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Intra-retinal visual cycle required for rapid and complete cone dark adaptation

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Daytime vision is mediated by retinal cones, which, unlike rods, remain functional even in bright light and dark-adapt rapidly. These cone properties are enabled by rapid regeneration of their pigment. This in turn requires rapid chromophore recycling that may not be achieved by the canonical retinal pigment epithelium visual cycle. Recent biochemical studies have suggested the presence of a second, cone-specific visual cycle, although its physiological function remains to be established. We found that the Müller cells in the salamander neural retina promote cone-specific pigment regeneration and dark adaptation that are independent of the pigment epithelium. Without this pathway, dark adaptation of cones was slow and incomplete. Notably, the rates of cone pigment regeneration by the retina and pigment epithelium visual cycles were essentially identical, suggesting a possible common rate-limiting step. Finally, we also observed cone dark adaptation in the isolated mouse retina.

Phototransduction in rods and cones begins with the light-triggered isomerization of the visual pigment chromophore from 11-*cis* to all-*trans* retinal[1]. Eventually, the photoactivated pigment dissociates into free opsin and all-*trans* retinal[2], which is then reduced to all-*trans* retinol[3]. Dark adaptation of both rods and cones requires the regeneration of the visual pigment from opsin and 11-*cis* retinal[4,5]. However, the speed of pigment regeneration and therefore sensitivity recovery is very different in rods and cones, with full recovery requiring only about 5 min in cones and up to 1 h in rods[6,7]. The fast turnover of cone visual pigment required for cones to rapidly dark-adapt and to remain functional in bright light imposes a need for rapid recycling of their chromophore from all-*trans* retinol back into 11-*cis* retinal. The canonical pathway for chromophore recycling[8] involves the pigment epithelium, where all-*trans* retinol is converted into 11-*cis* retinal via a series of enzymatic reactions and then transported back to the photoreceptors for incorporation into opsin. There are reasons to think that, apart from the epithelial pathway common for rods and cones, a separate, cone-specific chromophore-recycling pathway may exist. First, although rods are nonfunctional in bright light, their pigment continues to cycle through repetitive bleaching and regeneration, acting as a sink for 11-*cis* retinal. Thus, even saturated, rods continue to use 11-*cis* retinal, thereby reducing its availability to cones. In the mouse and human retina, cones constitute only 3–5% of all photoreceptors, and cone opsin has to compete with overwhelming levels of rod opsin for recycled 11-*cis* retinal. Second, unlike the extremely stable rod pigment, cone pigment can dissociate spontaneously into opsin and 11-*cis* retinal[9,10]. Thus, cone pigment might lose its chromophore to rod pigment even after regeneration[2]. Finally, the rate of pigment regeneration required for sustaining cone function in bright light exceeds the maximal reported rate of chromophore recycling by the pigment epithelium[11].

Recent biochemical studies have uncovered a series of enzymatic reactions in the retina that are consistent with a chromophore-recycling pathway[11–15]. This pathway is possibly cone specific, as it was characterized in cone-dominant retinas such as those of chicken and ground squirrel. The emerging theory[11] is that the all-*trans* retinol released from cones is converted into 11-*cis* retinol in the retina independently of the pigment epithelium. The 11-*cis* retinol is then used by the cones, which, unlike rods, can convert 11-*cis* retinol into 11-*cis* retinal (at least in salamander)[4]. This pathway is capable of turning over chromophore 20-fold faster than the canonical pigment epithelium pathway[11]. Although these biochemical studies are widely accepted, the functional validation of this separate pathway *in situ* has not been carried out and it is not known whether this pathway can promote cone pigment regeneration or dark adaptation. Equally important, the question remains of whether such a visual cycle exists in rod-dominant retinas. In fact, two recent studies, albeit indirect, failed to find any evidence for chromophore recycling in the rod-dominant mouse retina[16,17]. Finally, it is not known what, if any, role this putative retina visual cycle has in the dark adaptation of cones. We examined these questions by combining microspectrophotometric measurements with single-cell and whole-retina recordings from amphibian and mouse photoreceptors *in situ* in the retina.

RESULTS

The retina promotes cone pigment regeneration

We used the rod-dominant salamander retina, which consists of 35% cone cells[18], to investigate whether a cone-specific visual cycle is present

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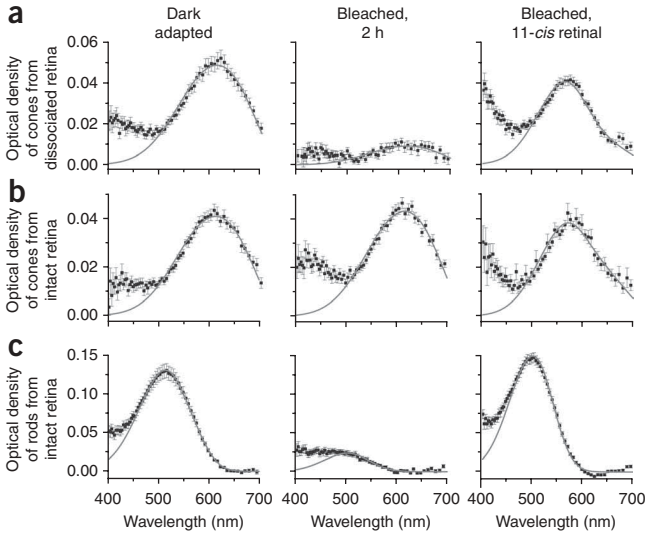

Figure 1 Effect of bleach on pigment content in salamander photoreceptors in dissociated and intact retina. (**a**–**c**) The average absorbance spectra of cones from dissociated retina (**a**, $n = 20$), cones from intact retina (**b**, $n = 20$) and rods from intact retina (**c**, $n = 10$) are shown under three different conditions: in dark-adapted state (left), 2 h after a bleach (middle) and following treatment with exogenous 11-*cis* retinal (right). In all cases, photoreceptors were bleached by 40 s of white light. The optical densities in dark, bleached and 11-*cis* treated conditions were 0.049 ± 0.004, 0.010 ± 0.002 and 0.042 ± 0.002 for cones of dissociated retina, respectively, 0.040 ± 0.002, 0.039 ± 0.002 and 0.040 ± 0.002 for cones of intact retina, respectively, and 0.130 ± 0.009, 0.024 ± 0.003 and 0.149 ± 0.006 for rods of intact retina, respectively. The fraction of bleached pigment was 82% in rods of intact retina and 79% in cones from dissociated retina. Note the recovery of pigment content after bleach of cones from intact retina (**b**), but not of cones from dissociated retina (**a**) or rods from intact retina (**c**). Error bars represent s.e.m.

in the vertebrate retina and is functional under physiological conditions. Following bleaching by bright light, such a pathway would be expected to exclusively promote the regeneration of cone pigment, independently of the pigment epithelium. Using single-cell microspectrophotometry, we compared the pigment content in dark-adapted cones with that of cones that had been bleached and then allowed to recover for 2 h in darkness without pigment epithelium. We measured the pigment recovery in cones that had been dissociated from the retina before the bleach and in cones that were bleached while still in the intact retina. All measurements were performed at the end of the recovery period from individual isolated cells.

We found that exposure of dissociated red cones to 40 s of white bleaching light induced a 4.7-fold decrease in their optical density (**Fig. 1a**), corresponding to a loss of 79% of the cone pigment (**Table 1**). The bleached cone pigment could be partially regenerated with exogenous 11-*cis* retinal chromophore (**Fig. 1a**). In contrast, identical bleach of cones from whole isolated retina produced only a 4.3% decrease in their optical density (**Fig. 1b**). Thus, the retina was able to regenerate 95% (74.7%/79%) of all bleached cone pigment, whereas pigment regeneration in isolated cones was not detectable. Treating such cones with exogenous 11-*cis* retinal fully restored their pigment content (**Fig. 1b**). Thus, the incomplete recovery of cone pigment content in isolated retina was a result of insufficient recycled chromophore. It is possible that some chromophore released from the bleached photoreceptors was lost to the bath; alternatively, retina integrity could have been compromised during the dissection, reducing the efficiency of its visual cycle. The ability of the retina to promote pigment regeneration was restricted to cones, as we found no pigment regeneration in rods from intact retina (**Fig. 1c**).

The retina promotes cone dark adaptation

Using single-cell suction recordings, we investigated whether the salamander retina could promote cone dark adaptation independently of the pigment epithelium. To allow for comparison with our microspectrophotometric results, we carried out these experiments under identical conditions. As expected, the bleach induced a substantial persistent desensitization in the absence of pigment regeneration in dissociated cones (**Fig. 2a**). Photosensitivity decreased from 10.4 ± 1.5 × 10^{-3} pA per photon μm^2 (mean ± s.e.m., $n = 19$) in dark-adapted cones to 2.0 ± 0.4 × 10^{-4} pA per photon μm^2 ($n = 20$) in cones dissociated from the retina before the bleach. Using the relation between the percentage of bleached cone pigment and decrease in the sensitivity of salamander red cones[9], this decrease in sensitivity corresponds to bleaching of 83% of the cone visual pigment (**Table 1**), a value that is similar to the 79% pigment loss from microspectrophotometric measurements. Consistent with the adaptation induced by the bleaching light, the average integration time of the cone dim flash response was also accelerated from 445 ± 42 ms ($n = 19$) for dark-adapted cones to 183 ± 15 ms ($n = 20$) for bleached cones. Exogenous 11-*cis* retinal restored the sensitivity of bleached dissociated cones to 8.2 ± 2.0 × 10^{-3} pA per photon μm^2 ($n = 7$; **Fig. 2a**). Similar results were obtained with 11-*cis* retinol (data not shown), confirming that salamander cones can oxidize it to 11-*cis* retinal for pigment regeneration[4].

In contrast, cones from whole isolated retina recovered their sensitivity following identical bleach in the absence of pigment epithelium and without exogenous chromophore (**Fig. 2b**). On average, the flash sensitivity changed from 7.3 ± 0.8 × 10^{-3} pA per photon μm^2 ($n = 14$) for dark-adapted cones to 4.8 ± 0.4 × 10^{-3} pA per photon μm^2 ($n = 14$) for cones bleached in the intact retina. This represents a 1.5-fold decrease in sensitivity, corresponding to a loss of only 7.5% of the pigment. Consistent with the substantial recovery of cone sensitivity in isolated retina following a bleach, the flash response acceleration was also largely reversed and the integration time of the dim flash response changed from 496 ± 48 ms ($n = 14$) for dark-adapted cones to 451 ± 39 ms ($n = 14$) for bleached cones. Exogenous 11-*cis* retinal produced only a small further increase in sensitivity to 8.5 ± 1.1 × 10^{-3} pA per photon μm^2 ($n = 7$), which was just higher than the initial dark-adapted level (**Fig. 2b**), consistent with the presence of free opsin in dark-adapted salamander cones[9]. Thus, the intact amphibian retina, removed from the pigment epithelium, was able to reverse the effects of bleaching in cones and promote their dark adaptation. The ability of the retina to promote dark adaptation was restricted to cones, as we found no dark adaptation in rods from intact retina (**Fig. 2c**).

Table 1 Percentage of cone pigment content following a bleach

	Dissociated retina	Intact retina	Intact retina + L-α-AAA
Microspectrophotometry	21 ± 4 ($n = 20$)	96 ± 5 ($n = 20$)	31 ± 5 ($n = 15$)
Single-cell recordings	17 ± 2 ($n = 20$)	93 ± 2 ($n = 14$)	32 ± 3 ($n = 15$)
Whole-retina ERG	—	92 ± 2 ($n = 11$)	23 ± 2 ($n = 4$)

For microspectrophotometry, cone pigment content was estimated from optical density. For single-cell and whole-retina ERG recordings, cone pigment content was estimated from the bleach-induced desensitization using the relationship between pigment content and sensitivity in salamander red cones (see text for details). All values are the mean ± s.e.m.

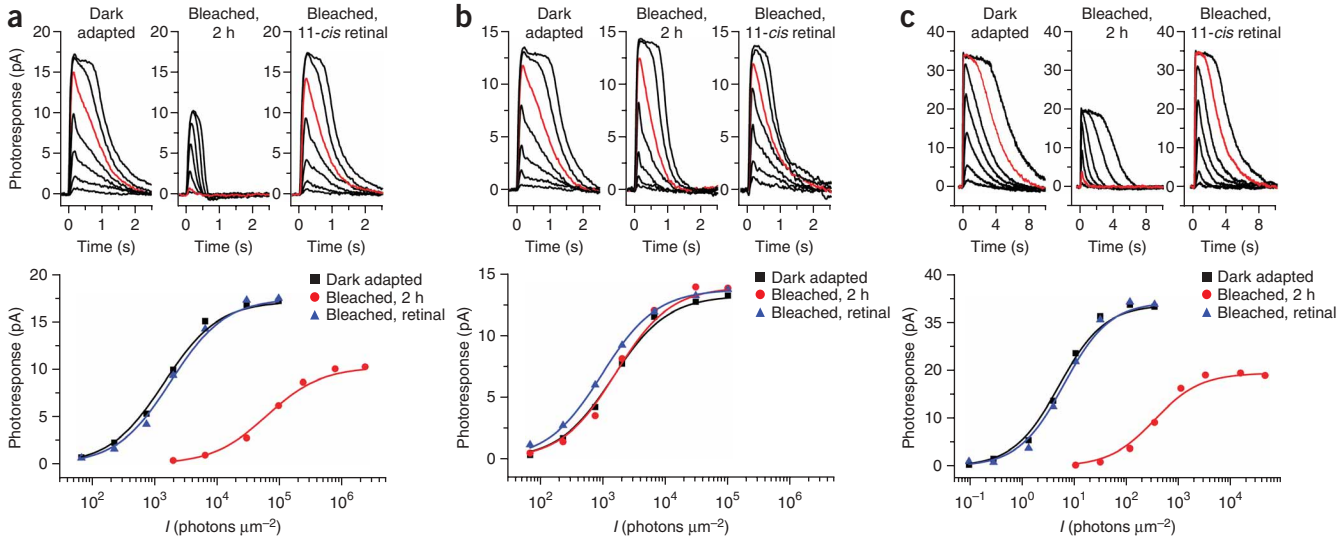

Figure 2 Effect of bleach on sensitivity in salamander photoreceptors in dissociated and intact retina. (**a**–**c**) Suction recordings of flash intensity-response families from single dissociated cones (**a**), cones from intact retina (**b**) and rods from intact retina (**c**). Cells were stimulated at time 0 with 20-ms flashes of intensity increasing in 0.5 log unit steps. The top panels show the test flash responses from cells in dark-adapted state (left), following a 40-s white light bleach (middle) and after treatment with exogenous 11-*cis* retinal (right). For cones (**a**,**b**), red traces represent photoresponses to 6,550 photons μm^{-2} (620 nm). For rods (**c**), red traces represent photoresponses to 119 photons μm^{-2} (520 nm). The bottom panels show the corresponding intensity-response relation for each cell, fit with Michaelis-Menten function $R/R_{max} = I/(I + I_0)$, where R/R_{max} is the normalized response amplitude, I is the flash intensity and I_0 is the intensity required to produce half-saturating response. Note the recovery of sensitivity of bleached cones from intact retina (**b**), but not of cones from dissociated retina (**a**) or rods from intact retina (**c**).

Müller cells are part of the retina visual cycle

We next investigated the role of Müller cells in the regeneration of cone pigment using the Müller cell–specific gliotoxin α-aminoadipic acid (L-α-AAA)[19]. L-α-AAA inhibits cysteine uptake through the cysteine/glutamate antiporter, which is primarily localized in glial cells, leading to depletion of the antioxidant glutathione and eventually causing cellular damage[20]. As in the adult retina L-α-AAA does not affect retinal neurons, including photoreceptors[21,22], it is widely used for selectively abolishing Müller cells. As expected from the lack of L-α-AAA uptake by neurons[23], 48-h incubation of isolated salamander retina in 10 mM L-α-AAA before recordings did not noticeably affect its morphology (**Supplementary Fig. 1** online) or the function of cones (but see Discussion). However, a subsequent bleach induced a substantial decrease in sensitivity, from $5.7 \pm 0.9 \times 10^{-3}$ pA per photon μm^2 ($n = 12$) in dark-adapted cones to $3.3 \pm 0.6 \times 10^{-4}$ pA per photon μm^2 ($n = 15$) following the bleach (**Fig. 3a**). This 17-fold decrease in sensitivity corresponds to a loss of 68% of the pigment (**Table 1**), ninefold more than that following identical bleach in control solution. In parallel microspectrophotometry measurements, the bleach induced a loss of 69% of the pigment (**Table 1**).

L-α-AAA did not affect considerably cone pigment content or sensitivity in darkness. Furthermore, both cone pigment loss (data not shown) and desensitization (**Fig. 3a**) in bleached L-α-AAA–treated retinas were readily reversed by exogenous 11-*cis* retinal, confirming that L-α-AAA did not affect the function of dark-adapted cones, whereas it largely inhibited cone pigment regeneration and dark adaptation in isolated retina. In contrast, in eyecup with the retina attached to the pigment epithelium, L-α-AAA did not block recovery of

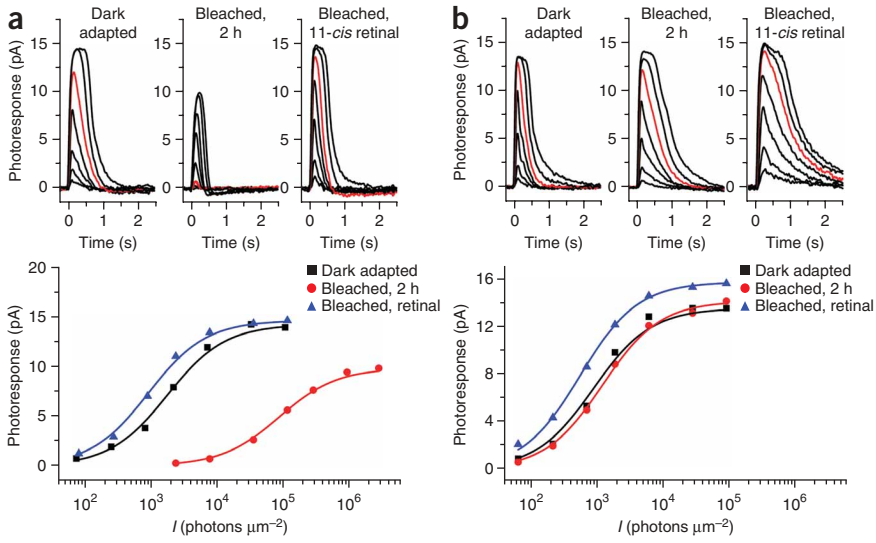

Figure 3 Effect of the Müller cell inhibitor L-α-AAA on the recovery of cone sensitivity following a bleach. (**a**,**b**) Recordings from cones bleached in isolated intact retina (**a**) and in eyecup, with retina still attached to the pigment epithelium (**b**). All retinas were treated for 48 h with 10 mM L-α-AAA and then transferred to Ringer solution before recordings. Red traces represent photoresponses to 6,550 photons μm^{-2} (620 nm). Inhibiting the function of Müller cells blocked the recovery of sensitivity of cones from isolated retina, but not of cones from eyecup following exposure to 40-s white bleaching light.

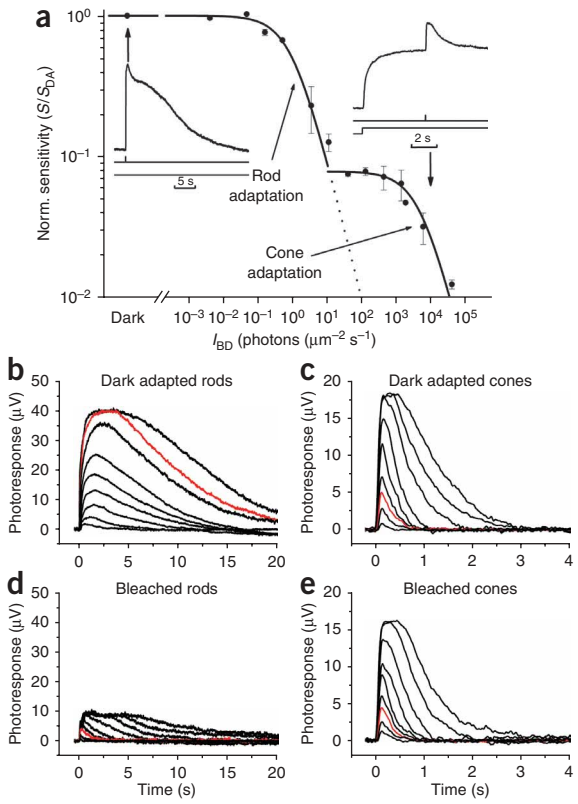

Figure 4 Rod and cone responses from salamander whole-retina ERG recordings. (**a**) Background adaptation of isolated salamander retina showing distinct rod and cone components. Data are fit with the Weber-Fechner relation, $S/S_{DA} = (1 + I_B/I_0)^{-1}$, where S is the light-adapted sensitivity, S_{DA} is the dark-adapted sensitivity, I_B is the intensity of the background and I_0 is the background that reduced sensitivity to 0.5 S_{DA}. I_0 was 0.94 photons μm^{-2} s^{-1} for rods and 4,840 photons μm^{-2} s^{-1} for cones. Insets show a combined rod and cone response in darkness (left) and a cone-only response in background saturating the rods (right). (**b**–**e**) Time course of test flash and background under each trace. Rod (**b**,**d**) and cone (**c**,**e**) responses from one retina in darkness (top) and following a 40-s white light bleach (bottom) are shown. For each trace, a 20-ms flash was delivered at $t = 0$. Red traces represent photoresponses to 530 photons μm^{-2} (520 nm) for rods and 2,100 photons μm^{-2} (620 nm) for cones. Note the substantial desensitization of rods and the recovery of sensitivity in cones from the same retina following the bleach. Error bars represent s.e.m.

sensitivity of bleached cones (**Fig. 3b**) and had no effect on the dark adaptation of rods (see below). Thus, the Müller cell gliotoxin specifically inhibited the retina visual cycle and not the canonical pigment epithelium visual cycle.

Role of the retina visual cycle in cone dark adaptation

To observe the recovery of cone sensitivity in real time and determine the kinetics of chromophore recycling by the cone-specific retina visual cycle, we recorded rod and cone photoresponses from whole salamander retina. We isolated the photoreceptor component (a-wave) of isolated retina electroretinogram (ERG) responses by pharmacologically blocking synaptic transmission (see Methods). The photoreceptor response from salamander retina contained both rod (slow) and cone (fast) components (**Fig. 4a**). Using backgrounds that were bright enough to saturate the rods while minimally affecting the cone component of the response (**Fig. 4a**), we were able to record cone test-flash responses from intact retina (**Fig. 4**). By subtracting this cone response from the rod and cone response elicited by an identical flash in darkness, we were able, in turn, to obtain the rod component of the retina responses (**Fig. 4**). Although the bleach reduced the rod sensitivity by over three orders of magnitude, its effect on the sensitivity of cones from the same retina was a reduction of only 1.7-fold, corresponding to a loss of

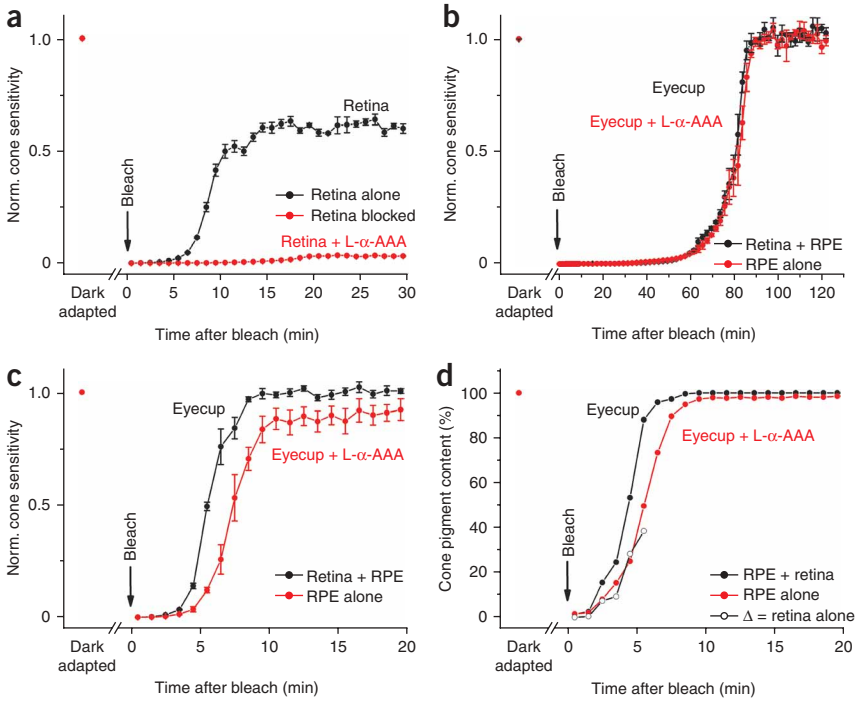

Figure 5 Kinetics of cone dark adaptation from whole-retina ERG recordings. (**a**) Recovery of salamander cone sensitivity driven by isolated retina in Ringer solution (black, $n = 4$) or following 48-h treatment with L-α-AAA (red, $n = 3$). Cone sensitivity in Ringer solution recovered to 64% of its dark-adapted value, corresponding to a regeneration of 93% of cone pigment. In contrast, bleached cones from retina treated with L-α-AAA recovered only 3.2% of their dark-adapted sensitivity. (**b**) Recovery of rod sensitivity in eyecup in Ringer solution (black, $n = 4$) and following 48-h treatment with L-α-AAA (red, $n = 4$). L-α-AAA did not affect the rate or final level of recovery of sensitivity of rods driven by the pigment epithelium. (**c**) Recovery of cone sensitivity in eyecup in Ringer solution (black, $n = 5$), driven by both retina and pigment epithelium visual cycles, or following 48-h treatment with L-α-AAA (red, $n = 5$), driven by the pigment epithelium alone. The recovery of cone sensitivity was substantially accelerated and driven to completion by the addition of the retina visual cycle. (**d**) Recovery of cone pigment content, estimated from **c**, using the relation between cone pigment loss and desensitization[9]. The initial rate of pigment regeneration by the retina visual cycle (open circles) was estimated by subtracting the pigment regenerated by the pigment epithelium (red) from the total regenerated pigment (black). Note the comparable rates of pigment regeneration by the two cycles. In all cases, 40 s of white bleaching light was delivered at $t = 0$. Error bars represent s.e.m.

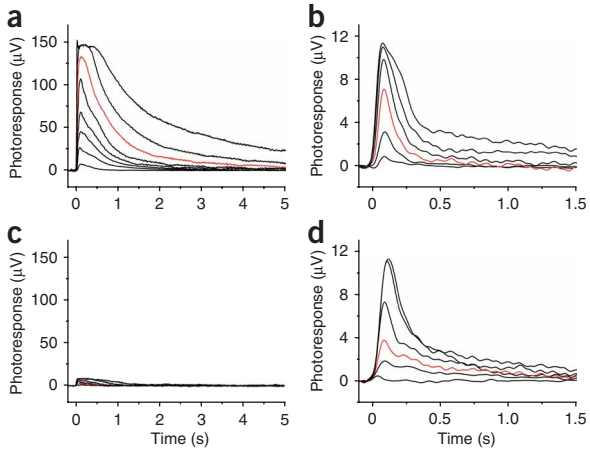

Figure 6 Rod and cone responses from mouse whole-retina ERG recordings. (**a**–**d**) Rod (**a**,**c**) and cone (**b**,**d**) responses from one retina in darkness (top) and following a 40-s 9.7 × 10^6 photons μm^{-2} s^{-1} 500 nm bleach (bottom) are shown. Test flashes of intensity increasing in 0.5 log unit steps were delivered at $t = 0$. Red traces represent photoresponses to 1,977 photons μm^{-2} for rods and 22,850 photons μm^{-2} for cones, both at 500 nm. Note the substantial desensitization of rods and the recovery of sensitivity in cones from the same retina following the bleach.

only 8.0% cone pigment (**Table 1**), a value that is similar to the 7.5% pigment loss from single-cell recordings.

Using ERG recordings from isolated salamander retina, we were able to observe in real time the recovery of cone sensitivity following a bleach (**Fig. 5a**). Consistent with our single-cell results, this recovery was inhibited by 48-h incubation in L-α-AAA (**Fig. 5a**), whereas similar incubation in control solution had no effect on recovery (data not shown). In contrast, L-α-AAA did not affect recycling of chromophore by the pigment epithelium, as demonstrated by the recovery of rod (**Fig. 5b**) and cone (**Fig. 5c**) sensitivity in retina from eyecup, still attached to the pigment epithelium. Notably, the recovery of cone sensitivity was fastest in eyecup, where both retina and pigment epithelium recycled chromophore for cones (**Fig. 5c**), so that 5 min following a bleach, cones regained 32% of their dark-adapted sensitivity (pigment epithelium and retina), as opposed to only 8% (pigment epithelium alone). Thus, the addition of chromophore recycled in the retina substantially accelerated the dark adaptation of cones.

The pigment epithelium alone was not sufficient for complete cone pigment regeneration; in the L-α-AAA–treated eyecup, cone sensitivity following bleach recovered to 92% (**Fig. 5c**), corresponding to regeneration of 98% of cone pigment (**Fig. 5d**). The cone pigment regeneration driven by the isolated retina was somewhat lower at 93% (64% sensitivity recovery; **Fig. 5a**), probably because of dissection damage to the retina and loss of chromophore to the bath. However, combining the pigment epithelium and the retina visual cycles in the bleached intact eyecup fully restored cone sensitivity, indicating 100% pigment regeneration (**Fig. 5c**,**d**). Thus, both visual cycles were required for full recovery of cone sensitivity following a bleach. Notably, the initial rates of pigment regeneration by the two visual cycles were similar (**Fig. 5d**, see Discussion).

A retina visual cycle is functional in the mouse retina

Finally, to determine whether a visual cycle functions in the mammalian retina, we recorded ERG responses from isolated mouse retina. As in the case of the salamander, we used the difference in light adaptation between rods and cones to isolate their responses in the same retina. For simplicity, we used 500-nm light for both bleaching and test-flash stimulation, effectively excluding mouse cone S-opsin and limiting our studies to mouse cone M-opsin. This allowed us to investigate the possible function of a visual cycle in the mouse retina without considering the coexpression of two cone pigments in mouse cones.

As expected, the bleach induced a substantial reduction in the rod response amplitude (**Fig. 6**) and a 140-fold ($n = 16$) decrease in rod sensitivity. In contrast, following an identical bleach, the amplitude of the cone response recovered fully and the cone sensitivity recovered to one-third ($n = 8$) of its prebleach dark-adapted level (**Fig. 6**) indicating substantial pigment regeneration in cones from isolated retina. Thus, similar to the case in salamander, the mouse retina was able to promote dark adaptation independently of the pigment epithelium in cones, but not in rods.

DISCUSSION

Functional cone-specific visual cycle in vertebrate retina

We investigated whether the vertebrate neural retina is able to recycle chromophore and promote cone pigment regeneration independently of the pigment epithelium. Using microspectrophotometry, we observed cone-specific pigment regeneration in the isolated salamander retina following exposure to bright bleaching light. In parallel physiological experiments using single-cell and whole-retina recordings, we observed cone-specific dark adaptation in the same conditions. Recordings from mouse photoreceptors revealed that the isolated mouse retina is also able to promote cone-specific dark adaptation. Together, these results demonstrate that a functional visual cycle is present in the vertebrate retina, where it provides recycled chromophore, presumably 11-*cis* retinol, exclusively to cones and independently of the pigment epithelium (**Supplementary Fig. 2** online). We also found that both the canonical pigment epithelium visual cycle and the retina visual cycle are capable of independently promoting pigment regeneration in cones. Thus, although rods rely on chromophore recycled solely in the pigment epithelium, cones utilize chromophore supplied by both the pigment epithelium and the retina. Our results help to explain the long-standing, but controversial, observation of recovery of cone early receptor potential following a bleach in isolated frog retina[24]. The implications of the addition of a retina visual cycle for cone function are discussed below.

How is the cone specificity of the retina visual cycle achieved? Previous physiological studies from salamander photoreceptors provide two possible mechanisms. First, exogenous 11-*cis* retinol can produce dark adaptation in cones, but not rods[4]. As 11-*cis* retinol is the presumptive form of recycled chromophore produced by the retina, this will provide one mechanism for the cone specificity of the retina visual cycle. Second, chromophore can diffuse from the inner to the outer segment in cones, but not in rods[25]. Because Müller cell processes surround photoreceptor cell bodies, but do not reach their outer segments[26] (see also **Supplementary Fig. 1**), the recycled chromophore released from Müller cells will diffuse to the outer segment and combine with opsin in cones, but not in rods.

As a first step in dissecting the physiology of the retina visual cycle, we examined whether Müller cells are involved in chromophore recycling. First, we found that the retina visual cycle functions only in intact retina, where contacts between cones and Müller cells are preserved. Even simply drawing the outer segment of a single cone, still attached to a small piece of retina, into the recording electrode was sufficient to prevent retina-driven pigment regeneration (**Supplementary Fig. 3** online). Thus, preserving retina morphology and proper contact between cones and adjacent inner neurons and Müller cells is paramount for the function of the retina visual cycle. Second, we found

that preincubation of the retina in the Müller cell–specific gliotoxin L-α-AAA selectively blocked the retina-driven cone pigment regeneration and dark adaptation (**Figs. 4a** and **5a**). In the adult frog and chicken retina, treatment with L-α-AAA at concentrations between 60 and 300 mM (higher than the one used in this study) has no effect on retinal elements other than Müller cells[22]. Indeed, we found that treatment of salamander retina with L-α-AAA had no effect on photoreceptor morphology (**Supplementary Fig. 1**) or on the ability of bleached cones to regenerate their pigment and dark-adapt using exogenous 11-*cis* retinal. Together, these results indicate that Müller cells are an essential part of the retina visual cycle.

The inhibition of chromophore recycling by L-α-AAA resulted in ~50% reduction in the sensitivity of dark-adapted cones. This desensitization probably reflects the gradual accumulation of free opsin produced as a result of the high rate of spontaneous thermal activation of pigment in salamander red cones[9]. Indeed, subsequent treatment of such cells with exogenous 11-*cis* retinal resulted in substantial overshoot of sensitivity (**Fig. 3**), indicating a larger than normal level of free opsin. L-α-AAA did not completely block the retina visual cycle, suggesting that some chromophore released from the gradual 'thermal bleach' of cone pigment would have been recycled to regenerate cone pigment. These results indicate that, similar to activation by a photon, thermal activation of the cone pigment probably results in its decay to free opsin.

Functional role of the retina visual cycle

What are the advantages of having two separate sources of chromophore for cones, but only one for rods? Rapid cone pigment regeneration is crucial for the rapid dark adaptation of cones and for their persistent function in bright light, where cone pigment is continuously bleached at a high rate. The rapid turnover of cone pigment facilitated by its rapid formation and decay[2] also imposes the need for rapid recycling of chromophore.

Our results demonstrate that, although both the pigment epithelium and retina visual cycles are able to promote substantial cone pigment regeneration, the combined action of the two visual cycles is required for the complete dark adaptation of cones. Furthermore, the addition of the retina visual cycle to the canonical pigment epithelium visual cycle accelerated cone dark adaptation fourfold (**Fig. 5c**), indicating that that the retina visual cycle is required for the rapid and complete dark adaptation of cones. Rod pigment regeneration and dark adaptation, on the other hand, were not affected by the retina visual cycle, and the action of the pigment epithelium visual cycle alone was sufficient for complete rod pigment regeneration and dark adaptation (**Fig. 5b**).

Rates of the retina and pigment epithelium visual cycles

Previous biochemical experiments have suggested that the retina might be able to recycle chromophore 20-fold faster than the pigment epithelium[11]. Our whole-retina ERG recordings allowed for a direct comparison of the rates of the two visual cycles under physiological conditions. Because chromophore loss and retina damage reduce the efficiency of the retina visual cycle, as measured from isolated retina, we used instead measurements from intact eyecup to estimate the rate of the retina visual cycle. By subtracting the fractional pigment regenerated by the pigment epithelium alone (eyecup + L-α-AAA) from the pigment regenerated by both pigment epithelium and retina (eyecup), we obtained the fractional pigment regenerated by the retina alone (**Fig. 5d**). This estimate is based on the lack of effect by L-α-AAA on the pigment epithelium cycle (**Fig. 5b**). To our surprise, we found that the initial rates of cone pigment regeneration by the pigment epithelium and by the retina were essentially equal. Thus, the additional action of the retina visual cycle doubles the amount of recycled chromophore available to cones during dark adaptation. Because of the nonlinear relation between bleached pigment and desensitization[9], this results in a fourfold increase in cone sensitivity and corresponding acceleration of cone dark adaptation.

How can we explain the discrepancy between the biochemical estimate of the maximum rate of enzymatic recycling of chromophore in ground squirrel retina[11] and our physiological measurement of pigment regeneration kinetics in amphibian retina? A simple explanation could be the differences between species. It is more likely, however, that cone pigment regeneration via the two cycles could be rate limited, not by their enzymatic reactions, but rather by a common step such as the delivery of recycled chromophore to cones, as has been recently proposed for rods[27].

Ruling out contamination from the pigment epithelium

We took extreme care to remove all visible pigment epithelium from the retina and carried out multiple control experiments to demonstrate that residual pigment epithelium was not the source of chromophore in isolated retina. First, only cones in close proximity to a piece of pigment epithelium would be expected to undergo pigment regeneration and dark adaptation. Instead, we found uniform pigment regeneration (**Fig. 1b**) and dark adaptation (**Fig. 2b**) in individual cones and almost complete recovery of whole-retina cone sensitivity (**Fig. 5a**), indicating that all bleached cones underwent dark adaptation. Second, pigment epithelium contamination would be expected to promote both rod and cone pigment regeneration. However, we found no rod recovery of pigment content (**Fig. 1c**) or single-cell (**Fig. 2c**) and whole-retina (**Fig. 4d**) sensitivity in bleached isolated retina, indicating that rods did not receive chromophore. Third, the Müller cell inhibitor L-α-AAA blocked cone pigment regeneration and single-cell (**Fig. 3a**) and whole-retina (**Fig. 5a**) cone dark adaptation. This treatment specifically affected chromophore recycling in the retina and had no effect on the pigment epithelium, as shown by the dark adaptation of rods and cones in eyecup (**Figs. 3b** and **5b,c**). Fourth, cone pigment regeneration (**Fig. 1a**) and dark adaptation (**Fig. 2a**) were not observed in dissociated solitary cones or in cones from retina in which proper contact with Müller cells was disturbed by a suction electrode (**Supplementary Fig. 3**). Thus, if any pigment epithelium contaminants were present in our retina preparation, their role in cone pigment regeneration was negligible.

Interaction between the two visual cycles

Recently, two independent studies of *Rpe65*$^{-/-}$; *Nrl*$^{-/-}$ mice found no evidence for chromophore recycling in the retina, raising doubts about the functional presence of such a pathway in rod-dominant species[16,17]. Both studies found that ablation of RPE65, a chromophore-binding protein that is essential for the pigment epithelium visual cycle[28], leads to the absence of 11-*cis* retinal in the *Nrl*$^{-/-}$ retina, which is populated exclusively by Nrl cones. The conclusion from these results was that the pigment epithelium is the only substantial source of chromophore in the rod-dominant retina, ruling out chromophore recycling in the retina. However, as our findings clearly demonstrate the function of a visual cycle in the mouse retina (**Fig. 6**), the results from these previous studies most likely reflect an interaction between the two visual cycles. Specifically, as chromophore is not synthesized de novo in the eye, the pigment epithelium pathway is probably required for its initial uptake from the circulation. Furthermore, the lack of chromophore in *Rpe65*$^{-/-}$; *Nrl*$^{-/-}$ mice is not surprising if one considers that, as we show here, chromophore is recycled in Müller cells and supplied only to a small fraction of all photoreceptors (cones) in the retina. Thus, simply

increasing the mouse cone population would not be expected to increase the chromophore recycling capacity of the retina. Finally, if RPE65 itself is expressed in cones (see ref. 17 for discussion), its deletion might directly impair the retina visual cycle. Our results demonstrating the function of a cone visual cycle in the rod-dominant amphibian and mouse retinas settle this controversy.

Finally, unlike in primates, the salamander and mouse retinas do not have central cone-rich foveal regions. Notably, although the inner retina neurons are sparse in the central primate retina, presumably to minimize light scattering, the ratio of cones to Müller cells in the fovea of primates is 1:1 (ref. 29), indicating that they may have a function in the cone-rich central area of the retina. Experiments to determine whether a similar retina visual cycle is present in human and subhuman primates are currently underway.

METHODS

Electrophysiology. Single-cell and whole-retina ERG recordings were carried out as previously described[9,30]. Briefly, we decapitated dark-adapted larval tiger salamanders (*Ambystoma tigrinum*) in dim red light, double-pithed them and enucleated and hemisected the eyes. The retina was removed from the eyecup and any visible residual pigment epithelium was cleaned with forceps. For single-cell recordings, the retina was chopped with a razor blade and a small aliquot of cell suspension was placed in the recording chamber. Under infrared illumination, the outer segment of an isolated single cone or rod photoreceptor was drawn into the tip of a tight-fitting glass pipette for recording. For ERG recordings, the retina was transferred to a recording chamber on filter paper (photoreceptor side up). In both cases, tissue was perfused with amphibian Ringer solution containing 110 mM NaCl, 2.5 mM KCl, 1.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10 mM dextrose, 10 mM HEPES (pH 7.8) and bovine serum albumin (100 mg l^{-1})[9]. For ERG recordings, to isolate the photoreceptor component (a-wave) of the retina, we added 5 μM L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) to the solution to block on-bipolar cell signals[31], 5 μM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) to block AMPA/kainate signals[32] and 50 μM D-2-amino-5-phosphonovalerate (D-AP5) to block NMDA signals[33]. ERG recordings were carried out between a microelectrode that was built into the bottom of the chamber and a capillary microelectrode that was positioned above the retina. To suppress any glial components of the retina response, we added 10 mM barium chloride, which blocks potassium channels on Müller cells[34], to the chamber electrode. ERG recordings from retina in eyecup were performed in a modified recording chamber containing a small well in which the eyecup was placed. For mouse ERG recordings, C57/BL6 mice were killed by CO_2 asphyxiation or cervical dislocation and the retina was prepared as described above for salamander. The mouse retina was perfused using a 36–38 °C bicarbonate-buffered solution containing 140 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 3 mM HEPES (pH 7.4), 0.02 mM EDTA, 10 mM glucose, 0.1× MEM amino acids, 0.1× MEM vitamins, 5 μM D,L-2-amino-4-phosphonobutanoic acid, 2 μM NBQX and 10 μM D-AP5.

We stimulated the photoreceptors with calibrated 20-ms flashes. The signals were amplified, low-pass filtered at 20 Hz (8-pole Bessel) and digitized at 100 Hz for further analysis. Photosensitivity was calculated from the linear region of the intensity-response curve as the ratio of response amplitude and flash intensity. Integration time was calculated as the integral of the dim-flash response with the transient peak amplitude normalized to unity.

Calculation of pigment content from electrophysiology. We used the previously derived dependence of flash sensitivity on the percentage of pigment bleached by light for salamander red cones[9] (**Fig. 4c**) to estimate pigment content from the level of cone desensitization. This method was used for both single-cell and whole-retina ERG recordings. The pigment content values derived from sensitivity measurements very closely matched the values measured directly using microspectrophotometry (**Table 1**). The effect of substituting the native A1/A2 chromophore mix with pure A1 following a bleach and regeneration with 11-*cis* retinal was not considered (**Supplementary Methods** online).

Microspectrophotometry. Isolated photoreceptors were prepared as described above for electrophysiology. Microspectrophotometric measurements were carried out as previously described[35,36]. The optical density of a rod or cone outer segment was measured over the wavelength range of 400–700 nm with a rectangular slit smaller than the outer segment, where optical density = $\log_{10}(I_o/I_t)$, with I_o being the transmitted light in the absence of a sample and I_t being the transmitted light through the outer segment. The collected optical density spectra for each photoreceptor were fitted by the 11-*cis* A1 and A2 retinal spectral templates derived previously[37]. Ringer solutions containing chromophore were prepared daily from 300-μg dry aliquots of retinoid by dissolving the chemical in 0.1% ethanol as described previously[9].

Note: Supplementary information is available on the Nature Neuroscience website.

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