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cis Retinol oxidation regulates photoreceptor access to the retina visual cycle and cone pigment regeneration

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Key points

- This study explores the nature of the *cis* retinol that Müller cells in the retina provide to cones for the regeneration of their visual pigment.
- We report that the retina visual cycle provides cones exclusively with 11-*cis* chromophore in both salamander and mouse and show that this selectivity is dependent on the 11-*cis*-specific cellular retinaldehyde binding protein (CRALBP) present in Müller cells.
- Even though salamander blue cones and green rods share the same visual pigment, only blue cones but not green rods are able to dark-adapt in the retina following a bleach and to use exogenous 9-*cis* retinol for pigment regeneration, suggesting that access to the retina visual cycle is cone-specific and pigment-independent.
- Our results show that the retina produces 11-*cis* retinol that can be oxidized and used for pigment regeneration and dark adaptation selectively in cones and not in rods.

Abstract Chromophore supply by the retinal Müller cells (retina visual cycle) supports the efficient pigment regeneration required for cone photoreceptor function in bright light. Surprisingly, a large fraction of the chromophore produced by dihydroceramide desaturase-1, the putative all-*trans* retinol isomerase in Müller cells, appears to be 9-*cis* retinol. In contrast, the canonical retinal pigment epithelium (RPE) visual cycle produces exclusively 11-*cis* retinal. Here, we used the different absorption spectra of 9-*cis* and 11-*cis* pigments to identify the isoform of the chromophore produced by the visual cycle of the intact retina. We found that the spectral sensitivity of salamander and mouse cones dark-adapted in the isolated retina (with only the retina visual cycle) was similar to that of cones dark-adapted in the intact eye (with both the RPE and retina visual cycles) and consistent with pure 11-*cis* pigment composition. However, in mice lacking the cellular retinaldehyde binding protein (CRALBP), cone spectral sensitivity contained a substantial 9-*cis* component. Thus, the retina visual cycle provides cones exclusively with 11-*cis* chromophore and this process is mediated by the 11-*cis* selective CRALBP in Müller cells. Finally, despite sharing the same pigment, salamander blue cones, but not green rods, recovered their sensitivity in the isolated retina. Exogenous 9-*cis* retinol produced robust sensitivity recovery in bleached red and blue cones but not in red and green rods, suggesting that *cis* retinol oxidation restricts access to the retina visual cycle to cones.

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Abbreviations CRALBP, cellular retinaldehyde binding protein; DES1, dihydroceramide desaturase-1; ERG, electroretinography; Gnat1, rod transducin α-subunit; LED, light-emitting diode; λ_{max}, wavelength of maximum absorbance; M-cone, middle wavelength-sensitive cone; RDH, retinol dehydrogenase; RPE, retinal pigment epithelium.

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Introduction

The vertebrate retina has two distinct classes of ciliary photoreceptors, rods and cones, for dim light and bright light vision, respectively (Baylor, 1987). Both photoreceptors have visual pigments (rhodopsin in rods and cone pigment in cones) composed of protein moiety, opsin and chromophore, typically 11-*cis* retinal. Upon absorbing light, visual pigment is activated to trigger the phototransduction cascade and subsequently decays into apo-opsin and photoisomerization product, all-*trans* retinal (Ebrey & Koutalos, 2001). Thus, regeneration of the pigment is required for continuous light perception. This is achieved by a biochemical process known as the visual cycle (or retinoid cycle) that recycles all-*trans* chromophore back into *cis* form in retinal pigment epithelium (RPE visual cycle; for both rods and cones) or in retinal glial Müller cells (retina visual cycle; only for cones) (Wang & Kefalov, 2011; Saari, 2012). The retina visual cycle has been suggested to enable cones to adapt to darkness rapidly and to function under bright light by providing *cis* retinal exclusively to cones faster than the RPE pathway (Mata *et al.* 2002; Miyazono *et al.* 2008; Kolesnikov *et al.* 2011; Xue *et al.* 2015). In this pathway, all-*trans* retinal released from the visual pigment upon its decay is reduced to all-*trans* retinol in cones (Ala-Laurila *et al.* 2006; Miyazono *et al.* 2008) and transported to adjacent Müller cells. There, all-*trans* retinol is isomerized to *cis* retinol (Kaylor *et al.* 2013) and returned to cones where it is oxidized to *cis* retinal to regenerate visual pigments (Jones *et al.* 1989; Miyazono *et al.* 2008; Wang *et al.* 2009; Wang & Kefalov, 2009).

Recently, dihydroceramide desaturase-1 (DES1) was identified as the putative retinol isomerase in Müller cells that converts all-*trans* retinol to *cis* retinol isomers (Kaylor *et al.* 2013). Surprisingly, 9-*cis* retinol represents a larger fraction of the DES1 isomerization products than 11-*cis* retinol. In addition, purified carp cones have both 11-*cis* and 9-*cis* retinol oxidation activity (Sato *et al.* 2013), and rod and cone opsins readily bind 9-*cis* retinal to form photosensitive pigment (Pepperberg *et al.* 1976; Fukada *et al.* 1990; Makino *et al.* 1999). Therefore, the 9-*cis* isomer may be a physiologically relevant chromophore supplied by the retina visual cycle. Consistent with this notion, 9-*cis* retinoids have been detected in dark-adapted mouse and chicken retinas, with 9-*cis* retinal 26-fold more abundant in the cone-dominant chicken retina compared to the rod-dominant mouse retina (Kaylor *et al.* 2013). Dark-reared mice with deficient RPE visual cycle also slowly accumulate 9-*cis* retinal in rods (Fan *et al.* 2003). However, the possibility that Müller cells provide 9-*cis* chromophore to cones has not been investigated in the intact retina and its significance for the function of cones remains unknown.

The mechanisms regulating access to the retina visual cycle are not well understood. Considering that *cis* retinol is the probable product of the retina visual cycle (Mata *et al.* 2002), it would be expected that only photoreceptors capable of oxidizing *cis* retinol to *cis* retinal will be able to use it for pigment regeneration (Wang & Kefalov, 2009; Wang *et al.* 2014). However, investigating the oxidation of 11-*cis* retinol in intact photoreceptors has been hampered by the difficulty of obtaining this retinoid and by its low stability (Parker *et al.* 2011). Thus, identifying a stable and readily available alternative to 11-*cis* retinol, such as 9-*cis* retinol, would be of substantial value for investigating the molecular mechanisms regulating the retina visual cycle.

Here, we first addressed the possible involvement of 9-*cis* retinol in the retina visual cycle by analysing the chromophore content of salamander and mouse cones dark-adapted with chromophore provided by the Müller cells in the isolated retina. Next, we examined which of four types of salamander photoreceptors, red and blue cones, and red and green rods, can access the retina visual cycle and whether such access requires the ability to utilize *cis* retinol for pigment regeneration. Our findings extend our understanding of the molecular mechanisms that regulate the regeneration of pigment and the dark adaptation of cone photoreceptors.

Methods

Ethical approval

All experiments were performed in accordance with the principles of UK regulations as described by Grundy (2015) and were approved by the Washington University Animal Studies Committee.

Single-cell suction recordings

Single-cell recordings were carried out as previously described (Wang *et al.* 2009). Briefly, larval tiger salamanders (*Ambystoma tigrinum*, Charles D. Sullivan Co. Inc., Nashville, TN, USA) were dark-adapted overnight in darkness, decapitated and double-pithed. Their eyes were then enucleated under dim red light and subsequently hemisected under infrared illumination. The retina was peeled off from the eyecup in Ringer solution containing 110 mM NaCl, 2.5 mM KCl, 1.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, 100 mg l^{-1} BSA, 10 mM glucose and 10 mM Hepes (pH 7.8). Visible residual pigment epithelium was removed with forceps. The retina was chopped with a razor blade and photoreceptors were dissociated mechanically by trituration with a wide-bore Pasteur pipette. When necessary, the isolated intact retina or dissociated photoreceptors thus prepared were fully bleached by white light (150 W halogen lamp for 40 s) and then kept in darkness for 1–2 h prior to the recordings.

Retinoid solutions were prepared by dissolving 300 μg of dry retinoid (11-*cis* retinal: a generous gift from the National Eye Institute and Rosalie Crouch; 9-*cis* retinoids: Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) into 4 μl ethanol and then gradually adding 4 ml Ringer solution or reference electrode solution (110 mM NaCl, 2.5 mM KCl, 1.6 mM $MgCl_2$, 1.0 mM $CaCl_2$ and 10 mM Hepes, pH 7.8). When treating with retinoid, the retinoid solution was added at a final concentration of ~60 μM to a Petri dish containing bleached dissociated photoreceptor cells and then incubated in darkness for 1–2 h before the recordings.

A small aliquot of dissociated photoreceptors was placed on a recording chamber and perfused with Ringer solution. The outer segment or inner segment of a single photoreceptor was drawn into a tight-fitting glass pipette and stimulated with calibrated test flashes generated by a white light-emitting diode (LED; SR-01-WC120, Quadica Developments Inc., Brantford, Ontario, Canada) or a halogen lamp (FCR 12 V 100 W, Ushio, Cypress, CA, USA). In the LED system, flash duration and intensity were controlled by an LED driver (LDC210C, Thorlabs, Newton, NJ, USA). In the halogen lamp system, they were controlled by a set of calibrated natural density filters and a computer-controlled shutter. In both cases, light wavelength was controlled by an interference filter. The light sources were calibrated after each experiment. The signals generated by the photoreceptor were amplified with an amplifier (Axopatch 200B, Axon Instruments, Inc., Union City, CA, USA) and tunable active filter (Model 3382, Krohn-Hite Corporation, Brockton, MA, USA), low-pass filtered at 30 Hz (8-pole Bessel, Model 3382, Krohn-Hite Corporation), digitized at 1 kHz (Digidata 1322A, Axon Instruments, Inc.) and stored on a computer using pClamp9 software (Molecular Devices, Sunnyvale, CA, USA). Rod and cone subtypes were identified based on their morphology and characteristic spectral sensitivities.

Transretinal electroretinography (ERG) recordings

Transretinal ERG recordings from mouse middle wavelength-sensitive cones (M-cones) were performed as previously described (Vinberg *et al.* 2014) with rod transducin a-subunit knock-out [Gnat1$^{-/-}$) mice (BALB/c background, Leu450 variant of RPE-specific protein 65 kDa (RPE65)] that lack rod signalling (Calvert *et al.* 2000). Young adult animals of either sex (1.5–3 months old) were used in all experiments. Animals were provided with standard chow (LabDiet 5053; LabDiet, Purina Mills, St Louis, MO, USA) and maintained under a 12 h light (10–20 Lux)/12 h dark cycle. The mice were dark-adapted overnight prior to the experiments and were killed by asphyxiation with a rising concentration of CO_2. The eyes were enucleated and hemisected as described above. The retina was dissected in Locke's solution [112.5 mM NaCl, 3.6 mM KCl, 2.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM Hepes, 20 mM $NaHCO_3$, 3 mM sodium succinate, 0.5 mM sodium glutamate, 0.02 mM EDTA, 10 mM glucose, 0.1% minimum essential media vitamins and 0.2% minimum essential media amino acids, pH 7.4]. When treating with 9-*cis* retinal, dissected retinas were pre-incubated in a Petri dish containing Locke's solution supplemented with 100 mM 9-*cis* retinal and 1% (w/v) BSA for 10 min and then the endogenous pigments were fully bleached by strong light (1.7×10^7 photons μm^{-2} s^{-1}, 500 nm, 51 s) prior to the recordings. The retina was placed photoreceptor-side up onto the specimen holder (OcuScience, Henderson, NV, USA) modified with a piece of black filter paper (HABG01300, Millipore, Billerica, MA, USA) on the top of the holder dome. The retina was perfused at 1–2 ml min^{-1} with Locke's solution bubbled with 95% O_2/5% CO_2 and supplemented with 40 μM DL-AP4 (3699, Tocris Bioscience, Ellisville, MO, USA), 2 mM L-aspartate and 100 μM $BaCl_2$ to isolate the photoreceptor component of the ERG signal (a-wave). Before the recordings, the retina was allowed to stabilize for 15 min. The temperature of the retina was maintained at 33–36°C during the recordings by heating the perfusion tubing located immediately before the specimen holder with a ceramic heater (Shi *et al.* 2007). Bleaching and light stimulation were applied with calibrated light from the LED system (see above). For bleaching 90% of cone visual pigments, the retina was exposed to a 2.56 s step of 505 nm light. Bleaching light duration was determined from the relation $F = 1 - \exp(-IPt)$, where F is the fraction of pigment to be bleached (0.9), t is the duration of light exposure, I is the light intensity (1.2×10^8 photons μm^{-2} s^{-1}), and P is the photosensitivity of mouse cones at the wavelength of peak absorbance (7.5×10^{-9} μm^2), adopted from Nikonov *et al.* (2006). The photoresponse signal after light stimulation was amplified by a differential amplifier (DP-311, Warner Instruments) and tunable active filter (Model 3382, Krohn-Hite Corporation), low-pass filtered at 300 Hz (8-pole Bessel; model 3382, Krohn-Hite Corporation), digitized at 1 kHz (Digidata 1322A, Molecular Devices, LLC, Hamden, CT, USA) and stored on a computer using pClamp9 software (Molecular Devices).

Data analysis

Data were analysed using pClamp9 (Molecular Devices) and Origin 7.5 (OriginLab, Northampton, MA, USA). Flash sensitivities were determined from response amplitudes below 30% of the maximum recorded from a photoreceptor or retina, and are plotted as the ratio of response amplitude and flash intensity. For salamander photoreceptors, spectral sensitivity plots thus obtained were fitted with a mixed 11-*cis* retinal (A_1) and 11-*cis* 3,4-didehydroretinal (A_2) template (Govardovskii *et al.*

2000) and, if necessary, A_1 9-*cis* template with fixed wavelength of maximum absorbance (λ_{max}) values (Makino *et al.* 1999). The ratio of templates in the fitting was obtained by least-squares analysis fit to the spectral sensitivity data, with their ratio set as a free parameter, and used as the ratio of 11-*cis* A_1, A_2 and 9-*cis* visual pigments. For mouse retinas, plots were fitted with the A_1 11-*cis* template with λ_{max} set as a free parameter.

Statistical analysis

For all experiments, data are expressed as means ± SEM. Two-tailed unpaired Student's *t*-test was used to test for the significance of differences in the mean values of two sample groups, with an accepted significance level of $P < 0.05$.

Results

Isoform of the chromophore supplied to cones by the retina visual cycle

The recently identified putative retinol isomerase in Müller cells, DES1, produces preferably 9-*cis* retinol over 11-*cis* retinol (Kaylor *et al.* 2013). However, the nature of the chromophore supplied by Müller cells to cones in the intact retina has not been investigated. We addressed this issue by using spectral sensitivity measurements from salamander and mouse cones to identify the fraction of their pigment associated with 9-*cis* chromophore. We started by comparing salamander red cones that were (1) dark-adapted *in vivo* (with retinoid supplied by both the RPE and the retina visual cycles), (2) dissociated and fully bleached (without access to retinoid supply) and (3) dark-adapted in the isolated retina after a full bleach (with retinoid supplied only by the retina visual cycle). Response families of these red cones were collected by single-cell suction recordings (Fig. 1*A*–*C*). As previously reported (Wang *et al.* 2009), compared with cones dark-adapted *in vivo* (Fig. 1*A*), bleached dissociated cones were substantially desensitized (Fig. 1*B*), whereas cones in the isolated retina partially recovered their sensitivity after the bleach (Fig. 1*C*; see also Table 1). To examine the fraction of 9-*cis* analogue regenerated pigment, we determined the sensitivity of cones at six different wavelengths and compared their action spectra (Fig. 2*A*–*C*). Salamander red cones with 9-*cis* analogue pigments have substantially blue-shifted spectral sensitivity (20 nm shift for A_1, 38 nm shift for A_2) (Makino *et al.* 1999). Thus, if 9-*cis* isomer is provided by the retina visual cycle, cones dark-adapted in the retina would be expected to have a blue-shifted λ_{max}. However, cones dark-adapted in the retina (Fig. 2*C*) showed spectral sensitivity identical to that from cones dark-adapted *in vivo* (Fig. 2*A*) or bleached without access to chromophore (Fig. 2*B*), with respective λ_{max} values of 586, 584 and 585 nm (Table 2). In addition, the spectral sensitivities in all three cases could be fit well by mixed A_1 and A_2 11-*cis* visual pigment templates (Govardovskii *et al.* 2000), without need of a 9-*cis* component. The A_1/A_2 ratios were estimated at ~40:60 in all three cases, showing no preference for one subtype of chromophore by the retina visual cycle. Together, these results demonstrate that, similar to the canonical RPE visual cycle, the retina visual cycle in salamander supplies cones exclusively with the 11-*cis* isomer chromophore.

As some of the biochemical experiments with DES1 have been performed in mouse tissue (Kaylor *et al.* 2013), we also examined the nature of the chromophore provided by Müller cells to cones in mice. We determined the spectral sensitivity of mouse M-cones by *ex vivo* transretinal recordings (Vinberg *et al.* 2014), for simplicity using isolated *Gnat1*$^{-/-}$ retinas that lack rod signalling (Calvert *et al.* 2000). The ERG a-wave (cone response) component was isolated by suppressing pharmacologically all post-photoreceptor ERG components (see Methods for details). Response families were recorded from mouse cones dark-adapted *in vivo* (Fig. 3*A*) and from cones that were bleached by 90% and then dark-adapted in the isolated retina (Fig. 3*B*). As expected, M-cones largely recovered from the bleach but remained slightly desensitized compared to dark-adapted cones due to the absence of RPE (Kolesnikov *et al.* 2011). Importantly, cones in the two conditions showed similar spectral sensitivity (Fig. 3*D*). In both cases the cone action

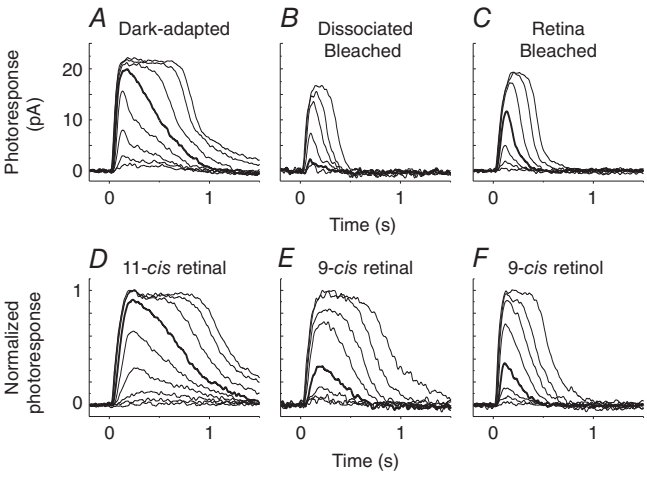

Figure 1. Single-cell suction electrode recordings from salamander red cones
A–C, representative flash response families from cones dark-adapted *in vivo* (*A*), dissociated and bleached (*B*), or bleached and dark-adapted in the retina isolated from the RPE (*C*). *D–E*, normalized flash response families from bleached dissociated red cones incubated with 11-*cis* retinal (*D*), 9-*cis* retinal (*E*) or 9-*cis* retinol (*F*). Test flashes at 620 nm and 20 ms duration of intensity increasing with a step of ~0.5 log units were delivered at time 0. Each trace is the averaged response from 2–10 flash trials. Bold traces represent responses to a flash of 5400–5700 photons μm^{-2}.

Table 1. Estimated flash sensitivity (pA photons^{-1} μm^2) at λ_{max} in single-cell suction recordings

	Dark-adapted	Dissociated Bleached	Retina Bleached	11-*cis* retinal	9-*cis* retinal	9-*cis* retinol
Red cone	$1.8 \times 10^{-2***}$ (100%, $n = 18$)	5.6×10^{-4} (3.2%, $n = 16$)	$3.1 \times 10^{-3*}$ (17%, $n = 20$)	$1.7 \times 10^{-2***}$ (98%, $n = 5$)	$4.1 \times 10^{-3*}$ (23%, $n = 7$)	$4.1 \times 10^{-3**}$ (23%, $n = 10$)
Red rod	$6.1 \times 10^{0***}$ (100%, $n = 7$)	1.7×10^{-2} (0.28%, $n = 9$)	1.5×10^{-2} (0.25%, $n = 5$)	ND	ND	2.5×10^{-2} (0.41%, $n = 11$)
Blue cone	$8.8 \times 10^{-2***}$ (100%, $n = 8$)	3.0×10^{-3} (3.4%, $n = 8$)	$2.0 \times 10^{-2*}$ (22%, $n = 10$)	ND	ND	$1.8 \times 10^{-2*}$ (21%, $n = 8$)
Green rod	$1.1 \times 10^{0**}$ (100%, $n = 20$)	5.0×10^{-3} (0.44%, $n = 10$)	8.6×10^{-3} (0.77%, $n = 7$)	ND	ND	9.1×10^{-4} (0.081%, $n = 5$)

Percentage values are relative sensitivity to that of cells dark-adapted *in vivo* (Dark-adapted). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ by two-tailed *t*-test *versus* measured sensitivity of dissociated and bleached cells (Dissociated Bleached) at 600 nm (red cones), 500 nm (red rods) or 440 nm (blue cones and green rods). ND, not determined.

spectrum could be fit well with 100% A_1 11-*cis* visual pigment template (Govardovskii *et al.* 2000), yielding an estimated λ_{max} of 518 nm for both dark-adapted and bleached cones. Interestingly, these values are about 10 nm longer than those previously determined by *in vivo* ERG (511 nm; Jacobs *et al.* 1991) or with recombinant visual pigment (508 nm; Sun *et al.* 1997). The reason for this difference is unclear although it is worth pointing out that in our case the test flash stimulation was applied to the retina from the photoreceptor side, opposite to its pathway *in vivo*. Thus, it is possible that the optical properties of the retina and photoreceptors might affect the effective spectral sensitivity of cones. Despite this issue, the essentially identical spectral sensitivities of M-cones dark-adapted *in vivo* and in an isolated retina suggest that, as in salamander, the retina visual cycle in mouse also provides 11-*cis*, and not 9-*cis*, chromophore to cones.

Next, to begin addressing the molecular mechanism of this 11-*cis* selectivity, we tested mice lacking the cellular retinaldehyde binding protein (CRALBP). CRALBP is a water-soluble 36 kDa protein expressed in Müller cells (Saari *et al.* 2001) that binds 11-*cis* retinoids with higher affinity than their 9-*cis* analogues (Saari & Bredberg, 1987; Golovleva *et al.* 2003) and plays an important role in the retina visual cycle (Collery *et al.* 2008; Fleisch *et al.* 2008; Xue *et al.* 2015). To determine the potential role of CRALBP in the 11-*cis* selectivity of the retina visual cycle, we compared the cone action spectra in dark-adapted control (*Gnat1*$^{-/-}$) and CRALBP-deficient (*CRALBP*$^{-/-}$ *Gnat1*$^{-/-}$) retinas. As shown in Fig. 3*E*, the deletion of CRALBP induced a shift in the cone spectrum λ_{max} from 518 nm in control *Gnat1*$^{-/-}$ retina to 514 nm in *CRALBP*$^{-/-}$ *Gnat1*$^{-/-}$ retina. Such a blue spectral shift suggests the presence of 9-*cis* chromophore in cones from retinas missing CRALBP. Consistent with this notion, treating bleached control mouse M-cones with exogenous 9-*cis* retinal resulted in a 12 nm blue shift in their action spectrum, with a corresponding λ_{max} of 506 nm (Fig. 3*E*, inset). Spectral templates for mouse cone pigments with 9-*cis* retinal have not been reported. Thus, to estimate the fraction of 9-*cis* pigment in CRALBP-deficient cones based on their spectral sensitivity, we used the salamander red rod templates (Makino *et al.* 1999) which undergo a 12 nm blue shift upon replacing 11-*cis* retinal with 9-*cis* retinal, identical to the 12 nm blue spectral shift observed empirically by us in mouse M-cones upon bleaching and regenerating their pigment with 9-*cis* retinal. We found that a 4 nm blue shift in the action spectrum corresponds to the replacement of the majority (60%) of the 11-*cis* pigment with 9-*cis* pigment. Thus, the 4 nm blue shift in the action spectrum of cones from CRALBP-deficient mice indicates the presence of a substantial amount (probably >50%) of 9-*cis* cone pigment.

Sensitivity recovery with 9-*cis* retinol in salamander red cones

The lack of detectable 9-*cis* visual pigment in cones dark-adapted in the isolated retina indicates that no 9-*cis* retinal was made available to the cones. However, this result could be due either to the lack of production of 9-*cis* retinol by the Müller cells, or, alternatively, to the inability of cones to oxidize 9-*cis* retinol to the 9-*cis* retinal needed for pigment regeneration. To distinguish between these two possibilities, we examined directly the ability of cones to use exogenous 9-*cis* retinol for pigment regeneration. To do that, we measured the absolute and spectral sensitivity of dissociated red cones after a full bleach and 2 h of dark incubation in the presence of exogenous A_1 9-*cis* retinol (Figs 1*F* and 2*F*). A_1 11-*cis* retinal and A_1 9-*cis* retinal were used as controls to confirm the increased sensitivity upon pigment regeneration and the blue shift induced by 9-*cis* pigment formation (Figs 1*D*, *E* and 2*D*, *E*). Spectral sensitivity plots were fitted with a mixed A_1 and A_2 11-*cis* spectral template (Govardovskii *et al.* 2000) for the unbleached pigment with native chromophores, and the A_1 11-*cis* retinal or A_1 9-*cis* retinal templates (Makino *et al.* 1999) for the fraction of pigment

regenerated with exogenous chromophore. In the fittings of cones with exogenous 9-*cis* isomer, the A_1/A_2 ratio of 11-*cis* templates was fixed at the 40:60 ratio estimated in cones without exogenous retinoids (Fig. 2*A*–*C*). Shiff-base formation between the exogenous retinal and proteins other than opsins was not likely to be a factor in our spectral sensitivity measurements as the stimulating light was orthogonal to the long axis of the cells (perpendicular to the outer segment), ruling out any masking effects due to absorption of light in the inner segment of the cell. Consistent with this notion, the action spectra could be fit well with a combination of 9-*cis* and 11-*cis* spectral templates for salamander red cone pigment (see below).

Compared to bleached cones (Fig. 2*B*), cones incubated with 11-*cis* retinal, 9-*cis* retinal or 9-*cis* retinol showed a respective 30- (Fig. 2*D*), 7.2- (Fig. 2*E*) and 7.2- (Fig. 2*F*) fold sensitivity recovery at their estimated peak wavelength. The absolute sensitivities (Table 1) and λ_{max} (Table 2) of red cones with 9-*cis* retinol (4.1×10^{-3} pA photons^{-1} μm^2, 543 nm) were essentially identical to those with 9-*cis* retinal (4.1×10^{-3} pA photons^{-1} μm^2, 542 nm), unlike those with 11-*cis* retinal (1.7×10^{-2} pA photons^{-1} μm^2, 562 nm). These results demonstrate that 9-*cis* retinol was oxidized in red cones into 9-*cis* retinal and utilized for efficient pigment regeneration. The 4-fold lower sensitivity of cones with 9-*cis* isomers could be largely explained by the 3.3-fold lower quantum yield of 9-*cis* pigment *vs.* 11-*cis* pigment (Hubbard & Kropf, 1958). In contrast to the substantial recovery of sensitivity of bleached red cones, treatment of red rods with 9-*cis* retinol failed to promote an increase in their sensitivity (Fig. 4*D* and *H*). Together, these results demonstrate that 9-*cis* retinol is able to promote pigment regeneration and recovery of sensitivity following a bleach in red cones but not in red rods.

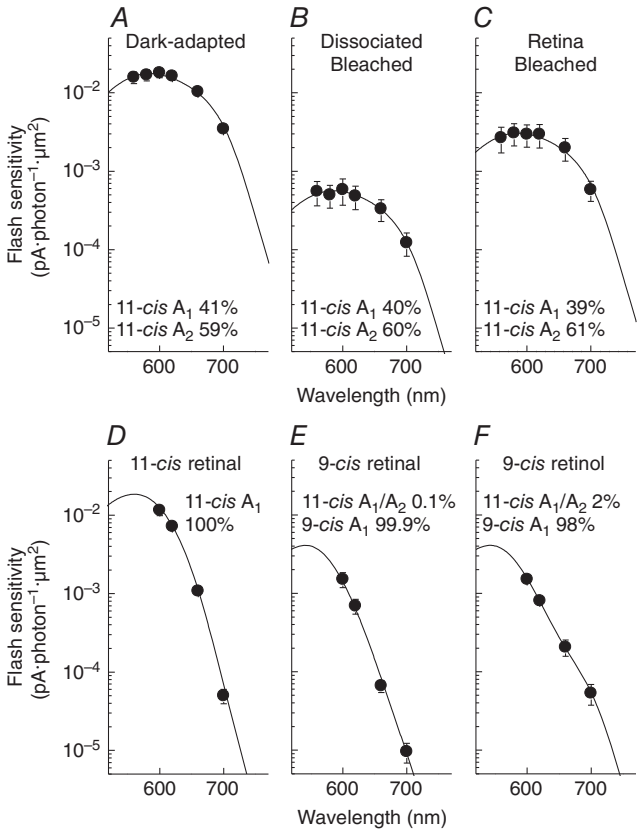

Figure 2. Spectral sensitivity of salamander red cones
A–*C*, averaged spectral sensitivities of cones dark-adapted *in vivo* (*A*, $n = 18$), dissociated and bleached (*B*, $n = 16$) or bleached and dark-adapted in the retina isolated from the RPE (*C*, $n = 20$). *D*–*F*, averaged spectral sensitivities of dissociated red cones bleached and incubated with 11-*cis* retinal (*D*, $n = 5$), 9-*cis* retinal (*E*, $n = 7$) or 9-*cis* retinol (*F*, $n = 10$). The fits to the data are sums of A_1 and A_2 11-*cis* spectral templates (*A*–*D*, A_1/A_2 ratios indicated in each panel), and A_1 9-*cis* spectral template (*E*, *F*). See Methods for details. Values show the fraction of each pigment template used in the fitting. Here and in all subsequent figures and tables, data are shown as mean ± SEM.

Interestingly, red cones treated with 9-*cis* retinol retained the small amount of unbleached native 11-*cis* pigment (Fig. 2*F*), whereas incubation with 9-*cis* retinal resulted in complete shift to 9-*cis* pigment with essentially no residual 11-*cis* pigment (Fig. 2*E*). This result is consistent with the relatively open chromophore binding pocket of red cone pigment which causes the gradual replacement of the native 11-*cis* chromophore in red salamander cones when treated with excess of exogenous 9-*cis* retinal (Matsumoto *et al.* 1975; Kefalov *et al.* 2005). Indeed, the incubation of bleached red cones with A_1 11-*cis* retinal resulted in a similar loss of the residual native A_2 11-*cis* component of the pigment (Fig. 2*D*).

Access to the retina visual cycle is independent of the pigment type

The salamander retina presents a unique opportunity to examine whether the nature of the visual pigment plays a role in regulating access to the retina visual cycle. In an unusual arrangement, blue cones and green rods in the salamander share the same visual pigment (Ma *et al.* 2001), providing a way to address this question. Thus, we next compared the dark adaptation in isolated salamander retina of its different rod and cone types. As previously shown (Wang *et al.* 2009), bleached red cones could utilize chromophore supplied by the Müller cells and as a result largely recovered their sensitivity even in the absence of RPE in the isolated retina (compare Fig. 2*A*–*C*). In contrast, salamander red rods could not recover their sensitivity when bleached in isolated retina (Fig. 4*A*–*C*, see also Fig. 4*E*–*G*) confirming the inability of these photoreceptors to regenerate their visual pigment by accessing the retina visual cycle. We then examined the recovery of blue cones from a bleach in the isolated retina. Similar to red cones, blue cones also recovered their sensitivity in the isolated retina (Fig. 5*A*–*C*, see

Table 2. Estimated λ_{max} (nm) of action spectra

	Dark-adapted	Dissociated Bleached	Retina Bleached	11-*cis* retinal	9-*cis* retinal	9-*cis* retinol
Red cone	584 (18)	585 (16)	586 (20)	562 (5)	542 (7)	542 (10)
Red rod	516 (7)	516 (9)	521 (5)			497 (11)
Blue cone	438 (8)*	438 (8)*	438 (10)*			422 (8)
Green rod	440 (20)	440 (10)	440 (7)			ND

Values were estimated by fitting spectral sensitivity plots with visual pigment templates except for those marked with an asterisk. Estimated endogenous A_1/A_2 11-*cis* chromophore ratios were different among red cones (40:60), red rods (18:82 or 0:100) and green rods (0:100) in cells not treated with exogenous chromophore (Dark-adapted, Dissociated Bleached and Retina Bleached), probably due to individual differences among salamanders. Numbers of cells are shown in parentheses. *Estimated from pigment templates with A_1/A_2 11-*cis* chromophore ratios in red rods dark-adapted *in vivo* (18:82) with a fixed λ_{max} at 438 nm (Makino *et al.* 1999). ND, not determined.

also Fig. 5*E–G*). Thus, similar to red cones, blue cones were able to utilize chromophore from the retina visual cycle for pigment regeneration and dark adaptation. In contrast, despite using the same visual pigment as blue cones, green rods were unable to recover their sensitivity following a bleach in the isolated retina (Fig. 6*A–C*, see also Fig. 6*E–G*) and remained permanently desensitized. We did not study the possible blue shift in the action spectrum of these cells because the expected spectral shift with 9-*cis* chromophore is relatively small (14 nm for A_1 and 18 nm for A_2; Makino *et al.* 1999) in blue cones and green rods compared with that in red cones (20 nm for A_1 and 38 nm for A_2; Makino *et al.* 1999). Together, these results demonstrate that despite sharing the same visual pigment, blue cones in the salamander can utilize chromophore from the retina visual cycle to regenerate their visual pigment and dark-adapt, whereas green rods cannot. Thus, the ability to access the retina visual cycle is restricted to cones and it is independent of the type of pigment expressed in photoreceptors.

Finally, we also investigated whether salamander blue cones and green rods can utilize 9-*cis* retinol for pigment regeneration. Coincident with their ability to access the retina visual cycle, blue cones also showed substantial sensitivity recovery with 9-*cis* retinol (Fig. 5*D* and *H*). Similar to the case of red cones, the recovery of sensitivity of blue cones treated with 9-*cis* retinol was only partial, probably due to the lower quantum yield of the resulting 9-*cis* pigment compared to the native 11-*cis* pigment. In contrast, 9-*cis* retinol was unable to promote pigment regeneration and dark adaptation in green rods (Fig. 6*H*). Indeed, the sensitivity of green rods treated with 9-*cis*

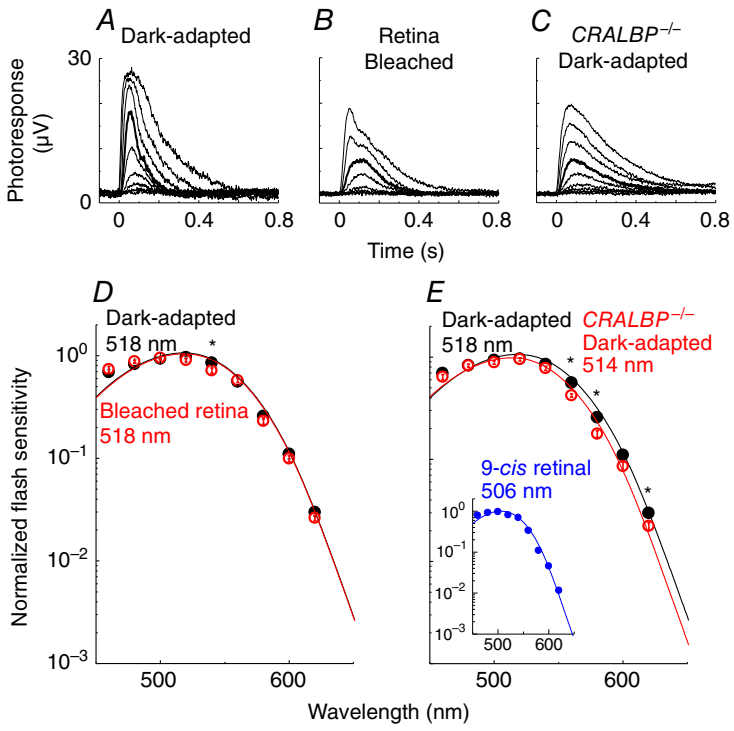

Figure 3. Transretinal recordings and spectral sensitivity of mouse M-cones
A–C, representative flash response families from cones in *Gnat1*$^{-/-}$ retina dark-adapted *in vivo* (*A*) or bleached and dark-adapted in the isolated retina (*B*), as well as in CRALBP$^{-/-}$ *Gnat1*$^{-/-}$ retina dark-adapted *in vivo* (*C*). Test flashes at 505 nm and 2 or 20 ms duration of intensity increasing with a step of ~0.5 log units were delivered at time 0. Each trace is the averaged response from 2–10 flash trials. Bold traces represent responses to a flash of 32,000 photons μm^{-2}. *D* and *E*, averaged and normalized spectral sensitivities of cones in *Gnat1*$^{-/-}$ retina dark-adapted *in vivo* (filled circles, $n = 16$), cones in isolated *Gnat1*$^{-/-}$ retina after a 90% bleach (*D*; open circles, $n = 9$), and cones in *CRALBP*$^{-/-}$ *Gnat1*$^{-/-}$ retina dark-adapted *in vivo* (*E*; open circles, $n = 13$). The inset in *E* shows a spectrum obtained from cones in *Gnat1*$^{-/-}$ retina incubated with 9-*cis* retinal after a >99% bleach. The data were fit with the A_1 11-*cis* pigment template with λ_{max} as a free parameter. *Statistically significant difference, $P < 0.05$.

retinol was lower than in their pre-treatment bleached state, consistent with increasing the rod opsin activity upon the non-covalent binding of chromophore analogues (Kefalov *et al.* 1999; Isayama *et al.* 2006) (but see also Ala-Laurila *et al.* 2009). The dark current of 9-*cis* retinol-treated green rods was also substantially reduced (Table 3), precluding us from obtaining reliable response family recordings. We confirmed the viability of these green rods by a subsequent treatment with 9-*cis* retinal, which resulted in substantial recovery of their sensitivity (Fig. 6*D*). Together, these results demonstrate that 9-*cis* retinol is able to promote pigment regeneration and recovery of sensitivity following a bleach in blue cones but not in green rods. Combined with our results from red cones and red rods above, these findings suggest that the oxidation of *cis*-retinol is restricted only to cones and is independent of the type of opsin expressed in the photoreceptors.

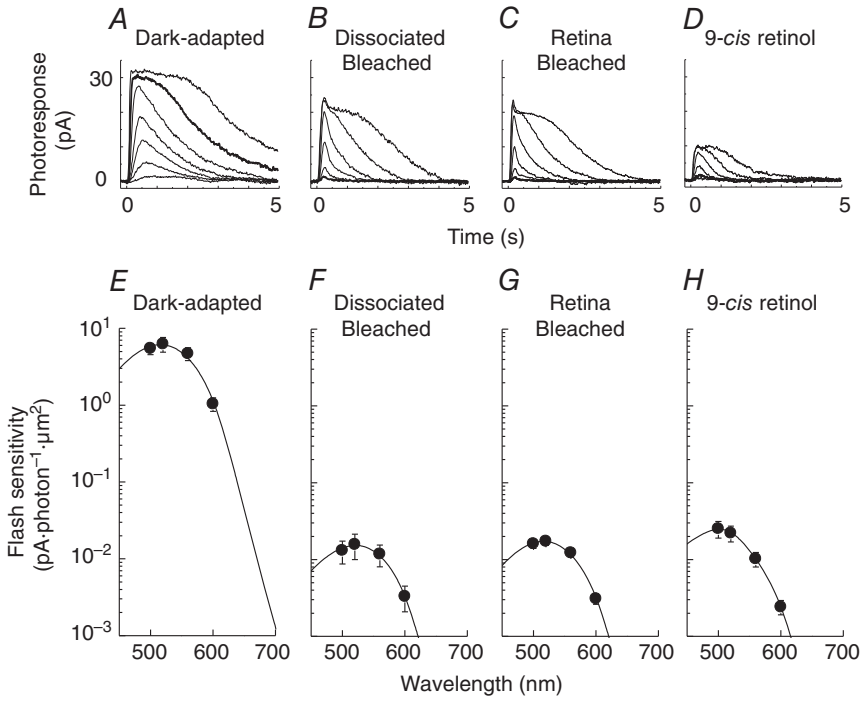

Figure 4. Single-cell suction electrode recordings and spectral sensitivity of salamander red rods
A–D, representative 500 nm flash response families from rods dark-adapted *in vivo* (*A*), dissociated and bleached (*B*), bleached and dark-adapted in the isolated retina (*C*), and dissociated, bleached and incubated with 9-*cis* retinol (*D*). Bold traces represent responses to a flash of 52 photons μm^{-2}. *E–G*, averaged spectral sensitivities of salamander red rods dark-adapted *in vivo* (*E*, $n = 7$), dissociated and bleached (*F*, $n = 9$), bleached and dark-adapted in the isolated retina (*G*, $n = 5$), and dissociated, bleached and incubated with 9-*cis* retinol (*H*, $n = 11$). Chromophore compositions of A_1 11-*cis*, A_2 11-*cis* and A_1 9-*cis* (for *H*) were estimated as 18:82 (*E*), 18:82 (*F*), 0:100 (*G*) and 5:20:75 (*H*).

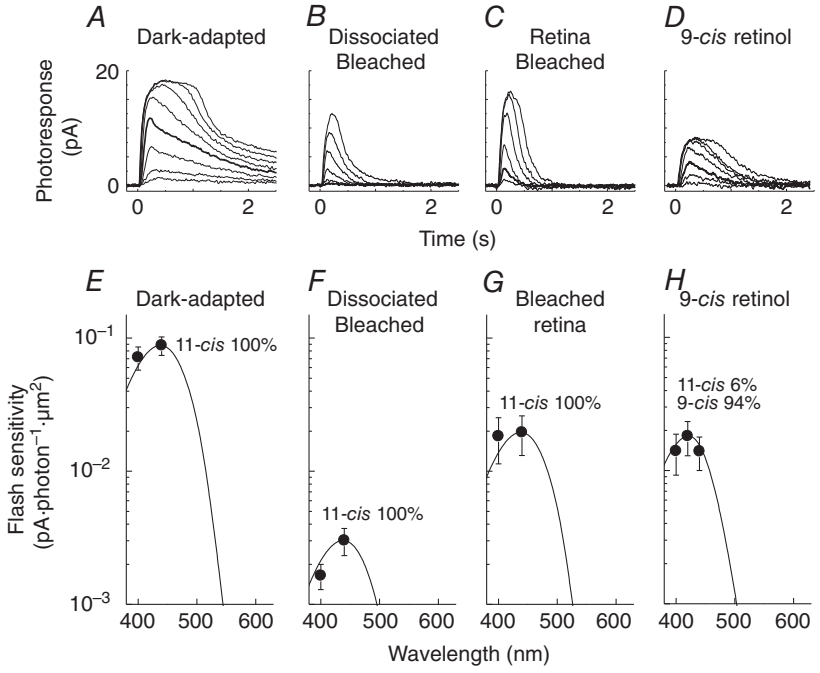

Figure 5. Single-cell suction electrode recordings and spectral sensitivity of salamander blue cones
A–D, representative 440 nm flash response families from cones dark-adapted *in vivo* (*A*), dissociated and bleached (*B*), bleached and dark-adapted in the isolated retina (*C*), and dissociated, bleached and incubated with 9-*cis* retinol (*D*). Bold traces represent responses to a flash of 200 photons μm^{-2}. *E–H*, averaged spectral sensitivities of salamander blue cones dark-adapted *in vivo* (*E*, $n = 8$), dissociated and bleached (*F*, $n = 8$), bleached and dark-adapted in the isolated retina (*G*, $n = 10$), and dissociated, bleached and incubated with 9-*cis* retinol (*H*, $n = 8$). Curves in *E–G* were obtained by fitting each 440 nm point with a combined 18% A_1 and 82% A_2 11-*cis* pigment template as estimated from red rods dark-adapted *in vivo* (Fig. 4*E*). The curve in *H* was obtained by fitting the 420 nm data point with a combined A_1/A_2 11-*cis* template (*F*) and the A_1 9-*cis* blue cone pigment template.

Discussion

Nature of the chromophore supplied by Müller cells to cones

The nature of the chromophore provided by Müller cells to cones as part of the retina visual cycle has been unclear. Biochemical studies have suggested that the putative Müller cell isomerase DES1 has a strong preference for 9-*cis* retinol over 11-*cis* retinol (Kaylor *et al.* 2013). Consistent with this possibility, 9-*cis* retinoid has been found in an array of species and conditions (Fan *et al.* 2003, Kaylor *et al.* 2013). On the other hand a recent study found that cultured rat Müller cells release 11-*cis* retinol (Betts-Obregon *et al.* 2014). We addressed this question directly by investigating the nature of the chromophore driving the regeneration of cone visual pigment in isolated salamander and mouse retinas, which is driven by the retina visual cycle. Our results demonstrate that retinal Müller cells provide cones exclusively with 11-*cis* and not with 9-*cis* chromophore in both salamander (Fig. 2*A*–*C*) and mouse (Fig. 3*D*). Furthermore, our results show that CRALBP in Müller cells plays a role in this process (Fig. 3*E*). As CRALBP binding favours 11-*cis* over 9-*cis* retinoids (Saari & Bredberg, 1987; Golovleva *et al.* 2003), the most likely explanation is that it enhances the conversion of all-*trans* retinol to 11-*cis* retinol by mass action law: as the DES1-catalysed isomerization of all-*trans* retinol produces a mix of retinoid isomers (Kaylor *et al.* 2013), CRALBP would bind to and remove selectively 11-*cis* retinol, thus promoting the production of 11-*cis* retinol. It is possible that in the absence of CRALBP a small fraction of 9-*cis* retinal is generated from all-*trans* retinal by dihydroflavin-catalysed reactions (Futterman & Rollins, 1973). However, as these reactions are slow and the levels of 9-*cis* retinal generated are miniscule, we believe that their contribution under our experimental conditions is negligible. Finally, a potential 11-*cis* selection mechanism that is yet to be explored *in vivo* involves the recently proposed 11-*cis*-specific retinyl ester synthase in Müller cells, multifunctional *O*-acyltransferase (Kaylor *et al.* 2014). The selective esterification of 11-*cis* retinol to 11-*cis* retinyl ester would also drive by mass action the production specifically of 11-*cis* retinoid in Müller cells. Such a scenario gives rise to the testable hypothesis that deletion of multifunctional *O*-acyltransferase will disinhibit the production of 9-*cis*

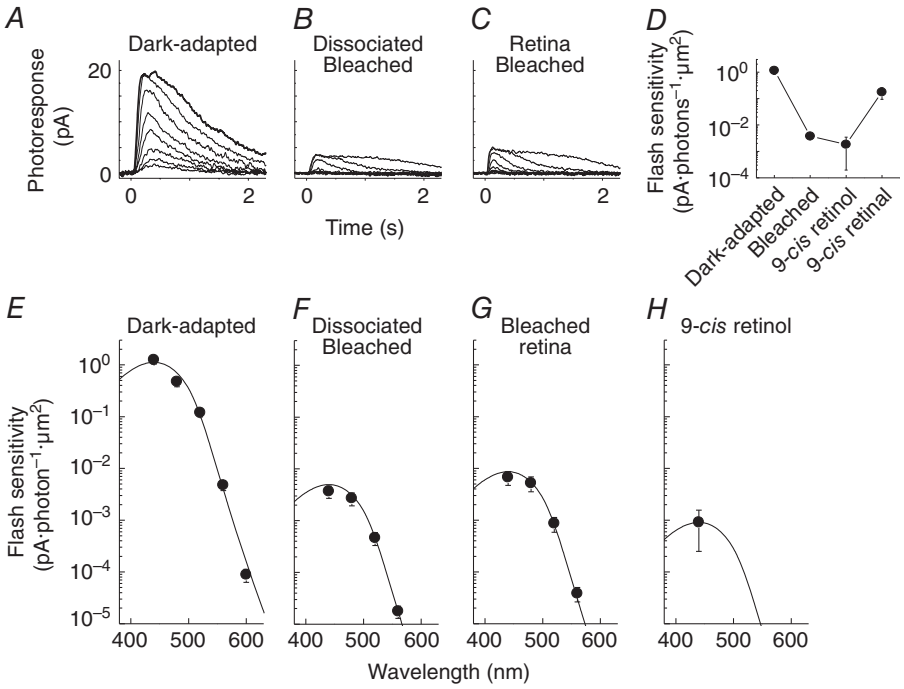

Figure 6. Single-cell suction electrode recordings and spectral sensitivity of salamander green rods
A–*C*, representative 440 nm flash response families from rods dark-adapted *in vivo* (*A*), dissociated and bleached (*B*), and bleached and dark-adapted in the isolated retina (*C*). Bold traces represent responses to a flash of 150 photons μm^{-2}. *D*, change in flash sensitivity of green rods at 440 nm after dark adaptation *in vivo* (Dark-adapted), full bleach (Dissociated Bleached), incubation with 9-*cis* retinol (9cROL) and incubation with 9-*cis* retinal after washing out 9-*cis* retinol (9cRAL) ($n = 2$ for all conditions). *E*–*H*, averaged spectral sensitivities of salamander green rods dark-adapted *in vivo* (*E*, $n = 20$), dissociated and bleached (*F*, $n = 10$), bleached and dark-adapted in the isolated retina (*G*, $n = 7$), and dissociated, bleached and incubated with 9-*cis* retinol (*H*, $n = 5$). Based on spectral templates fit, chromophore composition was estimated as 100% A_2 11-*cis* for *E*–*G* and the same template was used to fit the data in *H*.

Table 3. Dark current (pA), measured from saturated photoresponses in single-cell suction electrode recordings

	Dark-adapted	Dissociated Bleached	Retina Bleached	11-*cis* retinal	9-*cis* retinal	9-*cis* retinol
Red cone	21 ± 2 (18)	17 ± 3 (11)	21 ± 2 (12)	9.1 ± 1.5 (5)	15 ± 2 (5)	12 ± 1 (9)
Red rod	30 ± 3 (7)	15 ± 2 (9)	17 ± 3 (5)			7.5 ± 0.9 (11)
Blue cone	12 ± 2 (8)	12 ± 1 (8)	15 ± 1 (9)			10 ± 2 (7)
Green rod	24 ± 2 (20)	5.6 ± 1.0 (14)	8.2 ± 1.8 (7)			1.5 ± 0.5 (4)

Numbers of cells are shown in parentheses.

retinol, causing accumulation of 9-*cis* retinal-based cone visual pigment.

Cell type selectivity of the retina visual cycle

Our results demonstrate that not all photoreceptors can access the retina visual cycle. As previously shown, we found that salamander red cones (Figs 1 and 2) but not red rods (Fig. 4) can regenerate a substantial fraction of their pigment and largely recover their sensitivity following a bleach in the isolated retina. The ability of salamander blue cones and green rods to access the retina visual cycle had not been investigated. Whereas blue cones have cone-like morphology, their opsin, also expressed in green rods (Ma *et al.* 2001), has some rhodopsin-like properties so that binding of chromophore analogues, such as beta-ionone to blue cone opsin results in activation of the downstream phototransduction cascade (Isayama *et al.* 2006). This activation is typically observed for the opsin of red rods (Kefalov *et al.* 1999, 2003) but not red cones (Jin *et al.* 1993; Ala-Laurila *et al.* 2009). Thus, it was not clear whether blue cones will be able to regenerate their pigment via the retina visual cycle similar to red cones, or will be blocked from accessing this pathway similar to red rods. We found that blue cones undergo substantial recovery of their sensitivity following a bleach in the isolated retina (Fig. 5*C* and *G*). In contrast, green rods, despite using the same visual pigment, were unable to recover with the help of the retina visual cycle following a bleach (Fig. 6*C* and *G*). Thus, the type of the photoreceptor plays a critical role in regulating access to the retina visual cycle so that red and blue cones can readily regenerate their visual pigment in the isolated retina, whereas red and green rods cannot.

One of the mechanisms that possibly regulate access to the retina visual cycle is the ability to oxidize 11-*cis* retinol, the putative retinoid produced by the Müller cells (Jones *et al.* 1989; Wang & Kefalov, 2009; Wang *et al.* 2014). Considering that our results demonstrate that only cones but not rods can utilize the retina visual cycle for pigment regeneration, one would expect that oxidation of *cis* retinol will also be restricted to cones. Thus, we also investigated the ability of salamander photoreceptors to oxidize *cis* retinol and use it for pigment regeneration. As 11-*cis* retinol is difficult to obtain and unstable (Parker *et al.* 2011), we sought to determine whether its commercially available 9-*cis* analogue is a viable substitute. We found that 9-*cis* retinol produces robust sensitivity recovery comparable with that of 9-cis retinal in red cones (Fig. 2*F* and *E*) and blue cones (Fig. 5*H*), but not in red rods (Fig. 4*H*) or green rods (Fig. 6*H*). These results are consistent with the previous finding in a recent study that 11-*cis* retinol promotes pigment regeneration in salamander red and blue cones but not in red and green rods (Ala-Laurila *et al.* 2009). Together, our results demonstrate that only photoreceptors able to access the retina visual cycle can oxidize *cis* retinol and use it for pigment regeneration. Thus, this reaction appears to be one of the requirements for use of the retina visual cycle. The ability of 9-*cis* retinol to substitute for 11-*cis* retinol renders it an excellent experimental tool for investigating the mechanism driving the oxidation of *cis* retinol in cone photoreceptors and eventually identifying the enzyme responsible for this reaction. Indeed, 9-*cis* and 11-*cis* retinol oxidation are shown to be catalysed by the same enzyme in carp cones (Sato *et al.* 2015).

Candidate enzymes for the oxidation of *cis* retinol in cones

Our results presented here suggest that the oxidation of *cis* retinol is cone-specific. However, the identity of the enzyme driving this reaction remains unknown. The list of potential candidates includes retinol dehydrogenase 8 (RDH8), RDH13L (and its functional homologue in mice, RDH14) and RDH12. Recently, the deletion of RDH8 was found to block the 9-*cis* retinol-dependent sensitivity recovery in mouse M-cones (Kolesnikov *et al.* 2015). However, RDH8 is expressed in the outer segment of both rods and cones (Maeda *et al.* 2005; Miyazono *et al.* 2008) and it appears to be predominantly an all-*trans* retinal reductase (Rattner *et al.* 2000; see also Palczewski *et al.* 1994). RDH13L was identified as an 11-*cis*/9-*cis* RDH expressed in carp cone inner segments but not in rods (Sato *et al.* 2015). Although RDH13L is not present in amphibians and mammals, its functional homologue in mice, RDH14 (Sato *et al.* 2015), is conserved among vertebrates and appears to be expressed in mouse and bovine rods and cones (Haeseleer *et al.* 2002; Kanan *et al.* 2008) as well as in human retina (Zhang *et al.* 2015).

RDH12 belongs to the same RDH subfamily as RDH14 (Haeseleer *et al.* 2002) and catalyses both the reduction and the oxidation of all-*trans*/11-*cis*/9-*cis* retinoid (Belyaeva *et al.* 2005). RDH12 is expressed in the inner segments of both rods and cones in mouse retina (Maeda *et al.* 2006) and recombinant human RDH12 shows ∼2000-fold greater affinity for NADP(H) than NAD(H), implying its role predominantly as a retinal/aldehyde reductase (Belyaeva *et al.* 2005). In fact, RDH12 accounts for 30% of all-*trans* retinal reductase activity in mouse eye (Maeda *et al.* 2007).

Our results presented here demonstrate that in salamander retina access to the retina visual cycle and oxidation of *cis* retinol are restricted to red and blue cones and exclude red and green rods. In the case of mouse, access to the retina visual cycle is also restricted to cones and excludes rods (Wang & Kefalov, 2009). However, the hybrid rods in *rd7* mice that express a subset of cone genes (Corbo & Cepko, 2005) are able to regenerate a substantial fraction of their rhodopsin without assistance from the RPE (Wang *et al.* 2014). Together, these findings point to a future differential expression analysis in salamander rods *versus* cones and wild type *versus rd7* rods as a viable approach for identifying the elusive *cis* retinol dehydrogenase of the retina visual cycle.

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Additional information

Competing interests

The authors have no competing interests to declare.

Author contributions

Conception and design of the experiments: S.S. and V.J.K. Collection, assembly, analysis and interpretation of data: S.S. and V.J.K. Writing the article: S.S. and V.J.K. Both authors approved the final version of the manuscript.

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