11-cis-retinal is the light-sensitive component in rod and cone photoreceptors, and its isomerization to all-trans retinal in the presence of light initiates the visual response. For photoreceptors to function normally, all-trans retinal must be converted back into 11-cis-retinal through a series of enzymatic steps known as the visual cycle. The interphotoreceptor retinoid-binding protein (IRBP) is a proposed retinoid transporter in the visual cycle, but rods in Irbp<sup>-/-</sup> mice have a normal visual cycle. While rods are primarily responsible for dim light vision, the ability of cones to function in constant light is essential to human vision and may be facilitated by cone-specific visual cycle pathways. We analyzed the cones in Irbp<sup>-/-</sup> mice to determine whether IRBP has a cone-specific visual cycle function. Cone electroretinogram (ERG) responses were reduced in Irbp<sup>-/-</sup> mice, but similar responses from Irbp<sup>-/-</sup> mice at all ages suggest that degeneration does not underlie cone dysfunction. Furthermore, cone densities and opsin levels in Irbp<sup>-/-</sup> mice were similar to C57BL/6 (wild-type) mice, and both cone opsins were properly localized to the cone outer segments. To test for retinoid deficiency in Irbp<sup>-/-</sup> mice, ERGs were analyzed before and after intraperitoneal injections of 9-cis-retinal. Treatment with 9-cis-retinal produced a significant recovery of the cone response in Irbp<sup>-/-</sup> mice and shows that retinoid deficiency underlies cone dysfunction. These data indicate that IRBP is essential to normal cone function and demonstrate that differences exist in the visual cycle of rods and cones.

Introduction

Photoreceptors utilize the photosensitive properties of 11-cis retinal to detect light. The 11-cis retinal in photoreceptors covalently binds a G-protein-coupled opsin to form the visual pigment molecules in rods and cones. In the dark, 11-cis retinal functions as an opsin inverse agonist and reduces opsin activity, but when light strikes a visual pigment, 11-cis retinal isomerized to all-trans retinal, an opsin agonist (Wald, 1935, 1955). The photosomerization of retinal allows photoreceptors to generate a cellular response to light, but for photoreceptors to function in constant light, new 11-cis retinal must be available to bind the opsin when the all-trans photoproduction is released. To meet this requirement, all-trans retinal is efficiently converted back to 11-cis retinal through the enzymatic steps of the visual cycle. Our understanding of the visual cycle is largely derived from studies of rods that mediate vision in dim light. Cones, which function in bright light and mediate the bulk of human vision, appear to utilize the classical visual cycle also but may have access to unique sources of 11-cis retinal that facilitate their function in constant light (Mata et al., 2002, 2005).

The classical visual cycle is a compartmentalized cascade with steps occurring in the photoreceptors and retinal pigment epithelium (RPE). Through its steps, all-trans photoproducst from the photoreceptors are passed to the RPE, converted back to 11-cis-retinal, and returned to the photoreceptor for visual pigment regeneration. The compartmentalization of the visual cycle requires that poorly soluble and potentially toxic retinoids traverse the aqueous subretinal space between the photoreceptors and the RPE, and interphotoreceptor retinoid-binding protein (IRBP) is thought to facilitate this process (Bunt-Milam and Saari, 1983; Feng et al., 1984; Pepperberg et al., 1993; Lamb and Pugh, 2006).

IRBP binds retinoids (Adler and Spencer, 1991; Shaw and Noy, 2001) and is the most abundant soluble protein in the subretinal space (Loew and Gonzalez-Fernandez, 2002). In vitro studies have shown that IRBP promotes the release of all-trans retinol from photoreceptors (Ala-Laurila et al., 2006; Wu et al., 2007) and facilitates its delivery to the RPE (Okajima et al., 1994). Conversely, IRBP can promote 11-cis-retinal’s release from the RPE (Edwards and Adler, 2000), prevent its isomerization in the subretinal space (Crouch et al., 1992), and transfer 11-cis-retinal to photoreceptors (Jones et al., 1989).

If IRBP were essential for any of these steps, its absence should inevitably cause 11-cis-retinal deficiency, and the Irbp<sup>-/-</sup> mouse was expected to confirm IRBP’s importance to the visual cycle in vivo (Liou et al., 1998). Although rod function is diminished in Irbp<sup>-/-</sup> mice, the visual cycle in rods is surprisingly normal (Palczewski et al., 1999; Ripps et al., 2000), and the reduced rod function is thought to result from degeneration (Liou et al., 1998).
A 1 month WT 9 month Irbp $^{-/-}$

B 1 month WT 9 month Irbp $^{-/-}$

C 1 month WT 9 month Irbp $^{-/-}$

D 1 month WT 1 month Irbp $^{-/-}$ 9 month Irbp $^{-/-}$

Figure 1. Cone function is stable in aging Irbp $^{-/-}$ mice. A, Single-flash photopic ERG responses from individual WT and Irbp $^{-/-}$ mice to a 0.4 log cd*s/m$^2$ flash. B, Single-flash photopic ERGs of Irbp $^{-/-}$ mice to different flash intensities. C, Thirty hertz Flicker ERG responses from individual WT and Irbp $^{-/-}$ mice. D, Flicker responses in Irbp $^{-/-}$ mice (n = 3) were reduced relative to WT (n = 3), but remained stable from 1 to 9 months (n = 3). Data points represent mean amplitudes ± SD.

1998). Cone function in Irbp $^{-/-}$ mice is also diminished (Ripps et al., 2000), but the underlying cause remains unclear. Our purpose was to identify the cause of cone dysfunction in Irbp $^{-/-}$ mice and determine whether a cone-specific visual cycle defect exists.

Materials and Methods

Animals. Experimental procedures and animal care protocols adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina. Control wild-type (WT) mice were adult C57BL/6j mice (The Jackson Laboratory). The Irbp $^{-/-}$ mice were generated on a C57BL/6 background (Liou et al., 1998). Samples of each strain were genotyped to confirm the presence of the Leu450Met Rpe65 variant using the protocol described by Wenzel and coworkers (Wenzel et al., 2005).

Peanut agglutinin-stained retina flat-mounts. Eyes were dissected and placed in PBS (10 mM sodium phosphate, 0.15 M sodium chloride, pH 7.2). The retina-lens complex was fixed with 5% formaldehyde in PBS (4 h, 4°C) and washed with PBS (three times, 30 min, 4°C). 1:100 dilutions of FITC-conjugated peanut agglutinin (PNA) lectin (0.2 mg/ml; Sigma) were added (12 h, 4°C). After washing with PBS (as above), the retina was separated and mounted on a slide. Anti-fade solution (ProLong; Invitrogen) was applied and images were acquired with fluorescent microscopy. Images were taken from the mid-dorsal and mid-ventral regions of the retina. Counts from both eyes were averaged to create a dorsal and ventral density for each animal. Dorsal and ventral densities were then averaged to yield an overall cone density.

Western blots. Retinas were homogenized in 1% SDS buffer, and equivalent amounts of total retina protein (20 µg) were loaded onto 12% polyacrylamide gels following the XCell II Blot Module protocol (Invitrogen). Primary antibodies (1:500) for opsin detection were the rabbit anti-red/green opsin antibody (Millipore Bioscience Research Reagents) and rabbit anti-blue cone antibody (Millipore Bioscience Research Reagents). The secondary antibody (1:2000) was goat anti-rabbit IgG (Vector Laboratories). B-Actin was used as a loading control using mouse anti-β-actin (1:2000; Sigma) as a primary antibody and a goat anti-mouse IgG (1:5000; Vector Laboratories) secondary antibody. Blots were visualized with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific), and images were captured on a VersaDoc Imaging System (Bio-Rad).

Retina cross sections. At room temperature, eyes were fixed in 4% paraformaldehyde (2 h) and washed with PBS (three times, 15 min). Eyecups were separated and mounted on a slide. Anti-fade solution (ProLong; Invitrogen) and washed with PBS (three times, 30 min, 4°C). 1:100 dilutions of DRAQ5 (10 µM; Biostatus Limited) for 10 min. Images were acquired on a Leica confocal microscope. Sections used were made through the central retina (optic nerve visible). Images of the short-wavelength-sensitive (SWS) and medium-wavelength-sensitive (MWS) cone opsins were taken from the ventral and dorsal retina, respectively.

Electroretinogram analysis. Mice were anesthetized with xylazine (20 mg/kg) and ketamine (80 mg/kg), and pupils were dilated with phenylephrine hydrochloride (2.5%) and atropine sulfate (1%). Contact lens electrodes were placed on both eyes with a drop of methylcellulose. Electroretinograms (ERGs) were recorded with the UTAS E-2000 system (LKC Technologies) using 10 ms flashes of increasing light intensities under scotopic or photopic conditions. Cone recordings were made in the presence of a 30 cd/m$^2$ background light after 3 min of light adaptation.

Preparation and injection of 9-cis-retinal. 9-cis-retinal (Sigma) was prepared for injection under dim red light. The retinal was dissolved in ethanol (10% final injection volume) before adding the vehicle solution (10% BSA in 0.9% NaCl). After recording baseline ERGs, mice were rested 12 h, administered 0.375 mg of 9-cis-retinal intraperitoneally, and dark-adapted 12 h before recordings were repeated.

Statistical analysis. Statistical tests for each experiment were chosen after performing tests of normality for each sample (Kolmogorov–Smirnov test, Q-Q plots, and box plots). Photopic ERG responses of aging Irbp $^{-/-}$ mice were analyzed by one-way ANOVA. Kruskal-Wallis and Mann–Whitney U tests were used for cone density analysis. Intensity response functions between control and 9-cis-retinal injected Irbp $^{-/-}$ mice were analyzed with a paired sample two-way ANOVA and a post hoc analysis using a paired samples t test and the Bonferroni correction for multiple comparisons. Intensity–response functions from Irbp $^{-/-}$ and WT mice were compared with a two-way ANOVA.

Results

Cone function in Irbp $^{-/-}$ mice with photopic ERGs

Cone responses in Irbp $^{-/-}$ mice are diminished at 1 month (Ripps et al., 2000) and would decline further if a degenerative process underlies cone dysfunction. Cone function was analyzed in aging Irbp $^{-/-}$ mice with single-flash photopic and flicker ERGs. In agreement with previous findings (Ripps et al., 2000), cone responses were reduced in Irbp $^{-/-}$ mice at 1 month but were similar between Irbp $^{-/-}$ mice at 1 and 9 months of age (Fig. 1A). Between 1 and 8 months of age, there was no difference in the cone responses of Irbp $^{-/-}$ mice to a 0.4 log cd s/m$^2$ stimulus (p = 0.28) (Fig. 1B). Similar patterns of cone function were seen in flicker ERGs. Flicker ERG responses in Irbp $^{-/-}$ mice were
Cone densities counted from PNA-stained retina flat-mounts of MWS or SWS cones are uniquely affected in similar in the dorsal and ventral retina, suggesting that neither the densities declined in both n/H11005 mice had similar cone densities at 1 month (Fig. 1C,D). Scotopic ERGs showed reduced rod function in agreement with previous findings (Liou et al., 1998; Ripps et al., 2000). However, the constant level of cone function seen in both single flash and flicker ERGs from aging Irbp−/− mice suggests that degeneration is not the primary cause of cone dysfunction.

Cone density in aging Irbp−/− mice
While ERGs suggest that cone dysfunction is not secondary to degeneration, IRBP has proposed developmental functions (Gonzalez-Fernandez and Healy, 1990; Liou et al., 1994), and its absence could impair normal cone development. To determine if abnormalities in the cone population are present in Irbp−/− mice, retina flat-mounts were stained with PNA, a lectin that binds the glycoprotein sheath surrounding cones (Lotan et al., 1975; Johnson et al., 1986), and cone densities were compared in Irbp−/− and WT mice. For each retina, cone densities from the mid-dorsal and mid-ventral retina were used to generate an overall cone density. Figure 2A shows representative flat-mounts from Irbp−/− and WT mice at 1 and 8 months. Irbp−/− and WT mice had similar cone densities at 1 month (Irbp−/−, 256 ± 4.3, n = 4; WT, 261 ± 11, n = 4; p = 0.47) and 8 months (Irbp−/−, 218 ± 16, n = 3; WT, 216 ± 13, n = 3; p = 1.0) (Fig. 2B). Cone densities declined in both Irbp−/− and WT mice showed a decline in cone density between 1 and 8 months. To determine whether specific cone subpopulations were affected in Irbp−/− mice, densities were calculated for the dorsal and ventral retina of aging Irbp−/− mice. In Irbp−/− mice from 1 to 9 months, the overall cone densities dropped from 1 to 2 months (256 ± 4.3, n = 4; 222 ± 5.3, n = 4; p = 0.03) but remained stable between 2 and 9 months (p = 0.14) (Fig. 2C). At all ages, cone densities were similar in the dorsal and ventral retina, suggesting that neither the MWS or SWS cones are uniquely affected in Irbp−/− mice. In agreement with our ERG findings, cone densities were stable in aging Irbp−/− mice, and the similar cone densities between Irbp−/− and WT mice suggest that abnormal development does not underlie cone dysfunction.

Cone function in Irbp−/− mice after exogenous 9-cis-retinal injections
While neither the cone densities or opsin expression account for cone dysfunction in Irbp−/− mice, reduced cone function could be caused by a failure to promote normal outer segment development. We analyzed the localization and expression of MWS and SWS cone opsins in Irbp−/− mice. Western blots were used to identify MWS and SWS cone opsin levels from 20 μg of total retina protein. At 4 months of age, levels of the MWS and SWS cone opsins were similar in Irbp−/− and WT mice. After staining for either the MWS or SWS cone opsins, membranes were stripped and reprobed for β-actin as a loading control.

Cone opsins levels in Irbp−/− mice
Retina flat-mounts showed normal cone densities in Irbp−/− mice, but cone dysfunction could result from reduced or mislocalized opsin expression. Opsin levels and localization were analyzed with Western blots and immunohistochemistry. Western blots were performed for both cone opsins using equivalent levels of total retina protein and β-actin as a loading control. Representative blots from Irbp−/− and WT mice at 4 months of age showed equivalent levels of MWS and SWS opsins (Fig. 3). Retina cross sections were also used to confirm the correct localization of cone opsins. Images showing the MWS and SWS opsins were taken from the mid-dorsal and mid-ventral retina, respectively, of Irbp−/− and WT mice at 3 and 10 weeks. In both ages, the SWS and MWS cone opsins were localized to the outer segments of Irbp−/− mice, and both cone populations were comparable to WT mice (Fig. 4). While loss of the outer nuclear layer evident between 3 and 10 weeks is consistent with the rod degeneration reported to occur in Irbp−/− mice (Liou et al., 1998), the proper cone opsin localization and apparently healthy cone population suggest that cone abnormalities do not underlie cone dysfunction.

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result from visual cycle deficits in IRBP’s absence. We tested for 11-cis-retinal deficiency in the cones of Irbp−/− mice by analyzing photopic ERGs before and after intraperitoneal injections of 9-cis-retinal, an 11-cis-retinal analog that forms visual pigments (Crouch and Katz, 1980). Baseline responses from Irbp−/− mice were reduced relative to WT mice at all intensities but recovered dramatically after treatment with 9-cis-retinal (Fig. 5A). Intensity response plots from Irbp−/− mice (n = 8) show that cone responses increased significantly with 9-cis-retinal treatment at intensities above −0.8 log cd*s/m² (p = 0.005) (Fig. 5B). While baseline responses from Irbp−/− mice at each intensity were 50–60% of the WT responses, treatment with 9-cis-retinal resulted in responses that were 80–100% of WT values (Fig. 5C). Furthermore, the responses of Irbp−/− mice treated with 9-cis-retinal did not differ significantly from the responses of treated WT mice (p = 0.25) (Fig. 5D). 9-cis-Retinal had no effect on rod function in Irbp−/− mice (a-wave, p = 0.70; b-wave, p = 0.55) (Fig. 5E), and did not significantly alter the rod or cone responses in WT mice. Thus, the cones of Irbp−/− mice were uniquely sensitive to exogenous 9-cis-retinal, and the recovery of cone function to WT levels suggests that cone dysfunction in Irbp−/− mice results from an 11-cis-retinal deficiency.

Discussion

Rod and cone populations in Irbp−/− mice

IRBP is thought to link the photoreceptors and RPE in the classical visual cycle. While its absence does not alter retinoid metabolism in rods (Palczewski et al., 1999; Ripp et al., 2000), our current findings show that IRBP is important to cone function. In vitro studies have shown that IRBP can facilitate photoreceptors by enhancing the removal of all-trans photoproducts (Ala-Laurila et al., 2006; Wu et al., 2007) and the delivery of 11-cis-retinal (Jones et al., 1989; Edwards and Adler, 2000). Yet, the flow of all-trans photoproducts between the rods and RPE is unimpeded in Irbp−/− mice, and even in the presence of the Leu450Met Rpe65 variant, 11-cis-retinal is regenerated at normal rates (Palczewski et al., 1999). Furthermore, normal rhodopsin regeneration rates in Irbp−/− mice suggest that the return of 11-cis-retinal to rods is normal (Ripp et al., 2000). The lack of response to 9-cis-retinal confirms that adequate 11-cis-retinal is available for rhodopsin regeneration (Fig. 5E) and provides additional evidence that the rod visual is normal in Irbp−/− mice.

As IRBP is not essential to the rod visual cycle, rod dysfunction in the model is likely the result of degeneration and could result from the accumulation of toxic photoproducts in the absence of IRBP’s buffering abilities (Ho et al., 1989; Crouch et al., 1992; Wu et al., 2007). However, it is also likely that IRBP has important functions beyond retinoid transport. IRBP binds key outer segment components, such as docosahexanoic acid, that are essential for normal outer segment formation (Chen et al., 1993; Shaw and Noy, 2001). With disorganized outer segments common in the surviving rods of Irbp−/− mice (Liou et al., 1998), shortages of essential outer segment components could underlie rod dysfunction. Additionally, its size, abundance, and light-dependent movement with the interphotoreceptor matrix (IPM) suggest that IRBP is an important structural component of the IPM (Uehara et al., 1990), and it is possible that the formation and support of rod outer segments is disrupted by an altered IPM in IRBP’s absence.

While both rod and cone responses are reduced in Irbp−/− mice, our findings demonstrate a unique mechanism behind cone dysfunction. Stable cone ERGs (Fig. 1) and densities in aging Irbp−/− mice (Fig. 2) suggest that a significant degenerative process does not mediate cone dysfunction, and similar cone densities and opsin levels to WT mice (Figs. 2–4) show that the cone population develops normally. Thus, cone dysfunction exists in the presence of surprisingly healthy cone population. Interestingly, an IRBP mutation has been identified in a subpopulation of retinitis pigmentosa patients, who showed a profound loss of rod function but retained residual cone function (den Hollander et al., 2008). Similarly, our findings show that Irbp−/− mice undergo rod degeneration but that cones are less profoundly affected.

Recovery of cone function in Irbp−/− mice with 9-cis-retinal

In the absence of significant degeneration or developmental abnormalities, reduced cone function in Irbp−/− mice is likely due to cone-specific visual cycle deficits. Rod and cone ERGs performed before and after 9-cis-retinal injections confirmed that visual cycle deficits in Irbp−/− mice are cone-specific (Fig. 5). Rod responses were unchanged by 9-cis-retinal administration in Irbp−/− and WT mice, but the cone response of Irbp−/− mice recovered to WT levels. While our findings do not differentiate between the two cone subpopulations, the use of a standard Xenon stimulator may have been more effective at eliciting responses from the MWS cones, which are similar to human L/MWS cones. However, rescue of the cone response with 9-cis-retinal shows that at least the MWS cones survive with significant levels of unregenerated cone opsin and demonstrates that cones are uniquely dependent on IRBP for normal retinoid levels.

Functions of IRBP in cone visual cycle

Despite identifying a cone-specific visual cycle disruption, a number of questions remain surrounding IRBP’s contribution to cone function. Two explanations are plausible based on our findings. First, in vitro studies suggest that IRBP contributes to the classical visual cycle by promoting 11-cis-retinal’s release from
the RPE (Edwards and Adler, 2000) and delivery to photoreceptors (Jones et al., 1989). While the flow of 11-cis-retinal to rods is normal in Irbp−/− mice (Ripps et al., 2000), the flow to cones has not been analyzed, and it is possible that cones require IRBP to efficiently draw 11-cis-retinal from the RPE. A second explanation is that IRBP functions as the retinoid transporter in a proposed cone-specific visual cycle. Evidence in chicken and amphibian models (Hood and Hock, 1973; Jones et al., 1989; Das et al., 1992) suggests that cones have unique pathways for regenerating 11-cis-retinal through Müller cells (Das et al., 1992; Bustamante et al., 1995; Mata et al., 2002, 2005). Central to this theory is the unique ability of cones to regenerate 11-cis-retinal from 11-cis-retinol supplied to the inner segment (Jones et al., 1989). Müller cells can generate 11-cis-retinol (Das et al., 1992) and contain CRALBP in microvilli located near the cone inner segments (Bunt-Milam and Saari, 1983). IRBP colocalizes with the microvilli (Uehara et al., 1990), binds 11-cis-retinol endogenously (Saari et al., 1985), and is found in high concentration around cones (Carter-Dawson and Burroughs, 1992a,b). Although a functional relationship between IRBP, Müller cells, and cone inner segments has not been proven, the presence of retinoid deficient cones in Irbp−/− mice provides additional evidence for this pathway.

Summary
IRBP is not essential to the rod visual cycle, and the reduced rod function in Irbp−/− mice is likely from degenerative processes. Our current work shows that reduced cone function in Irbp−/− mice is due to 11-cis-retinal deficiency and implies that IRBP is essential to the normal cycling of retinoids in cones. These findings represent the first in vivo evidence implicating IRBP as a retinoid transporter in the visual cycle, and indicate a critical role for IRBP in the cone function that is essential for human vision.

References