Deletion of Aryl Hydrocarbon Receptor AHR in Mice Leads to Subretinal Accumulation of Microglia and RPE Atrophy

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Submitted: June 23, 2014

Accepted: August 13, 2014

Citation: Kim S-Y, Yang H-J, Chang Y-S, et al. Deletion of aryl hydrocarbon receptor AHR in mice leads to subretinal accumulation of microglia and RPE atrophy. *Invest Ophthalmol Vis Sci.* 2014;55:6031–6040. DOI: 10.1167/iov.14-15091

PURPOSE. The aryl hydrocarbon receptor (AHR) is a ligand-activated nuclear receptor that regulates cellular response to environmental signals, including UV and blue wavelength light. This study was undertaken to elucidate AHR function in retinal homeostasis.

METHODS. RNA-seq data sets were examined for *Ahr* expression in the mouse retina and rod photoreceptors. The *Ahr*^{−/−} mice were evaluated by fundus imaging, optical coherence tomography, histology, immunohistochemistry, and ERG. For light damage experiments, adult mice were exposed to 14,000 to 15,000 lux of diffuse white light for 2 hours.

RESULTS. In mouse retina, *Ahr* transcripts were upregulated during development, with continued increase in aging rod photoreceptors. Fundus examination of 3-month-old *Ahr*^{−/−} mice revealed subretinal autofluorescent spots, which increased in number with age and following acute light exposure. *Ahr*^{−/−} retina also showed subretinal microglia accumulation that correlated with autofluorescence changes, RPE abnormalities, and reactivity against immunoglobulin, complement factor H, and glial fibrillary acidic protein. Functionally, *Ahr*^{−/−} mice displayed reduced ERG c-wave amplitudes.

CONCLUSIONS. The *Ahr*^{−/−} mice exhibited subretinal accumulation of microglia and focal RPE atrophy, phenotypes observed in AMD. Together with a recently published report on another *Ahr*^{−/−} mouse model, our study suggests that AHR has a protective role in the retina as an environmental stress sensor. As such, its altered function may contribute to human AMD progression and provide a target for pharmacological intervention.

Keywords: orphan nuclear receptor, retinal degeneration, RPE atrophy, subretinal deposits, microglia

Nuclear receptors serve as key sensors and effectors that translate endocrine and metabolic cues to control diverse cellular functions, including metabolism, development, and apoptosis.^{1,2} The function of nuclear receptors can be modulated by specific ligands. In the retina, nuclear receptors have been implicated in differentiation and pathological change.³ The aryl hydrocarbon receptor (AHR) is a nuclear receptor that belongs to the basic-helix-loop-helix-Per-Arnt-Sim (bHLH/PAS) family of transcription factors. Aryl hydrocarbon receptor can bind to natural and synthetic ligands, polycyclic aromatic hydrocarbons such as those found in cigarette smoke, and polychlorinated dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.⁴ The search for endogenous and physiological AHR ligands has identified light-induced tryptophan metabolites such as 6-formylindolo [3,2-*b*] carbazole (FICZ), 6,12-diformylindolo [3,2-*b*] carbazole and 2-(1H-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid,^{5,6} and

tryptophan-derived phytochemicals such as indole-3-carbinol (I3C) from edible plants.⁷ AHR activation by UV radiation or FICZ and dietary I3C is reported to play protective roles in skin epidermal keratinocytes^{8,9} and intestinal intraepithelial lymphocytes, respectively.¹⁰

Visible light initiates the phototransduction cascade in photoreceptors of the mammalian retina; however, UV and blue wavelength light can damage the retina through complex phototoxic mechanisms that are still poorly understood.^{11,12} Reduced AHR expression and activity in aging human RPE^{13,14} suggest a protective role of AHR against light and/or oxidative damage. Here, we report that *Ahr* is expressed in the neural retina as well. We hypothesize that loss of AHR signaling increases the susceptibility of the retina to environmental stress such as intense light. We show that the retina of *Ahr*^{−/−} mice¹⁵ exhibits subretinal microglia accumulation that is coincidental with autofluorescence (AF) changes, RPE degeneration, and

immune activation. While our manuscript was in preparation, a recent report described age-related macular degeneration (AMD)-like pathology involving the presence of drusen in *Ahr*^{-/-} mice¹⁴ generated by the targeted deletion of exon 2,¹⁶ in contrast to the exon 1-targeted mice examined in our study. Together, these studies establish the relevance of AHR in retinal physiology and functional maintenance.

METHODS

Animals

We used *Ahr*^{-/-} mice generated by disruption of *Ahr* exon 1 (see Ref. 15) on a C57BL/6N genetic background. Age-matched wild-type (WT) C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) were used as control, except in ERG experiments, where C57BL/6N (DCT/Charles River, Frederick, MD, USA) mice were used. All WT C57BL/6J and *Ahr*^{-/-} mice were negative for *rd8* mutant allele by PCR, as described.¹⁷ All studies adhered to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committee of the National Eye Institute.

Transcriptome Analysis by RNA-Seq

Total RNA from mouse retina (15–50 ng) and purified rod photoreceptors (20 ng) was used to construct libraries for next generation sequencing on Illumina GAIIx (Illumina, Inc., San Diego, CA, USA), as previously described.^{18–20} For isolation of rod photoreceptors by fluorescence-activated cell sorting (FACS), *Nlrp*-GFP (Nrl promoter driving green fluorescent protein expression) transgenic mouse²¹ retinas were dissociated at 37°C for 10 minutes using Accutase (GIBCO/BRL, Grand Island, NY, USA). Green fluorescent protein-positive cells were collected using FACSaria II (Becton Dickinson, San Jose, CA, USA) with precision mode to prevent contamination from different retinal cell types. For each time point, four biological replicates of >97% purity were used for RNA-seq experiments. Transcript quantitation was performed with eXpress v1.3.1 (see Ref. 22) by streaming Bowtie2 v2.1.0 (see Ref. 23) aligned pass filter reads to GRCm38.p2 Ensembl release 73 annotation. This study utilized the high-performance computational capabilities of the Biowulf Linux cluster at the National Institutes of Health, Bethesda, Maryland, United States (<http://biowulf.nih.gov>).

Fundus, AF, and Optical Coherence Tomography (OCT) Imaging

Mice were anaesthetized by ketamine (61 mg/kg body weight) and xylazine (12 mg/kg body weight) and subjected to pupillary dilation with tropicamide (0.5%; Bausch & Lomb, Tampa, FL, USA) and phenylephrine hydrochloride (Mydrin 2.5%; Alcon, Fort Worth, TX, USA). Fundi were photographed using Micron II Rodent Fundus Imaging System (Phoenix Research Labs, Pleasanton, CA, USA) with a drop of hypromellose solution (2.5% Gonak; Akorn, Lake Forest, IL, USA) for corneal hydration. For AF and OCT imaging, Spectralis HRA+OCT system (Heidelberg Engineering, Heidelberg, Germany) was used. Fundus AF images were acquired using a 488-nm laser as excitation source. For AF measurements, we focused on the retina with a 55° angle lens with optic disc positioned in the center of the field. In cases when both AF spots and OCT images were obtained, fundus and OCT images were taken with a 30° angle lens in the same field, with the focus toward the inner retina for OCT images. The number of eyes examined under Micron Fundus system is as follows:

Ahr^{-/-} eyes, 1 to 2 months, *n* = 14; 6 to 9 months, *n* = 18; 12 months, *n* = 12; WT eyes, 1 to 3 months, *n* = 8; 6 to 9 months, *n* = 16; 12 months, *n* = 6. The number of the eyes used for quantification of AF spots is as follows: *Ahr*^{-/-} eyes, 1 month, *n* = 10; 3 months, *n* = 14; 6 months, *n* = 30; 9 months, *n* = 30; 12 months, *n* = 18; WT eyes, 3 months, *n* = 10; 12 months, *n* = 16.

Light Exposure

Two-month-old mice were dark-adapted overnight. After pupil dilation and anesthesia with 2.5% Avertin solution (15 µL/g body weight), animals were exposed to 14,000 to 15,000 lux of diffuse fluorescent white light (Phillips 36096-TLD bulbs; Phillips, Amsterdam, The Netherlands) in a foil-lined reflective cage for 2 hours.²⁴ Mice were then returned to a dark room for 16 hours and then to regular day/night cycle. Seven days after light exposure, AF spots from 10 to 12 eyes were counted for each group using the Spectralis system.

Retinal Histology and Immunohistochemistry

Mouse eyes were enucleated, fixed in 4% glutaraldehyde for 30 minutes and transferred to 4% paraformaldehyde in PBS until processing. Fixed eyes were embedded in methacrylate. Sections were collected at five locations near the optic nerve and stained with hematoxylin and eosin. Methacrylate sections were studied to determine the morphological appearance of the retina, as follows: *Ahr*^{-/-} eyes, 1 to 3 months, *n* = 22; 6 to 9 months, *n* = 21; 12 months, *n* = 8; WT eyes, 1 to 3 months, *n* = 12; 6 to 9 months, *n* = 8; 12 months, *n* = 13. For quantification of ectopic subretinal cells in 12-month-old retinas, one randomly chosen section per eye was photographed using a Zeiss Axio Imager Z1 microscope equipped with a motorized stage (Carl Zeiss Meditec, Jena, Germany) and a 20× objective. A series of tiled mosaic images of the entire retinal section were created, coded to mask their identity, and manually counted. For immunohistochemistry, eyes were fixed with 4% paraformaldehyde in PBS for 2 to 4 hours, and the anterior part of the eye was removed prior to embedding in agar blocks, which were then cut at 100 µm thickness using a vibrating microtome (Leica VT1000S; Leica, Wetzlar, Germany). The sections were incubated for 30 minutes with bovine serum albumin (3%) and Triton X-100 (0.5%) in PBS. Primary antibodies were applied for 2 days at 4°C. After washes with PBS, secondary antibodies were incubated overnight at 4°C. For whole mount F-actin RPE/sclerochoroidal staining, phalloidin-488 (Life Technologies, Carlsbad, CA, USA) was incubated for 3 days at 4°C. The following primary antibodies were used at 1 to 1000 dilution: rat monoclonal anti-CD11b (Serotec, Oxford, England, UK), goat polyclonal anti-complement factor H (CFH; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-rhodopsin,²⁵ rabbit polyclonal anti-cone arrestin (CAR; Millipore, Billerica, MA, USA), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; Chemicon, Billerica, MA, USA), and mouse monoclonal anti-ezrin (Thermo Scientific, Rockford, IL, USA). Relevant secondary antibodies were conjugated with Alexa Fluor 488 or 568 (Life Technologies). All antibody stainings were performed using at least three different eyes. For quantification of autofluorescent CD11b-positive cells, at least two areas from the superior and the inferior half of the eye was chosen randomly.

Electroretinogram

ERG was performed on 1- and 6-month-old mice using Espion e² Visual Electrophysiology System (Diagnosys, Lowell, MA, USA), as previously described.²⁶ After overnight dark-adaptation, the eyes of anesthetized mice were dilated with a drop of tropicamide and phenylephrine, and tetracaine (0.5%) was

FIGURE 1. *Ahr* expression in the retina and rod photoreceptors. (A) Time-course analysis of *Ahr* transcripts in mouse whole retina measured by RNA-seq. (B) *Ahr* transcript level detected by RNA-seq analysis in P2 and P21 WT, *Crx*^{-/-} and *Nrl*^{-/-} retinas. (C) Time-course analysis of *Ahr* expression in purified mouse rod photoreceptors by RNA-seq. (D) University of California, Santa Cruz (UCSC) genome browser view of *Ahr* showing CRX and NRL ChIP-seq peaks based on RPM. Whole retina from P28 WT mice was used for ChIP experiments. The y-axis in (A–C) represents average FPKM ± SEM from two to four biological replicates. **P < 0.01; *P < 0.05, Student's *t*-test. FPKM, fragments per kilobase of exon per million; RPM, reads per million.

applied before ERG. Body temperature was maintained at 37°C with a heating pad. Electroretinograms were recorded from both eyes using gold wire loops. A gold wire loop placed in the mouth was used as a reference electrode. Dark-adapted ERG was performed using flashes with intensities ranging from 0.0001 to 10 cd.s/m². For c-wave recording, stimulus was presented for 10 seconds with intensities from 0.01 to 1000 cd/m². Electroretinogram recordings were performed on five mice per age group.

RESULTS

Ahr Expression in the Retina and in Rod Photoreceptors

As part of our ongoing studies, we have generated global expression profiles of developing and mature mouse retina and of purified rod photoreceptors by microarrays and RNA-seq.^{18,21,27–29} The analysis of retinal gene expression profiling data revealed an increase in *Ahr* transcripts from P0 to P21 (Fig. 1A). However, *Ahr* expression was significantly reduced in adult *Crx*^{-/-} mouse retina³⁰ and in cone-only *Nrl*^{-/-} retina³¹ (Fig. 1B). We also observed up-regulation of *Ahr* expression during postnatal development in FACS-sorted rod photoreceptors from *Nrl*-GFP mice (Fig. 1C). Consistent with a reduction in *Ahr* transcripts in *Nrl*^{-/-} and *Crx*^{-/-} retina (Fig. 1B), we identified NRL and CRX binding sites in the promoter and intronic region of the *Ahr*

gene (Fig. 1D) in published ChIP-seq data sets.^{32,33} These findings suggest a role of AHR in rod photoreceptors in addition to the RPE, as reported.¹³

Fundus AF and Subretinal Ectopic Cells in *Ahr*^{-/-} Mice

To investigate the role of AHR, we obtained previously generated *Ahr*^{-/-} mice¹⁵ and validated the absence of Ahr and its downstream targets in *Ahr*^{-/-} retina by RT-PCR (Supplementary Fig. S1). The fundi of 3 months and older *Ahr*^{-/-} mouse eyes showed yellowish-white spots (Fig. 2A, arrows), and at 12 months, *Ahr*^{-/-} fundi displayed contiguous patches of RPE atrophy, reminiscent of geographic atrophy lesions in AMD (Fig. 2A, asterisk). We then evaluated *Ahr*^{-/-} and age-matched WT mouse retina by AF imaging. In *Ahr*^{-/-} mice, AF spots were absent at 1 month but detected as early as 3 months. These spots increased in number with age and were more numerous than those observed in WT controls (Figs. 2B, 2C). AF spots in *Ahr*^{-/-} mice were consistently more prevalent in the superior half of the fundus (Fig. 2D). In addition, acute light exposure augmented the number of AF spots in the superior retina of 2-month-old *Ahr*^{-/-} mice when assessed 7 days following exposure, while no significant change was detected in age-matched WT controls (Fig. 2E). Coincidentally, RPE/sclerochoroidal whole mounts from 1 month and older *Ahr*^{-/-} mice showed areas of aberrant pigmentation and choroidal thinning, largely in the superior part (Supplementary Fig. S2), as reported recently in another *Ahr*^{-/-} mouse.¹⁴ Optical coherence tomog-

FIGURE 2. Autofluorescence (AF) spots and RPE atrophy in *Ahr*^{-/-} mouse. **(A)** Color fundus images of 12-month-old WT retina and 1-, 3-, 6-, and 12-month-old *Ahr*^{-/-} retina taken by Micron system. Yellowish-white spots (arrows) and atrophic regions (asterisk) are observed in *Ahr*^{-/-} fundi. **(B)** Representative AF images of WT and *Ahr*^{-/-} fundi, showing AF spots predominantly in the superior half of mutant retina, taken by Spectralis system. **(C–E)** Graphs showing quantification of AF spots in 3- and 12-month-old WT and 1-, 3-, 6-, 9-, and 12-month-old *Ahr*^{-/-} fundus images with positioning the OD in the center **(C)**, in the superior or inferior half of 3-, 6-, 9-, and 12-month-old *Ahr*^{-/-} fundus images **(D)**, and 7 days after 2-hour acute light (14,000–15,000 lux) exposure in 2-month-old WT and *Ahr*^{-/-} mice **(E)**. Data represent average ($\pm$ SEM). * $P < 0.05$, Student's *t*-test. **(F)** Representative AF image of the 12-month-old *Ahr*^{-/-} fundus and the corresponding OCT image through the plane of section shown by the thick green horizontal line. Arrows in all panels point to the same retinal location. AF spots can be correlated with defects in OS/IS and RPE layer (see arrow in the bottom). **(G)** Representative OCT image of 12-month-old WT and *Ahr*^{-/-} eye. Numerous tiny vitreous opacities (possibly inflammatory cells; thin arrow) and epiretinal membrane (arrowhead) are observed in *Ahr*^{-/-} OCT. INL, inner nuclear layer; OD, optic disc; ONL, outer nuclear layer.

FIGURE 3. Histological examination of *Ahr*^{-/-} retina. (A) Pink deposit (thick arrow), ectopic or/and pigmented nodules (thin arrows), RPE vacuolization (asterisks), and RPE degeneration (arrowheads) in 6- to 12-month-old *Ahr*^{-/-} retina. The boxed areas are shown in the insets with higher magnification. (B) Retinal pigment epithelium-choroidal focal involution (center) and outer retinal folds (right) in 12-month-old *Ahr*^{-/-} retina. Areas delimited in yellow are shown at higher magnification in the lower panels. (C) Low (center) and high (right) magnification of areas containing ectopic cells, potentially microglia, in 12-month-old *Ahr*^{-/-} retina (arrowhead). CRD, choroid; INL, inner nuclear layer; IS, inner segments of photoreceptors; ONL, outer nuclear layer. Scale bars: 50 μ m ([A, C] and lower panels of [B]); 100 μ m ([B], upper panels).

raphy examination of *Ahr*^{-/-} retina at corresponding AF spot regions revealed attenuation of the hyper-reflective signal at both inner segment/outer segment junction of photoreceptor (IS/OS) and RPE layers (Fig. 2F, arrow in bottom panel), suggesting focal defects in IS/OS layers of photoreceptors and RPE. Finally, in almost all (94%) of the 1 month and older *Ahr*^{-/-} mice, OCT revealed numerous hyper-reflective dots in the posterior vitreous (Fig. 2G, arrow) and the presence of an epiretinal membrane (Fig. 2G, arrowhead).

Histology of *Ahr*^{-/-} eyes aged 6 months and older showed the presence of light pink (Fig. 3A, thick arrow) and pigmented deposits (Fig. 3A, thin arrows) over the RPE layer (63%, 32 of 51), as well as RPE vacuolization (Fig. 3A, asterisks) and degeneration (Fig. 3A, arrowheads). Furthermore, we observed RPE-choroidal focal involution (10%, 5 of 51) and outer retinal folds toward RPE (Fig. 3B; 22%, 11 of 51), as well as choroidal thinning (not shown; 61%, 31 of 51). The frequency of each histological phenotype was surveyed from at least two sections per eye at the level of the optic nerve head. Notably, we detected significantly higher number of ectopic cells, potentially microglia,³⁴ in the OS layer of *Ahr*^{-/-} retina compared to WT (4.00 ± 0.97 vs. 2.00 ± 0.47 cells per section $\pm$ SEM, respectively, $P < 0.05$; Fig. 3C).

Accumulation of Autofluorescent Subretinal Microglia in *Ahr*^{-/-} Mice

To examine the presence of microglia, we stained 12-month-old retinal vertical sections with CD11b antibody (Fig. 4A). We observed CD11b-positive long processes from outer plexiform layer (OPL) and ramified pseudopodia in IS extending from cell bodies in both WT and *Ahr*^{-/-} inner retina (Fig. 4A, white arrowheads). However, we detected subretinal accumulation of CD11b-positive microglia in *Ahr*^{-/-} retina but not in age-matched WT controls, suggesting that subretinal microglia in *Ahr*^{-/-} mice migrated from the inner retina. In RPE/sclerochoroidal whole mounts, CD11b-staining revealed a large number of microglia on the apical surface of 12-month-old *Ahr*^{-/-} RPE, while only occasional CD11b-positive cells were detected in the WT RPE (Fig. 4B). These autofluorescent subretinal microglia, which are coincidental to fundus AF spots, were found generally in larger numbers in the superior half of the *Ahr*^{-/-} RPE/sclerochoroid whole mounts (Figs. 4B, 4C). Over 96% of the CD11b-positive cells in the superior half of the RPE displayed autofluorescent signals compared to only approximately 20% in the inferior half. At high magnification, subretinal microglia located in the superior half displayed

FIGURE 4. Autofluorescent subretinal microglia in the *Ahr*^{-/-} retina. (A) Twelve-month-old retinal vertical sections stained for CD11b. Subretinal microglia were observed in *Ahr*^{-/-} retina while only microglia processes and ramified pseudopodia (arrowheads) were detected in WT. Left end of each panel is overlaid with DAPI counterstain (blue). (B) Twelve-month-old RPE/sclerochoroidal whole mounts showing CD11b immunoreactivity (red) and AF (white). Areas marked with a square are shown at a higher magnification (bottom). (C) Higher magnification of CD11b-positive subretinal microglia (red) in phalloidin-stained (green) RPE/sclerochoroidal whole mounts of WT and *Ahr*^{-/-} mice. Activated amoeboid microglia (arrow) with AF are seen in a patch of RPE degeneration. *Ahr*^{-/-} whole mount have large cell bodies and short pseudopodia. INL, inner nuclear layer; IS, inner segments of photoreceptors; ONH, optic nerve head; ONL, outer nuclear layer. Scale bars: 50 μ m (A), 200 μ m (B), 25 μ m (C).

FIGURE 5. Retinal pigment epithelium atrophy and immune activation. (A) Alexa-488-conjugated phalloidin staining (green) in whole mount RPE/sclerochoroidal preparations of 12-month-old WT and *Ahr*^{-/-} eyes. IgG immunoreactivity (red) lines the rim of the degenerating *Ahr*^{-/-} RPE (right end). Z-projection images. (B–E) IgG-immunoreactive areas (red) in the photoreceptor OS layer and apical RPE of 12-month-old *Ahr*^{-/-} retina, overlaid on DIC images (B) and coimmunostained with antibodies against CFH (green in [C]), rhodopsin (Rho; green in [D]), and CAR (green in [E]). Cone arrestin immunoreactivity is absent from IgG-positive areas. (F) Immunostaining of GFAP in 1- and 7-month-old WT and *Ahr*^{-/-} retinas. All retinal sections are generated by vibratome, counterstained with DAPI (blue) and labeled with indicated antibodies. DIC, differential interference contrast; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer. Scale bars: 50 μ m.

activated amoeboid morphology and were associated with areas of RPE atrophy, in contrast to more ramified microglia located in the inferior half with more intact RPE (Fig. 4C).

RPE Damage and Immune Activation in *Ahr*^{-/-} Retina

We detected patches of abnormal RPE characterized by uneven distribution of F-actin, loss of hexagonal morphology, and defective apical microvilli (Fig. 5A). The peripherally located patches of abnormal RPE in *Ahr*^{-/-} mice were first observed around 8 months. All 12-month-old *Ahr*^{-/-} mice exhibited severe RPE atrophy in the central region, as well as focal patches of damaged RPE in the periphery. Activated subretinal microglia (Fig. 4) and RPE atrophy (Fig. 5A) observed in *Ahr*^{-/-}

eyes suggested possible local immune reaction and prompted us to examine IgG and CFH immunoreactivity. Central patches of severe RPE atrophy were surrounded by a rim of prominent IgG-immunoreactivity (Fig. 5A, right panel). Consistent with this, we observed patchy IgG-positive signal in 12-month-old *Ahr*^{-/-} in the subretinal space (OS layer) but not in age-matched WT retina (Figs. 5B–E). These IgG-positive areas in the *Ahr*^{-/-} retina were also immunoreactive for CFH (Fig. 5C). The accumulation of IgG- and CFH-positive deposits in the subretinal space showed rhodopsin positive immunostaining, but no labeling was detected with anti-CAR antibody (Figs. 5D, 5E), in concordance with a previous report of human subretinal drusenoid deposits.³⁵ Furthermore, uneven rhodopsin distribution in *Ahr*^{-/-} retina suggests the disruption of OS layer. Additionally, GFAP immunoreactivity in Müller cell

FIGURE 6. Defective RPE apical microvilli and reduced ERG c-wave in *Ahr*^{-/-} mice. **(A)** Ezrin (red) and rhodopsin (green) immunostaining in vertical vibratome sections of 1-month-old WT and *Ahr*^{-/-} eye. Z-projection images (WT, $z = 5$; *Ahr*^{-/-}, $z = 12$ at 0.5- μ m intervals) are shown. Scale bar: 25 μ m. **(B)** Dark-adapted ERG recordings of 1-month-old WT and 1- and 6-month-old *Ahr*^{-/-} mice. Averaged a- and b-wave amplitudes of flash ERG are shown in *left* and *middle* panels, and c-wave amplitudes in response to 10 seconds long light stimuli are shown in the *right* panel. Data indicate the average ($\pm$ SD) of five animals per group. * $P < 0.05$, Student's *t*-test.

processes emerged in 3-month-old *Ahr*^{-/-} mice with a patchy distribution (data not shown), which expanded to all retinal regions by 7 months (Figs. 5E, 5G).

RPE Apical Microvilli Defect and Reduced ERG C-Wave in *Ahr*^{-/-} Retina

We examined ezrin immunoreactivity in vertical sections of *Ahr*^{-/-} retina since AHR is implicated in cell adhesion and matrix metabolism.³⁶ The *Ahr*^{-/-} retina exhibited patches of reduced ezrin immunolabeling as early as 1 month (Fig. 6A, asterisks) in contrast to age-matched WT retina that showed continuous ezrin staining throughout the RPE microvilli in close contact with rhodopsin-positive OS of photoreceptors. In concordance with RPE atrophy, we detected reduced ERG c-wave amplitude response in 1-month-old *Ahr*^{-/-} retina compared to WT; however, the c-wave amplitude did not decrease further at least until 6 months of age. It should be noted that c-wave is composed of RPE and Müller cell subcomponents,³⁷ thus, activation of GFAP in *Ahr*^{-/-} retina starting at 3 months may mask further c-wave changes. Amplitudes of a- and b-wave for dark-adapted flash ERG were comparable between *Ahr*^{-/-} and WT retina (Fig. 6B).

DISCUSSION

Photoreceptors are dependent on RPE for phototransduction and survival since RPE, among other functions, serves as the conduit for two-way transport to and from choroidal capillaries. Retinal pigment epithelium constitutes the blood-retina barrier, and altered RPE function under conditions of stress, advanced age, and/or due to genetic susceptibility can lead to accumulation of toxic metabolites, immune response, microglia activation, and atrophy.^{38–41} Our findings of age-related accumulation of autofluorescent subretinal microglia, RPE atrophy, and immune activation in *Ahr*^{-/-} mice indicate a protective role of AHR in countering adverse stress conditions to maintain the photoreceptor support system. The nuclear

receptor AHR has previously been implicated in the regulation of developmental pathways and biological responses to xenobiotics and phytochemicals.^{5,42} Together with a recent report on another allele of *Ahr*^{-/-} mice,¹⁴ we propose that AHR plays a significant role in maintaining photoreceptor and RPE homeostasis.

The findings in *Ahr*^{-/-} mice, presented here and by Hu et al.,¹⁴ are reminiscent of some of the ocular changes observed in AMD.^{41,43,44} In humans, advanced age is associated with the accumulation of autofluorescent lipofuscin particles in RPE and of lipid-rich drusen between the RPE and Bruch's membrane; large drusen between RPE and the Bruch's membrane are considered a hallmark for AMD.⁴⁵ Recently, reticular pseudodrusen in the form of subretinal drusenoid deposits have also been recognized as an additional feature in AMD.^{46–49} The fundus phenotype, subretinal microglia, and IgG deposits observed in *Ahr*^{-/-} retina appear similar to reticular drusen⁵⁰ and subretinal drusenoid deposits,^{46,48} suggesting that some *Ahr*^{-/-} phenotypes could be correlated to subretinal phenotypes in human AMD. Our observation of AF subretinal microglia primarily in the superior part of apical RPE suggests that the spots in the fundus may be correlated to subretinal microglia with AF. The presence of AF might also reflect abnormal rod OS ingestion and lipid accumulation.^{39,51} The identification of IgG and CFH immunoreactivity in subretinal region of the *Ahr*^{-/-} retina indicates the presence of immune complexes and probably autoantibodies, similar to those observed in AMD patients.^{52,53}

Several possible mechanisms could explain the observed ocular phenotypes in *Ahr*^{-/-} mice. First, aberrant RPE function in *Ahr*^{-/-} mice could delay the clearance of photoreceptor byproducts and promote the recruitment of microglia in subretinal region. Second, loss of AHR function could disrupt the expression of adhesion molecules in photoreceptors and/or RPE, leading to altered interaction between these two cell types, which may impact phagocytosis and produce subretinal debris. Third, AHR deficiency could alter functions of retinal microglia, which also express *Ahr*.³⁹ The loss of AHR or AHR ligands is reported to affect the

inflammatory property of dendritic cells^{54,55} and brain microglia.⁵⁶ Furthermore, the loss of AHR may induce a systemic inflammatory response since AHR activation is involved in differentiation of regulatory T cells⁵⁷⁻⁵⁹ and suppression of autoimmune diseases.^{54,60,61} Finally, the protective role of AHR could be mediated by light-induced or dietary tryptophan metabolites in different retinal cell types, including RPE, photoreceptors, and microglia.

We noted that *Ahr*^{-/-} retina in our study exhibits primarily subretinal deposits, different from the phenotype reported earlier.¹⁴ The latter study showed drusen-like deposits between the RPE and Bruch's membrane in *Ahr*^{-/-} mice at a relatively older age.¹⁴ This discrepancy in phenotypes may reflect the use of *Ahr*^{-/-} mice that were produced independently using different strategies and targeting different exons.^{4,15} Difference in age at the time of phenotyping and histological methods may also contribute to somewhat distinct retinal phenotypes observed in the two *Ahr*^{-/-} lines. Both common and unique phenotypes have been reported in nonocular tissues of the two knockout lines.⁶² Common phenotypes such as fundus geographic atrophy, RPE dystrophy, and local choroid atrophy identified in the two different *Ahr*^{-/-} mice (reported here and by Hu et al.¹⁴) validate the functional relevance of AHR in maintaining the integrity of retina/eye during stress conditions. Although AHR has not directly been associated to AMD in genetic studies,^{38,63,64} the *Ahr*^{-/-} mice exhibit a subset of AMD features. Thus, AHR ligands may represent an attractive target for pharmacological intervention in AMD by potentiating tissue-intrinsic protective mechanisms.

Acknowledgments

We are grateful to Frank J. Gonzales and Linda Byrd of the National Cancer Institute for *Ahr*^{-/-} mice. We thank Raphael Villasmil, Chun Gao, Yichao Li, Chi-Chao Chan, Yide Mi, Christine Park, and Wenxin Ma for technical advice and assistance. Supported by the Intramural Research Program of the National Eye Institute, National Institutes of Health.

Disclosure: **S.-Y. Kim**, None; **H.-J. Yang**, None; **Y.-S. Chang**, None; **J.-W. Kim**, None; **M. Brooks**, None; **E.Y. Chew**, None; **W.T. Wong**, None; **R.N. Fariss**, None; **R.A. Rachel**, None; **T. Cogliati**, None; **H. Qian**, None; **A. Swaroop**, None

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