (C), and plotted as a function a-wave and b-wave versus light intensities under dark-adapted conditions (B) and light-adapted conditions (D). Error bars indicate the standard error of the mean.

Figure 7: Measurements of a-wave recovery with double-flash ERG. ERG trace from WT mouse demonstrating the double-flash technique (left upper panel); recovery of a-wave after a test flash from WT mouse (left lower panel). The dark-adapted mice were conditioned first with the test flash (0.4 log cd·s·m⁻²) followed by a probe flash (1.6 log cd·s·m⁻²) with the delay time varied from 200 to 2000 ms. Each trace represents the average of recordings from n=8 eyes. Normalized a-wave recovery of the probe flash at different times after the test flash is shown. Error bars indicate the standard error of the mean.

Figure 8: Measurements of a-wave recovery after constant light stimulation. The represent-
tive wave forms during recovery phase from each genetic background (A). The dark-adapted mice were bleached with intense constant illumination (500 cd·m⁻²) for 3 min and the recovery of a-wave amplitudes was monitored with single-flash ERG (-0.2 cd·s·m⁻²) for 60 min; the recovery ratio is plotted (B). The ratio was significantly attenuated in rdh11⁻/⁻ (p<0.01), rdh5⁻/⁻ (p<0.0001), and rdh5⁻/⁻ rdh11⁻/⁻ (p<0.0001) compared with WT. Error bars indicate the standard error of the mean.

**Figure 9: Kinetics of retinoids after intense light bleaching.** Prior to the experiment, mice were dark-adapted for 48 h and bleached with background light at 500 cd·m⁻² for 3 min. HPLC retinoid analysis was performed right after the intense light stimulation. Error bars indicate the standard error of the mean (n = 3).
Table 1. Retinoid contents in dark-adapted mice from different genetic backgrounds.

| Retinoid                   | WT pmol/eye | rdh11−/− pmol/eye | rdh5−/− pmol/eye | rdh5−/−rdh11−/− pmol/eye |
|----------------------------|-------------|------------------|------------------|--------------------------|
| 11/13-cis-retinyl esters   | 18.8±1.3    | 17.7±0.5         | 189.6±48.2       | 186.4±34.5               |
| all-trans-retinyl esters   | 80.2±5.8    | 68.1±14.2        | 47.8±10.3        | 71.6±5.2                 |
| 11-cis-retinal             | 527.1±20.3  | 510.4±14.8       | 510.4±35.1       | 514.1±36.9               |
| all-trans-retinal          | 34.3±1.5    | 26.9±0.5         | 32.1±2.6         | 46.1±2.1                 |
| all-trans-retinol          | 13.6±4.7    | 5.8±2.1          | 4.8±1.5          | 3.7±2.2                  |
| 11-cis-retinol             | 8.4±5.8     | 7.5±2.3          | 9.2±1.4          | 9.8±4.2                  |

aMice were dark-adapted for more than 48 h. The results are presented with standard error, and n was between 3 and 5.

bFor calculations assumed as 1:1 mixture of 11-cis-retinyl and 13-cis-retinyl esters.
Table 2. Quantitative parameters of ERG a-wave\textsuperscript{a}. Maximum a-wave amplitude, sensitivity parameters, and time for 50% recovery of a-wave in \textit{rdh5}\textsuperscript{-/-} mice, \textit{rdh11}\textsuperscript{-/-} and \textit{rdh5}\textsuperscript{-/-}\textit{rdh11}\textsuperscript{-/-} mice compared with WT mice.

| Parameters                              | WT            | \textit{rdh11}\textsuperscript{-/-} | \textit{rdh5}\textsuperscript{-/-} | \textit{rdh11}\textsuperscript{-/-}\textit{rdh5}\textsuperscript{-/-} |
|-----------------------------------------|---------------|-------------------------------------|-------------------------------------|---------------------------------------------------------------------|
| maximum a-wave amplitude (µV)           | 964.6 ± 48.4  | 902.9 ± 152.4                       | 1055.5 ± 113.6                      | 885.5 ± 112.8                                                       |
| sensitivity (log cd\textsuperscript{-1} m\textsuperscript{2} s\textsuperscript{-3}) | 6.2 ± 0.3     | 5.8 ± 0.8                           | 5.7 ± 0.7                           | 5.7 ± 0.4                                                           |
| time between flashes for 50% recovery a-wave amplitude recovery (ms) | 893.3 ± 47.8  | 1039.0 ± 133.8                      | 912.4 ± 66.9                        | 1230.3 ± 121.3*                                                    |

\textsuperscript{a}ERG were recorded as described in Materials and Methods. Leading edges (initial 5-20 ms depending on response) of dark-adapted ERG photoresponses evoked by 2.8 log cd·s·m\textsuperscript{-2} flashes are fitted with a model of phototransduction. The results are presented with standard error, and \textit{n} was between 4 and 8.
REFERENCES

1. Okada, T., Ernst, O. P., Palczewski, K., and Hofmann, K. P. (2001) Trends Biochem Sci 26, 318-324
2. Pugh, E. N., Jr., Nikonov, S., and Lamb, T. D. (1999) Curr Opin Neurobiol 9, 410-418
3. Polans, A., Baehr, W., and Palczewski, K. (1996) Trends Neurosci 19, 547-554
4. Filipek, S., Stenkamp, R. E., Teller, D. C., and Palczewski, K. (2003) Annu Rev Physiol 65, 851-879
5. McBee, J. K., Palczewski, K., Baehr, W., and Pepperberg, D. R. (2001) Prog Retin Eye Res 20, 469-529
6. Saari, J. C. (2000) Invest Ophthalmol Vis Sci 41, 337-348
7. Mata, N. L., Radu, R. A., Clemmons, R. C., and Travis, G. H. (2002) Neuron 36, 69-80
8. Imanishi, Y., Gerke, V., and Palczewski, K. (2004) J Cell Biol 166, 447-453
9. Imanishi, Y., Batten, M. L., Piston, D. W., Baehr, W., and Palczewski, K. (2004) J Cell Biol 164, 373-383
10. Yamamoto, H., Simon, A., Eriksson, U., Harris, E., Berson, E. L., and Dryja, T. P. (1999) Nat Genet 22, 188-191
11. Driessen, C. A., Winkens, H. J., Hoffmann, K., Kuhlmann, L. D., Janssen, B. P., Van Vught, A. H., Van Hoosier, J. P., Wieringa, B. E., Deutman, A. F., Palczewski, K., Ruether, K., and Janssen, J. J. (2000) Mol Cell Biol 20, 4275-4287
12. Shang, E., Lai, K., Packer, A. I., Paik, J., Blaner, W. S., de Morais Vieira, M., Gouras, P., and Wolgemuth, D. J. (2002) J Lipid Res 43, 590-597
13. Haeseleer, F., Jang, G. F., Imanishi, Y., Driessen, C. A., Matsumura, M., Nelson, P. S., and Palczewski, K. (2002) *J Biol Chem* **277**, 45537-45546

14. Lin, B., White, J. T., Ferguson, C., Wang, S., Vessella, R., Bumgarner, R., True, L. D., Hood, L., and Nelson, P. S. (2001) *Cancer Res* **61**, 1611-1618

15. Kasus-Jacobi, A., Ou, J., Bashmakov, Y. K., Shelton, J. M., Richardson, J. A., Goldstein, J. L., and Brown, M. S. (2003) *J Biol Chem* **278**, 32380-32389

16. Simon, A., Hellman, U., Wernstedt, C., and Eriksson, U. (1995) *J Biol Chem* **270**, 1107-1112

17. Kedishvili, N. Y., Chumakova, O. V., Chetyrkin, S. V., Belyaeva, O. V., Lapshina, E. A., Lin, D. W., Matsumura, M., and Nelson, P. S. (2002) *J Biol Chem* **277**, 28909-28915

18. Jang, G. F., Van Hooser, J. P., Kuksa, V., McBee, J. K., He, Y. G., Janssen, J. J., Driessen, C. A., and Palczewski, K. (2001) *J Biol Chem* **276**, 32456-32465

19. Belyaeva, O. V., Stetsenko, A. V., Nelson, P., and Kedishvili, N. Y. (2003) *Biochemistry* **42**, 14838-14845

20. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. (1993) *Proc Natl Acad Sci U S A* **90**, 8424-8428

21. Driessen, C., Winkens, H., Haeseleer, F., Palczewski, K., and Janssen, J. (2003) *Vision Res* **43**, 3075-3079

22. Van Hooser, J. P., Liang, Y., Maeda, T., Kuksa, V., Jang, G. F., He, Y. G., Rieke, F., Fong, H. K., Detwiler, P. B., and Palczewski, K. (2002) *J Biol Chem* **277**, 19173-19182

23. Van Hooser, J. P., Aleman, T. S., He, Y. G., Cideciyan, A. V., Kuksa, V., Pittler, S. J., Stone, E. M., Jacobson, S. G., and Palczewski, K. (2000) *Proc Natl Acad Sci U S A* **97**, 8623-8628
24. Maeda, T., Van Hooser, J. P., Driessen, C. A., Filipek, S., Janssen, J. J., and Palczewski, K. (2003) *J Neurochem* **85**, 944-956

25. Howes, K. A., Pennesi, M. E., Sokal, I., Church-Kopish, J., Schmidt, B., Margolis, D., Frederick, J. M., Rieke, F., Palczewski, K., Wu, S. M., Detwiler, P. B., and Baehr, W. (2002) *Embo J* **21**, 1545-1554

26. Moore, S., Pritchard, C., Lin, B., Ferguson, C., and Nelson, P. S. (2002) *Gene* **293**, 149-160

27. Romert, A., Tuvendal, P., Simon, A., Dencker, L., and Eriksson, U. (1998) *Proc Natl Acad Sci U S A* **95**, 4404-4409

28. Palczewski, K., Van Hooser, J. P., Garwin, G. G., Chen, J., Liou, G. I., and Saari, J. C. (1999) *Biochemistry* **38**, 12012-12019

29. Saari, J. C., Garwin, G. G., Van Hooser, J. P., and Palczewski, K. (1998) *Vision Res* **38**, 1325-1333

30. Batten, M. L., Imanishi, Y., Maeda, T., Tu, D. C., Moise, A. R., Bronson, D., Possin, D., Van Gelder, R. N., Baehr, W., and Palczewski, K. (2004) *J Biol Chem* **279**, 10422-10432

31. Cideciyan, A. V., Haeseleer, F., Fariss, R. N., Aleman, T. S., Jang, G. F., Verlinde, C. L., Marmor, M. F., Jacobson, S. G., and Palczewski, K. (2000) *Vis Neurosci* **17**, 667-678

32. Wu, B. X., Chen, Y., Fan, J., Rohrer, B., Crouch, R. K., and Ma, J. X. (2002) *Invest Ophthalmol Vis Sci* **43**, 3365-3372

33. Mata, N. L., and Tsin, A. T. (1998) *Biochim Biophys Acta* **1394**, 16-22

34. Su, J., Chai, X., Kahn, B., and Napoli, J. L. (1998) *J Biol Chem* **273**, 17910-17916

35. Chai, X., Zhai, Y., and Napoli, J. L. (1997) *J Biol Chem* **272**, 33125-33131

36. Zhang, M., Chen, W., Smith, S. M., and Napoli, J. L. (2001) *J Biol Chem* **276**, 33
44083-44090

37. Zhuang, R., Lin, M., and Napoli, J. L. (2002) *Biochemistry* **41**, 3477-3483

38. Duester, G. (2001) *Chem Biol Interact* **130-132**, 469-480

39. Martras, S., Alvarez, R., Martinez, S. E., Torres, D., Gallego, O., Duester, G., Farres, J., de Lera, A. R., and Pares, X. (2004) *Eur J Biochem* **271**, 1660-1670

40. Bliss, A. F. (1951) *Arch Biochem* **31**, 197-204

41. Matschinsky, F. M. (1968) *J Neurochem* **15**, 643-657

42. Weng, J., Mata, N. L., Azarian, S. M., Tzekov, R. T., Birch, D. G., and Travis, G. H. (1999) *Cell* **98**, 13-23

43. Nasonkin, I., Illing, M., Koehler, M. R., Schmid, M., Molday, R. S., and Weber, B. H. (1998) *Hum Genet* **102**, 21-26

44. Maw, M. A., Kennedy, B., Knight, A., Bridges, R., Roth, K. E., Mani, E. J., Mukkadan, J. K., Nancarrow, D., Crabb, J. W., and Denton, M. J. (1997) *Nat Genet* **17**, 198-200

45. Saari, J. C., Nawrot, M., Kennedy, B. N., Garwin, G. G., Hurley, J. B., Huang, J., Possin, D. E., and Crabb, J. W. (2001) *Neuron* **29**, 739-748

46. Morimura, H., Saindelle-Ribeudeau, F., Berson, E. L., and Dryja, T. P. (1999) *Nat Genet* **23**, 393-394

47. Redmond, T. M., Yu, S., Lee, E., Bok, D., Hamasaki, D., Chen, N., Goletz, P., Ma, J. X., Crouch, R. K., and Pfeifer, K. (1998) *Nat Genet* **20**, 344-351

48. Allikmets, R. (2004) *Ophthalmic Genet* **25**, 67-79

49. Baehr, W., Wu, S. M., Bird, A. C., and Palczewski, K. (2003) *Vision Res* **43**, 2957-2958

50. Janecke, A. R., Thompson, D. A., Utermann, G., Becker, C., Hubner, C. A., Schmid, E.,
51. McHenry, C. L., Nair, A. R., Ruschendorf, F., Heckenlively, J., Wissinger, B., Nurnberg, P., and Gal, A. (2004) Nat Genet 36, 850-854

52. Perrault, I., Hanein, S., Gerber, S., Barbet, F., Ducroq, D., Dollfus, H., Hamel, C., Dufier, J. L., Munnich, A., Kaplan, J., and Rozet, J. M. (2004) Am J Hum Genet 75, 639-646

53. Haeseleer, F., Huang, J., Lebioda, L., Saari, J. C., and Palczewski, K. (1998) J Biol Chem 273, 21790-21799

54. Rattner, A., Smallwood, P. M., and Nathans, J. (2000) J Biol Chem 275, 11034-11043

55. Nakamura, M., Hotta, Y., Tanikawa, A., Terasaki, H., and Miyake, Y. (2000) Invest Ophthalmol Vis Sci 41, 3925-3932

56. Swain, P. K., Chen, S., Wang, Q. L., Affatigato, L. M., Coats, C. L., Brady, K. D., Fishman, G. A., Jacobson, S. G., Swaroop, A., Stone, E., Sieving, P. A., and Zack, D. J. (1997) Neuron 19, 1329-1336

57. Kelsell, R. E., Gregory-Evans, K., Payne, A. M., Perrault, I., Kaplan, J., Yang, R. B., Garbers, D. L., Bird, A. C., Moore, A. T., and Hunt, D. M. (1998) Hum Mol Genet 7, 1179-1184

58. Payne, A. M., Downes, S. M., Bessant, D. A., Taylor, R., Holder, G. E., Warren, M. J., Bird, A. C., and Bhattacharya, S. S. (1998) Hum Mol Genet 7, 273-277

59. Sokal, I., Li, N., Surgucheva, I., Warren, M. J., Payne, A. M., Bhattacharya, S. S., Baehr, W., and Palczewski, K. (1998) Mol Cell 2, 129-133

60. Cremers, F. P., van de Pol, D. J., van Driel, M., den Hollander, A. I., van Haren, F. J., Knoers, N. V., Tijmes, N., Bergen, A. A., Rohrschneider, K., Blankenagel, A., Pinckers, A. J., Deutman, A. F., and Hoyng, C. B. (1998) Hum Mol Genet 7, 355-362
60. Stecher, H., Gelb, M. H., Saari, J. C., and Palczewski, K. (1999) *J Biol Chem* **274**, 8577-8585
Figure 2

A

WT

vector

targeted

B

Southern

C

D

E

ES DNA

ES DNA

ES DNA

O7/ O8

O9/ O10

O9/ O12

3.0

2.0

389

300

kb

bp

kb

/+ /

+/

+/−

−/

3.0

2.0

1.6

309
Figure 3

A

Immunoblot

RDH 11

MW (kD)

+/+

+/–

–/–

B

rdh 11

rdh 12

rdh 13

rdh 14

mRNA (%)

+/+

+/–

–/–

RT PCR

MW (kD)

62

49

38

28

17
Figure 4

Dark adapted

15 min after flash

Absorbance at 325 nm (mAU)

Time (min)
Figure 5

WT  rdh11^-/-  rdh5^-/-  rdh5^-/-rdh11^-/-
Figure 6

A

WT  rdh11⁻/⁻  rdh5⁻/⁻  rdh5⁻/⁻ rdh11⁻/⁻

flash intensities (log cd·s·m⁻²)

-3.7

-2.0

-0.2

0.4

2.8

500 µV

50 ms

B

WT  rdh11⁻/⁻  rdh5⁻/⁻  rdh5⁻/⁻ rdh11⁻/⁻

a-wave amplitude (µV)

0 500 1000 1500

-4 -2 0 2

b-wave amplitude (µV)

0 500 1000 1500 2000

-4 -2 0 2

intensities (log cd·s·m⁻²)
Figure 6

C

|           | WT             | rdh11⁻/⁻        | rdh5⁻/⁻         | rdh5⁺⁻rdh11⁻/⁻ |
|-----------|----------------|-----------------|-----------------|----------------|
| a-wave amplitude (µV) |               |                 |                 |                |
| b-wave amplitude (µV) |               |                 |                 |                |
| flash intensities (log cd·s·m⁻²) | -2.0          | 1.6             | 2.8             | 2.0            |

D

|         | a-wave amplitude (µV) | b-wave amplitude (µV) |
|---------|-----------------------|-----------------------|
| WT      |                       |                       |
| rdh11⁻/⁻|                       |                       |
| rdh5⁻/⁻|                       |                       |
| rdh5⁺⁻rdh11⁻/⁻|               |                       |

Intensities (log cd·s·m⁻²):
-4 -2 0 2 4 6 8

Flash intensities (log cd·s·m⁻²):
-2.0 1.6 2.8

WT: wild type
rdh11⁻/⁻: rdh11 knockout
rdh5⁻/⁻: rdh5 knockout
rdh5⁺⁻rdh11⁻/⁻: rdh5 and rdh11 double knockout

a-wave amplitude (µV)

b-wave amplitude (µV)
Figure 7

**, p<0.02

WT

rdh11^-/

rdh5^-/

rdh5^-/rdh11^-/**
Figure 8

A pre-bleach

WT

rdh11−/

rdh5−/

rdh5+/−rdh11−/

500 µV

0

10

20

30

60

time after bleach (min)

0 10 20 30

time (ms)

0 10 20 30

time (ms)

0 10 20 30

time (ms)

0 10 20 30

time (ms)
Figure 8

B

![Graph showing the recovery of a-wave amplitude (%)](image)

- **WT**: Solid line with filled circles
- **rdh11-/-**: Dashed line with open circles
- **rdh5-/-**: Dotted line with filled triangles
- **rdh5-/-rdh11-/-**: Dashed-dotted line with open triangles

* p<0.01; ***, p<0.0001

- time after bleaching (min)
- recovery of a-wave amplitude (%)
Figure 9

0 min dark adaptation

30 min dark adaptation

WT  rdh11/-  rdh5/-  rdh5-/-rdh11-/-
Delayed dark adaptation in 11-cis-retinol dehydrogenase deficient mice: A role of RDH11 in visual processes in vivo
Tom S. Kim, Akiko Maeda, Tadao Maeda, Cynthia Heinlein, Natalia Kedishvili, Krzysztof Palczewski and Peter S. Nelson

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