Function of mammalian M-cones depends on the level of CRALBP in Müller cells

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Cone photoreceptors mediate daytime vision in vertebrates. The rapid and efficient regeneration of their visual pigments following photoactivation is critical for the cones to remain photoresponsive in bright and rapidly changing light conditions. Cone pigment regeneration depends on the recycling of visual chromophore, which takes place via the canonical visual cycle in the retinal pigment epithelium (RPE) and the Müller cell–driven intraretinal visual cycle. The molecular mechanisms that enable the neural retina to regenerate visual chromophore for cones have not been fully elucidated. However, one known component of the two visual cycles is the cellular retinaldehyde-binding protein (CRALBP), which is expressed both in the RPE and in Müller cells. To understand the significance of CRALBP in cone pigment regeneration, we examined the function of cones in mice heterozygous for Rlbp1, the gene encoding CRALBP. We found that CRALBP expression was reduced by ∼50% in both the RPE and retina of Rlbp1+/− mice. Electoretinography (ERG) showed that the dark adaptation of rods and cones is unaltered in Rlbp1+/− mice, indicating a normal RPE visual cycle. However, pharmacologic blockade of the RPE visual cycle revealed suppressed cone dark adaptation in Rlbp1+/− mice in comparison with controls. We conclude that the expression level of CRALBP specifically in the Müller cells modulates the efficiency of the retina visual cycle. Finally, blocking the RPE visual cycle also suppressed further cone dark adaptation in Rlbp1+/− mice, revealing a shunt in the classical RPE visual cycle that bypasses CRALBP and allows partial but unexpectedly rapid cone dark adaptation.

Introduction

The regeneration of photobleached visual pigments in retinal photoreceptors, rods and cones, underlies sustained vision in all vertebrate species. In these primary visual neurons, the initial absorption of a photon by the rod pigment (rhodopsin) or different cone pigments triggers the photoconversion of the visual chromophore, 11-cis-retinal, to its all-trans form. This isomerization activates the phototransduction cascade and generates the physiological light response. All-trans-retinal is then released from photoreceptors and recycled back to 11-cis-retinal via either the canonical retinal pigment epithelium (RPE)-driven visual cycle (which delivers chromophore to both rods and cones) or the Müller cell–mediated retina visual cycle serving the cones exclusively (reviewed by Wang and Kefalov, 2011; Tang et al., 2013; Kiser et al., 2014). Direct photoisomerization of phospholipid-bound all-trans-retinal to 11-cis-retinal with blue light, similar to that occurring within invertebrate opsins, has also been reported (Kolesnikov et al., 2006; Kaylor et al., 2017).

The cone-specific pathway and its critical molecular players are active areas of research (Kaylor et al., 2013; Kaylor et al., 2014; Sato and Kefalov, 2016; Sato et al., 2017; Xue et al., 2017; Kiser et al., 2019). A common feature of the classic RPE and intraretinal visual cycles is their complement of chromophore-binding proteins facilitating the process of recycling of very hydrophobic retinoids. One of these, cellular retinaldehyde-binding protein (CRALBP), is essential for mammalian cone pigment regeneration (Saari et al., 2001; Xue et al., 2015). CRALBP is abundantly expressed, both in Müller cells of the retina and in the RPE (Bunt-Milam and Saari, 1983; Saari et al., 2001), and its complete elimination severely compromises the cone-mediated daylight vision and dark adaptation of M-cones in mice (Xue et al., 2015). Structurally, CRALBP is a 36-kD globular protein capable of adopting two distinct conformational states for the binding and intracellular transport of both 11-cis-retinol and 11-cis-retinal (Futterman et al., 1977; Saari and Bredberg, 1987; Liu et al., 2005; He et al., 2009).

Numerous mutations in human CRALBP (encoded by the RLBP1 gene) have been implicated in several retinal diseases, including autosomal recessive retinitis pigmentosa (Maw et al.,...
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Study we performed for the Leu450 allele of Rlbp1+/− to assess whether the amount of CRALBP in the RPE modulates the expression and the contribution of newly regenerated chromophore. In addition, it is important to questioning by using mice with reduced (halved) or ablated CRALBP expression and/or pharmacologically blocked regeneration of visual chromophore. 

Western blotting
Whole mouse eyes or isolated retinas were retrieved from storage at −80°C and placed in PBS containing a 1× concentration of protease inhibitors (Bimake). Individual eyes were subjected to 30 passes in a Dounce homogenizer, and isolated retinas were lysed by sonication. Protein concentrations of the resulting lysates were determined using the Bradford assay (Pierce) by reading the sample 595-nm absorbances along with those of BSA standards together in a FlexStation 3 plate reader (Molecular Devices). The protein concentrations were equalized across each of the samples by the addition of lysis buffer. The lysates were then stored at −80°C until needed for further analysis. Protein samples of 35 μg (whole eyes) or 7 μg (isolated retinas) in standard loading buffer were separated by SDS-PAGE on a 12% gel in Tris-glycine running buffer. The proteins were then transferred onto a 0.45-μm polyvinylidene difluoride membrane using an eBlot semidry transfer buffer. The proteins were then transferred onto a 0.45-μm polyvinylidene difluoride membrane using an eBlot semidry transfer system (GenScript) according to the manufacturer’s instructions. The membrane was dried for 1 h and then rewetted with 100% methanol followed by the addition of a blocking mixture consisting of 5% nonfat milk in PBS containing 0.05% Tween 20 (PBST; Sigma-Aldrich). After 1-h incubation at room temperature (RT) with gentle rocking, the membrane was transferred into 2 ml of 5% nonfat milk in PBST. Rabbit anti-CRALBP (UW55) and mouse anti-tubulin (Ab7291; Abcam) antibodies were then added to the membrane incubation mixture at dilutions of 1:2,000 and 1:5,000, respectively. After a 1-h incubation at RT with gentle rocking, the primary antibody mixture was decanted, and the membrane was washed three times with 5 ml PBST, each time for 5 min. The membrane was then incubated in 5% nonfat milk PBST mixture containing goat anti-rabbit (IRDye 800CW; LI-COR Biosciences) and donkey anti-mouse (IRDye 680RD; LI-COR Biosciences) secondary antibodies, both at 1:20,000 dilution. After a 1-h incubation at RT with gentle rocking, the secondary antibody mixture was decanted, and the membrane was washed three times with 5 ml PBST, each time for 5 min. The membrane was then incubated in PBS for 3 min and then analyzed for fluorescent labeling using the 700-nm and 800-nm channels on a LI-COR Odyssey blot imager (LI-COR Biosciences). The image contrast and coloring were globally adjusted using Adobe Photoshop. CRALBP signals were normalized to those of the tubulin loading controls using ImageJ software (Schneider et al., 2012).

Materials and methods

Animals
CRALBP-deficient (Rlbp1−/−) mice were described previously (Saari et al., 2001). Rod transducin α-subunit–knockout (Gnat1−/−) mice lacking rod signaling (Calvert et al., 2000) were used as controls in all cone physiological experiments. The two lines were also crossed to generate Rlbp1−/−Gnat1−/− and Rlbp1−/−Gnat1−/− animals (for cone recordings) or Rlbp1−/−/Gnat1−/− and control Rlbp1+/+Gnat1−/− lines (for rod recordings). All mice used in this study were homozygous for the Leu450 allele of Rpe65 as determined by a genotyping protocol published elsewhere (Grimm et al., 2004) and free of Crlb1/rdi8 mutation (Mattapallil et al., 2012). Young adult animals of either sex (2–4 mo old) were tested in all experiments. Mice were provided with standard chow (LabDiet 5053; LabDiet, Purina Mills) and maintained under a 12-h light (10–20 lux)/12-h dark cycle. All experiments were approved by the Washington University Animal Studies Committee.

Transretinal (ex vivo electroretinography [ERG]) recordings from isolated retinas
Mice were dark adapted overnight and then euthanized by asphyxiation with a rising concentration of CO2, and a whole retina was removed from each mouse eye cup under infrared illumination and stored in oxygenated aqueous L15 (13.6 mg/ml, pH 7.4; Sigma-Aldrich) solution containing 0.1% BSA at RT. The
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sought to use a mouse model in which the total amount of this important visual cycle protein is reduced substantially. Therefore, we generated a mouse line heterozygous for the gene encoding CRALBP (Rlbp1+/−). In addition, to facilitate cone recordings, these animals were derived on the Gnat1−/− genetic background. The lack of the transducin α-subunit eliminates the rod component of the light response in mice without affecting cone morphology or function (Calvert et al., 2000).

Western blotting analysis demonstrated that the expression of CRALBP in whole Rlbp1−/− mouse eyes was reduced by 50% (Fig. 1A). Because this protein is abundantly expressed in both the Müller cells of the retina and RPE (Bunt-Milam and Saari, 1983; Saari et al., 2001), we also quantified its level in isolated retina samples. Again, we found ~40% decrease in expression of CRALBP in retinas from Rlbp1−/− animals (Fig. 1B), suggesting similar reduction of its expression in RPE and Müller cells. As expected, no CRALBP protein was detected in the eyes or retinas from Rlbp1−/− mice (Fig. 1A and B, right).

We then recorded a series of M-cone transretinal (ex vivo ERG) responses elicited by test flashes of increasing light intensity (Fig. 2A and B). The recordings were performed following the overnight dark adaptation of tested animals from their isolated retinas in the presence of post-synaptic blockers. Our analysis was limited to M-cones, which can be selectively stimulated with visible green light. We found that the maximal cone response amplitude in retinas of 2-mo-old Rlbp1−/− mice was indistinguishable from that in age-matched Gnat1−/− controls (Fig. 2A, A–C). The cone photosensitivity in Rlbp1−/− mice (defined as I1/2) was also unaltered (Fig. 2C).

Surprisingly, we found that M-cone dim flash responses in CRALBP-reduced mice were accelerated as compared with those in control animals (Fig. 2D). While the activation phase of cone phototransduction estimated from the rising part of the responses was similar in the two strains, the inactivation phase of the cascade was noticeably faster in Rlbp1−/− mice. The time-to-peak values of cone dim flash responses were 87 ± 3 ms (control; n = 11) and 60 ± 2 ms (Rlbp1−/−Gnat1−/−; n = 9; P < 0.05); yet, both were within the range of 60–90 ms described for mouse cones previously (Nikonov et al., 2006). The average dim flash recovery time constants determined from single-exponential fits to the falling phase of cone photoreponses after their peak were 78 ± 5 ms (control; n = 11) and 61 ± 3 ms (Rlbp1−/−Gnat1−/−; n = 9; P < 0.05). Still, in both strains, the dim flash responses completely recovered to a baseline within 400 ms following the test flash of 2.4 × 10^3 photons μm^2. Similarly, M-cone saturated responses appeared to be slightly faster in the mutant line (Fig. 2 D, inset). The mechanism producing this subtle difference in response kinetics is unclear. It is likely that it originates in cone phototransduction, as the kinetics of cone transretinal responses closely follow those recorded from individual cells by a suction electrode (Sakurai et al., 2011). However, it is also possible that the transretinal ERG responses are affected by voltage changes originating in the photoreceptor inner segments and axons (reviewed by Robson and Frishman, 2014). Importantly, the deletion of CRALBP did not cause any cone desensitization in CRALBP-deficient animals (Fig. 2C).

Thus, the reduced CRALBP expression did not affect the overall health and function of dark-adapted cones and only accelerated moderately the inactivation of phototransduction in mouse M-cone photoreceptors in young adult mice. Overall, this allowed us to investigate the effect of decreased CRALBP level on dark adaptation of these cones as well as on their function in bright light.

**Suppressed M-cone dark adaptation in mice with reduced expression of CRALBP**

Cones constitute only a small fraction of the photoreceptors in most mammalian retinas, which makes it impossible to study their visual pigment regeneration and chromophore recycling by biochemical means. However, as the sensitivity of cones is
strongly dependent on the level of visual pigment regeneration (Jones et al., 1989), it is possible to study the supply of chromophore to cones by the two visual cycles using measurements of cone sensitivity by electrophysiological recordings. In the case of mice, the electrical response generated by the cones (photopic ERG a-wave) is too small to be readily and reliably measured in vivo. Instead, we used the photopic ERG b-wave, a voltage signal recorded from the downstream bipolar cells, to monitor the sensitivity and, indirectly, the regeneration of pigment in mouse M-cones.

To address the possible role of CRALBP expression level on cone function in mice, we performed physiological experiments in live mice (Fig. 3). The recovery of M-cone $S_T$ after acute (>90%) bleaching of cone visual pigment with 520-nm LED light (35-s exposure) was monitored in intact eyes with full-field ERG recordings. Under these noninvasive conditions, the photoreceptors remain in their native microenvironment and cone pigment regeneration is driven exclusively by the intraretinal (Müller cell) visual cycle. In our previous study, this drug effectively blocked the RPE visual cycle-driven component of cone sensitivity recovery (Kiser et al., 2018). Under these conditions, cone dark adaptation is driven exclusively by the intraretinal (Müller cell) visual cycle. The treatment did not change the $S_{SP}$ in both control Gnat1$^{+/+}$ mice (0.75 ± 0.03 m$^2$ cd$^{-1}$ s$^{-1}$; $n = 18$ in the treated cohort versus $0.61 ± 0.07$ m$^2$ cd$^{-1}$ s$^{-1}$; $n = 8$ in the untreated group; $P > 0.05$) and Rlbp1$^{−/−}$ mice (0.90 ± 0.04 m$^2$ cd$^{-1}$ s$^{-1}$; $n = 20$; $P > 0.05$) mice. We then used these respective dark-adapted values to normalize all subsequent measurements and derive relative recovery time courses. Following a nearly complete pigment bleach, cones initially were desensitized by ~1.3 log units and then gradually recovered the bulk of their sensitivity in the dark. In this experiment, we found that the dark adaptation of M-cones after such acute pigment bleach was unaltered in Rlbp1$^{−/−}$ mice (Fig. 3 A) suggesting that the removal of approximately one-half of the CRALBP protein was insufficient to suppress the recovery of cone responsiveness in vivo.

The M-cone dark adaptation in the intact mouse eye is generally biphasic and the retina visual cycle contributes to its initial rapid phase (Kolesnikov et al., 2011), while a second (slower) component reflects visual pigment regeneration driven by the RPE visual cycle (Kolesnikov et al., 2015). However, more recent research has suggested that the interplay between the two phases of cone dark adaptation could be more complex in vivo (Kiser et al., 2018). To better dissect the role of CRALBP in maintaining the function of the retina visual cycle in live animals, MB-001, a potent selective RPE65 inhibitor of the retinylamine/emixustat family, was IP-injected into a cohort of mice, 22–26 h before performing similar ERG experiments. In our previous study, this drug effectively blocked the RPE visual cycle-driven component of cone sensitivity recovery (Kiser et al., 2018). Under these conditions, cone dark adaptation is driven exclusively by the intraretinal (Müller cell) visual cycle. The treatment did not change the $S_{SP}$ in both control Gnat1$^{+/+}$ mice (0.75 ± 0.03 m$^2$ cd$^{-1}$ s$^{-1}$; $n = 18$ in the treated cohort versus 0.61 ± 0.07 m$^2$ cd$^{-1}$ s$^{-1}$; $n = 8$ in the untreated group; $P > 0.05$) and Rlbp1$^{−/−}$ mice (0.90 ± 0.04 m$^2$ cd$^{-1}$ s$^{-1}$; $n = 20$ in the treated group versus 0.85 ± 0.03 m$^2$ cd$^{-1}$ s$^{-1}$; $n = 8$ in the untreated cohort; $P > 0.05$). However, MB-001 unmasked the effect of the reduced CRALBP level on M-cone dark adaptation in Rlbp1$^{−/−}$ mice, which was suppressed under these conditions (Fig. 3 B). Thus, maintaining the proper level of CRALBP in Müller cells of the retina is important for the normal speed of visual pigment regeneration in mouse M-cones.

Prolonged bright light exposure further compromises the dark adaptation of M-cones in mice with reduced CRALBP expression

To gain further insight into the significance of CRALBP expression level for cone function in response to extended bright light in vivo, we performed the following ERG experiments. After recording the b-wave $S_T$ of dark-adapted M-cones, we

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Figure 2. Sensitivity ($I_{1/2}$) and kinetics of M-cone photoresponses in Rlbp1$^{−/−}$ mice. (A and B) Representative families of M-cone transretinal ERG responses from 2-mo-old control Gnat1$^{+/+}$ (A) and Rlbp1$^{−/−}$/Gnat1$^{+/−}$ (B) animals. Flash strengths were increased from 2.4 × 10$^3$ to 6.0 × 10$^5$ photons µm$^{-2}$ by steps of ~0.5 log units (505-nm light). (C) Averaged cone intensity–response functions (mean ± SEM) for isolated control Gnat1$^{+/−}$ ($n = 10$) and Rlbp1$^{−/−}$/Gnat1$^{+/−}$ ($n = 9$) retinas. Points were fitted with Naka-Rushton hyperbolic functions (see Materials and methods). The fits yielded $I_{1/2}$ values of 2.4 × 10$^4$ and 2.8 × 10$^4$ photons µm$^{-2}$ for control and mutant animals, respectively ($P > 0.05$). Error bars represent SEMs. (D) Population-averaged normalized dim flash cone responses ($R/R_{max}$) to test stimuli of 2.4 × 10$^5$ photons µm$^{-2}$ for control Gnat1$^{+/−}$ ($n = 11$) and Rlbp1$^{−/−}$/Gnat1$^{+/−}$ ($n = 9$) mice. The inset shows population-averaged normalized saturated cone responses to test stimuli of 6.0 × 10$^5$ photons µm$^{-2}$ in the same retinas of the two mouse lines. Error bars represent SEMs.
applied a bright 530-nm Ganzfeld light (300 cd/m²) for 30 min, which we estimated would bleach ~0.8% of the M-cone pigment per second. This induced a rapid 2-log-unit cone desensitization due to background light adaptation (Fig. 4 A). In accordance with our earlier studies (Kolesnikov et al., 2015), we observed a small transient increase in cone b-wave $S_b$ (presumably as a result of retina network adaptation) in both control and Rlbp1−/− animals. The short peak of $S_b$ stability observed after 2-4 min of continuous illumination was then followed by its gradual decline. Although starting with somewhat lower sensitivity at the light onset, by the end of the background light exposure 30 min later, the cones in Rlbp1−/− mice were desensitized to an identical degree (216-fold relative to their dark-adapted state) to those in control animals ($P > 0.05$).

We then applied an additional bright 35-s 520-nm LED light to bleach the remaining M-cone pigment and afterward followed the recovery of photopic ERG b-wave sensitivity in the dark (Fig. 4 A, right; and Fig. 4 B). This allowed us to dissect a possible role of CRALBP level in dark adaptation of cone photoreceptors in a situation where any potential pools of cis-retinoids (in Müller cells and/or the RPE) available to dark-adapted cones in a situation where any potential pools of cis-retinoids (in Müller cells and/or the RPE) available to dark-adapted cones should already be depleted. We observed that, similar to the case in human cones (Mahroo and Lamb, 2012), the overall rate of cone dark adaptation in control mice was decelerated substantially under these conditions, as compared with those treated with the equivalent 35-s bleaching under dark-adapted conditions (compare Fig. 4 B with Fig. 3 A). However, both the retina- and RPE-driven phases of cone dark adaptation were still unaffected in animals with reduced CRALBP expression in their eyes.

Finally, we repeated this experiment in mice pretreated with the blocker of the RPE visual cycle, MB-001 (Fig. 5). Although there was no overall statistically significant effect of this compound on M-cone sensitivity under extended bright light conditions over the 30-min period in Rlbp1−/− mice (Fig. 5 A), their subsequent dark adaptation was greatly compromised as compared with drug-treated controls (Fig. 5 B). Thus, our results demonstrate that the normal expression level of CRALBP in Müller cells is required for the proper function of the retina visual cycle and the efficient cone dark adaptation after intense illumination.

M-cones can maintain a moderate level of pigment regeneration in the absence of CRALBP

It was demonstrated recently that the complete elimination of CRALBP has a dramatic effect on the ability of the retina visual cycle to promote mouse cone dark adaptation, both in vivo and ex vivo (Xue et al., 2015). However, mouse M-cones were still
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Consistent with previous observations, we found a substantial (two- to threefold) decrease in photopic ERG b-wave $S_b$ even in fully dark-adapted mice lacking CRALBP (Fig. 6 A, left). As expected, their cone dark adaptation after an acute, nearly complete pigment bleach was also greatly compromised (Fig. 6 A, red circles) compared with controls (Fig. 6 A, black circles). Similar to what we found in Rlbp1$^{-/-}$ animals described above, MB-001 did not reduce the maximal ERG b-wave amplitude ($157 \pm 11 \mu V; n = 8$ in the treated group versus $166 \pm 18 \mu V; n = 6$ in the untreated mice; $P > 0.05$) or sensitivity ($0.40 \pm 0.05 \text{ m}^2 \text{ cd}^{-1} \text{ s}^{-1}; n = 8$ in the treated cohort versus $0.37 \pm 0.03 \text{ m}^2 \text{ cd}^{-1} \text{ s}^{-1}; n = 6$ in the controls; $P > 0.05$) of dark-adapted M-cones in CRALBP-deficient mice. However, MB-001 suppressed further the cone dark adaptation (Fig. 6 A, red squares), which could be visualized better after normalization of the data to the corresponding prebleach cone $S_b$ (Fig. 6 B). The final level of response recovery by 60 min post-bleach in MB-001-treated Rlbp1$^{-/-}$ mice was approximately fourfold lower than in the control group, to which the compound was not administered. A small initial rapid component of cone $S_b$ recovery (within 1 min after the bleach) still observed in treated mutant animals is likely due to the inactivation of the cone phototransduction cascade. Thus, the cone recovery from a bleach in CRALBP-deficient mice could be suppressed even further when the RPE visual cycle was blocked by MB-001. Together, these findings indicate that mouse M-cones can maintain a considerable level of their pigment regeneration through a yet to be identified shunt in the classical RPE visual cycle that bypasses CRALBP.

Dark adaptation of mouse rods is unaffected by reduced CRALBP expression

The results above clearly demonstrate the importance of maintaining the normal level of CRALBP protein in the eye for the function of mouse cones in bright light. Because the turnover of visual chromophore in the rod photoreceptors can affect pigment regeneration and dark adaptation in mammalian cones as well (Kolesnikov et al., 2015), we next investigated, with in vivo significant effect during the extended light exposure ($F_{(1,12)} = 33.0; P < 0.0001$). MB-001 (red squares) further suppressed cone dark adaptation in Rlbp1$^{-/-}$Gnat1$^{-/-}$ (n = 8) mice compared with untreated mutants ($F_{(1,22)} = 33.0; P < 0.0001$). The data in A were normalized to their own $S_{DA}$ in each particular case. The effects of genotype and drug treatment remained highly significant after normalization ($F_{(1,22)} = 17.0; P = 0.0005$; and $F_{(1,22)} = 14.4; P = 0.0025$, respectively). Error bars for some points in A and B are smaller than the symbol size. DA refers to sensitivity of dark-adapted cones.

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Discussion

Efficient pigment regeneration plays a critical role in the ability of cone photoreceptors to function throughout the day in bright and rapidly changing light conditions. Mounting evidence indicates that this is achieved via parallel supplies with chromophore from the canonical visual cycle involving the RPE and from the retina visual cycle involving the Müller glial cells (Fleisch et al., 2008; Wang and Kefalov, 2009; Kolesnikov et al., 2011; Xue et al., 2015; Sato et al., 2017; Kiser et al., 2019; Morshedian et al., 2019; Ward et al., 2020). However, even though the functional significance of the retina visual cycle has now been well documented in a wide range of species, the molecular components of this pathway still remain largely unknown and controversial (Kaylor et al., 2013; Xue et al., 2017; Kiser et al., 2019; Ward et al., 2020). Here, we examined the role of one of these putative components, CRALBP. This chromophore-binding protein is expressed in both RPE and Müller cells (Bunt-Milam and Saari, 1983; Saari et al., 2001). A key feature of CRALBP is its binding selectivity for 11-cis-retinoids (Saari, 2012). As a result, in the RPE, it binds to 11-cis-retinol produced by RPE65, thus enhancing the recycling of chromophore by the RPE visual cycle (Winston and Rando, 1998; Stecher et al., 1999; Saari and Crabb, 2005; see also Fig. 8). Consistent with such a role, patients lacking functional CRALBP experience delayed dark adaptation (Burstedt et al., 2001) and a wide range of visual disorders.

The possible role of CRALBP in modulating the chromophore supply to cones is not well understood. It has been proposed that, similar to its function in the RPE, CRALBP expressed in Müller cells promotes the isomerization of all-trans-retinol back into its 11-cis form (Kaylor et al., 2013), but this has not yet been demonstrated experimentally in the retina. Another, not mutually exclusive alternative is that CRALBP in Müller cells serves as a storage site for recycled 11-cis-retinol, ready to be released and rapidly supplied to cones. Consistent with this notion, we have previously estimated that up to five CRALBP-associated 11-cis retinoids per cone pigment could be stored in Müller cells (Kiser et al., 2018). Thus, here we sought to evaluate how the expression level of CRALBP in both RPE and Müller cells affects the function of cones in dark-adapted conditions as well as during and after exposure to bright light bleaching a significant fraction of the cone visual pigment. These experiments allowed us to determine whether the expression level of CRALBP rates limits the ability of the RPE or the Müller cells to supply chromophore to cones in a timely fashion and to drive the efficient cone pigment regeneration.

Our results demonstrate that reducing twofold the expression of CRALBP in both the RPE and the Müller cells of Rlpb1−/− mice does not affect the dark-adapted photosensitivity of M-cones or the kinetics of cone dark adaptation following an acute bleach (Fig. 3 A). Similarly, the ability of these cones to function in continuous bright light or their subsequent dark adaptation following an acute bleach are also not compromised in Rlpb1−/− mice (Fig. 4). These results indicate that halving the expression of CRALBP does not slow down substantially the overall supply of chromophore to cones. However, as cone pigment regeneration in vivo is driven by the parallel function of two visual cycles, it is difficult to interpret this result in the context of each of the two pathways. Thus, we also examined separately how cone function is affected by the expression level of CRALBP in each visual cycle. Selective inhibition of the RPE visual cycle by a potent blocker of RPE65 activity, MB-001, allowed us to directly examine the effect of CRALBP expression in Müller cells on the efficiency of the retina visual cycle. We found that under these conditions the dark adaptation of M-cones following an acute pigment bleach (Fig. 3 B) and, to an even greater degree, their dark adaptation following an exposure to
continuous bright light (Fig. 5 B) were suppressed in Rlbp1+/- mice compared with controls. Together, these results clearly demonstrate that the expression level of CRALBP in Müller cells modulates the retina visual cycle (Fig. 8). The exact mechanism by which CRALBP in Müller cells affects the efficiency of cone pigment regeneration could involve transport of retinoids between cones and Müller cells, the recycling of retinoids in Müller cells, or their storage there. Notably, following dark adaptation, the function of cones in Rlbp1+/- mice recovered to normal even when the RPE visual cycle was blocked by MB-001. This is in stark contrast to the case of full CRALBP deficiency, where cone opsin is mislocalized and cone function is suppressed even after overnight dark adaptation (Xue et al., 2015).

Our experiments also allowed us to determine whether the RPE visual cycle is affected by the reduction in CRALBP expression. Since we still lack the tools to block selectively the retina visual cycle in vivo in a manner that would be nontoxic for Müller cells and other neurons in the retina, we were unable to evaluate directly the effect of CRALBP expression in the RPE on the efficiency of this visual cycle in providing chromophore to cones. However, the overall normal dark adaptation of cones in vivo when driven by both the RPE and retina visual cycles...
(Figs. 3 and 4) suggests that the RPE visual cycle efficiency is not compromised by reducing the expression of CRALBP there by twofold. A more direct examination of this question could be performed based on rod measurements, as rod pigment regeneration and dark adaptation are driven exclusively by the RPE visual cycle. Based on our finding that rod dark adaptation was comparable in control and Rlbp1−/− mice (Fig. 7), we conclude that the efficiency of the RPE visual cycle is not affected by halving the expression of CRALBP. Thus, the expression of CRALBP in the RPE is not rate limiting the recycling of chromophore by the RPE visual cycle and the pigment regeneration in either rods or M-cones (Fig. 8). We conclude that the CRALBP-driven delivery/release of chromophore to cones from the Müller cells modulates the retinal visual cycle, whereas the corresponding step in the RPE does not regulate the canonical visual cycle. Interestingly, our observation of normal rod dark adaptation in Rlbp1−/− mice is in contrast to a previous study by Saari et al. (2001), who found a slight suppression in ERG a-wave recovery in Rlbp1−/− mice following a full bleach. The difference between these results is likely to originate in the RPE65 isoform of the mice used in the two studies. The mice in the original CRALBP-knockout mouse study were on the C57BL/6 background and most likely expressed the Met450 isoform of RPE65. In contrast, our mice were homozygous for the more active Leu450 isoform of RPE65, providing a more efficient chromophore regeneration by the RPE (Wenzel et al., 2001). A recent study demonstrated that short-wavelength fundus autofluorescence, which measures the presence of visual cycle-derived bisretinoids in the retina, was reduced in asymptomatic patients with heterozygous loss-of-function mutations in RLBPI (Lima de Carvalho et al., 2020). The lack of visual impairment in these individuals is consistent with our finding that classical visual cycle activity is normal in Rlbp1−/− mice. We speculate that the human fundus autofluorescence findings may reflect a subtle slowing of visual cycle activity that preferentially reduces off-pathway (i.e., bisretinoid-forming) reactions manifesting as an asymptomatic reduction in fundus autofluorescence.

Finally, our results also reveal a shunt in the classical RPE visual cycle that bypasses CRALBP and allows partial but surprisingly rapid M-cone dark adaptation (Fig. 6). This result is reminiscent of the finding that rods in CRALBP-deficient mice restore their visual chromophore and sensitivity following extended dark adaptation (Saari et al., 2001). Similarly, patients with compound heterozygous loss-of-function mutations in the RLBPI gene can also recover their rod-specific electroretinogram signals after overnight dark adaptation (Lima de Carvalho et al., 2020). Thus, the RPE visual cycle can function even in the absence of CRALBP and trickle-down chromophore not only to rods but also to cones (Fig. 8). The relatively fast dark adaptation of cones in CRALBP-deficient mice (Fig. 6) could likely be explained by the small amount of chromophore required for the regeneration of the visual pigment in these smaller and sparse (<3%; Carter-Dawson and LaVail, 1979) photoreceptors. In contrast, the much more abundant and larger rods require greater amounts of chromophore and thus take hours to dark adapt in the absence of CRALBP. Interestingly, this finding parallels the previously demonstrated ability of the retina visual cycle also to function, albeit suboptimally, even in the absence of CRALBP and to sustain cone survival in darkness in the absence of strong demand for chromophore (Xue et al., 2015).

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