Anatomy and Pathology/Oncology

Probing the Potency of Artificial Dynamic ON or OFF Stimuli to Inhibit Myopia Development

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Purpose. To determine whether equiluminant artificial dynamic ON or OFF stimuli on a computer screen can induce bidirectional changes in choroidal thickness (ChTh) in both humans and chickens, and whether such changes are associated with bidirectional changes in retinal dopamine release in chickens.

Methods. Experiment 1: Before and after ON or OFF stimulation for 1 hour, ChTh was measured with optical coherence tomography (OCT). Experiment 2: chicks ($n = 14$) were raised under ON or OFF stimulation for 3 hours. ChTh was determined by OCT. Experiment 3: chicks were raised for 7 days either under room light (500 lux, $n = 11$), dynamic ON stimulus (700 lux, $n = 15$), or dynamic OFF stimulus (700 lux, $n = 7$). In addition, negative lenses were attached to their right eyes. After experiments 2 and 3, retinal and vitreal dopamine (DA), and its metabolites, were measured by HPLC–electrochemical detection.

Results. Experiment 1: Dynamic ON stimuli caused thicker choroids (+5.3 ± 2.0 μm), whereas OFF stimuli caused choroidal thinning (−4.7 ± 0.5 μm) (right eye data only, $P < 0.001$). Experiment 2: After 3 hours, chickens developed thicker choroids with ON stimuli (+37.4 ± 12.4 μm) and thinner choroids with OFF stimuli (−11.3 ± 3.6 μm, difference $P < 0.01$). Vitreal DA, 3-methoxytyramine, and homovanillic acid levels were elevated after ON stimulation, compared with the OFF ($P < 0.05$). Experiment 3: After 7 days, chickens with lenses developed more myopia both with ON and OFF stimulation, compared with room light. ON stimulation increased vitreal DA compared with OFF.

Conclusions. Artificial dynamic ON or OFF stimuli had similar effects on ChTh in humans and chickens, but more work will be necessary to determine whether such stimuli can be used as novel interventions of myopia.

Keywords: ON OFF pathways, chicken, myopia, choroid, dopamine

The visual system is organized into ON and OFF pathways. In the temporal domain, many retinal neurons respond predominantly either to rapidly increasing luminances (ON cells), or to decreasing luminances (OFF cells).[1] In the spatial domain, bipolar and retinal ganglion cells have circular fields that are organized into a center ON and periphery OFF region, or vice versa (i.e., mice,[1] monkeys,[2] cats[3]). The biological explanation for this arrangement may be that homogenously illuminated areas in the visual field do not excite ganglion cells, which saves considerable capacity in information processing and storage. Different from digital cameras, which read out and store the RGB gray levels for each single pixel, retinal ganglion cells respond mainly to temporal and spatial differences in luminance. Due to this, and also by pooling photoreceptor signals outside the fovea, it is possible to compress the output of 125 million photoreceptors into 1 million ganglion cell axons in the optic nerve without giving up high spatial and temporal resolution.

ON and OFF pathways differ in their spatial signal processing. Whereas the OFF system has smaller receptive field sizes and therefore higher visual acuity,[4] the ON system is more sensitive to small differences in contrast.[5] Selective adaptation to dynamic OFF stimuli increased contrast sensitivity in young subjects at low luminances, perhaps because the ON input became more dominant due to adaptation of the OFF channels.[6]

ON and OFF pathways also have selective effects on emmetropization and myopia. Smith et al.[7] used D, L-2-amino-4-phosphonobutyric acid to inactivate retinal ON-channel in cats and found that all animals developed relative hyperopia. It was concluded that "the mechanisms responsible for normal axial elongation are dependent to some extent on ON-channel activity." Later, Crewther and Crewther[8] found that chickens with negative lenses developed less myopia when they were exposed to light stimuli that had a fast brightness onset and slow decay (an ON stimulus in the ERG), whereas chickens reared under the reversed temporal luminance profiles (slow brightness onset and fast decay, corresponding to an OFF stimulus in the ERG) developed less hyperopia with positive lenses. Crewther and Crewther[9] also found that pharmacological inhibition of the ON pathways with L-alpha-aminoadipic acid reduced myopia, whereas inhibition of the OFF pathways with D-alfa-aminoadipic acid produced relatively more myopia. Experiments with knockout mouse models have shown that inactivation of the ON channel enhanced the development of deprivation myopia,[10] whereas inactivation of the OFF produces similar amounts of deprivation myopia as in wild-type.[11]

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Dopaminergic pathways have been implicated in myopia development in various animal models, and dopamine release is controlled by light (review in Feldkaemper and Schaeffel[12]). In 2008, Pardue et al.[13] found that mice with a functional deficit in the ON pathway (nob mice) had lower retinal dopamine and 3, 4-dihydroxyphenylacetic acid (DOPAC) levels compared with wild mice. Similarly, Chakraborty et al.[10] found lower dopamine levels also in mice with functional loss in the ON pathways due to genetic knockout of the mGluR6 receptor. At the 15th International Myopia Conference (2015), Pardue and colleagues described that the primary driver of retinal dopamine release is the cone ON bipolar cell and, less so, the melanopsin containing intrinsically photosensitive retinal ganglion cells.[14]

It is well known that the choroid thickens when positive lenses are placed in front of the eye and thins when negative lenses are used (chickens,[15] marmosets,[16] rhesus monkeys,[17] humans[18]). It has been proposed that choroidal thickness may be related to future changes in axial eye growth and a predictor of myopia.[19,20] Recently, we found in young human subjects that 60 minutes of reading bright text on dark background made the choroid thicker, whereas the opposite happened when reading conventional text.[21] Using C++ software for realtime analysis of the visual world in terms of ON and OFF stimulus strengths (developed in our laboratory and available for downloading[21]), it was found that black text on white background predominantly stimulates the OFF pathway, whereas white text on black background stimulates the ON pathway. Accordingly, we proposed that reading bright text on a dark background may have an inhibitory effect on myopia development, whereas the conventional text may stimulate myopia. It is clear that a study on the progression of myopia in school children, reading either dark or bright text, needs to be done to confirm the validity of this idea.

Three major questions emerge: (1) In addition to reading text with different contrast polarities, can artificial dynamic ON or OFF stimulation change choroidal thickness in humans in both directions? (2) Can ON stimuli, which caused choroidal thickening in humans, also stimulate choroidal thickening in the chicken model of myopia and also stimulate more retinal dopamine release, a known inhibitor of myopia? (3) Do such stimuli also inhibit long-term myopia? In the current study, we have attempted to answer these questions. In the first experiment, we measured choroidal thickness changes in young human subjects after exposure to dynamic ON or OFF stimuli for 60 minutes. In the second experiment, we measured dopamine and DOPAC levels, as well as choroidal thickness in chickens after 3 hours of exposure to either dynamic ON or OFF stimuli. In the third experiment, we studied refractive development, as well as dopamine and DOPAC release in chickens treated with negative lenses during 7 days of stimulation.

Methods

Subjects

Human Subjects. Eleven young adult subjects participated. Seven were Asian, the others Caucasian. Seven were female and four were male. Ages ranged from 23 to 29 years. There were no known ocular pathologies other than myopic refractive errors (spherical equivalent ranging from –0.25 to –8 diopters [D]). Two subjects were near emmetropic (spherical equivalent > –0.75), five were mildly to moderately myopic (spherical equivalent between –0.75 and –4 D), and four were moderately to highly myopic (spherical equivalent between –5 and –8 D). During the experiments, myopic subjects wore their habitual corrections. Informed consent was obtained from all subjects before the experiments and the experiments complied with the Declaration of Helsinki. Experiments were approved by the Ethics Commission of the Medical Faculty of the University of Tuebingen.

Animal Subjects. Forty-seven, 1-day-old, male White Leghorn H&N (*Gallus domesticus*) chickens were obtained from a local hatchery in Kirchberg, Germany. They were raised in temperature-controlled animal facilities under a 12/12-hour light/dark cycle (8 AM to 8 PM) at an illuminance of approximately 500 lux during the light phase. Water and food were supplied ad libitum. All experiments were conducted in agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Commission for Animal Welfare of the Medical Faculty of the University of Tuebingen.

Treatments

ON and OFF Stimulation. Dynamic ON or OFF stimuli were developed under Visual C++ 8.0. Stimuli consisted of approximately 2000 small squares with an angular subtense of 49 arcmin on the computer screen in the experiments with human subjects, and ranged between 79 arcmin when the chick had the largest possible distances from the computer screen and up to approximately 50 degrees when they were very close. Squares had a repetitive sawtooth-shaped monochrome temporal luminance profile, either with a rapid ON and slow linear decay (ON stimulus) or vice versa (OFF stimulus). All squares were randomly phase shifted with respect to each other, using the rnd() function in C++. Cycle frequency was approximately 1 Hz (determined by the processing speed of the computers).

Experiment 1: One-hour Exposure of Human Subjects to the ON or OFF Stimuli. ON and OFF stimuli were presented on a large 65-inch 4K TV screen (SONY LED-TV KD 65XD7505; Sony, Tokyo, Japan), placed at 2.3 m (0.4 D) distance. The size of the squares on the screen was 33 × 33 mm. Average screen luminance was 34 cd/m^2, as measured with a Minolta luminance meter (LS-100; Minolta Camera Co., LTD, Tokyo, Japan). Subjects were asked to watch both, ON and OFF visual stimuli, for 60 minutes, on two different days (one day watching either ON or OFF, and the opposite stimulus on the consecutive day). The presentation order of the stimuli was randomized. During these experiments, the room illuminance was in the mesopic range (3.7 lux). Before and after 60 minutes of exposure to the stimulus, subfoveal choroidal area was analyzed with spectral-domain optical coherence tomography (SD-OCT) (HRA+OCT Spectralis; Heidelberg Engineering, Heidelberg, Germany). Because it is known that choroidal thickness varies over the day,[18,22] all measurements were done at the same time in the morning between 9:55 and 11:00 AM.

Experiment 2: Three-hour Exposure of Chickens to the Dynamic ON or OFF Stimuli. Chickens were raised in a transparent plastic cage (60 × 60 cm) without top cover. The floor consisted of white cardboard (with food on it). Two video projectors were used to project the stimuli on the entire floor of the arena from above. In addition, four large computer screens (Acer XF270H, 61 cm; Acer, New Taipei City, Taiwan) were placed behind the walls of the transparent perspex container and showed the same stimuli. The average illuminance in the arena was 700 lux, generated by the two video projectors and the four computer screens. To prevent chickens from jumping out of the arena, a transparent perspex plate was placed across the monitors, leaving the corners open to allow the exchange of air (Fig. 1). Water and food were supplied ad libitum.

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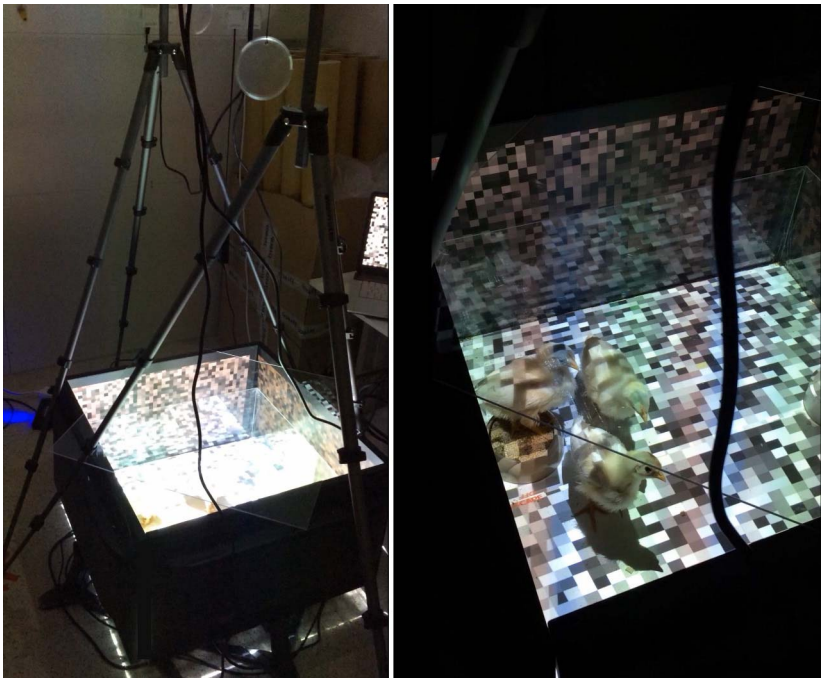

FIGURE 1. Stimulation arena in which chicks were continuously exposed to dynamic ON or OFF stimuli.

Seven 14-day-old chickens were treated with ON stimuli, and seven with the OFF stimuli for 3 hours, from 10:00 AM to 1:00 PM. Choroidal thickness was determined by SD-OCT before and after the exposure period. Subsequently, retina and vitreous were prepared for catecholamine (dopamine [DA], DOPAC, homovanillic acid [HVA], 3-methoxytyramine [3-MT]) measurements.

Experiment 3: Long-term ON or OFF Stimulation in Chickens. Thirty-three chicks were exposed to either ON or OFF stimulation for 7 days, starting at day 14 of age. During this time, they wore PMMA plastic lenses of −7 D power over their right eyes (treated eyes), leaving the left eyes untouched (control eyes). The lenses were attached to Velcro rings and the mating Velcro rings that were glued to the feathers around the chickens' eyes. Lenses were cleaned twice per day. After the lenses were first attached, chicks were randomly allocated to one of three light profile condition cages: room light ($n = 11$, "control"), dynamic ON stimulus ($n = 15$), or dynamic OFF stimulus ($n = 7$). In room light, illuminance was approximately 500 lux; the average illuminance in the arena was about 700 lux, as in experiment 2. Stimulation with dynamic ON or OFF stimuli was continued from 8 AM to 6 PM. After 6 PM, all chickens were kept in the dark. Refraction, axial length, and choroidal thickness were measured before and after the experiment. As choroidal thickness and axial length undergo diurnal modulation,[23] measurements were always taken between 8 and 10 AM. As in experiment 2, retina and vitreous were prepared for catecholamine (DA, DOPAC, HVA, 3-MT) measurements on the last day.

Measurements

Refraction and Axial Length. Refractions of the chickens were determined by automated infrared photoretinoscopy.[24] Axial lengths and vitreous chamber depths were measured in alert hand-held chickens, using a low coherence interferometer (Lenstar LS 900; Haag-Streit, Koeniz, Switzerland).[25]

Measurements of Human Subfoveal Choroidal Area and Thickness. Subfoveal choroidal area was imaged with SD-OCT (HRA+OCT Spectralis), as previously described.[21] Focus was adjusted for each subject depending on their spherical equivalent refractions. Because the choroid-sclera border was not well defined in some subjects, we used the EDI (enhanced depth imaging) mode of our OCT machine to improve visualization of the choroidal limits. Measurements of subfoveal choroidal area and subfoveal thickness were manually performed by carefully locating the margins of the choroid in the original images, using the public software package ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA), as previously described.[21] Because the images were slightly different due to eye movements during the measurements, a line was drawn perpendicular to the retinal surface from the floor of the foveal pit to the sclera. From there, an area of 260 pixels to the left and to the right of the image was analyzed. In this area, the borders of the choroid were marked by the operator and the area was measured in pixels, using ImageJ (Fig. 2A). Furthermore, choroidal thickness was also measured directly (Fig. 2A, red bar).

Measurements of Chicken Choroidal Thickness. The same SD-OCT instrument was used as in humans. Chicks were unanesthetized and alert for all measures. They were held by one of the investigators by hand for alignment. During the measurements, the position of the chicken's head was aligned with the camera lens so that the infrared laser beam of the OCT entered the eye through the center of the pupil. Ten to 20 images were collected for each chicken. Because chickens do not maintain stable fixation, 8 to 14 images were carefully selected. Only those scans in which the pupil was properly centered and the borders of the individual fundal layers were clearly visible (Fig. 3A) were analyzed. Again, ImageJ was used to manually determine choroidal thickness. Segmented lines were drawn into the original image files where the operator assumed the choroidal borders. The second author re-analyzed the OCT images from 14 chickens to determine the interobserver variance. The correlation coefficient was $R = 0.937$. This was a blind analysis and the operators did not know whether the chicks had been treated with ON or OFF stimuli. The

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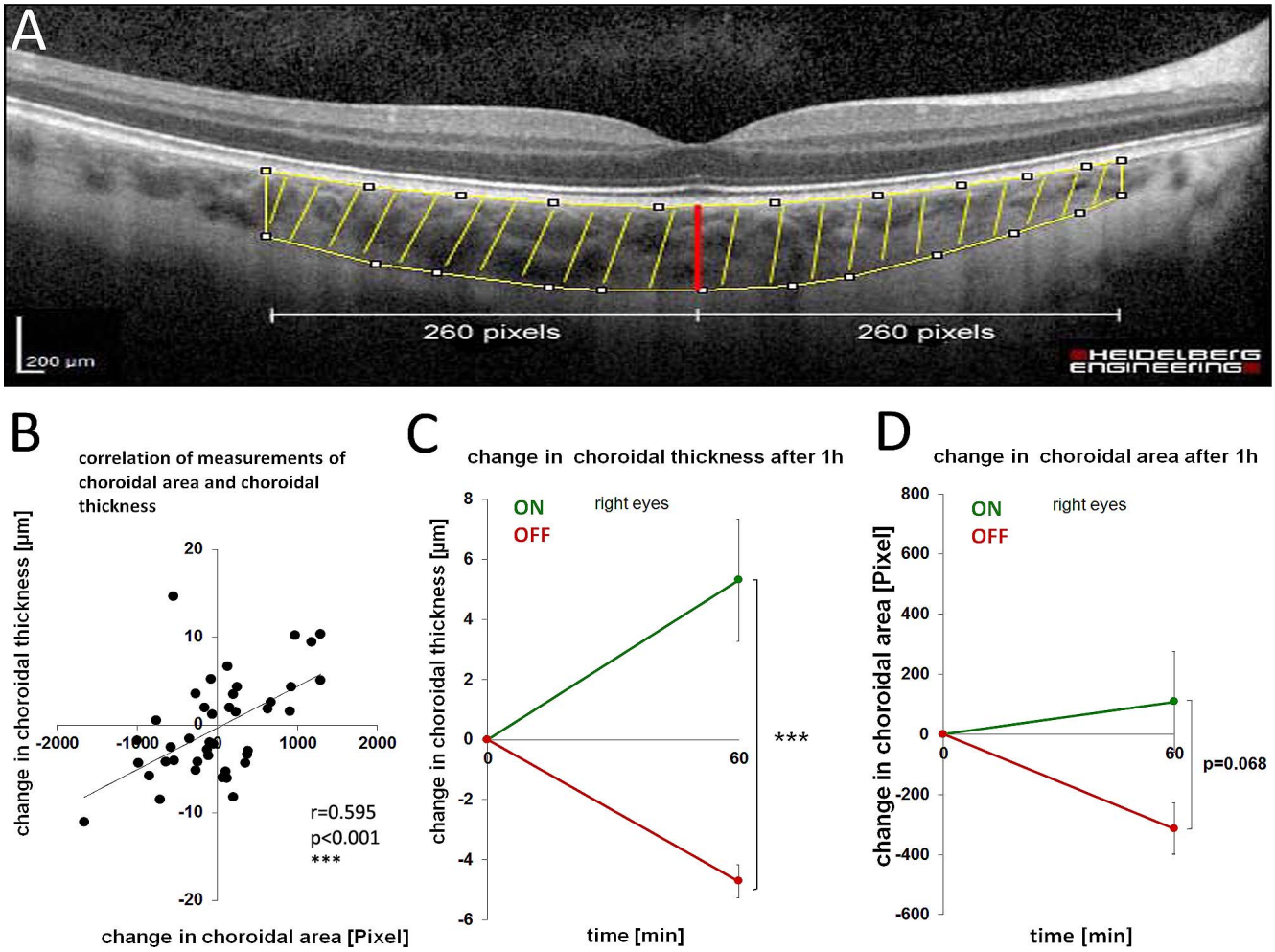

Figure 2. (**A**) Illustration of two procedures to evaluate changes in choroidal dimensions: subfoveal choroidal area (marked by *yellow lines*, measured as number of pixels) and subfoveal choroidal thickness (*red bar*, µm). (**B**) Correlation of the results of the two procedures ($R = 0.595$, $n = 41$, $P < 0.001$). (**C**) Subfoveal choroidal thickness (µm) and (**D**) subfoveal choroidal area (pixels) show similar effects after 60 minutes of exposure to the dynamic ON or OFF stimuli. ON stimuli caused choroidal thickening (*green lines*), whereas the OFF stimulus caused choroidal thinning (*red lines*). Only data of the right eyes are shown; for data of the left eyes, see text. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

variance of repeated measurements by the same observer was also determined in 104 OCT images from two chickens. The correlation coefficient was $R = 0.972$.

Measurements of Biogenic Amines by HPLC–Electrochemical Detection. Because DA levels are dependent on both illuminance and diurnal cycle,[26] preparation of retina and vitreous was done after 3 hours of stimulation between 1:00 PM and 2:00 PM in experiment 2, and between 10:00 and 12:00 AM in experiment 3 after long-term stimulation. Chicks were killed by an overdose of ether inhalation and decapitated. Eyes were enucleated and cut perpendicular to the anterior-posterior axis with a razor blade, approximately 1 mm posterior to the ora serrata. The anterior segment of the eye was discarded. Vitreous and retina were prepared as described before,[27] instantly frozen in liquid nitrogen, and stored at –80°C. Catecholamines were quantified with HPLC–electrochemical detection (Ultimate 3000 LC with electrochemical detection ECD3000RS; Thermo Fisher Scientific, Waltham, MA, USA) as previously described.[27] Vitreous was mixed with 750 µL mobile phase (Thermo Fisher Scientific) and homogenized with a tissue lyser (TissuesLyser LT; Qiagen, Hilden, Germany) at 50 Hz for 4 minutes, while the retina was disrupted with 500 µL mobile phase and 25 µL of the homogenate was saved for later protein measurements. Both the vitreous and retina homogenates were centrifuged at 14,000*g* for 10 minutes, the supernatant filtered through a 0.2-µm nylon membrane sample filter (Thermo Scientific, Rockwood, IL, USA), and 25 µL was directly injected into the HPLC system. Dopamine, DOPAC, HVA, and 3-MT concentrations were determined in vitreous and retina.

In the retina, biogenic amine content was determined as nanogram per milligram protein. The Pierce BCA Protein kit (Thermo Scientific) was used to measure retinal protein content, followed by the microplate procedure. In the vitreous, biogenic amines were determined relative to wet weight (ng/0.1 g wet weight).

Statistics

Statistical analyses were performed using the publically available software "JMP 14" (SAS Institute, Inc., Cary, NC, USA). Distributions of data on biogenic amines were tested for normality. To compare two independent groups in experiments 1 and 2, a two-tailed unpaired *t*-test was used. In experiment 3, to compare the effects of different light stimulation conditions with or without lenses, a 1-way ANOVA was used, followed by a Tukey-Kramer honestly significant difference (HSD) test for post hoc analysis. Average data are

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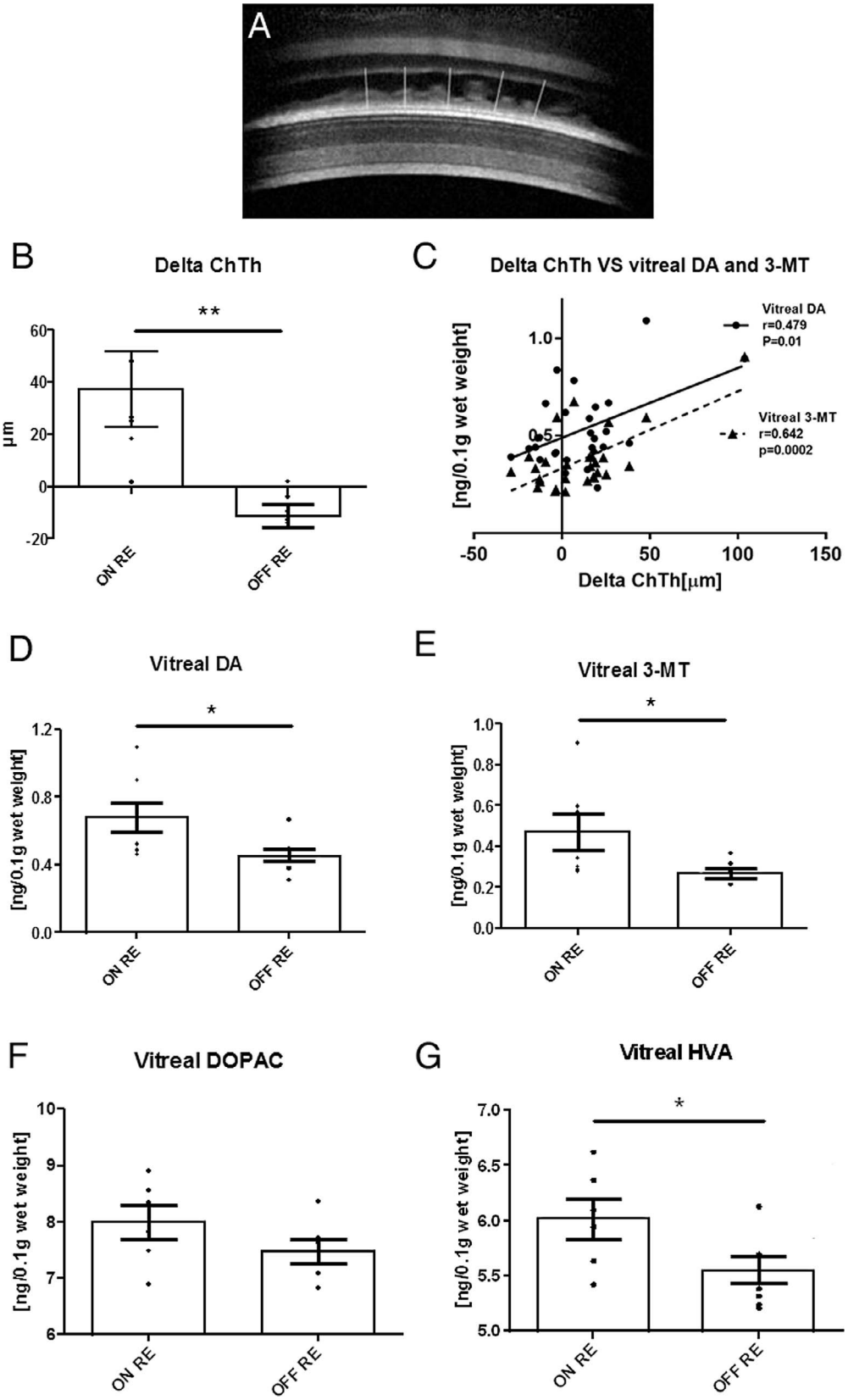

Figure 3. (**A**) Measurements of choroidal thickness in the chicken. In each image, the average of five measurements (*white lines*, µm) was determined, and at least eight images were analyzed from each eye. (**B**) ON stimulation caused choroidal thickening after 3 hours of exposure ($P < 0.023$), whereas OFF stimulation caused choroidal thinning ($P < 0.021$; paired *t*-test). (**C**) Choroidal thickness changes were correlated with changes in vitreal DA ($P < 0.01$) and 3-MT ($P < 0.0002$), although this correlation rested largely on two data points. With ON stimulation, there was a small increase in vitreal DA (**D**), 3-MT (**E**), HVA (**G**), and DOPAC (**F**), although it did not achieve significance for the last one. Only data of right eyes are shown. Significance levels as in Figure 2.

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Table 1. Concentration of Vitreal DA and Its Metabolites (3-MT, HVA, and DOPAC) (Mean ± SEM, ng/0.1 g Wet Weight) After 3 Hours of ON or OFF Stimulation

	ON, RE	OFF, RE	ON, LE	OFF, LE	ON, Both Eyes	OFF, Both Eyes
Vitreal DA	0.68 ± 0.09	0.45 ± 0.04	0.52 ± 0.06	0.46 ± 0.07	0.60 ± 0.06	0.45 ± 0.04
Vitreal 3-MT	0.47 ± 0.09	0.27 ± 0.02	0.40 ± 0.05	0.37 ± 0.04	0.43 ± 0.05	0.32 ± 0.03
Vitreal HVA	5.98 ± 0.16	5.54 ± 0.12	5.91 ± 0.10	5.86 ± 0.21	5.95 ± 0.09	5.69 ± 0.12
Vitreal DOPAC	7.87 ± 0.29	7.45 ± 0.19	7.67 ± 0.37	7.58 ± 0.35	7.77 ± 0.23	7.51 ± 0.19

Data for right eyes are graphically displayed in Figure 3. LE, left eyes; RE, right eyes.

expressed as mean ± SEM. The limit of significance was set to $P < 0.05$.

Results

Experiment 1: One-hour Exposure of Human Subjects to the ON or OFF Stimuli

Both subfoveal choroidal thickness (Fig. 2A, red bar, ChTh, μm) and subfoveal choroidal area (Fig. 2A, shaded yellow area, ΔChA, pixels) were measured. The results of both procedures were highly correlated ($R = 0.595$, $n = 41$, $P < 0.0001$; Fig. 2B). After 11 young human subjects had watched the dynamic ON and OFF stimuli for 1 hour, they displayed significant bidirectional changes in their subfoveal choroidal thickness. After 1 hour of ON stimulation, subfoveal choroid thickened from 258.4 ± 15.9 μm to 263.7 ± 17.0 μm ($P < 0.025$, paired *t*-test) in right eyes, and from 259.1 ± 14.8 μm to 263.1 ± 14.9 μm ($P < 0.003$) in left eyes. After 1 hour of OFF stimulation, subfoveal choroidal thickness decreased from 258.3 ± 4.8 μm to 252.8 ± 4.7 μm ($P < 0.001$) in right eyes, and from 256.7 ± 4.3 μm to 252.1 ± 4.2 μm ($P = 0.003$) in left eyes. Data from the right eyes are illustrated in Figures 2C and 2D.

Change in choroidal thickness differed clearly after ON and OFF treatment after 1 hour (right eyes: ON +5.31 ± 2.03 μm, OFF −4.71 ± 0.55 μm, $P < 0.001$; left eyes: ON +4.00 ± 1.04 μm, OFF −4.62 ± 0.87 μm, $P < 0.001$ [two-tailed unpaired *t*-test]). A similar trend was found when subfoveal choroidal area was analyzed, although the increase in area did not achieve significance in the case of the right eyes: ON +108 ± 166 pixels, OFF −314 ± 144 pixels, $P = 0.068$; left eyes: ON +636 ± 166 pixels, OFF −396 ± 187 pixels, $P = 0.0006$ (two-tailed unpaired *t*-test).

Experiment 2: Choroidal and Dopamine Changes After 3 Hours of Exposure to the Dynamic ON or OFF Stimuli in Chickens

Before and after 3 hours of exposure, choroidal thickness (Fig. 3A, white lines, μm) was measured with ImageJ. Three hours of ON stimulation caused choroidal thickening (from baseline 155.5 ± 6.8 to 193 ± 9.1 μm in the right eyes [$P = 0.023$, paired *t*-test] and 157.5 ± 5.3 to 173.9 ± 4.3 μm in left eyes [$P = 0.0001$, paired *t*-test]) and OFF stimulation caused choroidal thinning from 178.2 ± 8.1 to 166.8 ± 7.0 μm ($P = 0.021$, paired *t*-test) in right eyes and from 163.8 ± 6.6 to 163.7 ± 7.2 μm (not significant) in left eyes. Data of the right eyes are shown in Figures 3B and 3D to 3G.

Three hours of ON stimulation caused significantly higher vitreal DA levels (0.68 ± 0.09 vs. 0.45 ± 0.04 ng/0.1 g wet weight) than OFF stimulation (Fig. 3D). The same was true for its metabolites 3-MT (Fig. 3E) and HVA (Fig. 3G). Vitreal DOPAC was not significantly changed (Fig. 3F). In left eyes, none of these changes were significant (Table 1). If both eyes were pooled, only vitreal DA remained significant (ON versus OFF: 0.60 ± 0.06 vs. 0.45 ± 0.04, $P < 0.05$). Raw data are shown in Table 1. Retinal catecholamines were also compared after ON and OFF stimulation but no significant changes were observed (data not shown).

Experiment 3: Long-term ON or OFF Stimulation in Chickens

Fifteen chicks were exposed to dynamic ON stimulation for 7 days, 7 chicks were exposed to dynamic OFF stimulation, and 11 animals were kept under room light. Negative lenses (−7 D) were attached to the right eyes to induce myopia, whereas the left eyes remained untreated.

All lens wearing eyes developed more myopia than their fellow eyes ($P < 0.001$ for all conditions; Fig. 4A). Unexpectedly, chickens reared under room light developed less myopia in their lens-treated eyes than chicks reared with ON stimulation (−2.4 ± 0.4 D versus −3.7 ± 0.1 D, $P < 0.001$), or OFF stimulation (−2.4 ± 0.4 D versus −3.4 ± 0.2 D, $P < 0.05$). Also the fellow eyes without lenses became slightly more myopic with ON stimulation than under room light (−0.8 ± 0.1 D versus −0.4 ± 0.1 D; $P < 0.01$). However, with OFF stimulation, there was no significant difference (−0.6 ± 0.1 D versus −0.4 ± 0.1 D; ANOVA, followed by a Tukey-Kramer HSD test).

The changes in refraction were largely mirrored in the changes in axial length (with lenses: ON +1.30 ± 0.03 mm, room light +1.15 ± 0.06 mm, OFF +1.27 ± 0.04 mm, without lenses: ON +0.82 ± 0.02 mm, control +0.72 ± 0.02 mm, OFF: +0.81 ± 0.07 mm). Only the ON stimulus caused significantly more axial eye growth than the room light in lens-treated eyes ($P < 0.05$; Fig. 4B). Similar changes were found in vitreous chamber depth (Fig. 4C), but a significant difference was detected only in untreated eyes between ON stimulation and room light ($P < 0.05$, ANOVA, followed by a Tukey-Kramer HSD test). There were no significant interactions found between the effects of the dynamic light stimuli and lens treatment by 2-way ANOVA statistics.

As described originally by Wallman et al.,[15] treatment with negative lenses generated thinner choroids, compared with no lenses (ON +34.2 ± 8.1 versus ON+lens −7.8 ± 12.6 μm, $P < 0.05$; control +23.8 ± 7.5 versus control+lens −2.4 ± 9.1 μm, n.s; OFF +34.1 ± 9.6 versus OFF+lens −23.5 ± 9.3 μm, $P < 0.01$, ANOVA, followed by a Tukey-Kramer HSD test, Fig. 4D). However, no statistically significant effects of the light regimens on choroidal thickness were detected, either in eyes treated with lenses, or in fellow eyes. Raw data are shown in Table 2.

Vitreal DA and its metabolites (3-MT, DOPAC, and HVA) are established indicators of retinal DA release.[28,29] They were quantified after 7 days of exposure. The most prominent effects were generated by the negative lenses. Negative lenses induced a significant drop of vitreal DA under all three light regimens (ON 0.71 ± 0.08 versus ON+lens 0.32 ± 0.03 ng/0.1g wet weight, $P < 0.001$; control 0.47 ± 0.05 versus control+lens 0.21 ± 0.02 ng/0.1g wet weight, $P < 0.001$; OFF 0.33 ± 0.03 versus OFF+lens 0.17 ± 0.02 ng/0.1g wet weight,

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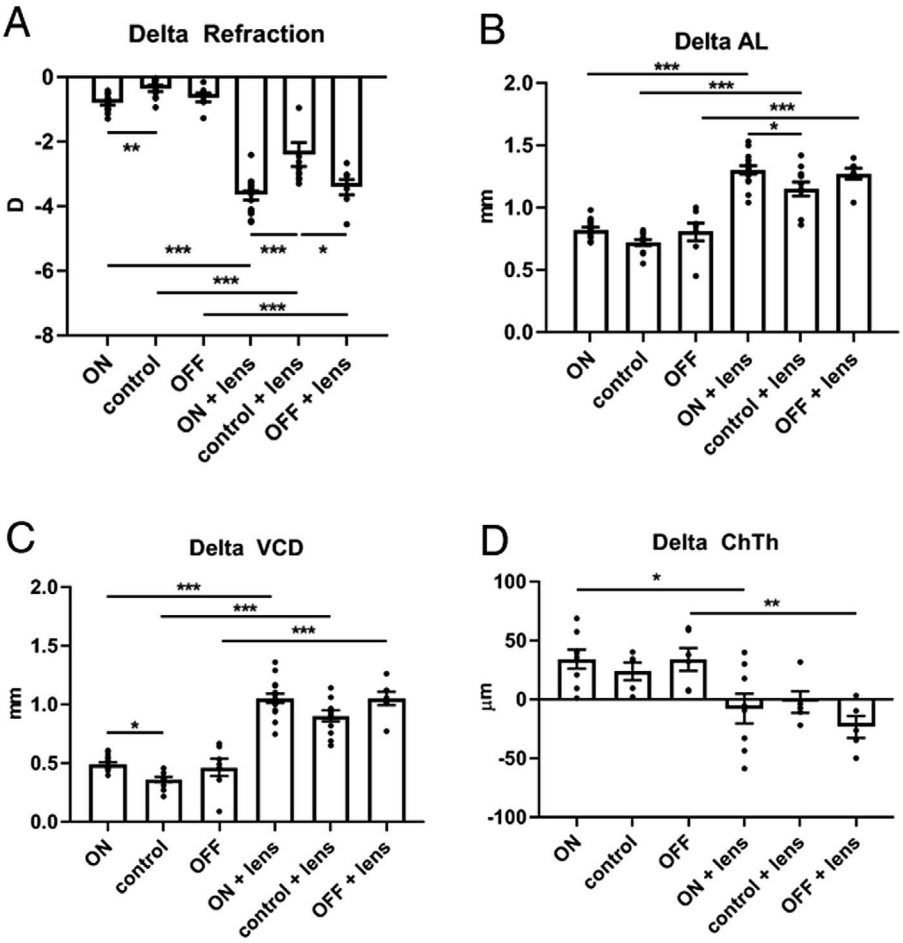

Figure 4. (**A**) Changes in refractive state in chickens after 7 days of exposure to dynamic ON stimuli, room light ("control") or dynamic OFF stimuli, without and with a −7 D negative lens. Myopia was generated by the negative lenses under all illumination conditions, compared with fellow eyes without lens. More myopia developed with the lenses under dynamic light stimulation, compared with under room light, no matter whether it was ON or OFF stimulation. A similar light stimulus-dependent effect was seen in eyes without lenses. (**B**) Axial lengths and (**C**) vitreous chamber depths mirrored the changes in refraction. (**D**) The choroid remained unchanged in negative lens-treated eyes under all light conditions, whereas it thickened in the fellow eyes without lenses. Overall, no changes were seen between ON and OFF stimulation. Significance levels as in Figure 2.

$P < 0.001$; ANOVA, followed by a Tukey-Kramer HSD test; Fig. 5A). These findings were in line with older studies.[29]

Novel and more interesting may be the effects of the different light regimens on retinal DA release. ON stimulation generated significantly higher retinal DA release than the room light or the OFF stimulation, both in lens-treated eyes (ON+lens versus control+lens and OFF+lens, both $P < 0.01$; ANOVA, followed by a Tukey-Kramer HSD test) and fellow eyes without lenses (ON versus control and OFF, $P < 0.05$ and $P < 0.001$, respectively; Fig. 5A).

Vitreal 3-MT was highly correlated with vitreal DA (correlation coefficient $r = 0.82$, $P < 0.001$; Fig. 5B). ON stimulation significantly increased vitreal 3-MT levels, compared with the control and OFF in both control (ON versus control and OFF, both $P < 0.001$; ANOVA, followed by a Tukey-Kramer HSD test) and lens-treated eyes (ON versus control and OFF, $P < 0.05$ and $P < 0.01$, Fig. 5B). Similar trends were found for vitreal DOPAC. ON stimulation increased vitreal DOPAC compared with control and OFF (Fig. 5C). Vitreal HVA was significantly lower in myopic eyes. ON stimulation caused higher content of HVA than under room light or OFF stimulation (Fig. 5D). Raw data are shown in Table 3.

Also, retinal catecholamines were measured (Fig. 6). Negative lenses caused a drop in retinal DOPAC, both with ON and OFF stimulation (Fig. 6C), but no changes were observed for DA, 3-MT, and HVA. However, ON stimulation

Table 2. Delta Refraction (D), Delta AL (mm), Delta VCD (mm), and Delta ChTh (µm) After 7 Days of Stimulation With ON or OFF Stimuli, or Room Light ("Control")

	ON	Control	OFF	ON+Lens	Control+Lens	OFF+Lens
Delta Refraction	−0.80 ± 0.06	−0.35 ± 0.06	−0.63 ± 0.13	−3.66 ± 0.14	−2.40 ± 0.37	−3.41 ± 0.23
Delta AL	0.82 ± 0.04	0.72 ± 0.03	0.81 ± 0.02	1.30 ± 0.03	1.15 ± 0.06	1.27 ± 0.04
Delta VCD	0.49 ± 0.02	0.36 ± 0.02	0.46 ± 0.07	1.05 ± 0.04	0.90 ± 0.05	1.05 ± 0.06
Delta ChTh	34.21 ± 8.12	23.80 ± 7.53	34.05 ± 9.55	−7.79 ± 12.60	−2.36 ± 9.06	−23.50 ± 9.31

Data are also graphically displayed in Figure 4. AL, axial length; VCD, vitreous chamber depth.

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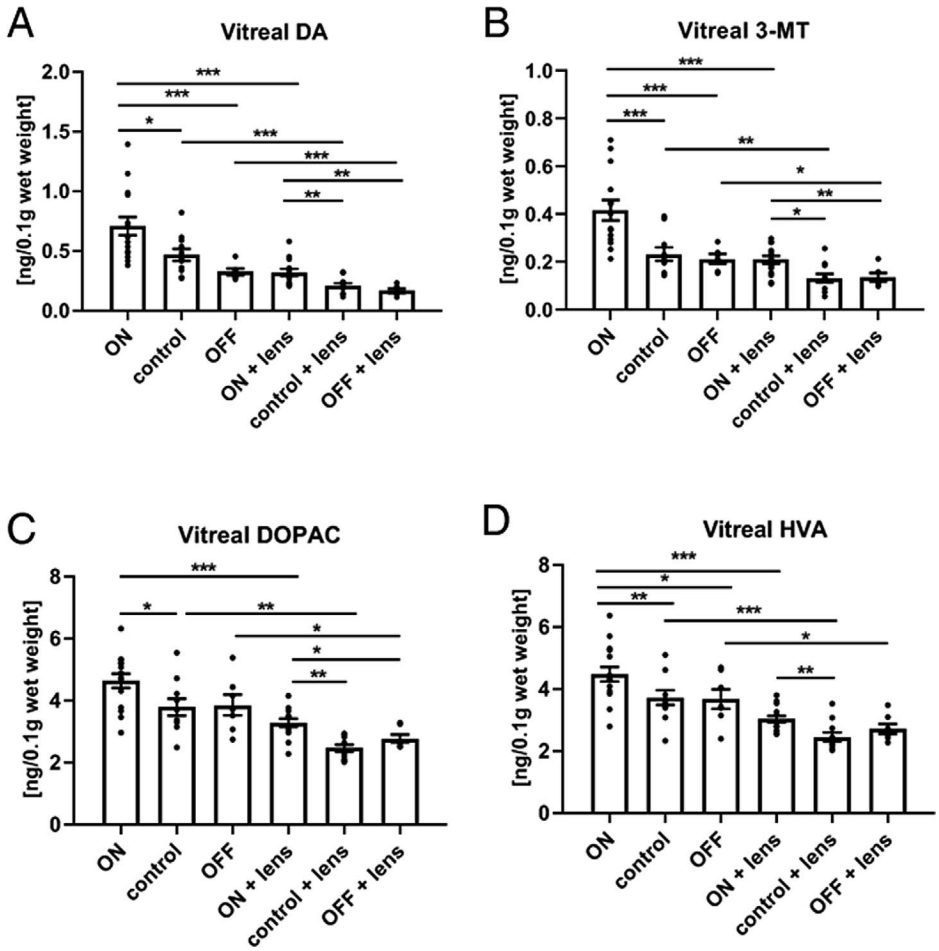

Figure 5. (**A**) Vitreal DA and its metabolites (**B**) 3-MT, (**C**) DOPAC, (**D**) HVA after 7 days of exposure to dynamic ON stimulation, room light ("control"), and OFF stimulation. Right eyes wore −7-D lenses; left eyes remained untreated. As expected, negative lenses induced a significant drop of vitreal DA (**A**) and its metabolites (**B–D**). ON stimulation generated significantly higher vitreal DA and 3-MT in both lens-treated and control eyes compared with room light, but also compared with OFF stimulation. Furthermore, ON stimulation generated significantly higher vitreal DOPAC and HVA levels in both lens-treated and control eyes, compared with room light. Significance levels as in Figure 2.

increased retinal DA in both lens-treated and control eyes compared with control illumination and OFF stimulation (Fig. 6A). No significant changes were observed for 3-MT, DOPAC, and HVA (Figs. 6B–D). Raw data are shown in Table 4.

Discussion

We have collected evidence supporting the hypothesis that the relative difference in the temporal integration of ON- and OFF-channel activity signals predictable bidirectional changes in choroidal thickness, dopamine release, and, perhaps, future eye growth. In detail, we found the following:

1. Exposing young human subjects to dynamic ON stimuli, presented on large monitor at 2.3 m distance, induced small but significant increases in choroidal thickness after 60 minutes. Exposure to a dynamic OFF stimulus for the same duration caused choroidal thinning.
2. Chickens exposed to a similar dynamic stimulus in a "stimulation arena" for 3 hours also developed thicker choroids with the dynamic ON stimuli than with the dynamic OFF stimuli in the right eye. The lack of significant effect in left eyes might have been because left eyes were always measured after right eyes. Vitreal DA, 3-MT, and HVA levels were elevated in right eyes

Table 3. Vitreal DA and Its Metabolites (3-MT, HVA, and DOPAC) (Mean ± SEM, ng/0.1 g Wet Weight) After 7 Days of Stimulation With ON or OFF Stimuli, or Room Light ("Control")

	ON	Control	OFF	ON+Lens	Control+Lens	OFF+Lens
Vitreal DA	0.71 ± 0.08	0.47 ± 0.05	0.33 ± 0.03	0.32 ± 0.03	0.21 ± 0.02	0.17 ± 0.02
Vitreal 3-MT	0.42 ± 0.04	0.23 ± 0.03	0.21 ± 0.02	0.21 ± 0.02	0.13 ± 0.02	0.14 ± 0.02
Vitreal HVA	4.48 ± 0.24	3.37 ± 0.23	3.67 ± 0.31	3.03 ± 0.10	2.46 ± 0.14	2.71 ± 0.16
Vitreal DOPAC	4.64 ± 0.23	3.79 ± 0.28	3.86 ± 0.34	3.22 ± 0.13	2.46 ± 0.11	2.77 ± 0.13

Data are also graphically displayed in Figure 5.

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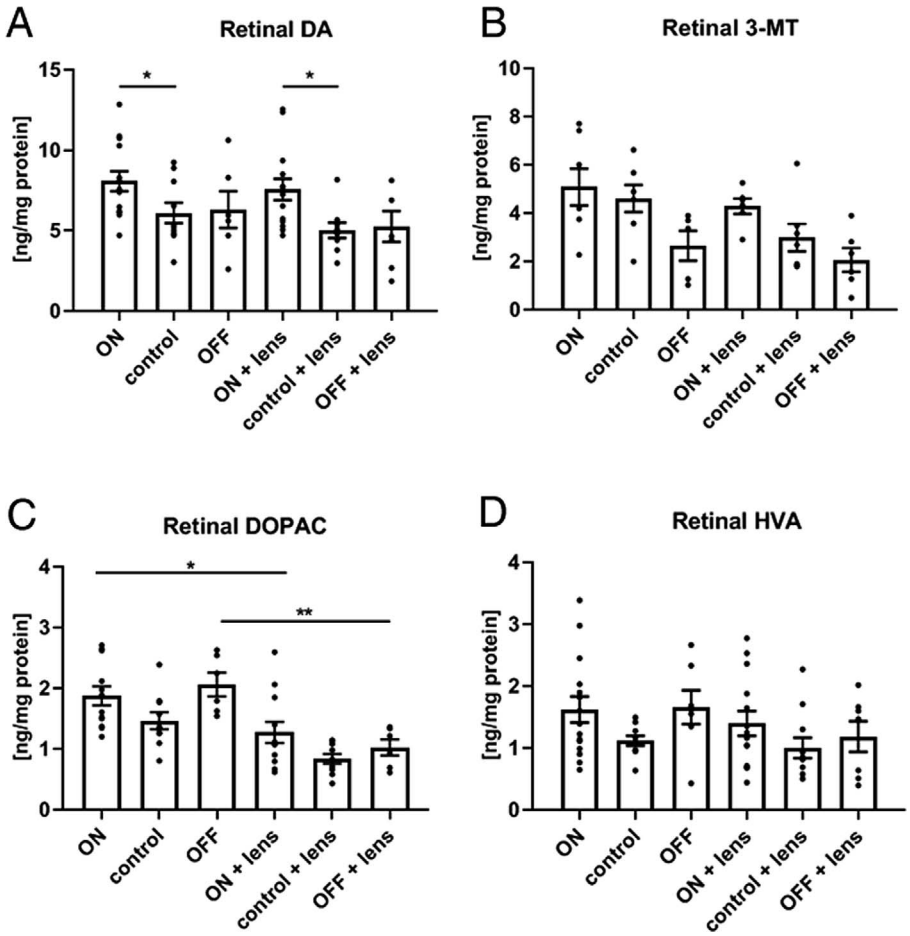

Figure 6. (**A**) Retinal DA, (**B**) 3-MT, (**C**) DOPAC, and (**D**) HVA content after 7 days of treatment with the three different light regimens. Negative lenses (−7 D) lowered retinal DOPAC after ON and OFF stimulation (**C**). The other three retinal compounds were not affected by the lenses. (**A**) ON stimulation increased retinal DA in both treated and control eyes, compared with room light ("control"). For retinal 3-MT (**B**) and HVA (**D**), no significant differences were found among the three light regimens. Significance levels as in Figure 4.

after exposure to ON stimuli, compared with the OFF stimuli.

3. Chickens with a negative lens in front of their right eyes developed more myopia when they were exposed to ON or OFF stimulation for 7 days, compared with when they were exposed to constant light of similar illuminance. The increase in myopia was mirrored by longer axial lengths and deeper vitreous chamber depths and was observed in both the lens-treated and the fellow eyes, which had unobstructed vision. After 7 days, eyes with lenses that were exposed to ON stimulation displayed elevated levels of vitreal DA and 3-MT, compared with those stimulated with the OFF stimuli, although there was no difference in vitreal HVA.

In conclusion, short-term artificial dynamic ON or OFF stimuli have similar effects on choroid thickness in humans and chickens. Furthermore, the direction of changes in choroidal thickness matched previous findings with text of different contrast polarities, assumed to stimulate either ON or OFF pathways.[21] Our study has also shown that ON stimulation increases retinal DA release. Because DA has been implemented in the visual control of axial eye growth and myopia, the changes in DA may be involved in the changes in choroidal thickness. Nickla et al.[30] proposed that "release of dopamine from the retina in response to visual stimuli triggers the release of NO from either the retina or choroid, leading to choroidal thickening and ocular growth inhibition." However, it also should be kept in mind that choroidal and scleral growth mechanisms can be partially dissociated with flickered spectral stimuli (i.e., Refs. 31–36). Also, Nickla and Totonelly[37] found

Table 4. Concentration of Retinal DA and Its Metabolites (3-MT, HVA, and DOPAC) (Mean ± SEM, ng/mg Protein) After 7 Days of ON and OFF Treatment

	ON	Control	OFF	ON+Lens	Control+Lens	OFF+Lens
Retinal DA	8.08 ± 0.62	6.09 ± 0.64	6.30 ± 1.15	7.55 ± 0.66	5.03 ± 0.48	5.26 ± 0.96
Retinal 3-MT	5.08 ± 0.76	4.60 ± 0.56	2.65 ± 0.62	4.50 ± 0.35	2.98 ± 0.57	2.06 ± 0.50
Retinal HVA	1.62 ± 0.21	1.12 ± 0.08	1.66 ± 0.27	1.40 ± 0.20	1.00 ± 0.17	1.18 ± 0.25
Retinal DOPAC	1.88 ± 0.16	1.46 ± 0.14	2.06 ± 0.19	1.27 ± 0.17	0.83 ± 0.08	1.02 ± 0.13

"Control" refers to room light. Data correspond to Figure 6.

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that the relationship between choroidal thickening and eye growth inhibition may be disrupted when eye growth was experimentally altered.

Magnitudes of Changes in Choroidal Thickness

Aleman et al.[21] found that reading white text that stimulates the ON pathway increases choroidal thickness by 10 μm after 60 minutes, whereas reading black text decreased choroidal thickness by 16 μm. The magnitudes of these effects are similar to those in studies with lenses in adults and children, as well as after application of high-dose atropine (reviewed in Aleman et al.[21]). The dynamic ON or OFF stimulus was apparently less effective than reading text with different contrast polarities. It should be kept in mind that the letter height of the text was 11.8 arcmin, whereas the individual squares in the dynamic ON or OFF stimuli were approximately 49 arcmin in diameter, suggesting that ON and OFF cells with different receptive field sizes were stimulated in both cases. Perhaps a dynamic stimulus with smaller squares would have been more effective. Another point is that receptive field sizes increase from the fovea to the periphery, and it is not clear which retinal areas are most stimulated with our dynamic ON/OFF stimuli. It is encouraging that also chickens with lower visual acuity than humans (approximately 7 cyc/deg)[38] respond to the dynamic ON/OFF stimuli. Because chickens had shorter viewing distances in their "stimulation arena," the squared fields in the stimuli had also much larger angular subtense. Furthermore, it could be that different sizes of the squares, as well as different frequencies of the dynamic ON/OFF stimuli would have been more effective. Chichilnisky and Kalmar[39] measured response properties of ON and OFF cells in the rhesus monkey retina in vitro. They found that receptive field sizes of ON cells were 20% larger than those of OFF cells and that ON cells had faster response kinetics than OFF cells, with a 10% to 20% shorter time to peak, trough, and zero crossing in the biphasic temporal impulse responses. In monkeys, the mean receptive field diameters of ON cells was 100 (±3.2) μm and for OFF cells was 87 (±4.5) μm. Converting linear distances on the retina into angular subtense using the rhesus monkey eye data,[40] an axial length of 17 mm and a posterior nodal distance of approximately 11 mm, leads to a visual angle of 48 arcmin, similar to the diameters of the stimulating squared fields used in the current study in experiment 1 (49 arcmin).

Increased Dopamine Release With ON Stimulation Versus OFF Stimulation Even With Equiluminance

In the vertebrate retina, dopamine is produced mainly by a subpopulation of amacrine cells that are immunoreactive to tyrosine hydroxylase.[41] It is well known that dopamine release from the retina drops when myopia is induced by lenses or diffusers.[29] On the other hand, temporally modulated light (flickering light) triggers dopamine release.[42,43] Schwahn and Schaeffel[44] found that chickens with occluders or lenses developed less myopia under flickering light of different duration (either 12 or 3 hours) and duty cycles (4%, 12%, 25%, 50%, and 75%) and stated that "possibly the loss of high spatial contrast during lens and occluder treatment might cause a decrease in the activity of retinal cells leading to the reported drop in the rate of dopamine release." In contrast, in guinea pigs it was described that flickering light for 12 hours and duty cycle of 50% triggered more myopia development and longer eyes, apparently paradoxically coupled with an increase in dopamine release.[45] However, flicker frequency is an important variable and it was very different in both studies: 6 and 12 Hz in the chicken experiments and 0.5 Hz in the guinea pig study.

The first demonstration that flicker light can interfere with the emmetropization process was presented at the 1987 Neuroscience meeting.[46] Two years later, Cremieux and colleagues[47] found that cats became more myopic when they were reared under low-frequency strobe flicker light of 2 Hz. Also mice became more myopic under 2 Hz flicker light.[48] In chickens, it was found that 1, 2, and 4 Hz flicker light induced more myopic refractions with deeper anterior and vitreous chambers in the eyes.[49] Rucker and colleagues[35,36] found that the blue light can suppress the effects of flicker light on emmetropization in chickens. Furthermore, they found that dynamic chromatic noise stimulation promotes myopia in chickens, which let the authors conclude that "emmetropization is further disrupted when randomized color information is combined with randomized luminance information" (Taylor CP, Rucker FJ. *IOVS* 2018;59:ARVO E-Abstract 749). In summary, these studies make clear that artificial dynamic ON OFF stimuli as used in the current study may also interfere with emmetropization, just based on their temporal frequency components.

Flicker light was also found to stimulate the release of retinal nitric oxide (NO) and dopamine compared with steady light stimulation.[50] The effects of NO on retinal ON bipolar cells and ON pathway has been described in a number of previous studies.[51,52] Zhang et al.[53] found that dopaminergic amacrine cells have synapses with ON cone bipolar cells. The association between dopamine levels and ON/OFF pathways has been reported in experiments with knockout mice as well. Mice with mutations in the Nyx gen (leading to a nonfunctional ON channel) had lower DA and more myopic refractions but also developed more deprivation myopia.[13] Mice with functional knockout of the mGluR6 receptor developed more myopia than wild-type mice and also had significantly lower retinal dopamine levels.[10] On the other hand, Vsx1$^{-/-}$ mice with a nonfunctional OFF pathway had similar refractions as their wild-type controls and showed no differences in dopamine levels.[11] In the current study, we found that vitreal dopamine levels were higher after dynamic ON stimulation compared with continuous light, or dynamic OFF stimulation. These effects added to the ones of the negative lenses.

Effects of Illuminances During Dynamic ON and OFF Stimulation

Several studies have shown that insufficient time spent outdoors represents a risk factor for myopia development in children (reviewed in French et al.[54]). Also in animal models it was found that high illuminances have an inhibitory effect on myopia development (chickens[55,56]; tree shrews, Siegwart JT, et al. *IOVS* 2012;53:ARVO E-abstract 3457; rhesus monkeys[57]). In 2015, Read and colleagues[58] found that myopic children spent less time in bright light above 1000 lux compared with nonmyopic children. On the other hand, there is also evidence for a role of dim light in myopia development. In a recent study, Landis et al.[59] found that myopic children spent less time in both scotopic and outdoor light, compared with nonmyopic children. They concluded that "there is a potentially protective effect of both dim and bright light exposure in myopia development."[59] Dopamine release is typically correlated with illuminance.[12] However, it is also related to the spatiotemporal structure of the light stimulus, as can be seen in the current study, at least under photopic conditions (approximately 700 lux). Rods make synapses to only ON bipolar but not to OFF bipolar cells, raising the possibility that the observed effects may vary with illuminance. Park et al.[60] have shown that mice with no functional rods no longer become myopic when they are treated with diffusers.

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Time Courses

The current experiments have shown that dopamine release is similarly changed after 7 days of stimulation with the ON or OFF stimuli and after 3 hours. Signals controlling DA production and release build up slowly. In contrast, changes in choroidal thickness were apparent after 3 hours (Fig. 3 versus Fig. 5) and had largely disappeared after 7 days; however, this was not unexpected. Already Wallman et al.[15] proposed that choroidal thickness changes represent a "third mechanism of focusing the eye, intermediate in speed between (fast) accommodation and (slow) eye growth."

It would be important to determine the time courses in more detail to find out how long subjects should be exposed to the novel stimuli to inhibit myopia progression. It was found that choroidal thickening induced by +5 D positive lenses for 30 minutes recovered to baseline after 10 minutes without lenses.[61]

Why Is Myopia Not Inhibited When Dopamine Release in Chicks Is Increased?

Unexpectedly, under both dynamic ON and OFF stimulation, more myopia was induced by the lenses than under continuous illumination with comparable brightness. There are at least three possible explanations:

1. Flickering light, as provided by the dynamic stimuli, may activate retinal circuits that make the retina more sensitive to defocus. However, the argument is rendered invalid because fellow eyes without lenses became also slightly more myopic. It has been shown that whole-field sawtooth-shaped temporal luminance flicker of approximately 1 Hz also induces myopia in chickens.[62] An alternative explanation could therefore be that our stimuli (also at approximately 1 Hz) had stimulated local retinal luminance detectors to induce myopia independently of the spatial features of the stimuli.
2. The closed loop system of emmetropization, triggered by lens defocus, could work against the inhibitory growth signal produced by the dynamic ON stimuli. An experiment under open loop conditions, that is, with diffusers, might provide a clearer answer to this question (although the diffuser should not be too frosted so that the dynamic ON/OFF stimuli can still be seen). In general, the interaction between growth signals derived from ON/OFF-channel activity and from defocus is not well understood. The current data with the negative lenses may indicate that the defocus signal could partially overwrite the signal from ON or OFF stimulation. Only a few available studies touch this topic: Crewther and Crewther[8] found that ON stimulation reduced the effects of negative lenses on eye growth whereas OFF stimulation reduced the effect of positive lenses. Schwahn et al.[44] found that 6- to 12-Hz flicker light was most effective in inhibiting myopia when duty cycles were short, whereas inhibition of hyperopia did not vary with duty cycle, indicating that different temporal processing modes are involved. Recently, Khanal et al.[63] found signal components in the global flash multifocal ERG were specific for myopic defocus and could even selectively be enhanced by atropine, which normally inhibits myopia. Their data show that there are components in the ERG that selective for the sign of defocus. However, the direct link to ON- or OFF-channel activity was not established.
3. It could be that prolonged stimulation of the ON or OFF pathways causes adaptation and a severe loss of sensitivity in these pathways, so that they no longer contribute much. Autoregulation of the blood vessels serves to maintain a constant blood flow and may adapt to prolonged flicker stimulation resulting in the loss of the choroidal response. However, this idea does not explain why DA was still elevated after 7 days.

Possible Implications of the Study

Our study shows that artificial dynamic stimuli can be developed to modify choroidal thickness in either direction and to stimulate dopamine release from the retina, without increasing illuminance. These stimuli do not dependent on their exact focus on the retina (which is hard to control with different viewing distances and accommodation) and can probably be further optimized. In the long-term, it might be possible to develop new dynamic stimuli on a computer screen to inhibit axial eye growth and myopia.

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