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The Wavelength Composition and Temporal Modulation of Ambient Lighting Strongly Affect Refractive Development in Young Tree Shrews

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Abstract

Shortly after birth, the eyes of most animals (including humans) are hyperopic because the short axial length places the retina in front of the focal plane. During postnatal development, an emmetropization mechanism uses cues related to refractive error to modulate the growth of the eye, moving the retina toward the focal plane. One possible cue may be longitudinal chromatic aberration (LCA), to signal if eyes are getting too long (long [red] wavelengths in better focus than short [blue]) or too short (short wavelengths in better focus). It could be difficult for the short-wavelength sensitive (SWS, “blue”) cones, which are scarce and widely spaced across the retina, to detect and signal defocus of short wavelengths. We hypothesized that the SWS cone retinal pathway could instead utilize temporal (flicker) information. We thus tested if exposure solely to long-wavelength light would cause developing eyes to slow their axial growth and remain refractively hyperopic, and if flickering short-wavelength light would cause eyes to accelerate their axial growth and become myopic. Four groups of infant northern tree shrews (*Tupaia glis belangeri*, dichromatic mammals closely related to primates) began 13 days of wavelength treatment starting at 11 days of visual experience (DVE). Ambient lighting was provided by an array of either long-wavelength (red, 626 ± 10 nm) or short-wavelength (blue, 464 ± 10 nm) light-emitting diodes placed atop the cage. The lights were either steady, or flickering in a pseudo-random step pattern. The approximate mean illuminance (in human lux) on the cage floor was red (steady, 527 lux; flickering, 329 lux), and blue (steady, 601 lux; flickering, 252 lux). Refractive state and ocular component dimensions were measured and compared with a group of age-matched normal animals ($n=15$ for refraction (first and last days); 7 for ocular components) raised in broad spectrum white fluorescent colony lighting (100–300 lux).

During the 13 day period, the refraction of the normal animals decreased from (mean $\pm$ SEM) 5.8 ± 0.7 diopters (D) to 1.5 ± 0.2 D as their vitreous chamber depth increased from 2.77 ± 0.01 mm to 2.80 ± 0.03 mm. Animals exposed to red light (both steady and flickering) remained hyperopic throughout the treatment period so that the eyes at the end of wavelength treatment were

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significantly hyperopic (7.0 ± 0.7 D, steady; 4.7 ± 0.8 D, flickering) compared with the normal animals ($p < 0.01$). The vitreous chamber of the steady red group (2.65 ± 0.03 mm) was significantly shorter than normal ($p < 0.01$). On average, steady blue light had little effect; the refractions paralleled the normal refractive decrease. In contrast, animals housed in flickering blue light increased the rate of refractive decrease so that the eyes became significantly myopic (-2.9 ± 1.3 D) compared with the normal eyes and had longer vitreous chambers (2.93 ± 0.04 mm). Upon return to colony lighting, refractions in all groups gradually returned toward emmetropia. These data are consistent both with the hypothesis that LCA can be an important visual cue for postnatal refractive development, and that short-wavelength temporal flicker provides an important cue for assessing and signaling defocus.

Graphical abstract

Keywords

Myopia; Hyperopia; Vitreous Chamber; Longitudinal Chromatic Aberration; Optical Blur; Retinal signaling

1. Introduction

At birth, mammalian eyes, including humans, are typically hyperopic (eye shorter than the focal length) (Bradley et al., 1999; Cook and Glasscock, 1951; Mayer et al., 2001; Mutti et al., 2005; Norton and McBrien, 1992; Pickett-Seltner et al., 1988). An emmetropization feedback mechanism uses visual cues to modulate the axial growth of the eye to reduce this initial refractive error and achieve in-focus images (*emmetropia*) (Norton, 1999; Schaeffel and Feldkaemper, 2015; Schaeffel and Howland, 1988; Smith et al., 2014; Wallman and Winawer, 2004; Wildsoet, 1997). The eye can detect the sign of the defocus (hyperopia: eye too short, myopia: eye too long) and adjust its growth to achieve and maintain a match of the axial length to the focal plane (Norton et al., 2006b; Park et al., 2003; Wallman and Winawer, 2004; Zhu et al., 2005). (However see (Schaeffel and Wildsoet, 2013)). Further evidence that the neural retina by itself can detect the sign of optical blur comes from a study in chick retina that found a class of amacrine cells which responds differentially to the

sign of defocus (Fischer et al., 1999). However, the specific visual cues that the retina uses to detect the sign of optical blur remain unclear.

Many researchers have suggested that the eye could use *longitudinal chromatic aberration* (LCA), the fact that long wavelengths are focused farther back in the eye than shorter wavelengths, to help guide emmetropization (Kroger and Wagner, 1996; Rohrer et al., 1992; Rucker and Kruger, 2006; Rucker and Wallman, 2008, 2009; Schaeffel and Howland, 1991; Seidemann and Schaeffel, 2002; Wildsoet et al., 1993). As calculated using the Zemax software and previously described data on surface curvatures (Norton and McBrien, 1992) and using refractive indices/dispersion data from human eyes (Atchison and Smith, 2005) (Dr. Alexander Meadway, personal communication) tree shrews have approximately 2.77 diopters of LCA between 428 and 555 nm, which should be sufficient to produce strong optical cues for relative defocus for different wavelengths in the biologically relevant range.

However, the short-wavelength sensitive (SWS “blue”) cones are only sparsely distributed on the retina (Muller and Peichl, 1989; Roorda and Williams, 1999). This would seem to make it impossible for the retina to tell if a blue image is in focus *relative to longer wavelengths* with any degree of precision because the blue cones are not packed densely enough to provide the needed spatial resolution through their retinal connections. We note that while humans accommodate to dynamic targets most accurately under broad-band white light, they can still typically achieve good static focus in blue light (Aggarwala et al., 1995; Seidemann and Schaeffel, 2002). This might suggest that the blue cones can achieve high spatial acuity, but we argue that this is because the visual system is using the sensitivity of longer-wavelength cones to blue light to optimize focus, not because blue cones on their own have high spatial acuity. The low density of blue cones should make it impossible for the visual system to determine the *relative* sharpness of short and long wavelength images with any precision.

We hypothesized that the retina also uses the temporal abruptness of changes in light at short wavelengths as a proxy for spatial image focus: when an image is in sharp focus, a photoreceptor will experience rapid changes in illuminance as the animal moves around in a patterned environment and produce sharp transients in bipolar and amacrine cell membrane potentials. It has previously been found that flickering light can reduce the myopia produced by form deprivation (Wallman, 1990), and that this effect may be mediated by flickering light restoring dopamine metabolism (Rohrer et al., 1995). Experiments in chicks have suggested that the motion of an image sweeping across the retinal surface is important for emmetropization (Bitzer and Schaeffel, 2006). If present, this temporal mechanism should be more important in detecting defocus for the short (blue) wavelengths, i.e., blue flicker should be an especially important cue. We also note that it has been demonstrated that some rabbit retinal ganglion cells can distinguish changes in contrast from changes in blur in white light; importantly, this is coded in the temporal pattern of action potentials (Risner et al., 2010).

There have been a significant number of studies examining the effects of temporally and chromatically modulated ambient light on emmetropization (Britton et al., 2013; Crewther and Crewther, 2002; Crewther et al., 2006; Kee et al., 2007; Liu et al., 2014; Liu et al., 2011;

Rohrer et al., 1995; Rucker et al., 2015; Rucker, 2013; Rucker and Wallman, 2012; Schwahn and Schaeffel, 1997; Yu et al., 2011), however the results have differed across labs, making them difficult to interpret. Some of this may be due to species differences between chicks, rodents, and primates/near primates, complicated by interactions between the specific wavelengths employed, the number of cones types, and their absorption peaks. We also suggest that these results may seem confusing because we don't fully understand how the retina might integrate both temporal and chromatic signals to infer the sign of focus.

Potentially the emmetropization mechanism could respond to the differential focus of short and long wavelengths in two fundamentally different ways. One possibility is that it might use LCA as a *target*, matching the focal plane of the dominant wavelength by growing longer when the ambient light is of long wavelength and growing less when the dominant wavelength is short. This is also generally what happens to accommodation under monochromatic light (Seidemann and Schaeffel, 2002), and some studies have indeed suggested that emmetropization can optimize focus to the dominant wavelength (Rohrer et al., 1992; Seidemann and Schaeffel, 2002). A second possibility is that emmetropization could use LCA as a *cue*: if the long wavelengths are in better focus than the short wavelengths, this could signal the eye that it is long enough/too long and should stop growing to achieve or maintain emmetropia, and vice-versa if the shorter wavelengths are in better relative focus. Some previous studies in chicks have found evidence for this (Rucker, 2013; Rucker and Wallman, 2008, 2009, 2012), although Rucker and Wallman 2009 also found that eyes in blue light could be more hyperopic than eyes in red light, suggesting that in this case LCA was used as a target. In support of the notion that eyes can use LCA as a cue, a recent study in infant monkeys showed that wearing filters that only transmitted long wavelengths produced hyperopia, not the myopia that would be expected if the eye was using the focal plane of the longer wavelength light as a target (Smith et al., 2015). It also needs to be pointed out that even if LCA is in fact used as a visual cue for emmetropization, it might not be the only visual cue (Wildsoet et al., 1993)

We hypothesized that narrow-band red light should signal that the eye is becoming too long and inhibit eye growth, and that flickering blue light should signal that the eye is too short and stimulate eye growth. We tested this hypothesis on infant tree shrews (a diurnal mammal closely related to primates) by raising them in red or blue ambient light that was either steady or flickering. Tree shrews are dichromats; the retina contains long-wavelength sensitive (LWS "red", peak absorption at 555 ± 6 nm) and short-wavelength sensitive (SWS "blue", peak absorption 428 ± 15 nm) cones (Petry and Harosi, 1990). We began treatment at an age (11 days after eye opening) when the eyes are elongating rapidly and refractions are normally decreasing rapidly from hyperopia towards emmetropia. We report here the results of exposing these animals to steady narrow-band red light, flickering red light, steady narrow-band blue light, and flickering blue light, on the maturation of refractive state and size of the vitreous chamber of the eye.

2. Materials and Methods

2.1 Experimental Groups

As in previous studies from this laboratory (Guo et al., 2013; He et al., 2014; McBrien and Norton, 1992) the infant tree shrews (*Tupaia glis belangeri*) used in this study were raised until weaning by their mothers on a 14 hour light/10 hour dark cycle in our breeding colony. Fluorescent colony lighting (F34CW RS WM ECO) provided illuminance of 100-300 lux on the floor of the cages. All procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham (UAB). Four experimental groups were balanced to include both males and females and avoided multiple pups from the same parents. A group of normal animals was exposed to only normal colony lighting for 14 hours a day throughout development. Seven of these had daily refractive measures for comparison with the wavelength groups. Eight additional animals, derived from previous experiments, had refractive measures age-matched to the start and end of wavelength treatment. (Norton et al., 2006a; Siegwart and Norton, 2010, 2013).

Tree shrews are born with their eyes closed. The first day both eyes are open, which occurs about three weeks after birth, is taken to be the first day of visual experience (DVE). Wavelength treatments in all groups began at 11 ± 1 DVE and continued for 13 days. The animals then were returned to fluorescent colony lighting.

During wavelength treatment, the long-wavelength (red, 628 ± 10 nm) or short-wavelength (blue, 464 ± 10 nm) LED lights (but never both wavelengths at the same time) were illuminated for 14 hours per day (10 hours of dark). These lights could be either flickering or steady. The four wavelength-treated groups were: steady red (n=5), red flicker (n=5), steady blue (n=5), and blue flicker (n=7). Mean illuminance at the cage floor was measured in human lux with a LX1330B digital illuminance meter (Hisgadget, Inc.). The mean luminance was red (steady, 527 lux; flickering, 329 lux), and blue (steady, 601 lux; flickering 252 lux). Based on the absorption spectra of the SWS (peak: 428 ± 15 nm) and LWS cones (peak: 555 ± 6 nm), the red light only stimulated the LWS cones; the blue light strongly stimulated the SWS cones as well as the LWS cones (Petry and Harosi, 1990), rods (peak: approximately 496 nm (Petry and Harosi, 1990)) and intrinsically photosensitive retinal ganglion cells (ipRGCs, peak not reported in tree shrew, but probably near primate, 482 nm (Dacey et al., 2005)). Multiplying the blue wavelength profile with nomograms based on the absorption spectra of tree shrew cones (Petry and Harosi, 1990) indicated that the blue LEDs produced a “cone catch” in the LWS cones that was approximately 51% of the SWS cones after accounting for pre-retinal absorption.

2.2 Visual Stimulators

The red LEDs were 16 type 40200R truck tail lights (ASL American Superlite, San Fernando, CA) arranged in a 4×4 array. The blue LEDs were NFLS-RGBX2 high power RGB flexible light strips (Superbright, St. Louis, Missouri) arranged in three rows, each strip 50 cm long (only the blue LEDs of these RGB light strips were used). The red and blue LEDs were affixed in a uniform array to a white-painted 60 cm by 60 cm piece of plywood that

was then placed on top of the cage, which was a cube 60 cm on a side. A Gray PVC tube with one open end served as a nest that the animals could enter and leave at will. A white resting board 28 cm above the cage floor was often used by the animals. Light intensity was approximately 40 percent greater on the shelf.

The red and blue LEDs were driven by a custom-designed power circuit controlled by Arduino Uno microcontrollers (Niwot, CO, USA). This produced a temporal pattern of light (Fig. 1A) designed to approximate the temporal dynamics of a point on the retina as an animal moves around and as the eyes exhibit small amounts of tremor. The large excursions occurred pseudo-randomly from 180 to 250 msec apart and randomly jumped to a value between 4% and 96% of maximum illuminance (uniformly distributed across this range and symmetrical around the mean luminance). The higher frequency flicker (not shown to scale on the diagram) was superimposed on this pattern, and consisted of random jumps of $\pm 2\%$ of maximum illuminance around the current level, every 10 milliseconds. The abruptness of the jumps, and the random intervals between the jumps, produced a relatively broad frequency distribution, without a single dominant temporal frequency (Fig. 1B). The amplitude decreased with frequency similarly to natural scenes (vanHateren and vanderSchaaf, 1996). This temporal configuration seemed preferable to sine-wave flicker because we did not know what temporal frequencies might be important, or even if the process is nonlinear (in which case sine waves of any frequency might be ineffective).

2.3 Pedestal Installation

As an aid to painlessly aligning the animals for the refractive and axial component measures, a dental acrylic pedestal was installed on the skull of all animals at 10 ± 1 DVE following procedures described previously (Siegwart and Norton, 1994). The animals were removed from their home cage where they had been in a dark whelping box and anesthetized (approximately 100 mg/kg ketamine, 7 mg/kg xylazine supplemented with 0.5 – 2.0% isoflurane as needed). After recovery from anesthesia, the animals were weaned and housed singly, or in pairs, in colony lighting. One day later, treatment was initiated by placing the LED array on top of the cage, activating the LED controller and turning off the fluorescent colony lights. The on/off cycle of the LEDs was separately controlled on the same schedule as the colony lighting.

2.4 Refractive and Axial Measures

The refractive measures were made with a Nidek ARK-700A infrared autorefractor (Marco Ophthalmic, Jacksonville, FL) (Norton et al., 2003). Non-cycloplegic measures were made because atropine may interfere with refractive development (McBrien et al., 2013; McKanna and Casagrande, 1981). However, previous studies have shown that non-cycloplegic measures in tree shrews provide a valid estimate of a wide range of refractive states (Norton et al., 2006b) All refractive values were corrected for the small eye artifact (Glickstein and Millodot, 1970) previously shown to be approximately +4 diopters (D) in tree shrews (Norton et al., 2006b).

Refractive state was measured daily during the 13 days of wavelength treatment in awake animals shortly after 9 AM, approximately 30 minutes after the LED lights turned on.

Wavelength-treated animals were kept in darkness while transported between their home cage and the measurement room. Measurements were initially made in darkness using the infrared autorefractor. When later animals were undergoing the wavelength treatments, the measurement room was illuminated with red or blue LEDs so that the animals did not have a period of visual stimulation that was different from the test conditions.

After wavelength treatment ended, the animals were returned to colony lighting. Frequent refractive measures were continued for 36 additional days to assess if animals that had developed refractive error “recovered” to age-normal refractive states once they were exposed to standard colony lighting.

Axial component dimensions were measured in all animals in the wavelength groups at the start (11 DVE) and end (24 DVE) of wavelength treatment. The axial component dimensions of the seven normal animals that had daily refractive measures were also measured at these two time points. Measures were made in awake animals with a LenStar LS-900 optical biometer (Haag-Streit USA, Mason, OH). The LenStar provides and stores waveforms with peaks that correspond to the front and back of the cornea, front and back of the lens, front and back of the retina and anterior surface of the sclera. Off-line, the analysis cursors (International software version) were moved to each pair of peaks to provide measures of corneal thickness, anterior chamber depth, lens thickness, vitreous chamber depth, retinal and choroidal thickness.

2.5 Statistical Analysis

Refractive and axial component measures were summarized in Excel spreadsheets. Data on refractions were plotted as a function of time. Non-parametric statistics (Kruskal-Wallis with Dunn’s post-hoc test) were run under SPSS to examine (1) differences in initial refraction (day 1) between the wavelength groups and 15 age-matched normal animals, (2) differences in refraction at the end of wavelength treatments (day 14) for the same groups and (3) day 1 and day 14 LenStar measures of ocular components (including vitreous chamber depth) for the wavelength groups and seven of the 15 normal animals on which LenStar measures were available. Alpha was set at 0.05.

3. Results

3.1 Refraction and Axial Dimensions

The refractive and ocular component dimensions of the right and left eye in each animal were very similar in both the normal and wavelength-treated animals. We therefore present the data as the average of the right and left eyes for each animal. At 11 DVE, before the start of wavelength treatments, there were no significant differences between the five groups in refraction or any ocular component (Kruskal-Wallis, $p > 0.05$).

Fig. 2A compares the refractive development of infant tree shrews in response to steady red light with normal animals exposed to colony lighting. Tree shrews developing in normal lighting (black circles) are initially hyperopic; the eyes (principally the vitreous chamber) then grow rapidly so that at 24 DVE they are only one to two diopters hyperopic (mean $\pm$ SEM, 1.5 ± 0.2 D). The small error bars for the normal group throughout this rapid

emmetropization period indicate the consistency of this process in colony lighting. In contrast to the normal pattern, the eyes of all five tree shrews exposed to steady red ambient lighting remained, or became, strongly hyperopic. At the end of treatment (Fig. 3A), the group refraction (mean $\pm$ SEM, 7.0 ± 0.7 D) was significantly higher than that of the normal animals at that age ($p < 0.005$). As shown in Fig. 3B, the depth of their vitreous chamber was the shortest of the four groups (2.65 ± 0.03 mm). This was significantly shorter than in normal colony-reared animals (2.80 ± 0.03 mm) ($p < 0.043$), indicating that the refractive hyperopia occurred because the eye was short. When exposed to regular fluorescent lighting, the hyperopia in all steady red-treated animals rapidly dissipated as the refractions decreased toward age appropriate levels, demonstrating that the red light exposure had not damaged the emmetropization mechanism.

Fig. 2B illustrates the results for the animals exposed to flickering red light. The refractive response was similar to that seen with steady red light. The refractive state at the end of treatment (Fig. 3A) was 4.7 ± 0.8 D, a lower hyperopia than in the steady red group but approximately 3.1 D above the normal group, a significant hyperopia ($p < 0.032$). In keeping with the slightly lower refraction, the vitreous chamber in this group (Fig. 3B, 2.75 ± 0.04 mm) was slightly deeper than in the group exposed to steady red light.

Fig. 2C shows that exposure to steady blue light did not have a strong overall effect on emmetropization: the time-course of the group average refractions paralleled normal refractions during most of the treatment period (although the variability was greater than in colony-reared eyes). The refractive state of one animal at the end of treatment was slightly hyperopic compared with normals (1.9 vs. 1.5 D). However, the group mean (Fig. 3A, 0.2 ± 1.2 D) was not significantly different from the refractions in the normal animals ($p < 0.41$). Vitreous chamber depth (Fig. 3B, 2.84 ± 0.04 mm) of the steady blue group was significantly longer than the vitreous chamber of the animals exposed to steady red light ($p < 0.01$) but was not significantly longer than the normal animals ($p < 0.442$).

Fig. 2D shows that the flickering blue light was myopiagenic. All animals in this group became more myopic than controls by the end of treatment, of which two were strongly myopic (-7.8 and -7.1 D). The group average (-2.9 ± 1.3 D) was significantly myopic compared with the animals raised in colony lighting ($p < 0.007$). The end-of-treatment refractive state with blue flicker (Fig. 2D) was more variable than for either group exposed to red light, but the refractive state of every blue-flicker treated animal at the end of treatment was myopic compared to all 15 of the normal animals. The vitreous chamber depth of this group (Fig. 3B, 2.93 ± 0.04 mm) was the largest of the four wavelength groups. It was significantly longer than vitreous chamber in the steady red ($p < 0.000$) and red flicker ($p < 0.012$) groups but not the normal animals ($p < 0.052$).

The complementary pattern of the refractive state and vitreous chamber depth in Fig. 3 shows that the refractive results were due primarily to differences in the amount of eye growth during the wavelength exposure period. Fig. 4 shows the association between the change in vitreous chamber depth and the change in refraction during the 13 day treatment period. A large increase in vitreous chamber depth in the blue flicker animals was associated with the myopic change in refraction, whereas the growth of the vitreous chamber in the

steady red group was slower than the normal animals, with a corresponding hyperopic change in refraction. The correlation between amount of change in vitreous chamber depth during treatment and the change in refraction was highly significant ($p < 0.001$, $r^2 = 0.78$). At the end of wavelength treatment, values of the other ocular components showed only very minor changes that were too small to account for the changes in refraction.

4. Discussion

In young tree shrews, at a time when the eyes' refractions normally are rapidly decreasing from hyperopia toward emmetropia, exposure to either steady or flickering narrow-band long-wavelength light that only stimulates the LWS cones, causes the eyes' vitreous chamber growth to slow and prevents the refractive decrease toward emmetropia, producing hyperopia. Exposure to flickering, but not steady, short-wavelength light, that stimulates SWS cones approximately twice as strongly as LWS cones, produces increased vitreous chamber elongation so that the eyes move past emmetropia and become myopic. In both the red-light conditions and in blue flicker, the eyes ignore other cues, such as refractive error, that might normally help to guide the eyes to emmetropia. Instead, they tolerate large amounts of hyperopia (in red) or myopia (in blue flicker). Thus, these stimuli powerfully affect the ability of the emmetropization mechanism to effectively guide emmetropization. However, none of the wavelength conditions produced a permanent effect. Upon return to standard colony lighting, the refractive state returned toward emmetropia, suggesting both that the effect was due to the wavelength treatments and that, once the treatment ended, refractive error cues could still guide normal eye growth.

Our results suggest that, under these conditions, the long and short-wavelength stimuli did not provide information about the *target* toward which eyes should grow. Rather, they were interpreted as *cues* by the emmetropization mechanism. The response to the red light seemed to be interpreted as a cue that the eye is too long and should slow its elongation rate. The blue flicker appeared to act as a cue that the eye is too short and should increase its elongation rate.

Although these responses are consistent with the suggestion that the retina can use LCA to guide emmetropization, the situation in which the tree shrews were placed was very different from a natural setting where LCA cues would change over time. In a normal visual environment, many wavelengths are present. Neurons in the retina might compare the image contrast at long vs. short wavelengths to generate signals that the eye should adjust its growth rate. Then, as the emmetropization mechanism responds to LCA cues by adjusting the growth of the vitreous chamber, the relative balance of short- vs. long-wavelength image contrast would change and assist the eye in stabilizing at emmetropia. This would be a closed loop feedback system. Our wavelength treatments may have created an open-loop condition: the wavelength stimulus did not change regardless of how the eye responded. Similar to the removal of refractive error cues by covering an eye with a translucent diffuser, the wavelength treatments prevented any possible LCA-mediated feedback loop. As seen in Fig. 2A, several of the animals began treatment with relatively low hyperopic refractions that moved toward increased hyperopia day after day. Animals whose initial hyperopia was greater tended to remain hyperopic. These results are consistent with an unabated presence

of retinal signaling to slow axial growth, despite the resulting large hyperopic refractive error.

Flickering blue light was strongly myopiagenic. The average change in refraction during the 13 days of treatment was -9.2 ± 1.1 D. For comparison, the normal animals' refraction decreased -3.7 ± 1.1 D, as expected during normal emmetropization. The effect of blue flicker is consistent not only with the suggestion that blue flicker acted here as a cue for guiding eye growth, but also with our hypothesis that temporal flicker can be used as a proxy for spatial image sharpness by the retina when stimulated by wavelengths that preferentially stimulate the blue (SWS) cone system.

The similarity of the response of the steady red and red flicker groups strongly contrasts with the difference between the steady blue and blue flicker groups. Steady blue light seemed to allow eyes to emmetropize relatively normally, at least on average. We speculate that this is because the steady blue light stimulates not only the SWS cones, but also the LWS cones approximately 51% as strongly as the SWS cones, measured as "cone catch" after accounting for pre-retinal absorption (Petry and Harosi, 1990). We would propose that, because the LWS cones get substantial activation from blue light, steady blue light might therefore provide a relatively balanced stimulation of LWS and SWS cones, and no strong signal for either hyperopia or myopia – although the lack of valid chromatic cues could make the system less stable/more likely to 'drift' off target. While flickering blue light would add a flickering stimulus to both the LWS and SWS channels, the absolute amount of flicker in the SWS channel would be significantly increased above that in the LWS channel (or the absolute flicker in the LWS channel reduced relative to the SWS channel), which would tend to be myopiagenic. Still, the variance for both blue groups is high, and in the long run we suspect that it is some aspect of *differential* flicker between LWS and SWS cones that is the ecologically normal visual cue.

The mean luminance of both the red flicker and blue flicker was approximately half of the steady red or blue. However, these lower levels were approximately the same as provided by normal colony lighting. The lower mean levels for flicker in both wavelength conditions cannot account both for the similarity of the response to the two red conditions and the substantial difference in response to steady blue vs. blue flicker. Rather, we suggest that the similar response of both red groups occurred because both stimuli stimulated only the LWS cones. The differing response to blue flicker vs. steady blue demonstrates that temporal cues may be (or were) important in guiding emmetropization involving the SWS cones.

4.1 Response Variability

Compared with normal animals, the variability of the refractive responses of the individual animals was greater in all treatment conditions. Although the refractions of the normal animals at 11 DVE (the age wavelength treatments started) were variable, similar to the initial refractions of the wavelength groups, the refractions of the normal animals generally decreased monotonically toward emmetropia. In contrast, the animals exposed to steady red and red flicker followed variable paths; although they generally moved toward hyperopia, the refractions of some animals moved toward emmetropia before reversing course. The responses of the animals to blue light (steady and flickering) was even more variable than to

red. For example, the refraction of one animal in the steady blue condition initially decreased rapidly, and then turned upward before again decreasing. The refraction of one of the blue-flicker animals became *more* hyperopic during the first two days of treatment before decreasing and become myopic. The reasons for these variable responses in both wavelength conditions are unclear, but may be related to the restricted range of wavelength cues present in the monochromatic lights which reduced the cues available to the emmetropization mechanism. Note also that these studies are limited to the time period of rapid emmetropization, and it is not known yet if similar effects will happen in older animals that have already achieved emmetropia. However, the powerful, opposite effect of red light versus flickering blue light seen here suggests that wavelength and (for blue) temporal fluctuations of ambient light directly engage fundamental retinal signaling mechanisms that guide emmetropization.

4.2 The Temporal Modulation

Although sinusoidally-modulated flicker has been commonly used in both psychophysical and neurophysiological studies, we avoided sinusoidal modulation of the light intensity in this study for several reasons. If temporal changes are important for the SWS cone system in guiding emmetropization, they would occur in a jerky, non-sinusoidal pattern as animals move their eyes across the edges of objects in their field of view. In addition, we were uncertain which temporal frequencies might be important. Thus, the pseudorandom flicker used here contained a wide range of temporal frequencies. Finally, we had no advance knowledge if temporal information would be summed linearly, an assumption behind the use of sinusoidal flicker.

Consideration must be given to what temporal frequencies the SWS cone system can respond. Psychophysical studies have found that the maximum flicker fusion frequency for blue is significantly lower than for longer wavelengths (Brindley et al., 1966). These results involve wavelength information that passed from the retina, through central chromatic pathways, to provide perception. However, in the retina, the temporal high frequency cut-off of blue cones is the same as for longer-wavelength sensitive cones (Kraft, 1988; Schnapf et al., 1990). Additionally, human psychophysics using blue-cone isolating stimuli have shown that even central mechanisms can detect blue flicker up to nearly 30 Hz (Stockman et al., 1991). Thus, many of the frequencies present in the blue flicker should have been encoded by retinal neurons.

It is possible, perhaps even likely, that the signals generated in the retina that guide emmetropization do not leave the retina through the geniculostriate pathway, so that the principles established for color vision and visual perception (such as color constancy and color opponency) may not pertain to the retinal signaling used specifically for emmetropization. It is well established that information important for emmetropization is communicated directly to the sclera via a signaling cascade involving retinal pigment epithelium, choroid and sclera (Guo et al., 2013; Guo et al., 2014; He et al., 2014a, b). This direct, spatially-local pathway is able to separately modulate emmetropization in the central or peripheral visual fields and produce emmetropia (albeit less effectively) if communication through the optic nerve is severed (Huang et al., 2011; Norton and Siegwart, 1991; Norton et

al., 1994; Smith et al., 2007; Wildsoet and McFadden, 2010). Whatever the retinal mechanisms that guide emmetropization through this direct retina-RPE-choroid-sclera signaling pathway, they likely have access to information about high temporal frequencies at short wavelengths.

4.3 Relationship with Other Studies

Even without manipulations of wavelength, studies of refractive development have varied in numerous ways, including the species, whether the animals are actively emmetropizing (as in newly-hatched chicks, young guinea pigs, tree shrews or monkeys) or older animals in which the task of the emmetropization mechanism is to maintain emmetropia, including studies in adolescent monkeys, tree shrews and chicks (Bradley et al., 1999; Norton et al., 2010; Papastergiou et al., 1998; Smith, III et al., 1999). In addition, the duration of the experimental conditions has varied. Occasionally, various types of flickering stimuli have been used (Crewther and Crewther, 1990; Gottlieb and Wallman, 1987; Schwahn and Schaeffel, 1997). Adding wavelength as a variable further complicates the experimental design, requiring, in addition, consideration of the number of cone types present in the retina, their peak sensitivity and the peak wavelength and bandwidth of the wavelength stimuli, which determines how they interact with the available cones. Given the many variables involved, it may not be surprising that recent studies of the effect of wavelength on refraction have produced differing and sometimes confusing results. For instance, similar to our results, Smith et al (Smith et al., 2015) found a slowing of axial elongation with a resulting hyperopia in macaque monkeys that wore filters that only allowed wavelengths in excess of approximately 600 nm to reach the retina. These conditions, like ours, should have only activated LWS cones. Another study in slightly older infant monkeys showed a slight trend of red light to create myopia (Liu et al., 2014). These differing results in the same species demonstrate clearly that we do not yet understand all of the relevant parameters governing the effect of wavelength on refractive development. In chicks, with shorter treatments and light that was sinusoidally flickered in either intensity or color, Rucker and Wallman also found that color and flicker had powerful effects on emmetropization (Rucker and Wallman, 2012): luminance flicker produced hyperopic refractions, and color flicker produced myopia.

Several recent studies in guinea pigs found opposite results to ours: monochromatic blue light inhibited axial eye growth, and monochromatic longer wavelength light stimulated it (Jiang et al., 2014; Liu et al., 2011; Qian et al., 2013). A study in chicks also showed that animals were more hyperopic in blue than in red light (Seidemann and Schaeffel, 2002). Another study in chicks also showed that red light caused progressive myopia and blue light progressive myopia – although interestingly, the effects on refractive error were much larger than can be accounted for by LCA i.e., the eyes were not adjusting to the focal plane of the dominant wavelength (Foulds et al., 2013). In cichlid fish, animals raised in blue light had shorter eyes while animals raised in red light developed longer eyes (Kroger and Wagner, 1996). In these studies, the refractive changes were often consistent with eyes using LCA as a *target* to adjust its growth to match the focal plane of the dominant wavelength, although when followed for long enough periods of time the refractions often go well beyond what would be required to account for LCA (see (Smith et al., 2015) for discussion). The different

results seen in the various studies may be due to specific experimental conditions and species that favor one mode of response over the other. We do speculate that one fundamental issue is that tree shrews are dichromats (and trichromatic primates *might* be functional dichromats when it comes to emmetropization), and chicks are tetrachromats. Thus, the chick may use differential wavelength cues between multiple photoreceptor classes with relatively closely-spaced absorption peaks, while the tree shrew is limited to a simple long vs. short discrimination. Clearly, more studies are needed to learn what aspects of the experimental conditions produce these dissimilar results.

4.4 The Tree Shrew Model

As a model for determining the role of wavelength in guiding refractive development, tree shrews have many advantages. They are diurnal mammals closely related to the primate line, have a cone-dominated retina (~95% cones), have relatively good visual acuity for a small animal (slightly less than a domestic cat (Pettry et al., 1984)), emmetropize rapidly and reliably in colony lighting and have the classical dichromatic mammalian pattern of both SWS and LWS cones (Bowmaker, 1998). Because emmetropization is presumably an evolutionarily ancient process, it seems likely that the fundamental mechanisms were developed before the onset of trichromatic retinas in primates and are conserved across many species. Indeed, human dichromats (red/green colorblind) may actually have a lesser incidence of refractive errors compared to trichromats (Qian et al., 2009): certainly the red/green opponent system is not required for the human emmetropization mechanism to function. If humans do use chromatic cues for emmetropization, they most likely rely on the same short- vs. long-wavelength systems as dichromatic mammals.

4.5 Conclusions

The incidence of myopia is increasing worldwide (Hrynychak et al., 2013; Sun et al., 2012). In addition, myopia is a major risk factor for eye disease, (Burton, 1989; Saw et al., 2005; Vongphanit et al., 2002; Zadnik, 2001) and has significant negative effects on the quality of life. (McCarty and Taylor, 2000; Roch-Leveq et al., 2008) The substantial hyperopia produced by the long-wavelength LEDs might eventually prove useful as a counter-measure against myopia. Interestingly, we recently have found that the red-light treatment can produce hyperopia in adolescent tree shrews that have completed normal emmetropization (Norton et al., 2016). Also, in young tree shrews, presenting the red light for as little as 2 hours per day slows the decrease in refraction toward emmetropia (Gawne et al., 2016). Conversely, the myopiagenic effect of the specific short-wavelength flicker used here raises the possibility that there may be similar stimuli present in some artificial lighting that should be avoided. Certainly, the dramatic effects observed here during early postnatal refractive development suggest that it is important to further explore the degree to which the chromatic and temporal content of ambient light may affect refractive state.

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Highlights

- Examined effect of long and short wavelengths on refractive development
- Studied tree shrews, cone-dominated dichromatic mammals closely related to primates
- Narrow-band red light (626 nm) slowed vitreous chamber growth, producing hyperopia
- Flickering narrow-band blue light (464 nm) produced elongated vitreous and myopia
- Wavelength exposure in young tree shrews has powerful effects on emmetropization

Fig. 1.

A. The temporal pattern of flickering light with superimposed large and small excursions (not to scale – see text). The pattern is pseudo-randomly generated and would repeat every 497 days if run continuously. B. Log magnitude of the Fourier transform of this temporal pattern indicating that a wide range of temporal frequencies were present in the flickering light arrays.

Fig. 2.

The effect on refractive development in young tree shrews of steady and flickering, and red and blue, ambient light compared with fluorescent colony lighting. Positive numbers on the y-axis indicate hyperopic refractive error, and negative numbers, myopic refractive error. Thin lines show individual animals (mean of the two eyes.) Error bars are $\pm$ SEM. Dashed, black line indicates emmetropia (0 D refractive error). Black circles (“normal eyes”) are from the normal group raised exclusively in colony lighting. A: Refractions (n=5) during exposure to 13 days of steady red light (red, triangle symbols) followed by colony fluorescent lighting “recovery” (inverted white triangle symbols). All five animals became hyperopic compared to normals. When transferred to colony lighting, refractions returned toward normal levels. B: Flickering red light. Similar to A, all five animals became hyperopic and “recovered” in colony lighting. C: Exposure to steady blue light. One animal became myopic, another remained hyperopic, and the remaining three appeared to emmetropize normally. The overall pattern of refractive change during treatment was essentially normal. D: Exposure to flickering blue light. All seven animals became myopic

relative to normals; two became highly myopic. Refractions returned toward normal after the animals were placed in colony lighting.

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Fig. 3.

A. Refractive state of the four wavelength-treated groups at the end of the 13-day treatment period. Values are the average of the right and left eyes, $\pm$ S.E.M. Age-matched normal refractions $\pm$ 95% confidence intervals are indicated by the solid and dashed horizontal black lines ($n=15$). Values significantly different from the age-matched normals are indicated by asterisks (Kruskal-Wallis with Dunn's post-hoc test). Steady and flickering red light both produced strong hyperopia; steady blue light, on average, had no effect and flickering blue light was myopiagenic. B. End of wavelength treatment vitreous chamber depths. The most hyperopic group had the shortest vitreous chambers. Vitreous chamber depth was greater in groups with lower refractive state. Age-matched normal refractions

$\pm$ 95% confidence intervals are indicated by the solid and dashed horizontal black lines (n=7).

Fig. 4.

Association between change in refractive state and change in the depth of the vitreous chamber during the 13-day treatment period. Values are the average of the right and left eyes. Vitreous chamber depth changed in parallel with refractive change.