The Retinal G Protein-coupled Receptor (RGR) Enhances Isomerohydrolase Activity Independent of Light**S

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Rod and cone visual pigments use 11-cis-retinal, a vitamin A derivative, as their chromophore. Light isomerizes 11-cis- into all-trans-retinal, triggering a conformational transition of the opsin molecule that initiates phototransduction. After bleaching all-trans-retinal leaves the opsin, and light sensitivity must be restored phototransduction. After bleaching all-

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interacts with transducin thereby activating the phototransduction cascade that leads to closure of cGMP-activated channels in the plasma membrane. The resulting hyperpolarization leads to a reduction of synaptic glutamate release, completing the conversion of a light stimulus into a neurochemical signal (reviewed in Ref. 1).

Light-induced activity of rhodopsin is quickly terminated by phosphorylation and capping of its transducin interaction site. The chromophore, now in the all-trans form, is hydrolyzed and released. To become photosensitive again, the bleached opsin molecule requires the re-introduction of a new molecule of 11-cis-retinal. The source of 11-cis-retinal for rod photoreceptors is the retinal pigment epithelium (RPE), where 11-cis-retinal is regenerated from all-trans-retinoids. This "classical" visual cycle involves formation of all-trans-retinol in the photoreceptor, transport to the RPE, several steps of metabolic conversion, and transport of 11-cis-retinal back to the photoreceptor (reviewed in Refs. 2 and 3). The retinal pigment epithelial protein 65 (RPE65, on mouse chromosome 3 (4)) is essential for this classical visual cycle: absence of RPE65 leads to undetectable levels of 11-cis-retinal (5) and dramatically reduced photoreceptor function (6).

Recently, the existence of an additional pathway providing 11-cis-retinal under photic conditions was proposed, based on studies of mice deficient in the retinal G protein-coupled receptor (RGR, on mouse chromosome 14 (7)). In vitro, RGR binds all-trans-retinal and upon illumination converts it into 11-cis-retinal (8–10). As RGR−/− mice have decreased steady-state levels of rhodopsin during light exposure, it was suggested that RGR-dependent generation of 11-cis-retinal represents an auxiliary "photic pathway" supporting the classical visual cycle in periods of high chromophore demand, e.g. during continuous light exposure (7, 11, 12).

Several lines of evidence indicate that this proposed photic pathway and the classical visual cycle might not be independent. RGR forms a protein complex with components of the classical visual cycle, namely cellular retinaldehyde-binding protein (CRALBP), 11-cis-retinol dehydrogenase (RDH5), and RPE65 (13, 51, 52). Additionally, RPE65 and RGR protein levels may influence each other (14) and most importantly the

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1 The abbreviations used are: RPE, retinal pigment epithelium; sER, smooth endoplasmic reticulum; RDH5, 11-cis-retinol dehydrogenase; REST, retinyl ester storage particle; sRPE65, soluble RPE65; CRALBP, cellular retinaldehyde-binding protein; WT, wild type; HPLC, high performance liquid chromatography; ERG, electroretinogram recordings; mRPE65, membrane-bound RPE65.
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MATERIALS AND METHODS

Mice—WT and Rgr−/− mice (7) were screened for variations at codon 450 of Rpe65 and bred to homozygosity for Rpe65-Met450. Rpe65 mice were derived from Rpe65+/− and Rpe65−/− mice (5) in F1 and carried Met at position 450 of the remaining allele. Genotyping for codon 450 of Rpe65 was performed as described recently (22).

BALB/c, C57BL/6, and 129/Ola wild-type mice were obtained from Harlan (Netherlands and United Kingdom) and Janvier (France). B6/129S hybrids were from Jackson Labs (Bar Harbor, ME). All mice were reared in cyclic light (12:12 h, 60 lux, lights on 6:00 a.m.). Experiments were performed with mice aged 6–10 weeks. Animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and according to the guidelines of the local veterinary authorities.

Western Blotting—Eyes of dark-adapted mice were prepared as follows: the eye was enucleated, attached tissue was thoroughly removed, and the retina was removed. The remaining eye cup (containing the retina) was homogenized in Tris NH2OH (pH 6.8) and 200 μl of methanol and was homogenized by probe sonication (sonicator, Bandelin, Berlin, Germany). Retinoids were extracted from the homogenate as described previously (28). HPLC analyses were performed on a Hypersil 3-μm column (Knaur, Germany) on a System Gold (Beckman) equipped with a multidiode array (model 166, Beckman) and Karat software. The individual retinoids were each determined by their retention times and spectral characteristics as described previously for authentic standards. For quantification of the molar amounts, peak integrals were scaled with defined amounts of reference substances. The reference substances all-trans-retinyl palmitate, and all-trans, 13-cis, and 9-cis-retinal were purchased from Sigma. 11-cis-Retinal was isolated from dark-adapted bovine eyes. The corresponding retinoids and oximes were obtained by the reduction of the retinoids with NaBH4 or their reaction with NH2OH. The proportion of a retinal isomer was determined by the total peak areas of both its syn- and anti-retinal oxime. For example, to calculate the amount of 11-cis-retinal from the HPLC profile shown in Fig. S3, the peak areas of syn-11-cis retinaloxime (peak 2) and anti-11-cis retinaloxime (peak 8) were scaled according to the corresponding areas of the peaks in the 11-cis-retinal reference, and the resulting amounts were summed to provide the total amount of 11-cis-retinal (in pmol) in the tissue.

Electroretinogram Recordings (ERG)—ERGs were obtained as described previously (6). Briefly, mice were dark-adapted overnight prior to the experiments and their pupils dilated. Anesthesia was induced by subcutaneous injection of ketamine (66.7 mg/kg), xylazine (11.7 mg/kg), and atropine (1 mg/kg). Silver needle electrodes served as ground (forehead or body), and gold wire rings as active electrodes. The ERG equipment consisted of a ganzfeld bowl, an amplifier (0.1 to 3,000 Hz), and a PC-based control and recording unit (Toennies Multiliner Vision, Jaeger/Toennies, Hochberg, Germany). Responses to trains of flashes (flicker) were obtained for a fixed frequency (6 Hz) with a range of intensities (0.00012 to 19 cd s/m2 in steps of 0.2 logarithmic units). For ERG recordings following light exposure, mice were exposed to 5,000 lux of
white light for 10 min and transferred into darkness for 2 or 4 h. During bleaching and recovery in the dark the mice were not anesthetized. Anesthesia was induced directly before the ERG was recorded. Therefore, anesthesia only interfered with regeneration for the final 5–10 min of the 2- or 4-h period.

RESULTS

Three genotypes of mice were used to study the role of RGR, and these are designated throughout as WT, Rgr<sup>−/−</sup>, and Rpe65<sup>−/−</sup>. These abbreviations represent the following genotypes: wild type ("WT = Rgr<sup>−/−</sup>/Rpe65<sup>+/+</sup>"), Rgr deficient ("Rgr<sup>−/−</sup><sup>−/−</sup> = Rgr<sup>−/−</sup>/Rpe65<sup>−/−</sup>"), and Rpe65 heterozygous ("Rpe65<sup>+/−</sup> = Rgr<sup>−/−</sup>/Rpe65<sup>+/−</sup>").

RGR and RPE65 Protein Levels Correlate in Different Strains of Mice—We measured RPE65 and RGR protein levels both in the transgenic mice and also in several strains of wild type mice. BALB/c and 129/Ola mice express the RPE65 variant carrying Leu at position 450. B6/129S hybrids and C57BL/6J may contain both the WT and RPE65<sup>−/−</sup> types: wild type ("WT" or Rgr<sup>−/−</sup>/Rpe65<sup>−/−</sup>), and BALB/c and 129/Ola mice express the RPE65 variant (7) we originally obtained were mixed with C57BL/6J mice (7) we involved blastocyst injection of transgenic C57BL/6JRpe65<sup>+/+</sup> mice and the resulting chimeric animals were bred with C57BL/6J mice. C57BL/6JRpe65<sup>+/+</sup> mice and RPE65<sup>−/−</sup> mice were likewise compared and found to be similar (Fig. 2B) and quantified RPE65 immunoreactivity using the Odyssey infrared imaging system. According to our comparisons, Rgr<sup>−/−</sup> and Rpe65<sup>−/−</sup> mice on average express the same amount of RPE65, which is 43 ± 10 and 46 ± 8% of wild type RPE65 levels, respectively (Fig. S2). Thus, our results are in line with previously published data: RPE65 immunoreactivity in Rgr<sup>−/−</sup> mice was reported to be "roughly half" that seen in WT (31) and to be reduced by 50% in Rgr<sup>−/−</sup> mice (14). Consequently, the effect of RGR ablation can be analyzed in Rpe65<sup>−/−</sup> and Rgr<sup>−/−</sup> mice without the confounding factor of different RPE65 expression levels.

Regeneration of Rhodopsin in Darkness: Role of RGR in the Classical Visual Cycle—Mice with different levels of RPE65 protein because of the expression of either the Met<sup>−/−</sup> or Leu<sup>−/−</sup> variant of Rpe65 show different regeneration kinetics for rhodopsin in darkness following a strong bleach (16, 19, 21). Therefore, we assumed that reduced levels of RPE65 in Rgr<sup>−/−</sup> mice should slow rhodopsin regeneration in darkness as compared with WT mice. To compensate for different expression levels of RPE65 and to analyze the pure effect of RGR deficiency, we also employed Rpe65<sup>−/−</sup>/Rgr<sup>−/−</sup> mice. These mice on average express wild type RPE65 levels (Fig. S2, mean = 80 ± 19%, no significant difference, n = 5, p > 0.3, two-tailed t test) and the same RPE65 levels as Rgr<sup>−/−</sup> mice (Figs. 2B and supplemental materials, Fig. S2).

WT, Rgr<sup>−/−</sup>, and Rpe65<sup>−/−</sup> mice with dilated pupils were exposed to white light of 5,000 lux for 10 min. This treatment caused bleaching of more than 90% of their rhodopsin. Animals were placed in darkness, and rhodopsin regeneration was measured. The regeneration of rhodopsin in the three genotypes is plotted in Fig. 3A, as a function of time after extinction of the bleaching light. Note that these recoveries have been measured without the use of anesthesia, which has been shown to slow the regeneration of visual pigment (reviewed in Refs. 3
and 53). In this figure, the rhodopsin level was measured spectrophotometrically; in a subsequent section (Fig. 6B) we will compare results determined both this way and by measuring the quantity of 11-cis-retinal.

The recovery for WT is virtually complete in 5 h, broadly similar to the kinetics obtained previously for C57BL/6 mice by Wenzel et al. (16); also see Ref. 3, Fig. 22). Recovery is very slightly slower for Rpe65<sup>−/−</sup> mice, but substantially slower for Rgr<sup>−/−</sup> mice, with complete recovery taking more than 15 h.

The curves in Fig. 3A plot the pigment regeneration kinetics predicted by the “rate-limited” MLP model of Mahroo and Lamb (32) and Lamb and Pugh (3) (relevant parameters are listed in Table I). The rhodopsin level, \( Rh(t) \), is given by Equation 1,

\[
Rh(t) = \frac{R_{\text{max}}}{1 - \frac{B}{Km}} \exp \left( \frac{B}{Km} \exp \left( -\frac{1}{Km} vt \right) \right) \tag{1}
\]

where \( B \) is the size of the bleach, \( v \) is the initial fractional rate of pigment regeneration following a total bleach, \( K_m \) is a semi-saturation constant that describes the degree of curvature, and \( W(x) \) denotes the Lambert W function; for details, see Refs. 3 and 32. For all curves in this paper the semisaturation constant was held at \( K_m = 0.2 \), as used previously.

To describe the results in Fig. 3, we took the total quantity of pigment to be \( R_{\text{max}} = 515 \) pmol for all three strains. The three curves in Fig. 3A were then calculated using \( v = 0.23 \) (WT), 0.078 \( Rgr^{-/-} \), and 0.20 \( Rpe65^{-/-} \) h<sup>−1</sup>, and clearly they provide a reasonable description of the data. These curves also provide a straightforward way of extracting the maximal rate of pigment regeneration for each strain, because the absolute magnitude of the initial rate of pigment regeneration after a full bleach is given by the fractional rate, \( v \), multiplied by \( R_{\text{max}} \). Hence the absolute rates of rhodopsin regeneration were initially 118 (WT), 40 \( Rgr^{-/-} \), and 103 \( Rpe65^{-/-} \) pmol h<sup>−1</sup>; these values show that the rate was roughly 3-fold higher in WT than in \( Rgr^{-/-} \) mice. Furthermore, absence of one Rpe65 allele (54% reduction of the RPE65 protein) in \( Rpe65^{-/-} \) mice reduced the rate of rhodopsin regeneration by less than 15% compared with WT.

**Lack of RGR Causes Slowed Recovery of Photoreceptor Function following Bleaching**—To verify our finding of slowed rhodopsin regeneration in \( Rgr^{-/-} \) mice in darkness and to assess the functional consequences, we recorded ERGs. A slowed regeneration of rhodopsin was expected to cause a desensitization of rods in \( Rgr^{-/-} \) mice in the early phase of dark adaptation. Retinal function was therefore analyzed using a scotopic 6-Hz flicker ERG intensity series (6) after 2 and 4 h of dark adaptation following a >90% bleach. At these time points of regeneration WT mice had recovered about 40 and 70% of their dark adapted levels of 11-cis-retinal on average; \( Rgr^{-/-} \) mice had recovered about 20 and 30%, respectively (Table II). As judged by the unresponsiveness of the retina to low stimulus

**Table I**

| Parameters used in curves for rhodopsin, ester, and sum, in Figs. 3, 5, and 6, and the ratios of extracted rates of regeneration and of RPE65 and RGR protein levels |
|---------------------------------------------------------------------------------|-----------------|------------------|-----------------|------------------|
| All conditions                                                                 | WT (red)        | \( Rgr^{-/-} \) (blue) | \( Rpe65^{-/-} \) (green) |
| \( R_{\text{max}} \)                                                          | \( R_{\text{max}} \) | \( R_{\text{max}} \) | \( R_{\text{max}} \) |
|                                 | 650             | 515              | 875             | 650              |
| \( K_m \)                                                                     | 0.2             | 0.95             | 0.9             |
| \( B \)                                                                       | 0.9             | 0.95             | 0.9             |
| \( v_{\text{Dark}} \)                                                         | 0.23            | 0.078            | 0.20            |
| \( v_{\text{Light}} \)                                                        | 0.96            | 0.36             | 0.84            |
| \( L \)                                                                       | 1.56            | 1.56             | 1.56            |
| \( v_{\text{Light}}/v_{\text{Dark}} \)                                       | 4.2             | 4.6              | 4.2             |
| \( v_{\text{Light}}/v_{\text{Dark}} \)                                       | 2.9             | 1                | 2.6             |
| \( v_{\text{Light}}/v_{\text{Dark}} \)                                       | 2.7             | 1                | 2.3             |
| **Amount of RPE65**                                                           | 2.3             | 1                | 1               |
| **Relative ratios**                                                           | 1.25            | 0                | 1               |

*WT mice on average express more RGR than do Rpe65<sup>−/−</sup> mice; however, the difference was found to be statistically insignificant (n = 5, p > 0.3, two tailed t-test).*
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**Table II**
Changes in rhodopsin and the major retinoid metabolites during regeneration in darkness following a large bleach

|                      | WT       | Rgr<sup>-/-</sup> | Rpe65<sup>-/-</sup> |
|----------------------|----------|-------------------|---------------------|
| Before bleach        |          |                   |                     |
| Rhodopsin            | 541 ± 55 | 532 ± 34          | 521 ± 9             |
| 11-cis-Retinal       | 532 ± 83 | 486 ± 93          | 472 ± 8.3           |
| Retinyl ester        | 88 ± 14  | 292 ± 64          | 101 ± 29            |
| All-trans-retinal    | 66 ± 33  | 58 ± 63           | 73 ± 8              |
| All-trans-retinol    | 4 ± 1    | 4 ± 5             | Not detectable      |
| Sum                  | 695 ± 79 | 859 ± 114         | 649 ± 34            |
| Immediately after bleach |         |                   |                     |
| Rhodopsin            | 24 ± 16  | 33 ± 24           | 36 ± 12             |
| 11-cis-Retinal       | 26 ± 19  | 42 ± 3           | 31 ± 8              |
| Retinyl ester        | 386 ± 86 | 509 ± 87          | 338 ± 47            |
| All-trans-retinal    | 119 ± 34 | 200 ± 78          | 152 ± 42            |
| All-trans-retinol    | 38 ± 7   | 67 ± 21           | 62 ± 30             |
| Sum                  | 578 ± 94 | 814 ± 120         | 586 ± 71            |
| 1 h after bleach     |          |                   |                     |
| Rhodopsin            | 184 ± 59 | 93 ± 7            | 144 ± 13            |
| 11-cis-Retinal       | 153 ± 23 | 66 ± 3            | 151 ± 12            |
| Retinyl ester        | 44 ± 32  | 613 ± 101         | 419 ± 6             |
| All-trans-retinal    | 13 ± 1   | 9 ± 1             | 30 ± 4              |
| All-trans-retinol    | 16 ± 1   | 13 ± 3            | 5 ± 1               |
| Sum                  | 646 ± 55 | 915 ± 101         | 592 ± 14            |
| 2 h after bleach     |          |                   |                     |
| Rhodopsin            | 264 ± 16 | 80 ± 0            | 245 ± 12            |
| 11-cis-Retinal       | 205 ± 40 | 102 ± 31          | 222 ± 11            |
| Retinyl ester        | 299 ± 27 | 810 ± 79          | 351 ± 18            |
| All-trans-retinal    | 58 ± 26  | 34 ± 6            | 34 ± 1              |
| All-trans-retinol    | 7 ± 3    | 15 ± 5            | 12 ± 1              |
| Sum                  | 599 ± 48 | 951 ± 82          | 631 ± 21            |
| 4 h after bleach     |          |                   |                     |
| Rhodopsin            | 424 ± 32 | 153 ± 25          | 391 ± 29            |
| 11-cis-Retinal       | 386 ± 108| 141 ± 22          | 376 ± 26            |
| Retinyl ester        | 167 ± 63 | 594 ± 39          | 274 ± 20            |
| All-trans-retinal    | 104 ± 43 | 38 ± 3            | 50 ± 1              |
| All-trans-retinol    | 6 ± 2    | 9 ± 7             | Not detectable      |
| Sum                  | 682 ± 110| 788 ± 46          | 648 ± 35            |
| 6 h after bleach     |          |                   |                     |
| Rhodopsin            | 424 ± 141| 231 ± 13          | 480 ± 6             |
| 11-cis-Retinal       | 455 ± 29 | 188 ± 19          | 444 ± 15            |
| Retinyl ester        | 121 ± 13 | 507 ± 104         | 127 ± 11            |
| All-trans-retinal    | 25 ± 2   | 13 ± 3            | 74 ± 3              |
| All-trans-retinol    | 8 ± 2    | 10 ± 3            | Not detectable      |
| Sum                  | 594 ± 102| 830 ± 134         | 663 ± 16            |
| 10 h after bleach    |          |                   |                     |
| Rhodopsin            | 608 ± 7  | 362 ± 50          | 526 ± 28            |
| 11-cis-Retinal       | 506 ± 53 | 401 ± 185         | 478 ± 26            |
| Retinyl ester        | 110 ± 5  | 446 ± 175         | 118 ± 43            |
| All-trans-retinal    | 0 ± 2    | 43 ± 24           | 72 ± 20             |
| All-trans-retinol    | 3 ± 3    | 11 ± 1            | 2 ± 3               |
| Sum                  | 720 ± 38 | 882 ± 223         | 694 ± 55            |
| 16 h after bleach    |          |                   |                     |
| Rhodopsin            | 503 ± 64 | 507 ± 32          | 506 ± 30            |
| 11-cis-Retinal       | 442 ± 73 | 560 ± 49          | 460 ± 28            |
| Retinyl ester        | 78 ± 12  | 296 ± 28          | 121 ± 34            |
| All-trans-retinal    | 120 ± 52 | 78 ± 19           | 73 ± 7              |
| All-trans-retinol    | 2 ± 2    | 5 ± 5             | 2 ± 2               |
| Sum                  | 673 ± 87 | 915 ± 54          | 679 ± 45            |

Intensities, rod function was almost completely absent in Rgr<sup>-/-</sup> mice 2 and 4 h after the bleaching, whereas in contrast, WT mice had recovered a substantial rod response after 2 h and had almost recovered completely after 4 h (Fig. 4). Thus, at a measurement time 2 h after the bleach, and for responses of small amplitude, the symbols for Rgr<sup>-/-</sup> are shifted to the right compared with WT, indicating a reduction in rod sensitivity in the Rgr<sup>-/-</sup> mice, at that time. Four hours after the bleach this difference has increased to more than 3 log<sub>10</sub> units. At higher stimulus intensities, supposed to evoke responses from the cone system, the differences between WT and Rgr<sup>-/-</sup> mice were diminished. On the other hand, we found no difference between WT and Rgr<sup>-/-</sup> mice dark adapted overnight and not challenged by light exposure. This supports the conclusion that the loss of photoreceptor sensitivity in Rgr<sup>-/-</sup> mice results from a slowed regeneration of 11-cis-retinal after bleaching.

Slow Rhodopsin Regeneration in Darkness Is Independent of the Bleaching Regime—A 3-fold slowed rhodopsin regeneration in darkness in Rgr<sup>-/-</sup> mice as observed here is in contrast to results reported by Chen et al. (7). Those authors reported no difference between Rgr<sup>-/-</sup> and WT mice in the rate of rhodopsin regeneration after a partial bleach accomplished by expo-
which most likely evoke cone responses. ERG responses are attributed was clearly slower in versus min darkness. Regardless of the bleaching regime (5,000 lux for 10 s and immediately returned to (7) in our mouse strains. For this purpose, mice were exposed to bleaching regime may influence the kinetics of rhodopsin re-
furtherance, we aimed at reproducing the results of Chen et al. (7) in our mouse strains. For this purpose, mice were exposed to bleaching regime did not influence the rhodopsin regeneration difference was recorded in mice of both genotypes (WT, Rgr−/−, dark gray versus H11002/H11002) or the level of bleaching (93% versus 52% here. Thus, the rhodopsin regeneration kinetics after ~50% do not differ from those after ~90% bleach. Individual data points represent the mean ± S.D. rhodopsin content of at least 4 eyes. Time 0 represents the end of the light exposure; all other values were taken from animals left in darkness for the times indicated.

measure to 30,000 lux for a short period. To analyze whether the bleaching regime may influence the kinetics of rhodopsin regeneration, we aimed at reproducing the results of Chen et al. (7) in our mouse strains. For this purpose, mice were exposed to 30,000 lux of white light for 10 s and immediately returned to darkness. Regardless of the bleaching regime (5,000 lux for 10 min versus 30,000 lux for 10 s) or the level of bleaching (93% versus 52%), the rate of rhodopsin regeneration in darkness was clearly slower in Rgr−/− mice (Fig. 5, compare withFig. 3).

Furthermore, the rhodopsin regeneration kinetics for the two genotypes could be described adequately by the MLP model for both bleaching regimes by adjusting only one parameter: the size of the bleach. Thus, in both genotypes, changing the bleaching regime did not influence the rhodopsin regeneration kinetics. Therefore, there is no straightforward explanation for the different results regarding rhodopsin regeneration in darkness as reported by Chen et al. (7) and the results presented here. Potentially the results in the previous study were influenced by the Rpe65 variant at codon 450 (see Fig. 1), which was not considered in that study. In addition Chen et al. (7) based their conclusion about rhodopsin regeneration in the dark on a lower number of data points.

Ester Levels and Evidence for a Closed System for Retinoids—To reveal the enzymatic step(s) in the visual cycle that is (are) affected in the absence of RGR, we analyzed visual cycle intermediates in mouse eyes following a bleach of ~90%. Characteristic HPLC profiles of the retinoid composition of eyes from WT mice subjected to different light regimes are shown in supplemental materials Fig. S3. Dark-adapted WT, Rgr−/−, and Rpe65−/− mice contained similar amounts of 11-cis-retinal and all-trans-retinal, whereas retinyl esters were elevated 3-fold in Rgr−/− mice. Other retinoids (11-cis-retinol, 9-cis-retinal, 13-cis-retinal, and all-trans-retinol) together made up 4–8% of the total retinoids in both genotypes during dark adaptation (summarized in Table II).

Measurements of rhodopsin, 11-cis-retinal, and ester levels during regeneration in darkness in mice of the three genotypes are plotted in Fig. 6, using the same color coding as previously. Fig. 6A plots ester levels, whereas Fig. 6B plots 11-cis-retinal levels as downward triangles and rhodopsin levels as upward triangles. For each mouse, the esters and retinoids were analyzed in one eye, and rhodopsin was analyzed in the other eye.

Fig. 6A shows that the basal (and final) levels of retinyl ester in the WT and Rpe65−/− animals were around ~100 pmol, whereas the basal (and final) levels of ester in the Rgr−/− animals were roughly 3 times higher, at around 300 pmol. In each strain, delivery of a near-total bleach was followed by a large increase in ester levels, with the increment in each case exceeding 320 pmol at 1 h after bleaching. The curves in Fig. 6B will be described shortly, but are based on the concept that the eye contains a fixed pool of retinoid, so that to a reasonable approximation the decline in the level of ester matches the increase in the level of rhodopsin (except for the single time point obtained immediately after the bleach, when the quantities of all-trans-retinal and retinol are significant fractions of the total retinoid).

In Fig. 6B, we compare the levels of rhodopsin (A, measured spectrophotometrically) and 11-cis-retinal (V, measured by HPLC) in fellow eyes. There is good agreement between these two measures, as would be expected if the HPLC measurement of 11-cis-retinal primarily reflects the quantity of rhodopsin in the eye.

The correlation between these measures is examined further in supplemental materials Fig. S4, which shows scatter plots of the individual measurements of rhodopsin and 11-cis-retinal for each animal. In animals of each genotype there is a very
The open symbols (○) in Fig. 6B plot the summed measurements of the levels of retinoid. Because we had separate measurements of rhodopsin and 11-cis-retinal, we chose to average these, in an attempt to reduce the experimental variability, and thereby obtain a more reliable estimate of the "rhodopsin" level. That value was then summed with the corresponding levels of ester, all-trans-retinal, and retinol, to give a "summed retinoid" level, which is plotted as the open symbols. These summed values for each color show no clear variation with time. Thus, in view of the level of noise (see S.D. values in Table II), these results are consistent with the notion that for animals of each genotype the summed measurements are independent of time; i.e., constant. This is indicated graphically in Fig. 6B by the horizontal dashed lines at total = 650 (WT), 650 (Rpe65+/−), and 875 (Rgr−/−) pmol.

The plotted sum, sum(t), can therefore be written as Equation 2,

\[ \text{Sum}(t) = \text{Rht}(t) + \text{ester}(t) + \Delta t\text{RAL}(t) + \Delta t\text{ROL}(t) = \text{total} \]  

(Eq. 2)

where \(\Delta t\text{RAL}(t)\) and \(\Delta t\text{ROL}(t)\) represent all-trans-retinal and retinol, respectively. This total should differ from the true total retinoid content of the eye only by the level of trace constituents, such as 11-cis-retinol and 9- and 13-cis-isomers. Given the approximate constancy found in Fig. 4B, it is useful to rearrange Equation 2 into Equation 3,

\[ \text{Ester}(t) = [\text{total} - \text{Rh}(t) - \text{other}_{\text{basal}} - \Delta \text{ther}(t)] \]  

(Eq. 3)

where \(\text{other}(t) = \text{other}_{\text{basal}} + \Delta \text{other}(t)\) represent all the other retinoids, primarily all-trans-retinal and retinol. Thus, to the extent that we are justified in regarding total as constant and in neglecting \(\Delta \text{other}(t)\), we can approximate ester(t) by the terms inside the square brackets in Equation 3.

Hence the predicted curves for the esters in Fig. 6A have been obtained as the difference between the dashed lines total and the solid curves Rh(t) of the same color in Fig. 6B, minus a small fixed level, of \(\text{other}_{\text{basal}} = 40\) pmol. These predicted curves provide a reasonable description of the ester measurements, in view of the absence of data during the first hour, where the levels of all-trans-retinoids would perturb the predictions.

This analysis suggests that the rate of regeneration of 11-cis-retinal in WT and Rgr−/− mice is limited by the rate of conversion of esters by the isomerohydrolase. Therefore, the slowed regeneration of 11-cis-retinal and rhodopsin (Fig. 6B) in the Rgr−/− mouse seems to be caused by a slowed mobilization of retinyl esters (Fig. 6A).

Furthermore, we conclude from these experiments that the sum of the molar amount of retinoid in rhodopsin, esters, and all-trans-retinal and retinol is approximately constant over the time course of these experiments. These findings may be consistent with the idea that the eye is behaving approximately as a closed system for retinoid. However, we cannot exclude the possibility of a balanced influx and efflux of all-trans-retinol to and from the ester pool (33).

The magnitude of the fixed level \(\text{other}_{\text{basal}}\) has been chosen so as to provide a good description of the basal ester levels, which can be obtained by subtraction, as ester_{basal} = total - \(\text{Rh}_{\text{max}}\) - \(\text{other}_{\text{basal}}\). Thus, the value of \(\text{other}_{\text{basal}} = 40\) pmol was selected, as it gives basal ester levels of ester_{basal} = 95 (WT), 320 (Rgr−/−), and 95 (Rpe65+/−) pmol, providing a good description of the pre-bleach and recovery data in Fig. 6A.

Bleaching and Formation of Rhodopsin in the Light—RGR deficiency in mice homozygous for the Met450 variant of Rpe65 caused a previously undiscovered 3-fold slower regeneration of rhodopsin in darkness. We therefore also re-evaluated the effect of Rgr deletion under photic conditions, where rhodopsin is being both bleached and regenerated.
WT, Rgr<sup>−/−</sup>, and Rpe65<sup>−/−</sup> mice with dilated pupils were exposed to constant white light of 100 lux, and rhodopsin was measured at different times after onset of the exposure. The time course of decline in rhodopsin level during exposure to steady illumination is plotted in Fig. 3B, using the same color convention as in Fig. 3A. Very importantly, the final steady level was reached far more rapidly in this case than for recovery in darkness in the upper panel; note the different time scales. Thus, for WT the final steady level had been reached within about 1 h, and even for Rgr<sup>−/−</sup> it had been reached within about 2.5 h.

From these results it is possible to estimate the rate of rhodopsin formation during the steady illumination. Thus, it can be shown that the maximal rate of pigment regeneration is obtained (to a reasonable approximation) as the final steady fractional pigment level, <em>P</em><sub>f</sub>, multiplied by the initial rate of pigment depletion, <em>L</em> (which is proportional to the light intensity); i.e. <em>v</em> = <em>P</em><sub>f</sub> <em>L</em> (see Equation A16 of Ref. 32). Hence, for the Rgr<sup>−/−</sup> (blue) symbols in Fig. 3B, the final steady level is <em>P</em><sub>f</sub> ≈ 115 pmol, or 22%; whereas for all conditions the initial rate of bleaching (dotted straight line) was approximately <em>L</em> ≈ 1.5 h<sup>−1</sup>, meaning that the initial decline of pigment intersects the time axis at about 40 min (i.e. at roughly 1/1.5 h). Hence, for Rgr<sup>−/−</sup>, an approximate value for the rate of rhodopsin formation is calculated as <em>v</em> = 120 pmol × 1.5 h<sup>−1</sup> = 180 pmol h<sup>−1</sup>. Perhaps surprisingly, this estimate of the rate of rhodopsin formation during illumination is more than 4-fold higher than the corresponding value obtained in Fig. 3A for the rate of rhodopsin regeneration in darkness, in the same strain.

This preliminary analysis can be extended, by plotting the time course of pigment depletion predicted theoretically by the MLP rate-limited model, as given in Equation A12 of Ref. 32. Curves for the three strains are shown (Fig. 3B), with each curve constrained to a common initial rate of pigment bleaching, <em>L</em> ≈ 1.56 h<sup>−1</sup>. The fractional rates of rhodopsin formation in the three traces were <em>v</em> = 0.96 (WT), 0.36 (Rgr<sup>−/−</sup>), and 0.84 (Rpe65<sup>−/−</sup>) h<sup>−1</sup>, corresponding to absolute rates of 495 (WT), 185 (Rgr<sup>−/−</sup>), and 433 (Rpe65<sup>−/−</sup>) pmol h<sup>−1</sup>. As during regeneration in darkness, the rate of regeneration was roughly 3-fold higher in WT mice as compared with Rgr<sup>−/−</sup>. However, each of the calculated rates of rhodopsin formation in steady light is 4–5-fold higher than the corresponding rate in darkness; the individual acceleration ratios are 4.2 (WT), 4.6 (Rgr<sup>−/−</sup>), and 4.2 (Rpe65<sup>−/−</sup>); see Table I. Hence, we conclude that the rate of rhodopsin formation during steady illumination is considerably faster than the rate of rhodopsin formation in darkness, in each of these three genotypes. Thus, irrespective of the presence of RGR, and despite variations in the level of RPE65, the rate of rhodopsin formation is accelerated by at least 4-fold in the presence of a steady light of only moderate intensity (i.e. a light that would take ~38 min to bleach the pigment to 1/e in the absence of any regeneration at all; see dashed black curve in Fig. 3B).

The regeneration of rhodopsin during light exposure may depend on three pathways: 1) the classical visual cycle requiring RPE65 (5); 2) the postulated photic visual cycle involving RGR-dependent photosomerization (7); and/or 3) on photoreversal (34, 35), the instantaneous, non-metabolic regeneration of rhodopsin (or P500 see below) by short wavelength light. For the results displayed in Fig. 3B we reject photoreversal as a significant factor on several grounds. First, the marked difference between Rgr<sup>−/−</sup> and other strains would not be expected if photoreversal were a major factor. Second, we calculated the expected rate of photoreversal in this experiment. From our measurement of the spectral composition of illumination (data not shown), together with the normalized spectral sensitivity profiles for metarhodopsin II and rhodopsin, we calculated the relative light absorption by metarhodopsin II to be less than 3% of that by rhodopsin (ignoring self-screening and absorption by the ocular media). When account is taken of the slightly higher extinction coefficient of metarhodopsin II compared with rhodopsin (~1.2 times), but the significantly lower quantum efficiency of isomerization (~0.3 times), then we estimate that with equal mole quantities of metarhodopsin II and rhodopsin the white light in our experiments would induce photoreversal at ~1% of the rate of photobleaching. For the basis of these calculations, see for example, Refs. 34–39. Third, the results of Bartl et al. (37) indicate that the chromophore extracted after photoreversal (from the species they term P500) is predominantly all-trans-retinal, rather than 11-cis-retinal. However, our measurements of retinoid content during steady illumination (not shown) showed the time course of formation of 11-cis-retinal to be indistinguishable from the time course of formation of rhodopsin. From these three arguments we conclude that photoreversal contributed minimally to the rate of pigment regeneration deduced from the results of Fig. 3B.

**DISCUSSION**

Deletion of RGR Slows 11-cis-Retinal Synthesis in Darkness and Light—In this study we analyzed the contribution of RGR to 11-cis-retinal synthesis, by measuring the regeneration of rhodopsin both in darkness after a bleaching exposure, and also in the presence of steady light, in WT and Rgr<sup>−/−</sup> mice. The regeneration of rhodopsin and the recovery of the total quantity of 11-cis-retinal exhibited indistinguishable kinetics under all conditions (Figs. 4 and supplemental materials, Fig. S4). Furthermore, under the experimental conditions applied, rhodopsin/11-cis-retinal regeneration in mice of all genotypes analyzed here appeared to be limited by the rate of conversion of retinyl esters (Fig. 6).

The rate of rhodopsin regeneration is known to be critically dependent on the level of RPE65, which in turn depends on whether the Leu<sup>450</sup> or Met<sup>450</sup> variant of RPE65 is expressed (16, 21). Therefore, to avoid complicating the comparisons, we were careful to use only mice expressing the Met<sup>450</sup> variant of RPE65. Furthermore, because the expression levels of RGR and RPE65 appear interdependent (Fig. 1), we also measured rhodopsin regeneration in mice of a second control line, Rpe65<sup>−/−</sup>, which nearly express wild type levels of RGR but have levels of RPE65 as Rgr<sup>−/−</sup> mice (Figs. 2B and supplemental materials, Fig. S2). Comparison of the rates of regeneration in the three lines establishes incontrovertibly that the much lower regeneration rates in Rgr<sup>−/−</sup> mice, both in darkness and light (Fig. 3), are due to the absence of RGR, and not to altered levels of RPE65. Future experiments have been designed to reveal the influence of the RPE65 variant with Leu<sup>450</sup>, as it is expected that increased levels of RPE65 could alter the outcome of such a comparison. Furthermore, using Rgr<sup>−/−</sup> mice with the Leu<sup>450</sup> variant of RPE65 might help to clarify how lack of RGR influences the levels of RPE65. Currently the best hypothesis is that RGR somehow stabilizes RPE65 by means of a protein-protein interaction. This hypothesis would be in line with the following: RGR and RPE65 seem to interact directly (52) or indirectly via other proteins of the visual cycle (13, 51). On the other hand, the amount of RPE65 or its variant at position 450 seems to influence the amount of RGR (Fig. 1). Nevertheless, complete lack of either RGR or RPE65 does not lead to complete loss of the respective partner as Rgr<sup>−/−</sup> mice express RPE65, and Rpe65<sup>−/−</sup> mice express RGR (14).

In addition to RPE65 (5, 40, 41), the conversion of retinyl esters to 11-cis-retinal involves two other proteins: 1) RDH5, converting 11-cis-retinol to 11-cis-retinal (25); and 2) CRALBP, a 11-cis-retinal-binding protein that pulls the reaction towards
its end product, 11-cis-retinal (24). Our comparison of WT and Rgr$^{-/-}$ mice revealed that CRALBP and RDH5 protein levels were largely unaffected by the deletion of RGR (Fig. 2). In addition, we found no accumulation of 11-cis-retinal in this mouse strain. Therefore, the absence of RGR directly or indirectly affects the isomerohydrolase reaction. This view is supported by the observation of elevated retinyl ester levels in Rgr$^{-/-}$ mice under photic conditions (7) and in darkness after bleaching (Fig. 6).

RGR Does Not Function as a “Photoisomerase” in Vivo—If the role of RGR is to accelerate rhodopsin regeneration in the presence of light, then lack of RGR should influence regeneration under photic conditions but not in darkness. Comparison of regeneration rates in mice expressing equal levels of RPE65, but either with or without RGR, shows that RGR plays a critical role in rhodopsin regeneration in both light and darkness. Our analysis in Fig. 3 and Table I shows that the rate of regeneration in mice with RGR is nearly 3-fold higher than in mice lacking the protein, and (as discussed in the next section) this factor applies not only during the presence of dim steady illumination, but also in darkness after a bleach. Accordingly, it must be concluded that in vivo, and under these conditions, RGR does not act as a photoisomerase, but rather as a cofactor in events preceding or attending the synthesis of 11-cis-retinal.

RGR Acts “Multiplicatively” to Increase the 11-cis-Retinal Synthesis Rate 2-3-fold—The effect of RGR on the synthesis of 11-cis-retinal can be characterized as multiplicative. Thus, comparing regeneration in Rgr$^{-/-}$ and Rpe65$^{-/-}$ mice (which differ in RGR levels, but have equal RPE65 levels), the presence of RGR is associated with a regeneration rate that is 2.6-fold higher in darkness, and 2.3-fold higher in the light. Comparing Rgr$^{-/-}$ with WT (which have 2.3-fold higher levels of RPE65), the presence of RGR is associated with accelerations of 2.9-fold in darkness and 2.7-fold in the light (Table I). Put another way, the ratios of the rates in the two control lines to the rates in Rgr$^{-/-}$ are approximately conserved quantities; thus, for Rgr$^{-/-}$:Rpe65$^{-/-}$:WT, the ratios are 1:2.6:2.9 (in darkness) and 1:2.3:2.7 (in the light). As the regeneration rates in light in all three lines are ~4-fold higher than those in darkness (Table I), the RGR-dependent multiplicative effect occurs over a substantial range of rates.

Constraints on Models for the Role of RGR—How can the novel results presented here be integrated into a theory of 11-cis-retinal synthesis? To address this question we first summarize the key constraints that such a theory must incorporate. 1) RGR is an integral membrane protein (a G protein-coupled receptor) that co-precipitates with various sER proteins (13, 51), including RPE65 (42, 52), RDH5 (43), and CRALBP; thus, RGR resides in the sER membrane. 2) Although the specific protein responsible for the isomerohydrolase activity has not yet been identified, there is compelling evidence that it also resides in the sER membrane; thus, RPE cell microsomal fractions in which isomerohydrolase activity is present include the sER (44, 45), the classic site of cellular processing of lipids. 3) All-trans-retinyl esters are undoubtedly the substrate for the isomerohydrolase (19, 46, 47). 4) RPE65 is an all-trans-retinyl ester-binding protein with picomolar affinity (40, 41), and is postulated to act by presenting the all-trans-retinyl ester substrate to the isomerohydrolase (19, 40, 41). 5) In RPE cells of the mouse, retinyl esters are stored in “retinyl ester storage particles” (RESTs), which are physically separate from the sER: the REST size co-varies with extractable esters in various strains of mice, and its size also expands during the influx of all-trans-retinyl after a bleaching exposure, and subsequently contracts (48). The physical separation of the localized REST from the sER suggests that a soluble carrier is required to transfer esters from the REST to the sER, where the isomerohydrolase resides. 6) RPE65 exists in two intercon-vertible forms, membrane-bound (mRPE65) and soluble (sRPE65) (49), and there appears to be a signal-dependent mechanism for switching between the two forms. 7) In mice lacking RGR, the rate of formation of 11-cis-retinal is lowered despite a substantially elevated level of retinyl esters. Slowed regeneration of 11-cis-retinal occurs although Rgr$^{-/-}$ mice express levels of RPE65 that in Rpe65$^{-/-}$ mice are sufficient to
regenerate 11-cis-retinal with rates close to those observed in WT mice (Fig. 6A).

New Hypotheses about the Role of RGR in 11-cis-Retinal Synthesis—Taken together, the observations presented in the previous paragraph suggest that RGR acts to facilitate the mobilization of all-trans-retinyl esters from storage, and/or their delivery by RPE65 to the isomerohydrolase. On the other hand, RPE65 itself might be the isomerohydrolase (54), and its activity might be enhanced by an interaction with RGR. A number of lines of evidence, however, have been presented that suggest this to be unlikely (40, 41). Accordingly, we also consider other possibilities.

Certain motifs in the sequence of RGR conform to a pattern typical for G protein-coupled receptors (50). Thus, one plausible hypothesis is that RGR is a functional G protein-coupled receptor that activates a cascade that mobilizes RPE65. Accordingly, when RGR binds its proper ligand (all-trans-retinal), it could activate a G-protein cascade, whose effector would mobilize RPE65, allowing it to become more effective in transporting all-trans-retinyl esters from the REST to the isomerohydrolase at the sER (Fig. 7A). The occurrence of a rate of 11-cis-retinal regeneration around 2.5-fold lower, and a dark level of retinyl esters around 3-fold higher, in Rgr−/− mice compared with Rpe65+/− controls would then be explained, because in the absence of RGR, the carrier RPE65 would remain predominantly in a form unable to mobilize the ester pool. Such a mechanism would be distinct from, but possibly related to, that proposed to regulate the interconversion of sRPE65 to mRPE65 (49).

Another plausible hypothesis is that RGR serves to coordinate the “docking” of RPE65 with the isomerohydrolase, perhaps in a manner dependent on the ligand state of RGR (Fig. 7B). Thus, RPE65 might be less efficient in delivering all-trans-retinyl ester substrate to the isomerohydrolase in the absence of the “helper” RGR. Both hypotheses are examples of ways in which RGR might facilitate delivery of substrate to the isomerohydrolase by RPE65, and in doing so satisfy the seven constraints listed above.

Light Triggers Increased 11-cis-Retinal Synthesis—We found that dim illumination had an ~4-fold effect in accelerating rhodopsin regeneration, independent of RGR (Fig. 3). One plausible candidate would be the light-dependent palmitoylation of sRPE65 to mRPE65: palmitoylation of RPE65 accelerates the delivery of retinyl esters to the isomerohydrolase (49). What remains unclear is whether this latter mechanism also serves to facilitate mobilization of esters by RPE65 from the RESTs. This light-dependent acceleration of visual pigment regeneration has to be analyzed in more detail, for example, regarding the influence of different wavelengths.

The Essential and Dynamic Role of the Retinyl Ester Pool in the Visual Retinoid Cycle—It is now firmly established that all-trans-retinyl esters are the substrate for the isomerohydrolase that produces the visual chromophore, 11-cis-retinal (19, 46, 47). The evidence presented here contributes to a refined view of the dynamic role of the retinyl ester pool of the RPE cell in the retinoid cycle of vision. First, the approximate conservation of total retinoid in the eye, for each mouse strain (Fig. 6). The occurrence of a rate of 11-cis-retinal regeneration as the rate is 2.3–to 2.9-fold lower in than in controls (Table I), despite Rgr−/− mice having a roughly 3-fold higher level of esters in the dark-adapted state, and close to 2-fold higher level at the beginning of regeneration in the dark (Fig. 6A). Accordingly, our results suggest that the rate of synthesis of 11-cis-retinal is set by factors other than the level of ester. This is consistent with the notion that a tightly regulated control system exists, which sets the concentration of 11-cis-retinal and the rate of rhodopsin regeneration (3).

Thus, it appears that 11-cis-retinal synthesis is switched “on” and “off” in a manner determined by the need for chromophore. Based on the essential role of retinyl esters as substrate for the isomerohydrolase, and the hypothesized role of RPE65 as a retinyl ester-binding protein that delivers the substrate, the simplest hypothesis is that the switch modulates the availability of RPE65 as a shuttle for ester toward the isomerohydrolase, as proposed by Xue et al. (49). Our work then adds the new insight that RGR may contribute to throwing the switch to its on position. The determination of m- and sRPE65 levels in WT and Rgr−/− mice under different light conditions might reveal this function of RGR.

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