Acute Ocular Hypertension Diminishes Flicker-Induced Enhancement in Retinal Blood Flow and Full-Field Electroretinogram in a Mouse Model

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Purpose. Transient flickering light stimulation (FLS) was previously demonstrated to enhance both retinal blood flow (RBF) and full-field electroretinogram (ffERG) responses in wild-type mice. This study aimed to investigate the effects of acute ocular hypertension (AOH) on these flicker-induced changes in mice.

Methods. Adult C57BL6J mice were randomly divided into three groups: the AOH, sham, and naïve groups. All animals underwent the following measurements, including scotopic and photopic ffERGs (pre- and post-12 hertz [Hz] FLS), optical coherence tomography (OCT), and Doppler OCT (pre- and post-12 Hz FLS), at baseline (day 0), day 16, and day 43. On day 8, intraocular pressure (IOP) was elevated to 80 millimeters of mercury (mm Hg) in the AOH group or maintained at 15 mm Hg in the sham group for 1 hour. The naïve group received no intervention.

Results. Transient FLS significantly enhanced both RBF and photopic b-wave amplitudes in all three groups at day 0 (baseline). The AOH group showed significant reductions in the positive scotopic threshold responses and both inner and middle retinal layer thicknesses at days 16 and 43. Significant flicker-induced increases in RBF and b-wave amplitudes were observed in the sham and naïve groups at days 16 and 43. However, significant reductions in flicker-induced enhancements of RBF and b-wave amplitude were observed in the AOH group at days 16 and 43 compared with the sham and naïve groups.

Conclusions. Flicker-induced enhancements in RBF and electro-retinal responses were impaired by AOH. This impairment suggests that the retinal functional hyperemia is sensitive to IOP elevation.

Keywords: neurovascular coupling, flickering light, electroretinogram (ERG), retinal blood flow (RBF), retinal ischemia

The proper functioning of the retinal cells requires an adequate supply of oxygen and metabolic nutrients. The robust retinal vascular system has the intrinsic ability to appropriately regulate its blood flow and perfusion pressure in response to varying metabolic demands of the tissue.[1–3] Numerous studies have reported an increase in retinal blood flow (RBF) in response to retinal stimulation with flickering lights of varying frequencies, including 4 hertz (Hz), 5 Hz, 8 Hz, 10 Hz, 12 Hz, 16 Hz, 18 Hz, and 24 Hz, under normal physiological conditions.[4–16] The largest increase in RBF was observed in the mouse retina during 2 to 3 minutes of flickering light stimulation (FLS) at a frequency of 12 Hz, followed by 16 Hz and 8 Hz.[14] Recently, we further demonstrated similar enhancement of electro-retinal activity after 12 Hz FLS.[16] In contrast, exposure to steady light did not result in significant changes in either RBF or electrical activity.[16] Our recent study demonstrated that both 8 Hz and 16 Hz FLS significantly increased both photopic b-wave amplitudes and RBF (Rai et al., our unpublished data). Notably, 12 Hz FLS produced a greater enhancement of RBF compared to the 8 Hz and 16 Hz conditions (Rai et al., our unpublished data). The increased blood flow is believed to supply additional oxygen and nutrients to the retina, supporting its high metabolic demands due to increased retinal activity[17]

in response to FLS. Based on these reported findings, it has been suggested that retinal neuronal activity and blood supply are dynamically coordinated in the healthy retina.

Diseased retinas, such as those affected by diabetic retinopathy and age-related macular degeneration, have been shown to exhibit significantly reduced flicker light-induced enhancements in retinal vascular caliber.[18–21] However, limited studies have investigated changes in RBF in response to FLS following retinal ischemia-reperfusion injury. Additionally, neither animal nor human studies have evaluated the alterations in electro-retinal activity in response to FLS in this injury condition that compromises the retinal physiology.

To address this question, a retinal ischemia-reperfusion injury mouse model, induced by acute ocular hypertension (AOH), was utilized in this study. When IOP is elevated to an extremely high level, it causes a significant reduction in RBF and subsequently induces retinal ischemia.[22] This particularly affects the middle and inner retinal layers, which are sensitive to hypoxia.[23,24] Furthermore, elevated IOP at such a high level, not only causes retinal ischemia but also imposes mechanical insult on retinal cells, leading to structural damage and functional impairment.[25] Therefore, the study first explored the longitudinal effects of AOH on functional and structural changes in mice. Then, the impact of AOH on flicker-induced enhancements in RBF and retinal electrical activity was investigated.

We hypothesize that the cascade of changes induced by the AOH insult disrupts the dynamic relationship between RBF and neuronal activity. This disruption leads to an impaired retinal functional hyperemia defined as increased local blood flow in response to localized neural activity, which subsequently affects the retinal physiological activity. To test this hypothesis, we longitudinally assessed the changes in RBF and retinal electrical activity in response to FLS using Doppler optical coherence tomography (OCT) and full-field electroretinogram (ffERG), respectively,[16] in an AOH mouse model over the period of 43 days.

Methods

Animals

Adult C57BL6J mice, obtained from the centralized animal facility of The Hong Kong Polytechnic University, were housed in a temperature-controlled room (20°C to 22°C) under normal lighting conditions (approximately 200 lux) with a 12-hour light/12-hour dark cycle. Mice were provided with food (Pico Lab Diet 20, 5053; PMI Nutrition International, Richmond, IN, USA) and water ad libitum. All experimental procedures and care adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the Animal Ethics Sub-committee of The Hong Kong Polytechnic University (Animal Subjects Ethics Sub-committee approval number: 18-19/58-SO-R-OTHERS). Following the completion of all experiments, the animals were euthanized using CO_2 asphyxiation, and the carcasses were disposed of according to standard safety protocols.

Experimental Design

Before AOH induction, standard scotopic ffERG, photopic ffERG, OCT, and Doppler OCT were recorded in 34 mice (8–10 weeks old, 20–25*g*, 18 male and 16 female mice) on day 0 (baseline), with photopic ffERG and Doppler OCT repeated before and after retinal stimulation with 12 Hz flickering light (0.1 cd·s/m^2). The mice were then randomly divided into three experimental groups: the AOH, sham, and naïve groups. On day 8 (8 days after baseline measurements), intraocular pressure (IOP) was elevated to 80 millimeters of mercury (mm Hg) in the AOH group via anterior chamber cannulation whereas the sham group underwent the same procedure with IOP maintained at 15 mm Hg in one randomly selected eye. Naïve animals were transported to the surgical table, anesthetized, and allowed to rest on the table for 60 minutes to acclimate to the surgical environment. Unlike the AOH and sham groups, the naïve group did not receive any intervention. On days 16 and 43, all animals underwent follow-up measurements of scotopic ffERG, photopic ffERG, OCT, and Doppler OCT, with photopic ffERG and Doppler OCT measurements repeated before and after FLS, consistent with the day 0 experimental protocol. A flow diagram depicting the experimental study design is shown in Figure 1A. Figure 1B presents a flowchart illustrating the experimental steps involved.

Acute Ocular Hypertension Induction

The protocols for inducing AOH were similar to those described by Lakshmanan et al.[25] Briefly, animals were anesthetized by intraperitoneal (IP) injection of a cocktail containing 90 mg/kg ketamine (Alfasan International B.V., Woerden, Netherlands) and 12 mg/kg xylazine (Alfasan International B.V.). The body temperature of the anesthetized animal was maintained at about 37°C using a heating mat. A drop of topical anesthetic (Provain-POS 0.5% wt/vol; URSAPHARM, Saarbrücken, Germany) and pupil dilating drops (Mydriacyl 1% eye drops; Alcon-Couvreue, Puurs, Belgium) were applied, and once full pupil dilation was observed, the anterior chamber of one randomly chosen eye was cannulated using a 33-gauge needle attached to a tubing system connected to a reservoir containing Gibco Hank's balanced salt solution (HBSS; Thermo-Fisher Scientific, Waltham, MA, USA) via a pressure transducer (60-3003; Harvard Apparatus, Holliston, MA, USA). The target IOP of 15 mm Hg for the sham group and 80 mm Hg for the AOH group was maintained for 60 minutes by manually adjusting the height of the reservoir. The IOP was also monitored regularly and measured every 10 minutes, using the Tonolab tonometer TV02 (Icare, Vantaa, Finland). Table 1 presents the mean values of induced and measured IOP (in mm Hg) at 10-minute intervals throughout the 60-minute period in the AOH-treated and sham-treated eyes.

Lacryvisc gel (Alcon, Rueil-Malmaison, France) was applied continuously, except during IOP measurements, throughout the experimental period to prevent corneal dehydration. There was no Lacryvisc left on the cornea during any of the IOP measurements. Following a 60-minute period of ocular hypertension, the height of the reservoir was gradually lowered (approximately 3 cm/sec) to approximately 15 mm Hg (in the AOH group) and the needle was carefully withdrawn. After this, topical antibiotic eye drops (gentamycin; Gibco, Thermo Fisher Scientific) were applied to the eyes and subsequently once daily for 7 days. Despite careful measures to avoid complications, such as corneal haze, lens puncture, and iris perforation, approximately 23% (8/34) of the mice developed complications and were excluded from further study.

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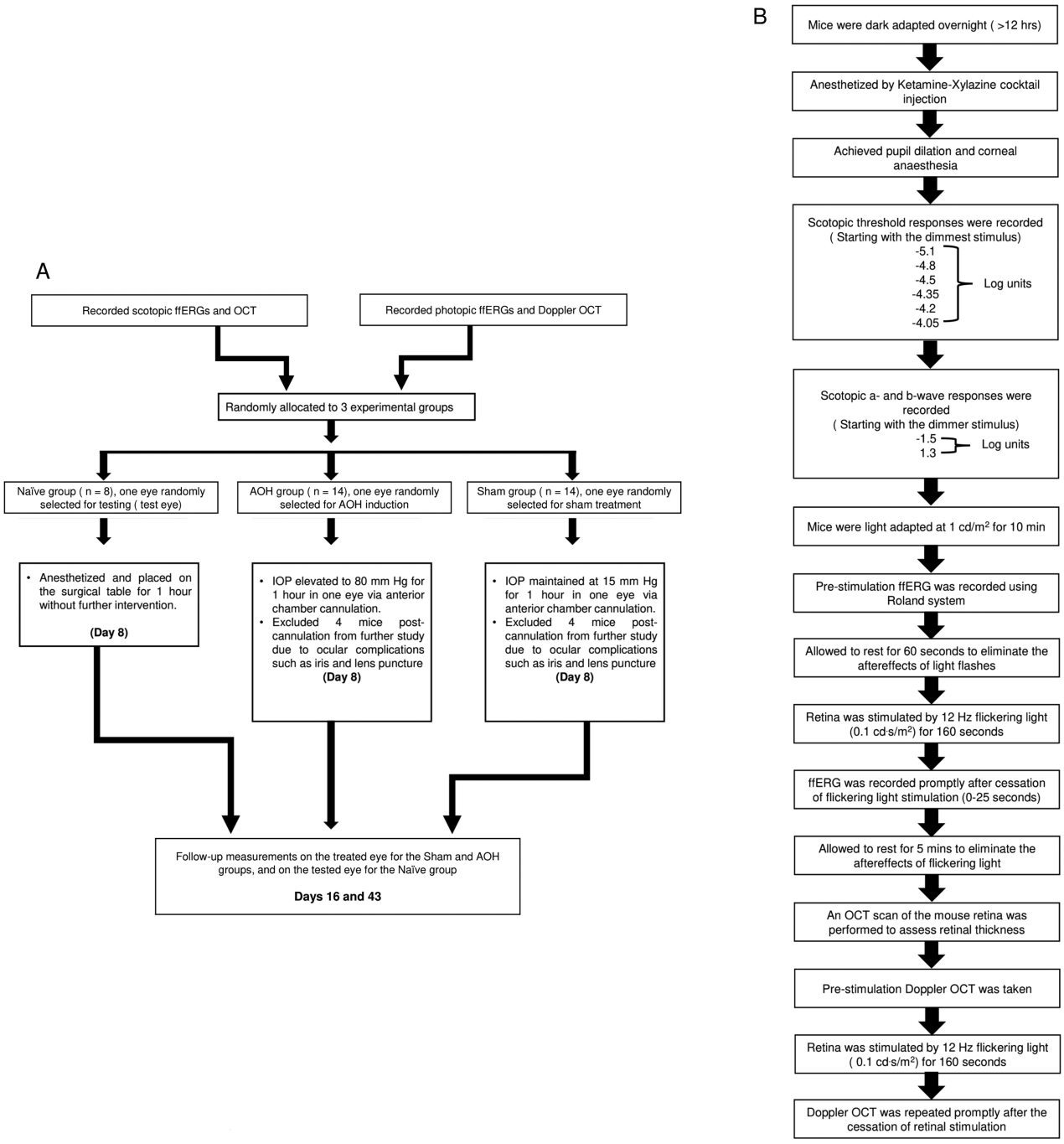

Figure 1. Schematic diagram. (A) Showing the experimental study design and the (B) experimental steps followed at each time point. Scotopic ffERG, photopic ffERG, OCT, and Doppler OCT were recorded at days 0 (baseline), 16, and 43. The photopic ffERG and Doppler OCT were measured before and after retinal stimulation with FLS in each time point. Mice were randomly divided into three groups: AOH, sham, and naïve.

Scotopic Full-Field Electroretinography

The electro-retinal responses were recorded using a Ganzfeld electroretinogram (ERG) system (Q450; RETI Animal, Roland Consult, Brandenburg an der Havel, Germany). Animals were dark-adapted overnight (>12 hours) and then prepared for ERG recording under dim red light. Following a similar corneal anesthetic and pupil dilation protocols used before AOH induction, the animal was placed on a heated pad to maintain the body temperature at approximately 37°C. To prevent corneal dehydration, a drop of Lacryvisc gel (Alcon, Rueil-Malmaison, France) was applied on the corneal surface. For ffERG measurements, a 2-mm diameter gold ring electrode (Roland Consult) was positioned on the cornea of each eye to serve as an active electrode. Needle electrodes (Item No. U51-426; GVB-geliMED,

Table 1. Induced and Measured IOP Values in the AOH-Treated and Sham-Treated Eyes Over Time

	AOH-Treated Eye		Sham-Treated Eye	
Time, Min	Induced IOP, mm Hg Mean ± SEM	Measured IOP, mm Hg Mean ± SEM	Induced IOP in mm Hg Mean ± SEM	Measured IOP in mm Hg Mean ± SEM
Immediately	80.30 ± 0.15	79.70 ± 0.31	15.25 ± 0.16	14.75 ± 0.45
10	79.40 ± 0.33	80.50 ± 0.34	14.63 ± 0.41	14.63 ± 0.18
20	80.00 ± 0.39	79.90 ± 0.31	14.63 ± 0.18	14.13 ± 0.22
30	80.40 ± 0.47	79.70 ± 0.26	14.63 ± 0.26	14.50 ± 0.18
40	80.00 ± 0.33	79.60 ± 0.22	14.75 ± 0.16	14.25 ± 0.17
50	79.70 ± 0.59	80.10 ± 0.40	14.38 ± 0.18	14.63 ± 0.18
60	79.80 ± 0.32	79.80 ± 0.32	14.88 ± 0.12	14.38 ± 0.26

The values represent the mean ± standard error of the mean (SEM), measured every 10 minutes over a duration of 60 minutes.

Bad Segeberg, Germany) were inserted in the subcutaneous tissue just under the skin at the lateral canthus of each eye and the upper base of the tail to act as reference electrodes and a ground electrode, respectively. An impedance of less than 5 kΩ was maintained for all electrodes throughout the recording period. The ERG responses were elicited by using a white light-emitting diode flash (delivered by the Ganzfeld bowl). The RETI-Port system (Roland Consult) was used for visual stimulation and data recording, starting with the stimulus from −5.1 to +1.3 log U (log U = log cd.s/m^2). The positive scotopic threshold (pSTR) responses were first measured at very dim light intensities from −5.1 to −4.05 log U. A total of 40 sweeps of response with an interstimulus interval of 2 seconds were averaged at the dimmest intensity (−5.1 log U). Thereafter, the number of sweeps was reduced to 30 (−4.8 to −4.05 log U). Following this, the scotopic a- and b-wave responses were then measured with a bright light stimulus of 1.3 log U. The amplitudes and implicit times of the pSTR and the scotopic a- and b-wave responses were extracted and analyzed. Scotopic ffERG responses were recorded to assess changes in the functional integrity of the retina in order to confirm the successful establishment of AOH in mice.[25]

Photopic Full-Field Electroretinography

The protocols and parameters for measuring photopic ffERG responses were adopted from our previous study.[16] Immediately after recording the scotopic ffERG, the animals were light-adapted for 10 minutes at a background luminance of 1 cd/m^2. Photopic ffERG responses were then measured by presenting a brief white LED flash (25 responses with an interstimulus interval of 1 second) at an intensity of 3.0 cd·s/m^2. The amplitudes and implicit times of the a-wave and the b-wave were extracted for analysis. The changes in flicker-induced photopic ffERG responses at each time point were compared to understand the effects of AOH induction on flicker-induced enhancement of retinal electrical activity.

Optical Coherence Tomography

The procedures for OCT imaging and measuring retinal thickness were similar to those described by Lam et al.[26] OCT was performed approximately 5 minutes after ERG completion using Bioptigen Envisu SD-OCT (R2210; Leica Microsystems, Morrisville, NC, USA) under the same anesthetic and mydriatic conditions in red dim light across all experimental groups. OCT rectangular scans (A-scans = 1000 lines; B-scans = 100 scans; frames per B-scan = 15) covering an area of 0.64 mm^2 (0.8 mm × 0.8 mm) were performed, with the optic disc centered to ensure consistent scanning location across animals and different time points. The thickness of the retinal nerve fiber layer (RNFL), inner retinal layer (IRL), middle retinal layer (MRL), outer retinal layer (ORL), and total retinal layer (TRL) was measured using the built-in software InVivoVue Diver (version 3.0.8; Bioptigen Inc., Morrisville, NC, USA). To confirm the successful establishment of an AOH mouse[25] model, these OCT measurements were compared to evaluate changes in the structure of various retinal layers following AOH induction.

Measurement of Retinal Blood Flow

RBF was measured using an annular Doppler blood flow B-scan from spectral-domain OCT (SD-OCT; Envisu R2210; Bioptigen, Morrisville, NC, USA), as described in previous studies.[16,27–29] This OCT system utilized the Doppler shift phenomenon to visualize blood flow toward and away from the imaging objective. First, the built-in Doppler OCT acquisition protocols were used to perform a circular scan with a diameter of 0.5 mm, centered on the optic nerve head. Doppler shifts were indicated by "red" and "blue" signals, with red denoting arterial blood flow and blue denoting venous blood flow. Ten OCT B-scan images, showing both "red" and "blue" signals, were captured in succession. Multiple images were collected for two main reasons: first, to account for the variability in blood flow due to the pulsatile nature of the cardiac cycle; and second, to enable averaging that helps to mitigate transient noise and potential artifacts during image acquisition. The acquisition protocol is standardized across all images and time points to ensure consistency and reproducibility of the data. The regions showing Doppler shifts were then cropped using FIJI software (https://imagej.net/software/fiji/) for further analysis. The regions were carefully selected to include only those areas displaying Doppler shifts, as indicated by the presence of "red" and "blue" signals. Major retinal vessels were used as references for the cropping process, ensuring that non-vascular regions are excluded. This procedure was performed to reduce background noise and to ensure that subsequent analyses focused exclusively on the major retinal vessels. In order to enhance the visibility of Doppler signals, the saturation of each cropped B-scan image was adjusted using the color threshold algorithm to ensure that all "red" and "blue" signals within the retinal blood vessels were included.

Investigative Ophthalmology & Visual Science

The same algorithm was utilized to exclude the pixels that fell below a predetermined noise threshold. The predetermined threshold values were determined using control images from healthy mice to establish a baseline representation of both actual Doppler signals with major retinal vessels and background noise within non-vascular regions. The analysis of pixel intensity distribution within these images was conducted using histograms for both vascular and non-vascular regions separately. True Doppler signals exhibit significantly high intensity values, whereas noise pixels tend to cluster at much lower intensity levels. The intensity of each pixel is proportional to the amplitude of the Doppler shift, which is a surrogate indicator of the magnitude of blood flow. A range of threshold values was identified based on this analysis to separate higher-intensity Doppler signals from the lower-intensity noise pixels. This was done to minimize the inclusion of background noise while retaining true vessel signals as much as possible. To maintain consistency in data analysis, saturation is applied uniformly to all images, and the same threshold settings were utilized for pre- and post-FLS scans. After removing noise from non-vascular areas in each image prior to averaging, the "red" and "blue" signals were quantified in terms of pixels using the "Measure" function in the "Analyze" category of the software. Only pixels within the vessel areas with intensity above the noise level were extracted for analysis. Then, the analyzed pixel counts from all 10 B-scans were averaged to determine the final red/blue pixel values. The mean pixel count served as a surrogate measure of retinal arterial (red pixels) and venous (blue pixels) calibers (per se, the blood flow), with higher counts reflecting greater magnitude of RBF and lower counts indicating lower magnitude of RBF. The alterations in flicker-induced RBF at each time point were compared to explore how AOH affected the flicker-induced enhancement of RBF.

Flickering Light Stimulation

In order to assess the transient changes in neuronal (functional) and hemodynamic (vascular) responses following FLS, photopic ffERG and Doppler RBF B-scans were recorded twice, both pre- and post-FLS, as described in our previous study.[16] After recording baseline measurements (photopic ffERG/Doppler B-scans), the animals were allowed to rest for 60 seconds to minimize any aftereffects of the flashes after photopic ffERG. This rest period was not required following Doppler B-scans. The retina was then stimulated with a 12-Hz square-wave flickering light (white LED source with intensity of 0.1 cd·s/m^2) for 160 seconds. Measurements were immediately repeated following the cessation of FLS. The background luminance of 1 cd/m^2 was maintained throughout the experiment, except during the 160-second FLS period, as described previously.[16] As Hanaguri et al. demonstrated that 12 Hz FLS for 2 to 3 minutes induced the largest increase in RBF, these parameters were selected for the current study.[14]

Data Analysis

Data are presented as mean and standard error of the mean (SEM). The longitudinal effects of AOH on retinal layer thickness, scotopic ffERG responses, and flicker-induced changes in RBF and photopic ffERG responses were analyzed separately as follows.

Effects of AOH on Retinal Functions and Structures

Scotopic ffERG responses and retinal thicknesses measured by OCT were assessed to validate the effects of AOH on overall retinal function and structure in a mouse model. A mixed-model ANOVA was used to assess the AOH-induced effects on overall retinal function and structure by evaluating differences in scotopic ffERG responses and OCT-measured retinal thicknesses among the three groups (considering AOH induction as a between-subjects factor and time as a within-subjects factor) across three time points. Post hoc pairwise comparisons were performed using the Bonferroni adjustment to control for multiple comparisons.

Effects of AOH on Flicker-Induced Percentage Changes of RBF and ffERG Responses

The relative changes in arterial blood flow (ABF), venous blood flow (VBF), and photopic ffERG responses measured pre- and post-FLS for that day are presented as the percentage changes from the respective baseline values. These percentage changes were used to assess the effects of retinal ischemia-reperfusion injury on flicker-induced enhancements of retinal hemodynamic responses and electrical activity. The mixed-model ANOVA was applied to compare the percentage changes in ABF, VBF, and photopic ffERG responses among the three groups (considering AOH induction as a between-subjects factor and time as a within-subjects factor) across three time points. To adjust for multiple comparisons, a Bonferroni correction was applied to all post hoc pairwise comparisons. All statistical analyses were conducted using SPSS 29.0 (IBM Corp., Armonk, NY, USA), with $P < 0.05$ considered as statistically significant.

Results

Effects of AOH on Retinal Layer Thickness

Figure 2A presents the segmentation and marking of the retinal layers from a representative wild-type mouse, and Figure 2B shows the OCT B-scans of one representative mouse from each experimental group at baseline (day 0), day 16, and day 43. The retina was divided into five regions, including RNFL, IRL (from RNFL to inner nuclear layer), MRL (from outer plexiform layer to outer nuclear layer), ORL (from external limiting membrane to retinal pigment epithelium (RPE), and TRL (from RNFL to RPE), for analyses. The mean values of RNFL, IRL, MRL, ORL, and TRL thicknesses are presented in Figures 2C–G, respectively.

As shown in Table 2, mixed-model ANOVA revealed no significant differences in RNFL or ORL thickness among the three groups over time. The AOH group showed significant reductions in IRL, MRL, and TRL thickness at both day 16 and day 43 compared to baseline. Furthermore, at both post-AOH time points, IRL, MRL, and TRL thicknesses in the AOH group were significantly thinner compared with those in the naïve and sham groups.

Effects of AOH on Scotopic ffERG Responses

Figure 3A depicts the traces of averaged pSTR and scotopic ERG responses from a representative mouse of each experimental group at baseline (day 0), day 16, and day 43. Figures 3B, 3D, and 3F show the averaged amplitudes of

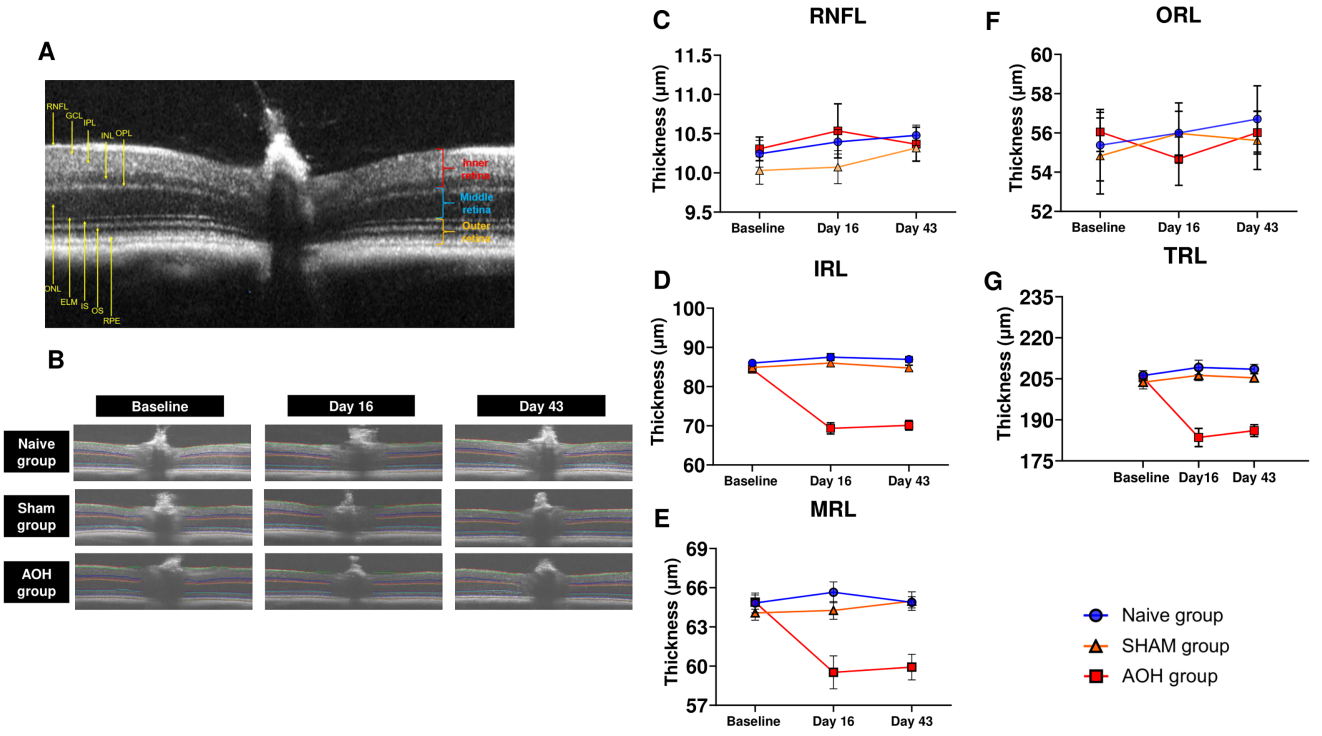

Figure 2. Assessment of retinal layer thickness using SD-OCT in the naïve, sham, and AOH-treated mice. (**A**) Segmentation and labeling of retinal layers in a representative mouse, showing the inner retina layer (IRL), middle retina layer (MRL), outer retina layer (ORL), and total retina layer (TRL). (**B**) SD-OCT B-scans of one representative mouse from each group at three time points. Mean values of (**C**) RNFL, (**D**) IRL, (**E**) MRL, (**F**) ORL, and (**G**) TRL thicknesses in the naïve, sham, and AOH-treated mice at baseline, day 16, and day 43.

pSTR, scotopic a-wave, and b-wave responses, respectively, and Figures 3C, 3E, and 3G illustrate the averaged implicit times of these responses, respectively.

Significant differences in pSTR amplitudes (mixed-model ANOVA: time: $P = 0.012$; group: $P = 0.008$; interaction effect: $P = 0.004$) were observed among the three groups over time. Although there were no significant differences in the pSTR amplitudes among the three groups at baseline ($P > 0.05$ for all), the pSTR amplitudes in the AOH group were significantly lower than those in the sham and naïve groups at both day 16 ($P < 0.001$ for both groups) and day 43 ($P < 0.05$ for both groups). There were no significant differences in pSTR amplitudes between the sham and the naïve groups at day 16 and day 43 ($P > 0.05$ for all). The AOH group demonstrated a significant reduction in pSTR amplitude at both days following AOH induction ($P < 0.001$ for both days 16 and 43) relative to its baseline. No significant differences were noted within the sham and naïve groups compared to baseline at day 16 and day 43 ($P > 0.05$ for all).

The amplitudes of the scotopic b-wave did not differ significantly among the three groups across the three time points (mixed-model ANOVA: time, $P = 0.115$; group, $P = 0.330$; interaction effect, $P = 0.06$). Although the AOH group showed a reducing trend in scotopic b-wave amplitude compared with respective baseline and the sham and naïve groups at days 16 and 43, this reduction was not statistically significant. No significant differences were detected in the amplitudes (mixed-model ANOVA: time, $P = 0.125$; group, $P = 0.060$; interaction effect, $P = 0.804$) and implicit times (mixed-model ANOVA: time, $P = 0.677$; group, $P = 0.441$; interaction effect, $P = 0.160$) of scotopic a-wave responses, pSTR implicit times (mixed-model ANOVA: time, $P = 0.126$; group, $P = 0.319$; interaction effect, $P = 0.212$), and scotopic b-wave implicit times (mixed-model ANOVA: time, $P = 0.365$; group, $P = 0.209$; interaction effect, $P = 0.748$) among the three groups measured at three time points.

Effects of AOH on Flicker-Induced Changes in Photopic ffERG Responses

Figure 4A shows a set of averaged photopic ERG response traces from a representative mouse of each experimental group, recorded at baseline, day 16, and day 43, with 2 recordings taken at each time point: one before and one after FLS. Figures 4B to 4E present the mean percentage changes in a-wave amplitude, a-wave implicit time, b-wave amplitude, and b-wave implicit time, respectively.

Significant differences in percentage changes in b-wave amplitudes (time: $P < 0.001$; group: $P < 0.001$; interaction effect: $P = 0.017$) were detected among the three groups over time. The b-wave amplitudes measured after FLS were significantly greater than the respective baseline values at all time points in the naïve group ($P < 0.01$ for baseline and $P < 0.01$ for both days 16 and 43) and the sham group ($P < 0.001$ for all time points). Although the b-wave amplitude showed a significant increase at baseline in the AOH group ($P < 0.001$), which was comparable to the corresponding baseline values in other two groups, no significant changes were noted on days 16 ($P > 0.05$) and 43 ($P > 0.05$). Although the percentage changes in b-wave amplitudes did not differ significantly among groups at baseline (naïve versus sham: $P = 0.142$; naïve versus AOH: $P = 0.860$; sham versus AOH:

Investigative Ophthalmology & Visual Science

Table 2. Mixed-Model Analysis of Retinal Layer Thickness Across Three Experimental Groups Over Time

Retinal Layers	Groups	*P* Value Within Group, D 0 vs. D 16	*P* Value Within Group, D 0 vs. D 43	*P* Value Within Group, D 16 vs. D 43	*P* Value Between Groups, D 16	*P* Value Between Groups, D 43	*P* Value Main Time Effect	*P* Value Main Group Effect	*P* Value Interaction Effect
RNFL	Naïve	>0.05	>0.05	>0.05	(>0.05) for both groups)	(>0.05) for both groups)	>0.05	>0.05	>0.05
	Sham	>0.05	>0.05	>0.05	(>0.05) for both groups)	(>0.05) for both groups)			
	AOH	>0.05	>0.05	>0.05					
IRL	Naïve	>0.05	>0.05	>0.05	**(<0.001) AOH vs. Naïve**	**(<0.001) AOH vs. Naïve**	**<0.001**	**<0.001**	**<0.001**
	Sham	>0.05	>0.05	>0.05	**(<0.001) AOH vs. Sham**	**(<0.001) AOH vs. Sham**			
	AOH	**<0.001**	**<0.001**	>0.05					
MRL	Naïve	>0.05	>0.05	>0.05	**(<0.001) AOH vs. Naïve**	**(<0.001) AOH vs. Naïve**	**<0.001**	**<0.001**	<0.05
	Sham	>0.05	>0.05	>0.05	**(<0.01) AOH vs. Sham**	**(<0.001) AOH vs. Naïve**			
	AOH	**<0.001**	**<0.001**	>0.05					
ORL	Naïve	>0.05	>0.05	>0.05	(>0.05) for both groups)	(>0.05) for both groups)	>0.05	>0.05	>0.05
	Sham	>0.05	>0.05	>0.05	(>0.05) for both groups)	(>0.05) for both groups)			
	AOH	>0.05	>0.05	>0.05					
TRL	Naïve	>0.05	>0.05	>0.05	**(<0.001) AOH vs. Naïve**	**(<0.001) AOH vs. Naïve**	**<0.001**	**<0.001**	**<0.001**
	Sham	>0.05	>0.05	>0.05	**(<0.01) AOH vs. Sham**	**(<0.01) AOH vs. Sham**			
	AOH	**<0.001**	**<0.001**	>0.05					

P values for main within-group, between-groups, and interaction effects are presented.
The *P* values in bold represent statistical significance.

$P = 0.825$), there were significant reductions in the AOH group at day 16 and day 43 compared to the sham (day 16: $P = 0.001$ and day 43: $P < 0.001$) and the naïve (day 16: $P = 0.026$ and day 43: $P = 0.031$) groups. No significant differences in percentage changes in b-wave amplitudes were noted between the sham and naïve groups at these time points (day 16: $P = 0.779$; day 43: $P = 0.371$).

Although there were no significant differences in the percentage increases in b-wave amplitudes (relative to the corresponding baseline value) within the sham (baseline versus day 16: $P > 0.05$; baseline versus day 43: $P > 0.05$; day 16 versus day 43: $P > 0.05$) and naïve (baseline versus day 16: $P > 0.05$; baseline versus day 43: $P > 0.05$; day 16 versus day 43: $P > 0.05$) groups, the percentage increases in b-wave amplitudes (relative to the corresponding baseline value) were significantly reduced in the AOH group at day 16 (baseline versus day 16: $P < 0.001$) and day 43 (baseline versus day 43: $P < 0.001$). No significant difference was detected between day 16 and day 43 in the AOH group ($P > 0.05$).

There were significant differences in a-wave amplitudes among the three groups measured over three time points (mixed-model ANOVA: time, $P = 0.407$; group, $P = 0.002$; interaction effect, $P = 0.654$). Pairwise comparisons revealed that only the a-wave amplitudes in the AOH group, measured after FLS on day 16, were significantly different from those measured at the same time point in the sham and naïve groups ($P < 0.05$ for both groups). There were no significant differences in the implicit times of a-wave (mixed-model ANOVA: time, $P > 0.05$; group, $P > 0.05$; interaction effect, $P > 0.05$) and b-wave responses (mixed-model ANOVA: time, $P > 0.05$; group, $P > 0.05$; interaction effect, $P > 0.05$) among the three groups. Similarly, percentage changes in a-wave amplitudes (mixed-model ANOVA: time, $P = 0.769$; group, $P = 0.266$; interaction effect, $P = 0.917$), a-wave implicit times (mixed-model ANOVA: time, $P = 0.956$; group, $P = 0.828$; interaction effect, $P = 0.031$) and b-wave implicit times (mixed-model ANOVA: time, $P = 0.946$; group, $P = 0.482$; interaction effect, $P = 0.884$) were not significantly different among the three groups over time.

Effects of AOH on Flicker-Induced Changes in RBF

Figure 5A shows the Doppler B-scans of one representative mouse retina recorded pre- and post-FLS in each experimental group. Figures 5B and 5C represent the mean percentage changes in ABF and VBF, respectively.

There were significant differences in the percentage changes in ABF among the three groups measured over time (mixed-model ANOVA: time, $P < 0.001$; group, $P < 0.001$; interaction effect, $P < 0.001$). In all three groups, pairwise comparisons showed that ABF measured after FLS was significantly greater than their respective baseline values at day 0 ($P < 0.001$ for all). Although there were significant differences in ABF measured before and after FLS in both the sham and naïve groups at day 16 ($P < 0.001$ for both groups) and day 43 ($P < 0.001$ for both groups), no significant differences were noted in the AOH group at these time points ($P > 0.05$ for both time points). Post hoc comparisons showed that the percentage increases in ABF were significantly reduced at day 16 and day 43 compared to baseline percentage increase in the AOH group ($P < 0.001$ for both time points). The percentage increases in ABF in the AOH

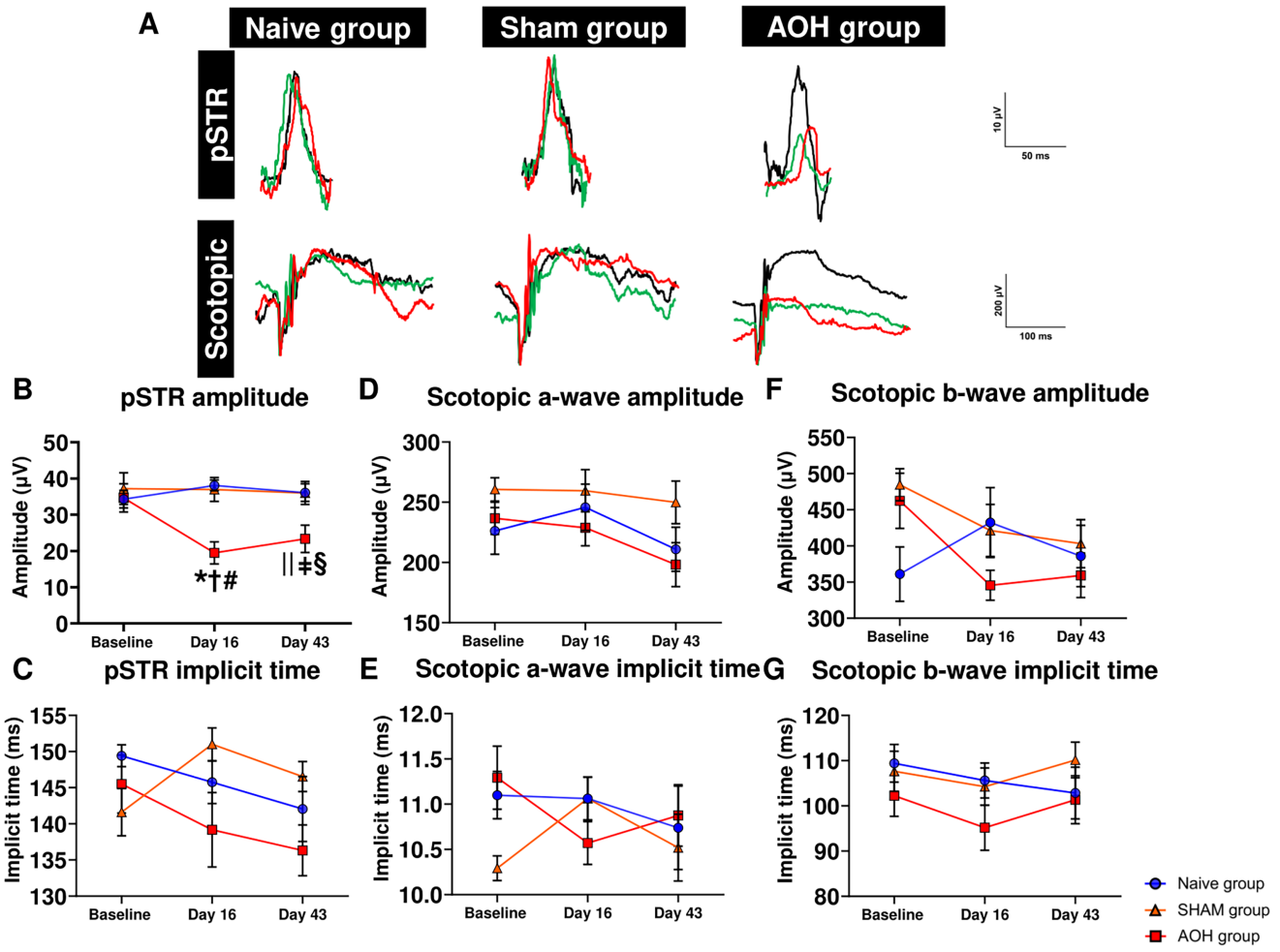

Figure 3. Scotopic full-field electroretinography (ffERG) responses in three experimental groups measured over time. (**A**) Representative traces of scotopic ffERG responses, showing positive scotopic threshold response (pSTR) and scotopic responses, recorded from three groups at baseline (day 0, *black*), day 16 (*red*), and day 43 (*green*). Mean amplitudes of (**B**) pSTR, (**D**) scotopic a-wave, (**F**) scotopic b-wave, and the corresponding (**C, E, G**) implicit times were compared before and after AOH induction at three time points. Data are expressed as mean ± SEM. Symbols indicate statistical significance: $^{*}P < 0.001$: AOH at day 16 versus AOH baseline, $^{\dagger}P < 0.001$: AOH versus naïve at day 16, $^{\#}P < 0.001$: AOH versus sham at day 16, $^{\S}P < 0.001$: AOH at day 43 versus AOH baseline, $^{\ddagger}P = 0.033$: AOH versus aïve at day 43, and $^{\|}P = 0.035$: AOH versus sham at day 43 (mixed-model ANOVA with Bonferroni post hoc adjustments).

group, measured at day 16 and day 43, were significantly lower than those in the sham and naïve groups at the corresponding time points ($P < 0.001$ for both groups at both time points).

In terms of percentage changes in VBF, significant differences were detected among the groups over time (mixed-model ANOVA: time, $P < 0.031$; group, $P < 0.001$; interaction effect, $P < 0.001$). The AOH group showed significant reductions in the percentage increases in VBF at day 16 and day 43 compared to the baseline percentage increase ($P < 0.001$ for both time points). Additionally, the percentage increases in VBF at day 16 and day 43 in the AOH group were significantly lower than those in the sham and naïve groups at the corresponding time points ($P < 0.001$ for both groups at both time points).

Discussion

The present study reports the detrimental effects of AOH on flicker-induced changes of RBF and retinal electrical activity in mice. Our findings demonstrated that AOH impaired retinal functional hyperemia, which is the increase in RBF that occurs in response to increased neuronal activity during visual stimulation.[2] These impairments were demonstrated by significant reductions in flicker-induced enhancements in RBF and photopic b-wave amplitudes in the AOH group compared to the sham and naïve groups. These impairments highlight the sensitivity of retinal functional hyperemia to short-term IOP elevation, which may have implications for understanding ocular diseases such as acute glaucoma and retinal vascular disorders.

In agreement with previous studies,[25,30] our findings revealed that AOH caused significant reductions in the pSTR amplitude and IRL thickness at days 16 and 43. Both OCT and ffERG findings confirmed that AOH led to significant structural and functional damage to the retina, which lasted over time, suggesting the successful establishment of an AOH mouse model utilized in this study.

All experimental groups exhibited significant enhancements in ERG amplitude and RBF in response to FLS at

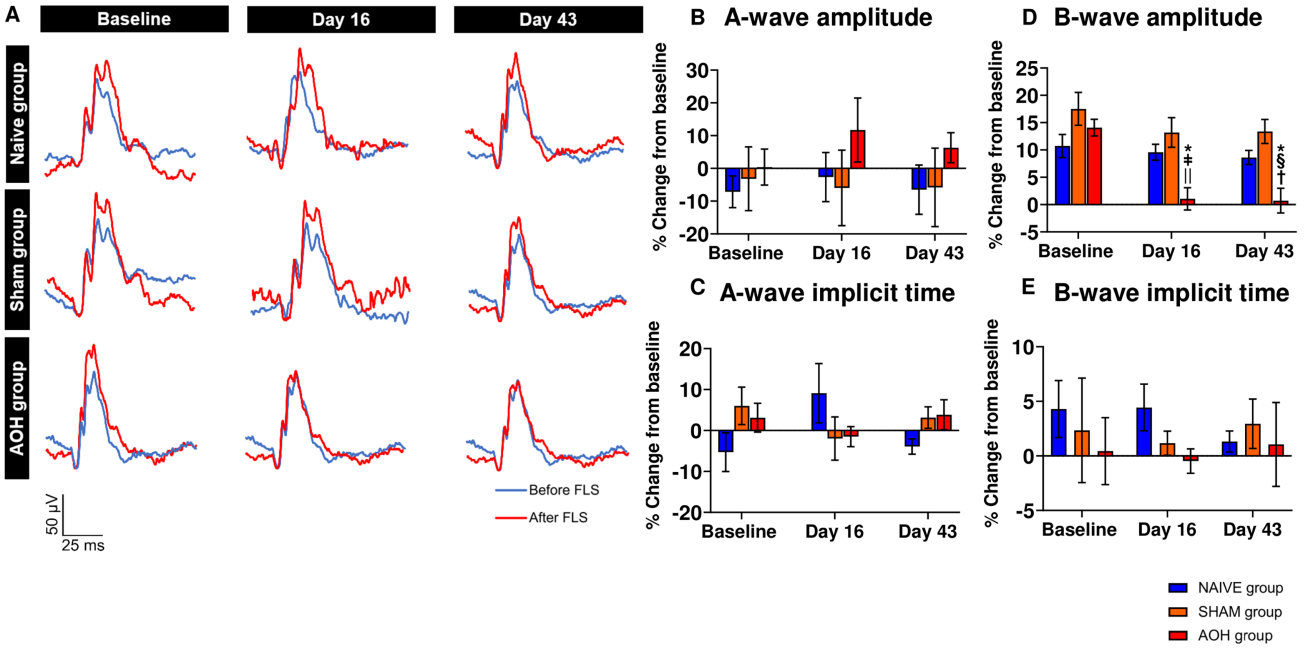

Figure 4. Percentage changes in photopic full-field electroretinography (ffERG) responses before and after FLS in naïve, sham, and AOH groups. (**A**) Traces of averaged photopic ffERG responses from a representative mouse in each experimental group, recorded at baseline, day 16, and day 43, with traces taken before (*blue*) and after (*red*) FLS. Mean percentage changes in (**B**) a-wave amplitudes, (**C**) a-wave implicit times, (**D**) b-wave amplitudes, and (**E**) b-wave implicit times from respective baseline values measured at three time points were compared. Statistical significance is denoted by symbols: $^{*}P < 0.001$ versus baseline (AOH), $^{\ddagger}P < 0.05$: naïve compared to AOH (day 16), $^{\|}P = 0.001$: sham compared to AOH (day 16), $^{\S}P < 0.001$: sham compared to AOH (day 43), and $^{\dagger}P < 0.05$: naïve compared to AOH (day 43) (mixed-model ANOVA followed by Bonferroni's post hoc tests).

day 0, which agreed with the findings from our recent study. Whereas the naïve and sham groups maintained relatively consistent enhancements at day 16 and day 43, the AOH group showed significant deterioration of the enhancements following AOH induction at those time points. These findings suggested that the short-term IOP elevation had a significant impact in both vascular and neuronal responses, which was demonstrated by the absence of significant post-FLS transient effect on retinal electrophysiological responses or RBF after AOH induction. Previous studies have widely reported the existence of neurovascular coupling that links retinal neuronal activity to local blood supply under normal physiological conditions.[1–3] An increase in neural activity raises immediate metabolic demands, signaling adjacent blood vessels to dilate. This vasodilation enhances local blood flow, ensuring an adequate supply of oxygen and nutrients. This dynamic regulation of blood vessel diameter, known as vascular autoregulation, facilitates improved local blood supply.[2] The impaired enhancements in RBF and photopic b-wave amplitude in response to FLS suggest that AOH may disrupt the retinal vascular autoregulatory mechanism. This disruption likely leads to a diminished capacity of retinal blood vessels to increase their diameters and enhance blood flow in response to FLS. Alternatively, damage to retinal neurons, specifically those in the inner retina, due to elevated IOP could impair their ability to respond to FLS. This lack of responsiveness may hinder the release of essential signals for vasodilation to the blood vessels, thereby disrupting effective vascular autoregulation. The prolonged deterioration suggests that even a single episode of high IOP (80 mm Hg) for 1 hour could lead to a significant lasting effect on retinal functional hyperemia. Several studies have shown that flickering light increases retinal vessel diameters[18,19] and RBF[4–16] as well as ERG responses,[16] to meet the increased metabolic demands of the retina during neuronal activation by FLS. These findings are in line with our results in the naïve and sham groups where FLS increased both RBF and photopic b-wave amplitudes at all time points.

Garhöfer et al.[19] reported that flicker-induced enhancements in retinal vessel dilation and optic nerve head blood flow at normal IOP levels were not significantly altered by elevated IOP up to 43 ± 4 mm Hg in healthy human subjects.[31] This indicated that the vasodilatory reserve was still present at such elevated IOP levels. However, the absence of significant flicker-induced enhancement in both ABF and VBF observed in the present study suggests that this vasodilatory reserve is compromised at the very high IOP of 80 mm Hg. Furthermore, the duration of IOP elevation was only 60 seconds in their study,[31] compared to 1 hour in the present study. This suggests that prolonged elevation at high IOP levels significantly diminishes flicker-induced enhancements in both retinal hemodynamic and functional responses. Supporting the findings of a previous study[32] which indicated an insufficiency of RBF autoregulation following moderate IOP elevation (approximately 30 mm Hg) in healthy eyes, the impaired flicker-induced enhancements in RBF and ERG responses in the AOH group further indicated that extremely high acute IOP elevation (80 mm Hg) compromised the robust intrinsic ability of the retina to adjust its blood flow to meet its metabolic demands during increased neuronal activity induced by FLS. Retinal vascular autoregulation, a physiological process that maintains sufficient blood flow to the retinal cells in response to the changes in retinal perfusion pressure or

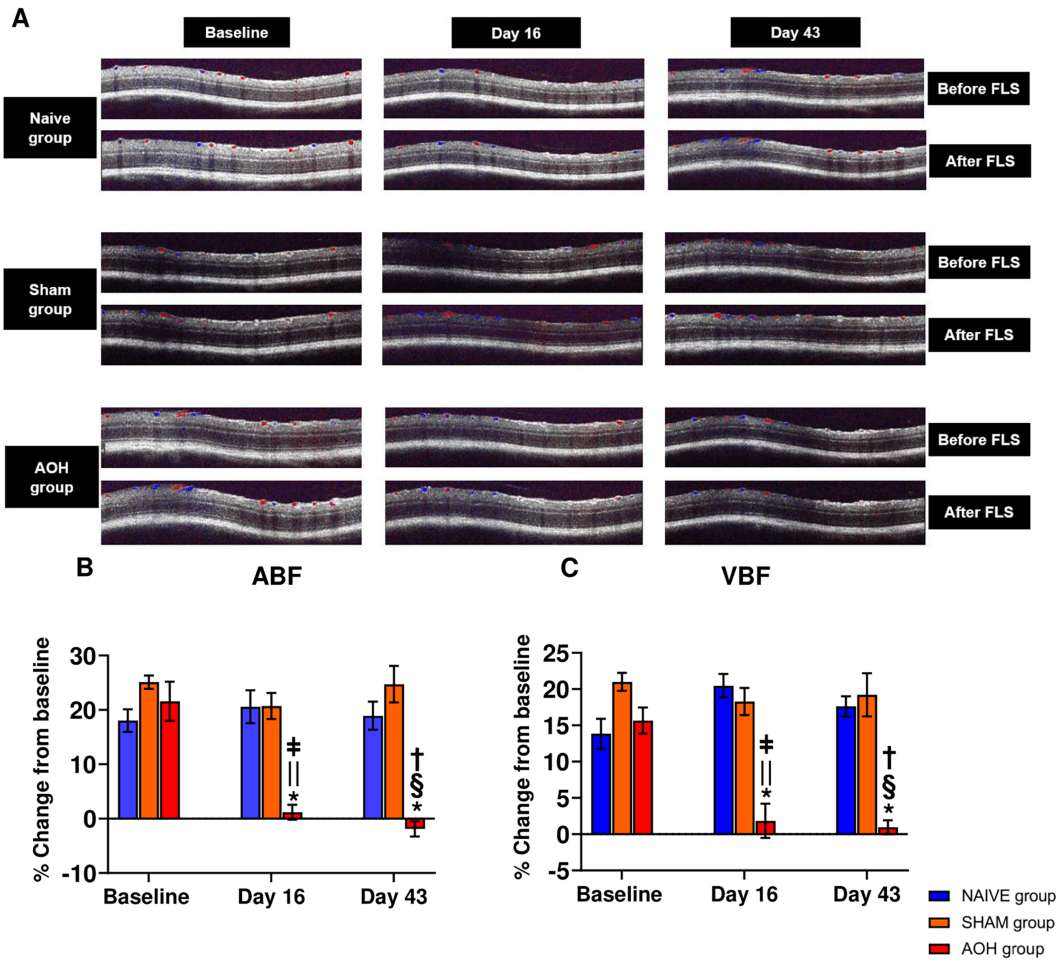

Figure 5. Doppler B-scans and percentage changes in arterial and venous blood flow in mouse retina pre- and post-FLS. (**A**) Doppler B-scans of a representative mouse retina from naïve, sham, and AOH groups, recorded before and after FLS at baseline, day 16, and day 43. Mean percentage changes in (**B**) arterial (ABF) and (**C**) venous blood flow (VBF) from respective baseline values were compared across experimental groups over three time points. Statistical significance is denoted by symbols: $^{\ddagger}P < 0.001$ naïve compared to AOH (day 16), $^{\|}P < 0.001$: sham compared to AOH (day 16), $^{\dagger}P < 0.001$ naïve compared to AOH (day 43), $^{\S}P < 0.001$ sham compared to AOH (day 43), and $^{*}P < 0.001$ compared to baseline (AOH; (mixed-model ANOVA followed by Bonferroni's post hoc tests).

metabolic demands,[33] forms an integral part of the retinal neurovascular coupling which involves a complex interaction and coordination among neuronal activity, glial cells, and vascular cells, mediated by signaling molecules such as nitric oxide, lactate, and adenosine.[2,34–39] The significant impairment in flicker-induced changes in both vascular and neuronal responses following IOP elevation suggested the multifactorial disruption of retinal functional hyperemia.

Disturbances in this interaction may be attributed to a combination of ischemic injury, mechanical, or structural damage to the retinal vasculature and cells, neurodegenerative changes, and dysregulation of key signaling pathways that mediate vascular autoregulation. The acute high IOP elevation would have a direct impact on the retinal vasculature, largely reducing the ocular perfusion pressure (OPP), defined as the difference between mean arterial pressure and IOP.[40]

Ocular blood flow autoregulation is a vital physiological mechanism that ensures stable perfusion of the retina and optic nerve head, despite fluctuations in OPP.[33] It is mediated through local vascular adjustments.[41] When OPP increases, vasoconstriction occurs, increasing vascular resistance to limit excessive blood flow and protect ocular tissues from potential damage due to high pressure or overperfusion.[41–43] In contrast, a decrease in OPP induces vasodilation to sustain adequate nutrient delivery to ocular tissues and prevents ischemia.[41–43] Although the retinal vascular system has intrinsic autoregulation of blood supply despite reduced OPP up to a certain extent, it fails to maintain this when it drops below the threshold.[44] Puchner et al. demonstrated that the retinal vascular system maintains autoregulation up to an OPP decrease of approximately 21 mm Hg, beyond which blood flow becomes pressure-passive, particularly when OPP drops by approximately 30 mm Hg.[44] He et al. demonstrated that ocular blood flow was reduced by approximately 50% when IOP was elevated from baseline (10 mm Hg) to 50 to 60 mm Hg in Long-Evans rats with mean arterial pressure maintained at 108 ± 4 mm Hg.[40] A subsequent increase in IOP to 80 mm Hg resulted in a 75% to 80% decrease in blood flow, showing a progressive decline linked to rising IOP levels.[40] Similarly, Zhi et al. reported that total RBF remained stable in Brown Norway rats with a mean arterial pressure of 102 ± 4 mm Hg when IOP was elevated successively from 10 mm Hg to 30 mm Hg.[45] However, they

noted a significant linear decline in blood flow beyond this threshold, with reductions of approximately 50% at 60 mm Hg IOP and 75% at 80 mm Hg IOP.[45] Human studies have also suggested that RBF autoregulation is sustained up to an IOP of approximately 30 mm Hg.[32,46]

Considering the 24-hour mean arterial blood pressure of approximately 110 mm Hg in conscious mice[47] and IOP of approximately 15 mm Hg in healthy C57BL/6J mice, the estimated mean OPP under normal physiological conditions is approximately 95 mm Hg. In the present study, acute elevation of IOP to 80 mm Hg for 1 hour resulted in a significant decrease in OPP by approximately 65 mm Hg, surpassing the autoregulatory threshold[44] and thereby potentially leading to ischemic changes in the retina.[22]

Ischemic insult results in a reduced supply of metabolic nutrients, such as oxygen and glucose, and impairs the release or function of vasoactive mediators such as nitric oxide,[22] adenosine,[48] and potassium ions[49] that are essential for vasodilation, thereby impairing the ability of retinal blood vessels to respond to varying metabolic demands due to FLS. Thus, extremely low OPP compromises the retinal metabolism, particularly in the middle and inner retinal layers, which are supplied by superficial, intermediate, and deep vascular layers in the rodent retina.[8]

Ischemia-reperfusion injury in mice has also been shown to induce significant endothelial dysfunction in retinal arterioles and elevated reactive oxygen species production in the retinal vasculature.[50] A recent study has shown that interpericyte tunnelling nanotubes (IP-TNTs), which connect pericytes across retinal capillary networks to facilitate neurovascular coupling and regulate blood flow,[3] were structurally and functionally damaged by ocular hypertension, leading to impaired light-evoked neurovascular responses.[51] Our findings are further supported by those of Gericke et al., who reported impaired vascular autoregulation and endothelial dysfunction in mouse retinal arterioles, mediated by oxidative stress and inflammation.[52] The impaired enhancements of flicker-induced RBF and ERG responses observed in our study could also be caused by the structural changes in the retinal vasculature due to extremely high IOP. Tao et al. demonstrated that a single transient elevation of IOP to approximately 50 mm Hg could result in a significant reduction in retinal vessel density, particularly in the superficial and intermediate capillary plexuses, with no recovery detected at 14 days post-insult.[53] In our AOH model, where IOP was maintained at 80 mm Hg for 1 hour, the vascular damage may be more pronounced and, thus, the ability of the vascular system to meet the increased metabolic demands of retinal cells in response to FLS may be more compromised.

Previous studies have reported reduced flicker-induced changes in retinal vascular calibers in ocular diseases such as diabetic retinopathy and age-related macular degeneration.[18–21] These conditions are reported to be characterized by impaired retinal vascular autoregulation,[20,21,54] similar to the effects observed in this study. Understanding the mechanisms underlying this impaired vascular autoregulation could provide valuable insights into the pathophysiology of ocular diseases characterized by elevated IOP, such as glaucoma and retinal ischemia. Further studies are required to investigate the underlying mechanisms and identify potential targets for preserving vascular autoregulation in diseased retina. Unlike previous studies that focused on flicker-induced retinal vascular caliber changes, this study also investigated the effects of AOH on both flicker-induced retinal vasculature and functional changes. In addition, the longitudinal results delineated the changes in flicker-induced RBF and retinal electrophysiological responses over time, which is crucial for understanding the temporal progression of AOH-induced impairments in retinal vascular autoregulation. Last, these flickering light-induced changes in ERG and RBF may be a potential biomarker for early detection of ocular diseases related to impaired retinal vascular autoregulation.

Although our study demonstrated how AOH impacts the flicker-induced retinal functional hyperemia, few limitations should be acknowledged. First, although mice share many aspects of the retina with humans, they still exhibit certain differences in terms of structure and function. For example, mice exhibit a rod-to-cone ratio of 49:1, reflecting extreme rod dominance in this nocturnal species.[55] In contrast, humans have a substantially lower rod-to-cone ratio of 20:1.[56] This suggests that even less intense flickering light sufficient to induce functional hyperemia in mice may not be adequate to produce the same response in humans. Additionally, there are notable differences in the types of cone cells, rod cells, and bipolar cells between the two species.[57] For instance, humans possess midget bipolar cells, which are absent in mice.[58] These types of differences may influence the mechanisms and extent of functional hyperemia in response to FLS under both normal and pathological conditions. Second, the induction of AOH in the current study may have altered the shape of the eyeball, potentially affecting the Doppler angle measured on day 16 and day 43 compared to day 0. However, given that both pre-FLS and post-FLS Doppler OCT measurements were conducted on the same day, with percentage changes calculated relative to the corresponding baselines at each time point, any variation is likely to be minimal. Third, whereas Doppler OCT provides surrogate measures of RBF, it does not measure absolute volumetric blood flow. Its accuracy is limited by potential signal saturation and noise that may bias intensity values. To minimize these limitations as much as possible, standardized imaging protocols were utilized, and all consecutive scans were averaged to mitigate pulsatile and noise effects. Furthermore, uniform thresholding was applied to eliminate background noise, and the analysis was performed strictly within the major retinal vessel areas. Despite these approaches, values obtained from Doppler OCT should be regarded as relative indicators of blood flow rather than precise measurements. Fourth, the effects of chronic IOP elevation on the parameters discussed above were not explored. Future studies should explore the long-term effects of chronic elevated IOP on these parameters. Fifth, blood pressure measurements were not obtained due to the potential complications arising from the simultaneous induction of AOH and the measurement of systemic blood pressure. In conclusion, the flicker-induced increases in both RBF (including both artery and vein) and retinal electrophysiological responses (specifically from bipolar cells of the middle retinal layer) are significantly reduced in the AOH mouse model, suggesting impaired vascular autoregulation in the retina.

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Investigative Ophthalmology & Visual Science

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