Mitochondrial Respiration in Outer Retina Contributes to Light-Evoked Increase in Hydration In Vivo

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PURPOSE. To test the hypothesis that mitochondrial respiration contributes to local changes in hydration involved in phototransduction-driven expansion of outer retina, as measured by structural responses on optical coherence tomography (OCT) and diffusion magnetic resonance imaging (MRI).

METHODS. Oxygen consumption rate and mitochondrial reserve capacity of freshly isolated C57BL/6 and 129S6/SvEvTac mouse retina were measured using a Seahorse Extracellular Flux Analyzer. Light-stimulated outer retina layer water content was determined by proton density MRI, structure and thickness by ultrahigh-resolution OCT, and water mobility by diffusion MRI.

RESULTS. Compared with C57BL/6 mice, 129S6/SvEvTac retina demonstrated a less robust mitochondrial respiratory basal level, with a higher reserve capacity and lower oxygen consumption in the light, suggesting a relatively lower production of water. C57BL/6 mice showed a light-triggered surge in water content of outer retina in vivo as well as an increase in hyporeflective bands, thickness, and water mobility. In contrast, light did not evoke augmented hydration in this region or an increase in hyporeflective bands or water mobility in the 129S6/SvEvTac outer retina. Nonetheless, we observed a significant but small increase in outer retinal thickness.

CONCLUSIONS. These studies suggest that respiratory-controlled hydration in healthy retina is linked with a localized light-evoked expansion of the posterior retina in vivo and may serve as a useful biomarker of the function of photoreceptor/retinal pigment epithelium complex.

Keywords: mitochondria, genetics, functional imaging, photoreceptor, water content

Sight-threatening disease of photoreceptor cells potentiate a substantial reduction in a person’s quality of life, with an increased risk of falls, an important predictive factor of long-term survival.1 Many photoreceptor diseases remain with inadequate or no therapies. One major reason for this limited progress has been the lack of high spatial resolution, noninvasive imaging of the morphology and function of photoreceptors. A conventional measure of photoreceptor physiology in vivo is electroretinography. However, the electroretinographic response is an integrated signal over the entire retina, and to a degree from different retinal layers, making it difficult to compare to localized structural changes, as measured by optical coherence tomography (OCT, the modality of choice for high spatial resolution imaging of photoreceptor anatomy in vivo). An alternative approach is to noninvasively measure aspects of focal photoreceptor cell subcompartment function by using high-resolution magnetic resonance imaging (MRI) and compare the results to OCT data.2 At present, the wide availability of OCT in the clinic is motivating work to encode information about photoreceptor function into OCT images.

One important aspect of photoreceptor function is the light-stimulated expansion of outer retina volume. Such a response was first measured by microelectrodes in frog and chick retina ex vivo, and in vivo in cat retina, by diffusion MRI in mice and rats, and more recently by ultrahigh-resolution OCT in mice and people.3–11 These studies imply, but do not establish, that outer retina (i.e., the photoreceptor/retinal pigment epithelium [RPE] complex) hydration is dynamically linked with its function and associated energy ecosystem. The health of photoreceptor cells is dependent on adequate hydration in order to regulate local ion and metabolite content, the composition of the interphotoreceptor matrix, and retinal adhesiveness to the RPE.8,9,12,13

Here, we test the hypothesis that mitochondrial respiration contributes to local changes in hydration involved in the phototransduction-driven expansion of the outer retina, as measured by structural responses on OCT and diffusion MRI.5–7,14 We compared C57BL/6 and 129S6/SvEvTac mice, two strains with different RPE65 variants regulating distinct outer retina reactions to light; different expressions of functional DISC1, a regulator of an electron transport chain, monoamine oxidase, and Ca2+ activity, located in the basal body...
complex of the apical region of the mitochondria-rich inner segment;\(^ {15}\) and different photoreceptor histopathology outcomes to similar oxidative stress-inducing insults.\(^ {16-22}\) Together, these strain differences suggest distinct energy ecosystems in their outer retina. Photoreceptors account over 70% of cell populations and are the largest consumer of oxygen in the retina. Oxygen consumption is directly proportional to water production. These considerations imply a differential posterior retinal hydration status between the two strains, a hypothesis tested herein.

In this study, we first compared C57BL/6 and 129S6/SvEvTac retinal oxygen consumption rate (OCR) and mitochondrial maximum reserve capacity (MRC) ex vivo, which are two important indices of activity/efficiency of mitochondria function.\(^ {23,24}\) Next, we associated these strain-specific respiratory indices to in vivo light-evoked responses in posterior retina water content (proton density MRI), layer thickness (ultrahigh-resolution OCT), and water mobility (diffusion MRI).

**Materials and Methods**

All animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and Institutional Animal and Care Use Committee authorizations at Wayne State University and National Eye Institute. We used male 2- to 3-month-old C57BL/6 (Jackson Labs, Bar Harbor, ME, USA) and 129S6/SvEvTac (Tacomic Labs, Rensselaer, NY, USA) mice. Animals were housed and maintained under 12-hour:12-hour light:dark cycle laboratory lighting, unless otherwise noted. The exact number of mice used in each experimental condition is given in the figure legends. The sample sizes used for each experimental condition are based on our previous experience with each technique.\(^ {4,5,23,25}\)

**Data Collection and Analysis**

**OCR and MRC.** OCR of retinal punches was performed ex vivo according to the published protocol.\(^ {25}\) Briefly, three punches of 1-mm diameter equidistant from the optic nerve head (ONH) were taken from each retina for the measurement. Ames’ buffer (120 mM NaCl) containing 10 mM HEPES (pH 7.35) was used in the assay. The mitochondrial uncoupler (2-fluorophenyl)(6-[(2-fluorophenyl)amino](1,2,5-oxadiazolo[3,4-c]pyrazin-5-yl))amine (BAM15; Timtec, Newark, DE, USA) and a complex I inhibitor rotenone were used at a final concentration of 5 \(\mu\)M and 1 \(\mu\)M, respectively. Raw OCR values were computed by the Seahorse KSV algorithm. Nonmitochondrial respiration, which is the residual reading obtained after the oxygen consumption is directly proportional to water production. These considerations imply a differential posterior retinal hydration status between the two strains, a hypothesis tested herein.

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of the ONH (after correcting for the surface coil inhomogeneity and linearizing the retinal exposure). In addition, the intraretinal water profiles were adjusted to be a percentage of total retinal thickness for each animal (with 0% thickness at the vitreous-retinal border, and 100% thickness at the retina-choroid border), the depth into the retina of 80% to 100% was considered to be the posterior retina. The accuracy of using MRI to estimate retinal water content was confirmed previously by agreement between MRI-derived water content and specific gravity measurements. We also previously validated the linearity of MRI signal intensity and water content in phantom studies.

**Diffusion MRI.** In separate subgroups of mice, anatomic and apparent diffusion coefficient (ADC) MRI data sets were collected; the diffusion sensitizing gradient was set parallel to the optic nerve based on our previous observations that this is a useful direction for detecting changes in the outer retina. In C57BL/6 mice, ADC was first acquired in the dark and then again 15 minutes after turning on the light; each ADC data set takes 10 minutes to collect, so we refer to the m-i-point in the ADC collection as 20 minutes of light exposure. For 129S6/SvEvTac mice, we collected ADC data in the dark and again at 13 minutes and 29 minutes (midpoints) after light exposure for a different study. However, we also used the light data herein by averaging these time points to estimate the ADC at 21 minutes of light. Anatomic images were acquired using a spin-echo sequence (slice thickness, 600 µm; TR, 1000 ms; TE, 11 ms; matrix size, 192 × 320; field of view, 8 × 8 mm²; number of acquisitions (NA), 2; axial resolution for central retina, 25 µm). Images sensitized to water diffusion were collected (TR, 1000 ms; slice thickness, 600 µm; TE, 33 ms; matrix size, 174 × 288; field of view, 8 × 8 mm²; axial resolution for central retina, 27.8 µm; b = 0, 100, 250, 500, 600, 750, 990 s/mm² [collected in pseudo-random order; NA 1 per b value]), registered to the anatomic image, and analyzed (using in-house code) to generate ADC profiles from the central retina. We have previously established that the present resolution in the central retina is sufficient for extracting meaningful changes in central retinal thickness on the anatomic images and significant ADC changes at 88% to 100% depth, as previously discussed.

Data, largely free of choroidal contamination, were thus analyzed as described in detail in prior publications.

**Statistical Analysis**

Seven mice from each strain were used, and three punches were taken from each retina in the seahorse experiment. All outcomes (OCT thickness, ADC, and water content) were measured multiple times within each animal. As such, we used linear mixed models to examine the differences between strains and between light exposures. A significance level of 0.05 was used for all analyses.

The model for OCT thickness included the fixed effects of strain and light exposure along with the interaction between strain and light exposure. This model also included the random effect of mouse within strain.

The relationship between ADC and retinal depth was modeled using restricted cubic splines for depth to compare mouse-specific profiles between groups. The number of “windows” with a relationship between ADC and retinal depth (i.e., “knots”) was initially evaluated separately for each group for any given analysis, and the Akaike and Schwarz Bayesian information criteria (AIC and BIC, respectively) were used to identify the model with the fewest knots needed to model all groups, leading us to select six knots for the final model. Random coefficients for the intercept, light exposure, depth-specific coefficients (cubic spline coefficients), and interactions for light exposure/depth were also evaluated using AIC and BIC.

The random coefficients for the intercept, light exposure, depth, the interaction between light exposure and depth, positive cubic distance of depth from knot 3, and positive cubic distance of depth from knot 4 were included in the final model. This model also included the fixed effects of strain, light exposure, location-specific values for the cubic splines, and all interactions among the main effects. The three-way interaction for strain/light-exposure/retinal depth was significant based on a likelihood ratio test (P < 0.0001). Given these results, we used the model with all three-way interactions in testing for differences between the strain/light-exposure groups. We used numeric integration to estimate the mean for any region identified as showing a response to light exposure.

We initially examined the relationship between water content and depth by using cubic splines for the entire retinal region. However, the mouse-specific water-depth profiles could not be fit well across the entire region using this approach. As such, we focused on the outer retina, with the relationship between water content and retinal depth being modeled using quadratic regression. Random coefficients for the intercept, light exposure, and depth (linear and quadratic coefficients), and interactions for light exposure/depth were evaluated using AIC and BIC. The random coefficients for the intercept, light exposure, depth, and the interaction between light exposure and depth were included in the final model. This model also included the fixed effects of strain, light exposure, depth, and all interactions among the main effects. The three-way interaction for strain/light-exposure/retinal depth was significant based on a likelihood ratio test (P = 0.0461). Thus, we included the interaction in testing for differences between strain/light-exposure groups. We used numeric integration to estimate the mean water content for any region identified as showing a response to light exposure.

**RESULTS**

**OCR and MRC**

Light-adapted retina from C57BL/6 mice ex vivo displayed higher (13%, P < 0.05) basal OCR than that of 129S6/SvEvTac mice (Figs. 1A, 1C). Mitochondria content, as measured by pooled cytochrome a (cyt a) levels, was 8.85 pmol/retina in C57BL/6 mice and 10.81 pmol/retina in 129S6/SvEvTac mice; alternatively, cyt a content was 0.44 pmol (C57BL/6) and 0.54 pmol (129S6/SvEvTac) per retinal punch assayed. Normalizing the OCR data to the strain-specific cyt a quantity also revealed a higher (59%; P < 0.01) basal OCR level in C57BL/6 than 129S6/SvEvTac retina (Figs. 1B, 1C). In addition, mitochondria in C57BL/6 retina exhibited a smaller MRC value than that of 129S6/SvEvTac mice (Fig. 1D). Both normalized and unnormalized results suggest that mitochondria in the retina of C57BL/6 mice function at higher capacity and consume more oxygen. As water production is directly proportional to oxygen consumption, our data suggest that the retina (and thereby rod photoreceptors, which constitute almost 75% of all retinal cells) in C57BL/6 mice produce more water in light than 129S6/SvEvTac mice.

**Water Content MRI**

The measurement of water content in vivo in C57BL/6 mice revealed that outer hydration was increased significantly (P < 0.05) with light exposure compared to that in darkness (Fig. 2). However, no light-evoked change in posterior retina hydration was noted in 129S6/SvEvTac mice (Fig. 2). Water content was not different in the dark between the two strains of mice but was different in the light (Fig. 2).
Optical Coherence Tomography

Figure 3 shows examples of OCT images captured from the same eye under light- and dark-adapted conditions in a C57BL/6 mouse (left panel) and a 129S6/SvEvTac mouse (right panel). Light-adapted OCT images of C57BL/6 mice have an additional hyporeflection band between photoreceptor tip layer and RPE layer compared to the mice in the dark (Fig. 3). On the other
hand, this hyporeflective band is not observed on light-adapted OCT images from 129S6/SvEvTac mice, and the photoreceptor tip layer is merged with RPE layer, a feature resembling OCT images from dark-adapted C57BL/6 mice (Fig. 3). In addition, light adaptation induced a relatively smaller increase in the thickness of the outer retina in 129S6/SvEvTac mice than in C57BL/6 mice (Fig. 4).5

Diffusion MRI

As shown in Figure 5, the posterior retina in C57BL/6 mice exhibited a significant ($P < 0.05$) increase in ADC relative to that in darkness, consistent with an increased outer retina volume/water mobility.3,4 In contrast, ADC values in light were not significantly different from those in darkness at any location across the retina of 129S6/SvEvTac mice (Fig. 5).

DISCUSSION

In this study, we provide evidence for different outer retinal energy ecosystems in C57BL/6 and 129S6/SvEvTac mice, in agreement with less direct previous reports.15–22 The differences in mitochondrial respiration suggested distinctions in strain-specific light-driven outer retina hydration in vivo, which we validated by using proton density MRI, ultrahigh-resolution OCT, and diffusion MRI.3–11,27 Our results support the interpretation of the local light-driven imaging responses in

**Figure 3.** Representative same-eye OCT images in light and dark for C57BL/6 and 129S6/SvEvTac mice. Layer assignments are NFL, nerve fiber layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, rod inner segment layer; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, rod outer segment layer; and RPE, retinal pigment epithelium, as previously published.47 Red arrow points to the hyporeflective band between the OS and RPE layer observed on the light-adapted OCT image of C57BL/6 mice.

**Figure 4.** Summary of outer retina thickness, measured from OCT image in light and dark for C57BL/6 ($n = 7$) and 129S6/SvEvTac ($n = 8$) mice.

**Figure 5.** Summary of modeled central retinal ADC as a function of retinal depth during dark (closed symbols) and at 20 to 21 minutes of ~500 lux light (open symbols), in C57BL/6 ($n = 23$ dark-light pairs) and 129S6/SvEvTac mice ($n = 6$ dark-light pairs). Horizontal range bar indicates the region with significant differences ($P < 0.05$) between profiles. A representative OCT image (above profiles) is shown here. Dashed vertical lines map the outer plexiform layer and retina/choroid boundary onto diffusion profiles.
normal retina as a function of respiratory-induced hydration of the posterior retina. Based on these findings, we suggest that light-evoked imaging response in retinal disease, such as diabetic retinopathy, may be a biomarker of changes in mitochondrial activity, a previously understudied component of the light-based expansion of the outer retina.4,6

Some aspects of the experimental designs of the MRI and OCT arms of the study need comment. First, we did not find evidence that the anesthesia substantially altered the light-evoked expansion of the outer retina because, in this study, the effect was observed using two different anesthetics (urethane for the MRI studies and ketamine/xylazine for OCT); and, in our other studies, light-induced changes in the outer retina are also seen on OCT images of B6 mice anesthetized with isoflurane (data not shown). In addition, the light/dark order was not randomized because it takes several hours to fully dark adapt the retina. As previously shown, after light exposure, outer retinal changes on OCT return to initial values.5

The present study identifies strain differences in energy regulation and imaging responses to light. One contributing factor may be the established difference in rhodopsin regeneration rate in C57BL/6 and 129S6/SvEvTac mice as a consequence of having different RPE65 variants.16 Notably, a previous study did not find evidence that the rate of rhodopsin regeneration per se contributes substantially to the light-evoked increases in posterior retinal thickness.7 Another likely factor is that the 129S6/SvEvTac strain contains a natural mutation of the DISC1 gene that prevents the production of the full-length protein, which plays an important role in regulating mitochondrial function in collaboration with the mitochondrial inner membrane protein mitofillin.20 Deficiency in DISC1 function has been reported to induce mitochondrial dysfunction, such as decreased NADH dehydrogenase activity, disrupted mitochondrial Ca21 dynamics, reduction in mitochondrial monoamine oxidase activity, and reduced cellular ATP contents.22 In this study, we observed higher basal OCR in C57BL/6 than 129S6/SvEvTac retina that is strongly weighted for C57BL/6 mice by using both OCT retinal thickness and ADC. OCR was not randomized because it takes several hours to fully dark adapt the retina. As previously shown, after light exposure, outer retinal changes on OCT return to initial values.5

C57BL/6 and 129S6/SvEvTac mice have been suggested to have distinct photoreceptor energy ecosystems in part because of different insult-induced morbidity outcomes.19,35–37 The energy bionetwork is important in this regard because it regulates large light-driven swings in photoreceptor ATP production, which is required to support phototransduction and modify ion channel function and outer retina ion content.19,36–44 In support of this view, mice genetically modified to prevent phototransduction exhibit no OCT and diffusion MRI responses.3,7 The results presented here support this energy ecosystem concept, but additional work is needed to determine, for example, the differences in mitochondria morphology, density, or function between C57BL/6 and 129S6/SvEvTac mice.

The reasons for disparities between light-stimulated changes of outer retinal water content, retinal thickness, and water mobility indices are currently unclear. One possibility is that subtle alterations are more sensitively detected using OCT retinal thickness measurements compared with MRI assessment of proton density and water mobility. For example, 129S6/SvEvTac mice in this study show light-evoked expansion based on OCT retinal thickness (Fig. 4), but we are not able to detect such expansion using ADC MRI (Fig. 5). However, the extent of light-evoked expansion is less for 129S6/SvEvTac than for C57BL/6 mice by using both OCT retinal thickness and ADC MRI. The lack of a statistically significant expansion in 129S6/SvEvTac mice based on ADC MRI (Fig. 5) may be due to a lack of statistical power and perhaps lower resolution of this method. In addition, the posterior retina water content measured herein reflects the net result of water production minus water removal. An adult mammalian retina is estimated to generate, by aerobic respiration and glycolysis, 1.8 times more water in dark than in the light.11 Rod photoreceptors being the most prevalent cell type are likely a major contributor to retinal water production.45 Water removal in the outer retina is largely controlled by RPE pump activity.11 Future studies could determine relative contributions of water production and removal in each strain studied herein. Nonetheless, all three imaging methods led to the same conclusion, that is 129S6/SvEvTac mice have a lower photoresponse in the outer retina than C57BL/6 mice.

MRI inherently measures the proton density associated with water, which is 110 M in equivalent protons. Proton density has been used to accurately assess retinal layer-specific water content after calibration against preretinal vitreous water signal.25,31,32 This is an advance over conventional imaging methods, such as OCT, that noninvasively evaluates retinal thickness, but not water content directly.25 Notably, MRI water content reflects a combination of intra- and extracellular hydration information because intracellular water resides in intracellular and extracellular compartments for short times (~msec) relative to the time it takes to acquire an image (~minutes).46 Nonetheless, we previously observed an agreement between MRI and specific gravity measurements of retinal water content.25

In summary, the present results support and extend previous findings of parallels between light-stimulated ultra-high-resolution OCT and functional diffusion MRI responses. The data herein suggest that both methods assess the same phenomena, that is increased posterior retina hydration in light linked with mitochondrial respiration. Furthermore, these imaging responses are strongly regulated by the genetic background, a previously unappreciated modifier that needs to be considered in future studies of retinal diseases both in animal models and patients.

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