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Opposing effects of atropine and timolol on the color and luminance emmetropization mechanisms in chicks

Laura A. Goldberg *, Frances J. Rucker

New England College of Optometry, 424 Beacon Street, Boston, MA 02115, United States

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ABSTRACT

This study analyzed the luminance and color emmetropization response in chicks treated with the non-selective parasympathetic antagonist atropine and the sympathetic β-receptor blocker timolol.

Chicks were binocularly exposed (8 h/day) for 4 days to one of three illumination conditions: 2 Hz sinusoidal luminance flicker, 2 Hz sinusoidal blue/yellow color flicker, or steady light (mean 680 lux). Atropine experiments involved monocular daily injections of either 20 μl of atropine (18 nmol) or 20 μl of phosphate-buffered saline. Timolol experiments involved monocular daily applications of 2 drops of 0.5% timolol or 2 drops of distilled H_2O. Changes in the experimental eye were compared with those in the fellow eye after correction for the effects of saline/water treatments.

Atropine caused a reduction in axial length with both luminance flicker (−0.078 ± 0.021 mm) and color flicker (−0.054 ± 0.017 mm), and a reduction in vitreous chamber depth with luminance flicker (−0.095 ± 0.023 mm), evoking a hyperopic shift in refraction (3.40 ± 1.77 D). Timolol produced an increase in axial length with luminance flicker (0.045 ± 0.030 mm) and a myopic shift in refraction (−4.07 ± 0.92 D), while color flicker caused a significant decrease in axial length (−0.046 ± 0.017 mm) that was associated with choroidal thinning (−0.046 ± 0.015 mm).

The opposing effects on growth and refraction seen with atropine and timolol suggest a balancing mechanism between the parasympathetic and β-receptor mediated sympathetic system through stimulation of the retina with luminance and color contrast.

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1. Introduction

The increasing occurrence of myopia in the population presents an important public health issue because of an association with elevated risk of ocular diseases including cataract, glaucoma, retinal detachment, and blindness (Saw, Gazzard, Shih-Yen, & Chua, 2005). In humans, the environmental effect of time spent outdoors has been implicated in a reduction in myopia development (Dirani et al., 2009; Guggenheim et al., 2012; Jones et al., 2007; Jones-Jordan et al., 2012; Onal et al., 2007; Parssinen & Lyyra, 1993; Rose, Morgan, Ip, et al., 2008; Rose, Morgan, Smith, et al., 2008; Wu, Tsai, Hu, & Yang, 2010), and recent work has helped to clarify the protective effects of factors such as high light levels (chicks: Ashby, Ohlendorf, and Schaeffel (2009), Backhouse, Collins, and Phillips (2013), Cohen, Belkin, Yehezkel, Avni, and Polat (2008) and Cohen, Belkin, Yehezkel, Solomon, and Polat (2011); monkeys: Smith, Hung, and Huang (2012)) and spatial and temporal changes in the retinal image (Rucker, 2013; Rucker, Britton, Spatcher, & Hanowsky, 2015; Rucker & Wallman, 2008, 2009; Rucker & Wallman, 2012) that may be involved. In the meantime, promising pharmacological interventions (e.g., atropine) can slow the development of myopia progression (Chia et al., 2014, 2015, 2012; Bedrossian, 1971; Lee et al., 2006; Li et al., 2014; Morgan, Ohno-Matsui, & Saw, 2012; Walline, 2016; Wu, Yang, & Fang, 2011), although the effects of these treatments under different environmental conditions have not been studied.

1.1. Color and luminance contrast affect emmetropization

As a result of dispersion, short-wavelength light has a shorter focal length than long-wavelength light, producing an effect called longitudinal chromatic aberration. The differences in focus of the different wavelengths produce changes in color of the retinal image with defocus (Rucker & Wallman, 2012), which in turn is reflected in changes in the stimulation of the retinal cones and the retinotectal color and luminance pathways (review: Rucker (2013)). A theoretical analysis of the change in the retinal image with defocus has indicated that with myopic defocus, the retina would experience changes in luminance contrast, whereas with

* Corresponding author.
E-mail address: goldbela30@gmail.com (L.A. Goldberg).

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hyperopic defocus the retina would also experience changes in color contrast (Rucker & Wallman, 2009). In the laboratory, flickering light of fixed frequency and waveform can be used to simulate the changes in luminance and color contrast of the retinal image that occur with changes in focus and changes in fixation in the natural environment. Rucker and Wallman (2012) tested their hypothesis by exposing chicks to 2 Hz, high contrast, sinusoidal changes in either luminance or color contrast for 3 days and found hyperopic shifts (2.01 D) with changes in luminance contrast and myopic shifts with changes in color contrast. Rucker et al. (2015) determined that the reduction in eye length was most pronounced with exposure to high temporal frequencies (5–10 Hz), confirming earlier research (Gottlieb & Wallman, 1987; Schwahn & Schaeffel, 1997), and that the more myopically defocused blue component of the light source provided protection against increases in eye growth at low temporal frequencies with luminance flicker. These results have confirmed that the eye utilizes signals arising from temporally-sensitive changes in luminance and color contrast to determine the emmetropization response.

1.2. Parasympathetic and sympathetic control of color and luminance pathways

The molecular pathways for these color and luminance signals are unknown. One possibility is that these light signals activate the parasympathetic and sympathetic nervous systems. The neurotransmitter acetylcholine (ACh) is released from parasympathetic axon terminals that innervate the ciliary body, iris, smooth muscle in the vasculature, but also from intrinsic interneurons in the retina. There are two categories of acetylcholine receptors: nicotinic (ionotropic; nAChR) and muscarinic (metabotropic; mAChR), which are coupled to heterotrimeric G-proteins (review: Nathanson, 1987). Atropine, which has been proposed as a treatment for myopia because of its effect in reducing eye growth, is a non-selective antimuscarinic. In mammals, five muscarinic receptor subtypes, M1 through M5, are present in the human eye (review: Mitchelson, 2012). Of these receptor types, the M3 receptor is the most predominant receptor type in human iris sphincter, ciliary body (causing accommodation), retina and sclera (Collison, Coleman, James, Carey, & Duncan, 2000; Gil, Krauss, Bogardus, & WoldeMussie, 1997; Ishizaka et al., 1998; Matsumoto, Yorio, DeSantis, & Pang, 1994; Pang, Matsumoto, Tamm, & DeSantis, 1994) with small amounts of M1, M4 and M5 in the iris sphincter and ciliary body (also M2) Mitchelson, 2012. There are also reports of mAChRs being expressed in the human RPE (Osborne, FitzGibbon, & Schwartz, 1991) and lens (Williams, Duncan, Riach, & Webb, 1993) with mainly M1 receptors in native human lens epithelium (Collison et al., 2000) and acetylcholinesterase on the lens surface (Michon & Kinoshita, 1968). Muscarinic receptors are found throughout the retina on amacrine, bipolar, horizontal and ganglion cells, though the only cholinergic cells in the adult retina are the starburst amacrine cells (Fischer, McKinnon, Nathanson, & Stell, 1998; McBrien, Jobling, Truong, Cottriall, & Gentle, 2009; Strang, Renna, Amthor, & Keyser, 2010; Townes-Anderson & Vogt, 1989; Yamada et al., 2003).

With regard to the chick animal model of myopia, four avian mAChR subtypes have been characterized: cm2 (Tietje & Nathanson, 1991), cm3 (Gadbut & Galper, 1994) cm4 (Tietje, Goldman, & Nathanson, 1990) and cm5 (Creason, Tietje, & Nathanson, 2000). Fischer et al. (1998) reported localization of three of the different isoforms of mAChRs (cm2–cm4) subtypes in the chick eye, in the retina, choroid, retina pigment epithelium (RPE) and ciliary body. It is important to note that chicks differ from mammals in that only nicotinic receptors are involved in accommodation (McBrien, Moghaddam, New, & Williams, 1993) and thus in chicks accommodation should not be affected by atropine.

In the human eye, the sympathetic nervous system innervates the ciliary muscle, ciliary epithelium, iris dilator muscle and smooth muscle of the vasculature. Innervation occurs through the action of the neurotransmitter noradrenaline on two subclasses of post-synaptic adrenergic receptor types: α- and β-adrenoceptors (review: Chen, Schmid, & Brown, 2003). Timolol maleate, which has been used as a clinical treatment for glaucoma since the late 70s, is a non-selective β-adrenoceptor antagonist (Airaksinen, Saari, Tiainen, & Jaanio, 1979). α-Adrenoceptors consist of two subtypes α1 and α2, which can be further subdivided into α2A, α2B and α2C subtypes (Regan & Cotecchia, 1992). Stimulation of α-adrenoceptors can regulate contraction of the iris dilator muscle (mydriasis) (van Alphen, 1976) and relaxation of the ciliary body (Garner, Brown, Baker, & Colgan, 1983; Zetterstrom, 1988). β-Adrenoceptors consist of two subtypes, β-1 and β-2. β-1 receptors are mainly found in cardiac tissues, but they also make up 10% of the receptors in human iris and ciliary body (Wax & Molinoff, 1987). Most of the receptors in the ciliary body are of the β-2 receptor subtype (Wax & Molinoff, 1987) and stimulation causes muscle relaxation. In addition, β-2 receptors control secretion from the non-pigmented ciliary epithelium, and blockade of these receptors by timolol reduces aqueous production (Zimmerman & Kaufman, 1977) and thus intraocular pressure (IOP). Many studies have reported that IOP readings are higher in human myopes than emmetropes (David, Zangwill, Tessler, & Yassur, 1985; Jensen, 1992; Maurice & Mushin, 1966; Parssinen, 1990; Quinn, Berlin, Young, Ziylan, & Stone, 1995), although the differences are small (2 mmHg) and not predictive of future myopia development (Goss & Caffey, 1999).

It is well established in the accommodation literature that dual excitatory parasympathetic and inhibitory sympathetic innervation to the ciliary muscle occurs (Toates, 1972; Tornqvist, 1967), though sympathetic innervation is much weaker (<−2D) and slower (maximal effect after 10–40 s) (Tornqvist, 1966). McBrien and Millodot (1986) suggested that late-onset myopes, with a reduced dioptric level of tonic accommodation, indicative of decreased parasympathetic tone, have a related decrease in inhibitory sympathetic tone. Furthermore, Gilmartin & Bullimore found that sympathetic blockade increases the decay time for accommodation after periods of extended near work (Gilmartin & Bullimore, 1987), particularly in late-onset myopes at high stimulus levels (5D) (Gilmartin & Bullimore, 1991). The authors' hypothesis that late-onset myopia may result from a deficit of the sympathetic nervous system has received considerable support (Chen et al., 2003; Ciuffreda & Lee, 2002; Ciuffreda & Wallis, 1998; Culhane, Winn, & Gilmartin, 1999).

In this study, we analyzed the effect of the non-selective parasympathetic antagonist atropine and the non-selective β-adrenergic receptor blocker timolol on the parasympathetic and sympathetic nervous systems' emmetropization responses to color and luminance flicker. We predicted that luminance and color stimulation may preferentially stimulate one or other of the autonomic nerve pathways, since exposure to high-frequency luminance flicker has been associated with a reduction in eye growth similar to that found with atropine.

2. Methods

2.1. Subjects

Subjects were white leghorn chicks (*Gallus gallus domesticus*) Cornell K strain (Cornell University, Ithaca, NY), hatched in an incubator and raised in temperature-controlled brooders. Upon

Table 1
Number of chicks used in data analysis for each experimental condition.

Treatment	LUM	Color	Steady
Atropine	8	9	8
Saline	8	15	8
Timolol	12	11	8
Water	6	7	7

hatching, the chicks were housed in 12 h light/12 h dark cycles under approximately 300 lux illumination fluorescent bulbs. Food and water were supplied *ad libitum*. The experiments were performed on chicks that were 5–7 days old at the start of the experiment. Care and use of the animals conformed to the Association for Research in Vision and Ophthalmology Resolution for the Care and Use of Animal Research.

2.2. Illumination conditions

Three illumination conditions were used: luminance flicker (LUM), blue/yellow color flicker (Color), or steady light (Steady), maintaining the same mean RGB lighting components in each condition. Chicks were free roaming in a 32 × 20 in. wire cage that was illuminated with a computer-controlled, sinusoidally modulated light source (mean 680 lux) using light-emitting diodes that consisted of independently controlled red, green, and blue components (Lamina Ceramics, Westhampton, NJ: Titan Light Engine; peak wavelengths: 619 nm, 515 nm, 460 nm; beam spread of 45°). The illuminants were driven by an eight-channel, 12-bit Access I/O, USB-DA12-8A digital-to-analog converter with waveform-generator functionality connected to a BuckPuck driver (LuxDrive: 3021 D-E-500) that provided a linear current output over a range of 1.6–4.3 V. Light output was calibrated and a sinusoidal pattern was digitally generated using lookup tables and confirmed by measurement of illuminance (Newport Model 818-SL serial number: 6915). Luminance flicker was produced with in-phase sinusoidal modulation (2 Hz) of the red (615 nm, half-bandwidth 20 nm), green (515 nm, half-bandwidth 35 nm), and blue (465 nm, half-bandwidth 25 nm) light-emitting diodes. Blue/yellow color flicker was created with counterphase modulation of the red and green components with the blue component. Steady light was produced by combining the red, green, and blue components without modulation. The mean irradiances of the individual components of the light source were set to 50 μW cm^{-2} for red, green and blue, which is equivalent to 214 "chick lux" for red, 191 "chick lux" for green, and 64 "chick lux" for blue. Illuminance was controlled with neutral density filters to maintain a mean illuminance equivalent to 680 human lux (Center Lightmeter 337).

2.3. Experimental groups and procedures (atropine and timolol experiments)

Chicks were randomly assigned to each of the four experimental groups (atropine, saline control, timolol, or distilled-water control) under each illumination condition (LUM, Color, or Steady). The number of chicks in each experimental condition is listed in Table 1. One eye was randomly selected and treated, while the fellow eye remained untreated and acted as a control for the illumination condition. Chicks with even-numbered tags received injections/drops in the right eye; chicks with odd-numbered tags were treated in their left eye (chicks were tagged at random). Treated chicks were randomly divided into the three lighting conditions.

In the atropine experiment, drug-injected chicks received 20 μl of atropine (18 nmol), while the saline-injected control chicks received 20 μl of phosphate-buffered saline in one eye. Injections were administered around mid-morning under anesthesia (isofluorane 1.5–2%) using a sterile Hamilton syringe with a sterile 30 G needle that was inserted through the lid on the temporal side of one eye (based on protocols by McBrien, Moghaddam, and Reeder (1993), Schmid and Wildsoet (2004)).

In the timolol experiment, drug-treated chicks received two drops of 0.5% timolol, while control chicks received two drops of distilled H_2O in one eye. The treatment was administered twice daily (10 am and 4 pm) under anesthesia (isofluorane 1.5–2%) (as described by Schmid, Abbott, Humphries, Pyne, and Wildsoet (2000)).

2.4. Measurements

Chicks were exposed (both eyes) to their assigned illumination condition for 4 days, 8 h/day (9 am to 5 pm). They were otherwise kept in the dark in a sound- and light-proof chamber. Measurements of the axial dimensions of the ocular components were performed with A-scan ultrasonography using a 30 MHz transducer sampled at 100 Hz and gain of 59 dB on Olympus NDT equipment (Nickla, Wildsoet, & Wallman, 1998). Measurements were taken prior to and immediately following the experimental period (after 4 days). Axial length was measured as the distance from the anterior cornea to the posterior sclera. In the timolol experiment, pupil reactions, accommodation fluctuations, and refraction were observed with an infrared photorefractor (Schaeffel, Farkas, & Howland, 1987), while in the atropine experiment, refractions were measured with a modified Hartinger refractometer (Zeiss, Jena, Germany). All measurements were carried out under anesthesia (1.5% isofluorane in oxygen, 2 L/min oxygen flow rate). Measurements of right and left eyes were randomized.

2.5. Data analysis

"Treatment Effect": Within each illumination condition, the change during the course of the experiment in the fellow eye (ΔN) was subtracted from the change in the treated eye (ΔX) in both the drug and saline/water control conditions to provide a measure of the Treatment Effect.

"Drug Effect": To separate the effect of administering the injection or drop from the effect of the drug itself, the mean Treatment Effect in the saline/H_2O treated eyes was then subtracted from that of the drug-treated eyes. Means and standard errors were calculated from these values:

$$\text{Drug Effect} = [\text{Drug}(\Delta X - \Delta N) - \text{Mean Saline}/H_2O(\Delta X - \Delta N)] \quad (1)$$

Averaging of the saline/water control reduces variability in these data sets, possibly incurring a Type I error. Therefore, the Treatment Effect was compared by two-way analysis of variance (ANOVA) in Data Desk (Data Description, Inc.; Version 6) before correction for saline injection/H_2O drop effects in order to confirm the presence of a Treatment Effect.

The Drug Effect was compared to zero with student *t*-tests to determine the effects of atropine and timolol on emmetropization, within each of the lighting conditions. Differences in the Drug Effect between drug or illumination conditions and their interactions were examined with ANOVA. *t*-Tests were performed if the *F* value was found to be significant.

3. Results

3.1. Effects of atropine

The effects of atropine injections and its saline control are seen in Fig. 1. Results of the effect of saline injections alone are described in Section 3.4, Fig. 1A and Table 4.

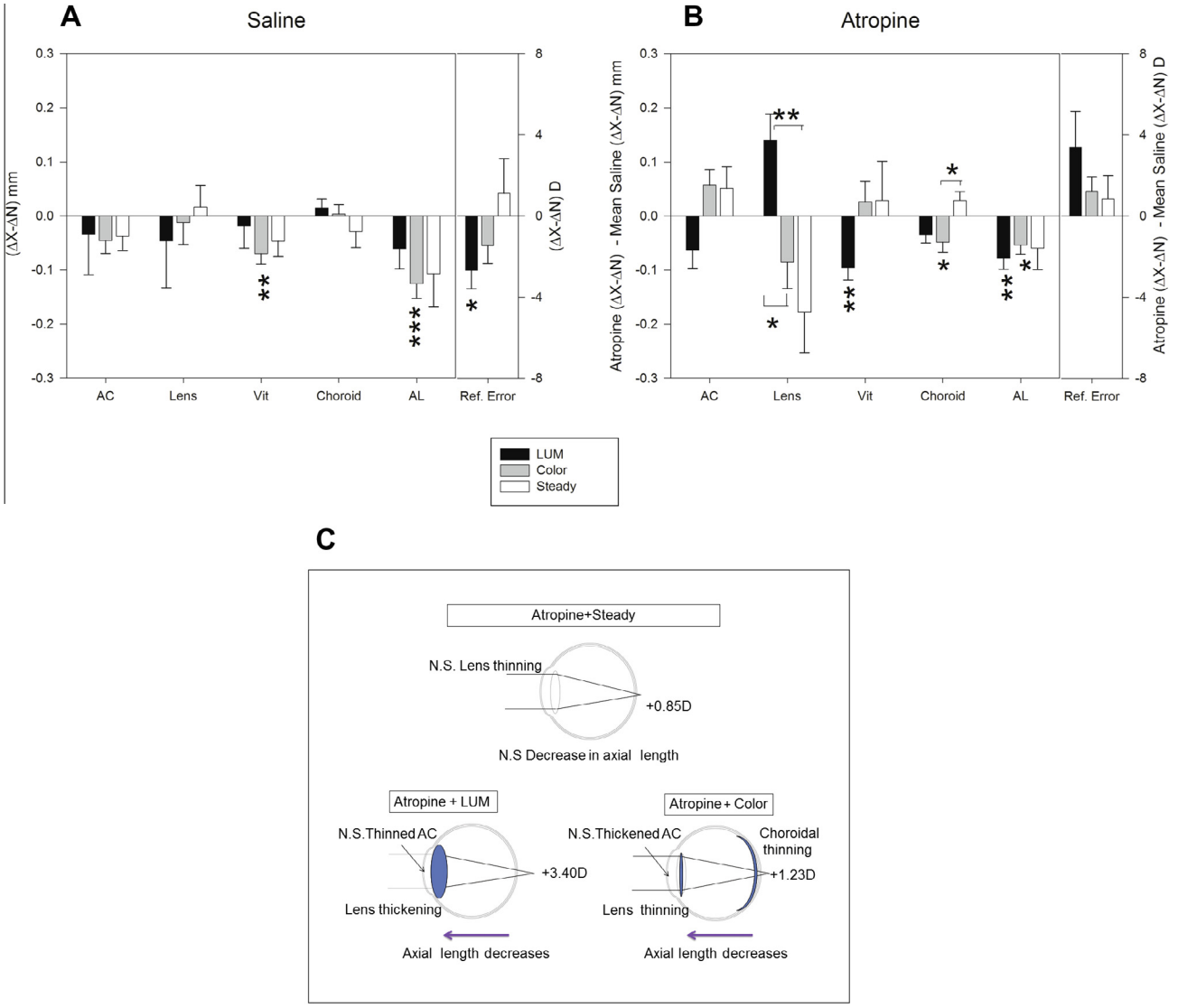


Fig. 1. (A) Treatment Effect of saline injections on ocular components over a four-day period. Saline-injected eyes demonstrated a myopic shift in refraction (Ref. Error) despite a decrease in axial length (AL). There was no difference in the injection effect between illumination conditions. (B) Drug Effect on ocular components with atropine over a four-day period. Eyes injected with atropine showed a significant reduction in axial length (AL) as well as vitreous chamber depth (Vit), resulting in small hyperopic shifts. An increase in lens thickness (Lens), and reduction in anterior chamber depth (AC) was also seen with luminance flicker, while color flicker produced choroidal thinning (Choroid), preventing a change in vitreous-chamber depth. (C) Diagrammatic representation of (B). Abbreviations as described in (A) and (B), N.S. = Non-Significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.1.1. Anterior eye – Treatment Effect

Atropine exerted one of its biggest effects on interocular lens changes. Our data demonstrate that lens thickness was strongly influenced by an interaction between the drug (atropine vs. saline) and illumination condition [ANOVA: $F = 3.56$ (df = 2), $p = 0.04$] (Fig. 1).

3.1.2. Anterior eye – Drug Effect

With LUM, chicks with atropine injections had significant thickening of the lens in experimental eyes (0.140 ± 0.049 mm, $p = 0.02$), which corresponded with a decrease in the anterior-chamber depth (−0.063 ± 0.034 mm, $p = 0.1$). In contrast, blue/yellow color flicker and steady light had a slight increase in anterior-chamber length (Color: 0.057 ± 0.029 mm, $p = 0.8$; Steady: 0.051 ± 0.040 mm, $p = 0.25$) along with a decrease in lens thickness (Color: −0.085 ± 0.049 mm, $p = 0.11$; Steady: −0.177 ± 0.076 mm, $p = 0.59$) (Fig. 1B, C; Table 2).

3.1.3. Posterior eye – Treatment Effect

Atropine induced a hyperopic shift in refraction through a reduction in eye growth when compared to saline [ANOVA: $F = 5.06$ (df = 1), $p = 0.03$].

3.1.4. Posterior eye – Drug Effect

The Drug Effect in atropine-treated eyes exposed to LUM was about three times more hyperopic (3.40 D) than that in eyes exposed to color flicker (1.23 D) or steady light (0.85 D) ($p < 0.05$) (Fig. 1B, C).

Refractive changes under LUM were associated with changes in vitreous chamber depth and axial length, while under Color only axial length changed. Atropine-injected eyes under LUM conditions exhibited a significant decrease in both the vitreous (−0.095 ± 0.023 mm, $p = 0.005$) and axial length (−0.078 ± 0.021 mm, $p = 0.007$). Atropine-injected eyes under color-flicker conditions also exhibited a decrease in

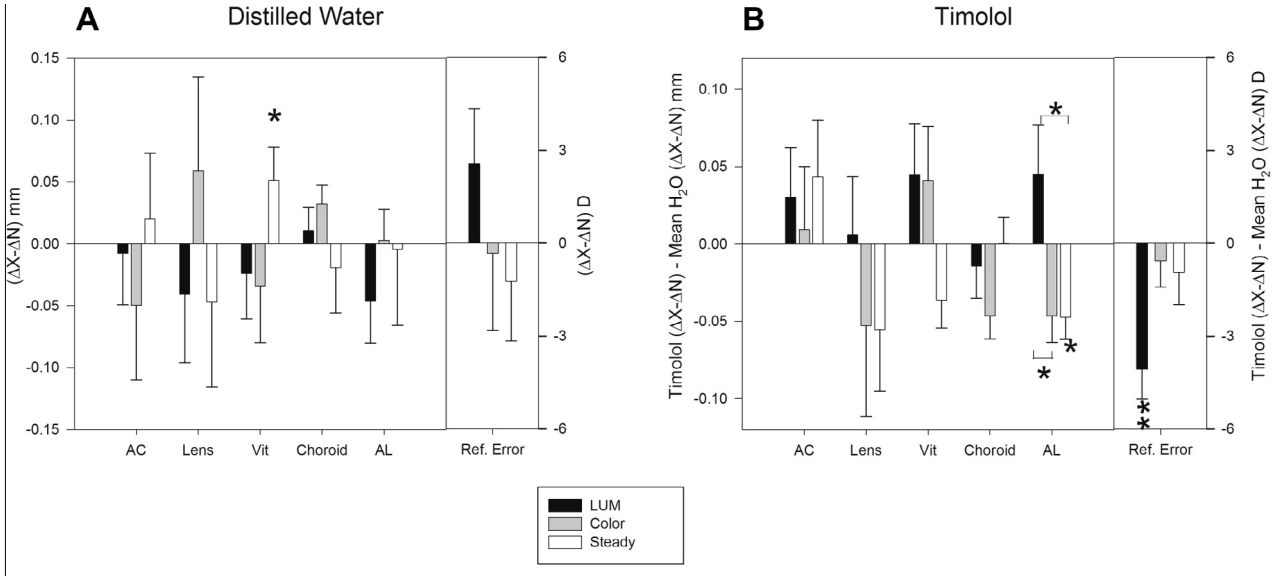
Table 2
Drug Effect on ocular components in atropine-injected chick eyes under luminance flicker, color flicker, and steady light conditions. Significant values are indicated in bold ($p < 0.05$). All units are expressed in either mm or D (for RE). p Values and standard errors are shown. *Abbreviations:* AC = anterior chamber, Vit = vitreous, AL = axial length, RE = refractive error.

Atropine						
	AC	Lens	Vit	Choroid	AL	RE
LUM	−0.063 ±0.034 $p = 0.1$	**0.140** **±0.049** ***p* = 0.02**	**−0.095** **±0.023** ***p* = 0.005**	−0.034 ±0.016 $p = 0.067$	**−0.078** **±0.021** ***p* = 0.007**	3.40 ±1.77 $p = 0.20$
Color	0.057 ±0.029 $p = 0.8$	−0.085 ±0.049 $p = 0.11$	0.026 ±0.039 $p = 0.53$	**−0.048** **±0.019** ***p* = 0.03**	−0.054 ±0.017 $p = 0.13$	1.23 ±0.71 $p = 0.12$
Steady	0.051 ±0.040 $p = 0.25$	−0.177 ±0.076 $p = 0.59$	0.029 ±0.072 $p = 0.77$	0.029 ±0.017 $p = 0.20$	−0.059 ±0.039 $p = 0.16$	0.85 ±1.14 $p = 0.48$

eye growth (−0.054 ± 0.017 mm, $p = 0.13$), but there were no vitreal or refraction changes, partially because of choroidal compensation (Fig. 1B, C; Table 2). Exposure to color flicker in atropine-injected eyes produced a marked thinning in choroidal thickness [ANOVA: $F = 4.85$ (df = 2), $p = 0.02$]. With color flicker, choroids thinned by −0.048 ± 0.019 mm ($p = 0.03$), slightly more than with LUM (−0.034 ± 0.016 mm, $p = 0.067$) and steady light (0.029 ± 0.017 mm, $p = 0.20$) (Table 2).

3.2. Effects of timolol

The effects of timolol and distilled water drops are seen in Fig. 2. Results of the effect of water drops alone are described in Section 3.5, Fig. 2A and Table 5.



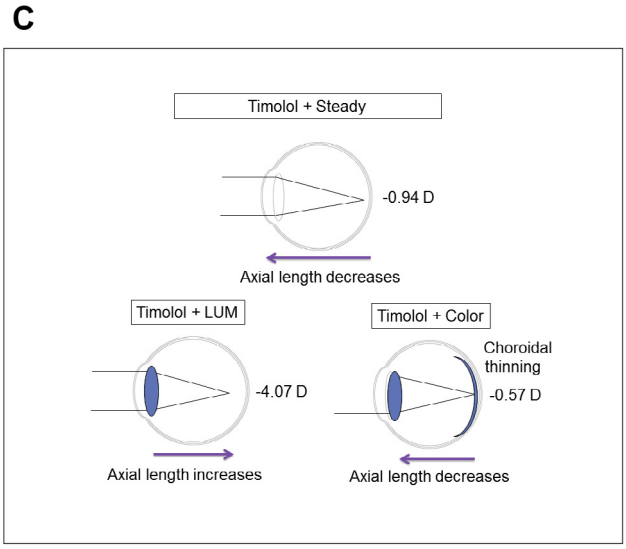


Fig. 2. (A) Treatment Effect of distilled water drops on ocular components over a four-day period. In steady light, a small increase in axial length combined with choroidal thinning produced an unexplained increase in vitreous chamber depth. There was no difference in the drop effect between illumination conditions. (B) Drug Effect on ocular components with timolol over a four-day period. Timolol-injected eyes exposed to luminance flicker produced an increase in axial length and a myopic shift in refraction. Color flicker and steady light caused a reduction in axial length that was compensated for by choroidal thinning in the color condition. (C) Diagrammatic representation of (B). Abbreviations as in Fig. 1.

Table 3
Drug Effect on ocular components in timolol-injected chick eyes under luminance flicker, color flicker, and steady light conditions. Significant values are indicated in bold ($p < 0.05$). Units and abbreviations as in Table 2.

Timolol						
	AC	Lens	Vit	Choroid	AL	RE
LUM	0.030 ±0.031 $p = 0.35$	0.006 ±0.036 $p = 0.88$	0.045 ±0.031 $p = 0.18$	−0.014 ±0.020 $p = 0.48$	0.045 ±0.030 $p = 0.17$	**−4.07** **±0.92** **$p = 0.001$**
Color	0.009 ±0.041 $p = 0.83$	−0.053 ±0.059 $p = 0.39$	0.041 ±0.035 $p = 0.27$	**−0.046** **±0.015** **$p = 0.01$**	**−0.046** **±0.017** **$p = 0.02$**	−0.57 ±0.84 $p = 0.51$
Steady	0.043 ±0.037 $p = 0.28$	−0.055 ±0.040 $p = 0.18$	−0.036 ±0.018 $p = 0.07$	0.000 ±0.017 $p = 0.98$	**−0.047** **±0.014** **$p = 0.01$**	−0.94 ±1.03 $p = 0.39$

Table 4
Interocular changes in ocular components ($\Delta X – \Delta N$) in saline-injected chick eyes under luminance flicker, color flicker, and steady light conditions. Significant values are indicated in bold ($p < 0.05$). Units and abbreviations as in Table 2.

Saline						
	AC	Lens	Vit	Choroid	AL	RE
LUM	−0.034 ±0.075 $p = 0.67$	−0.046 ±0.087 $p = 0.61$	−0.019 ±0.041 $p = 0.67$	0.015 ±0.016 $p = 0.40$	−0.060 ±0.038 $p = 0.15$	**−2.68** **±0.89** **$p = 0.02$**
Color	−0.045 ±0.024 $p = 0.132$	−0.012 ±0.040 $p = 0.742$	**−0.069** **±0.019** **$p = 0.002$**	0.003 ±0.018 $p = 0.75$	**−0.125** **±0.027** **$p = 0.000$**	−1.46 ±0.87 $p = 0.56$
Steady	−0.038 ±0.027 $p = 0.17$	0.016 ±0.041 $p = 0.69$	−0.047 ±0.029 $p = 0.13$	−0.029 ±0.029 $p = 0.32$	−0.107 ±0.061 $p = 0.10$	1.12 ±1.71 $p = 0.51$

3.2.1. Anterior eye – Treatment Effect

Timolol demonstrated no significant Treatment Effect of the drug or illumination condition on lens thickness nor anterior chamber [ANOVA: $F = 0.93$ (df = 2) and 0.35 (df = 1); $p = 0.55$ and 0.40, respectively] nor any significant interactions [$F = 0.004$ (df = 2), $p = 0.99$] (Fig. 2).

3.2.2. Anterior eye – Drug Effect

With timolol treatment, there was no significant change of the anterior segment of the eye for any of the illumination conditions. Anterior chamber depth increased slightly with LUM (0.030 ± 0.031 mm, $p = 0.35$) and steady light (0.043 ± 0.037 mm, $p = 0.28$), but no change occurred with color flicker (0.009 ± 0.041 mm, $p = 0.83$) (Table 3).

3.2.3. Posterior eye – Treatment Effect

Timolol, when compared to water, caused an increase in axial length that was dependent on the exposure to the LUM illumination condition [ANOVA: $F = 4.08$ (df = 2), $p = 0.02$].

3.2.4. Posterior eye – Drug Effect

While there was a myopic shift with LUM that was associated with an increase in axial length, there was no refractive change with Color since there was no change in vitreous chamber depth. A significant myopic shift in refraction was observed with LUM (−4.07 ± 0.92 D, $p = 0.001$), which coincided with an increase in axial length (0.045 ± 0.030 mm, $p = 0.17$) (Fig. 2B, C; Table 3). In contrast, color flicker and steady light caused no significant change in refraction (Color: −0.57 ± 0.84 D, $p = 0.51$; Steady: −0.94 ± 1.03 D, $p = 0.39$) despite a significant decrease in eye growth (Color: −0.046 ± 0.017 mm, $p = 0.02$; Steady: −0.047 ± 0.014 mm, $p = 0.01$). This lack of refractive change occurred because the vitreous chamber depth remained constant with choroidal thinning in the color-flicker condition (Vitreous: 0.041 ± 0.035 mm, $p = 0.27$) (Table 3).

3.3. Interactive effects of drugs and lighting

The Drug Effect on refraction (Rx), vitreous (Vit) and eye-length (AL) changes revealed an interaction between the lighting and drug conditions [ANOVA (df = 2): Rx: $F = 4.71$, $p = 0.0133$; AL: $F = 3.33$, $p = 0.04$; Vit: $F = 3.16$, $p = 0.0504$] (Tables 2 and 3).

When chicks were exposed to LUM, timolol rendered the eyes more myopic than atropine (difference: −7.47 D; $p < 0.001$), with more growth (difference: 0.123 mm, $p = 0.024$) and a greater increase in vitreous chamber depth (difference: 0.140 mm, $p = 0.04$). Timolol-treated eyes grew much more with LUM than with color flicker (difference: 0.91 mm; $p = 0.02$), making the LUM-treated eyes more myopic (difference: −3.50 D, $p = 0.045$) than Color-treated eyes.

Atropine-treated eyes showed more lens thickening with LUM than color flicker [ANOVA: $F = 6.82$ (df = 2), $p = 0.0024$; difference: 0.225 mm, $p = 0.046$] or steady light (difference: 0.317 mm, $p = 0.027$).

3.4. Saline injections (atropine experiment)

Saline injections produced a minimal effect on all ocular components in all three illumination conditions. Neither refractive nor axial changes were significantly different in any illumination condition [Rx ANOVA: $F = 2.48$ (df = 2), $p = 0.10$; Axial ANOVA: $F = 0.74$ (df = 2), $p = 0.49$] (Fig. 1A). Injected eyes exposed to the LUM condition became more myopic compared with fellow eyes (−2.68 ± 0.89 D) but without an increase in axial length. In Color and Steady, the injected eye was smaller than the fellow eye, particularly in eyes exposed to color flicker (AL: −0.125 ± 0.027 mm; Vit: −0.069 ± 0.019 mm). The relative myopic shift in LUM and the relative decrease in eye size after exposure to color flicker can be attributed to a combined injection and illumination effect (Table 4).

3.5. Distilled water drops (timolol experiment)

Treatment with water drops also led to minimal effects on the ocular components. Neither refractive nor axial changes were significantly different in any illumination condition [Rx ANOVA: $F = 1.15$ (df = 2), $p = 0.35$; Axial ANOVA: $F = 2.3$ (df = 2), $p = 0.14$] (Fig. 2A). In steady-light conditions, the vitreous of the experimental eye was longer than that of the control eye (0.051 ± 0.027 mm), probably as a result of the small increase in axial length combined with choroidal thinning. No injection was given in this experiment, and there is no obvious explanation why greater-than-normal vit-

Table 5
Interocular changes in ocular components ($\Delta X – \Delta N$) in distilled water-treated chick eyes under luminance flicker, color flicker, and steady light conditions. Significant values are indicated in bold ($p < 0.05$). Units and abbreviations as in Table 2.

Distilled water						
	AC	Lens	Vit	Choroid	AL	RE
LUM	−0.008 ±0.041 $p = 0.86$	−0.041 ±0.055 $p = 0.49$	−0.024 ±0.037 $p = 0.54$	0.010 ±0.019 $p = 0.60$	−0.046 ±0.034 $p = 0.24$	2.57 ±1.77 $p = 0.21$
Color	−0.050 ±0.061 $p = 0.443$	0.059 ±0.076 $p = 0.47$	−0.034 ±0.046 $p = 0.50$	0.032 ±0.015 $p = 0.08$	0.003 ±0.026 $p = 0.91$	−0.33 ±2.49 $p = 0.90$
Steady	0.020 ±0.053 $p = 0.39$	−0.047 ±0.068 $p = 0.47$	**0.051** **±0.027** **$p = 0.045$**	−0.019 ±0.037 $p = 0.77$	−0.004 ±0.061 $p = 0.24$	−1.23 ±1.93 $p = 0.30$

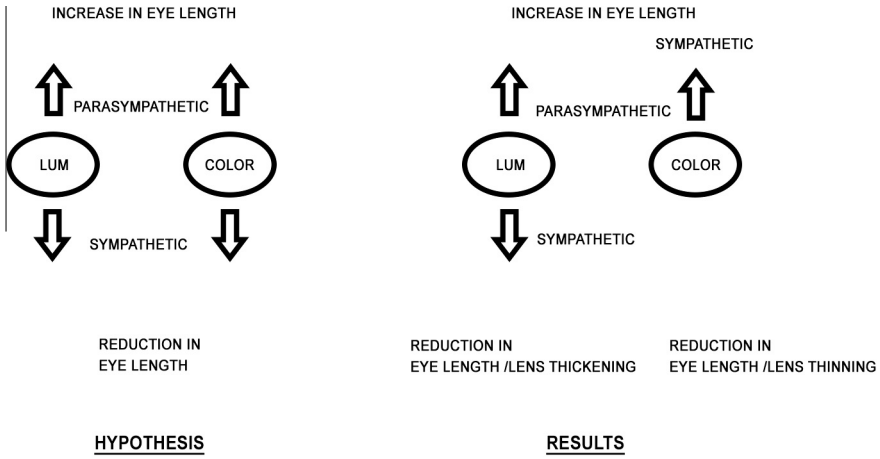


Fig. 3. Comparison of the results to the initial hypothesis. Atropine caused a reduction in eye growth with both luminance and color flicker, suggesting that parasympathetic stimulation increases eye growth and is independent of the environmental stimulus. Timolol caused an increase in eye growth with luminance flicker but a decrease with color flicker, suggesting that the effect of sympathetic stimulation on eye growth is dependent on the environmental stimulus.

reous chamber depth was observed without flicker in the steady-light condition (Fig. 2A, Table 5).

3.6. Correlation between choroid and axial length, and refractive error and axial length

No significant correlation was found between the change in choroidal thickness and the change in axial length for LUM and color flicker in the atropine (LUM: $r^2 = 0.04$, $p = 0.64$; Color: $r^2 = 0.21$, $p = 0.21$) and timolol (LUM $r^2 = 0.33$, $p = 0.051$; Color: $r^2 = 0.075$, $p = 0.41$) experiments. Furthermore, there was no significant correlation between the change in refractive error and the change in axial length for luminance and color flicker in the atropine (LUM: $r^2 = 0.02$, $p = 0.49$; Color: $r^2 = 0.1$, $p = 0.29$) and timolol (LUM $r^2 = 0.08$, $p = 0.65$; Color: $r^2 = 0.16$, $p = 0.34$) experiments.

4. Discussion

4.1. Summary of results

The experiments support our hypothesis that color and luminance changes in visual stimulation influence the activity of the parasympathetic and sympathetic nervous systems and affect emmetropization. Earlier experiments in untreated chicks showed that exposure to luminance flicker simulates myopic defocus and causes a reduction in eye length and hyperopia, while exposure to color flicker simulates hyperopic defocus causing an increase in eye growth and a myopic shift in refraction. In this experiment, inhibition of the parasympathetic nervous system with atropine resulted in further growth inhibition with both luminance and blue/yellow color flicker (Fig. 3). In contrast, timolol reversed the findings in untreated chicks, causing an increase in eye growth with luminance flicker and a reduction in eye growth with color flicker. These results suggest that with exposure to luminance contrast (myopic defocus) growth activity depends on the relative innervation of the parasympathetic and sympathetic nervous systems. On the other hand, with exposure to color contrast (hyperopic defocus) an increase in eye growth occurs through stimulation of the sympathetic nervous system and parasympathetic nervous system.

4.2. Atropine-induced changes

Current results agree with those of previous experiments in that they link atropine's anti-myopia effects with a reduction in axial length. Studies have investigated the role of non-selective parasympathetic antagonists atropine (Luft, Ming, & Stell, 2003; McBrien et al., 1993; Raviola & Wiesel, 1985; Schmid & Wildsoet, 2004; Schwahn, Kaymak, & Schaeffel, 2000; Stone, Lin, & Laties, 1991; Young, 1965) and oxyphenonium (Luft et al., 2003). In addition, studies have investigated pirenzepine (an M1 receptor antagonist that corresponds to cm^2 and cm^4 in chicks (Ellis & Seidenberg, 2000)) (Cottriall & McBrien, 1996; Leech, Cottriall, & McBrien, 1995; Luft et al., 2003; Stone et al., 1991), and himbacine and MT3 (M4 receptor antagonists: Cottriall, Truong, & McBrien, 2001; McBrien, Arumugam, Gentle, Chow, & Sahebjada, 2011), in effective reduction of form deprivation induced experimental myopia. Parasympathetic antagonists are also effective in reducing negative lens induced myopia (atropine: Diether, Schaeffel, Lambrou, Fritsch, & Trendelenburg, 2007; atropine, pirenzepine, but not MT3: Nickla, Zhu, & Wallman, 2013) in chicks.

It has been suggested that atropine acts in the retina by stimulating retinal dopamine release via its actions on dopaminergic amacrine cells (Schwahn et al., 2000). Retinal involvement is suggested through evidence that the highly selective muscarinic antagonist MT3 (M4 receptor antagonist) and MT7 (M1 receptor antagonist) can still inhibit myopia development even at concentrations close to their receptor affinity constants (Arumugam & McBrien, 2012). Dopamine has been associated with myopia development since the ratio of retinal dopamine to DOPAC levels changes with form deprivation (Schaeffel, Bartmann, Hagel, & Zrenner, 1995), and eye growth is reduced in form deprivation myopia with dopaminergic agonists (Cohen, Peleg, Belkin, Polat, & Solomon, 2012; Iuvone, Tigges, Stone, Lambert, & Laties, 1991; Rohrer, Spira, & Stell, 1993; Schmid & Wildsoet, 2004; Stone, Lin, Laties, & Iuvone, 1989). Form deprivation myopia is also reduced with exposure to 10 Hz stroboscopic flicker (Rohrer, Iuvone, & Stell, 1995; Schwahn & Schaeffel, 1997), causing upregulation of the expression of the *c-fos* gene in the retina. This gene promotes expression of tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis. Nevertheless, it is important to note that atropine still inhibits form deprivation in retinas in which most of the $NChAT^+$ (choline acetyltransferase-positive: acetylcholine-synthesizing) amacrine cells have been ablated (Fischer, Miethke, Morgan, & Stell, 1998) suggesting a non-retinal pathway, possibly through action on the M1 receptors in the sclera (Lind, Chew, Marzani, & Wallman, 1998; Luft et al., 2003), occurring as a result of the high concentrations used (Gallego et al., 2012; Lind et al., 1998). The effect is not thought to occur through an accommodative mechanism (McBrien et al., 1993).

In the current experiment, atropine-injected eyes showed a significant increase in lens thickness under luminance conditions compared with fellow eyes, as well as a decrease in anterior chamber depth. Lens thickening has also been observed in a previous experiment with atropine, in binocularly open chick eyes (McBrien et al., 1993). Although lens thinning was found in early biometric measures in a human atropine study at 4 months, this was followed by lens thickening at subsequent time points (Kumaran, Htoon, Tan, & Chia, 2015). Lens thickening causes light rays to converge in front of the retina, bringing the image into focus for a smaller eye, but also potentially creating a myopic defocus that may slow eye growth and produce hyperopia. On the other hand, activation of muscarinic M1 receptors present in the mammalian lens causes the release of intracellular [Ca^{2+}] ions and a calcium-induced blockade of lens K^+ channels (Williams et al., 1993). It remains to be determined whether lens thickening preceded the changes in axial length, whether they occurred in response to the relaxation of the lens zonules in a smaller eye, or if they occurred through changes in osmotic pressure of the lens, or as a result of cell proliferation.

4.3. Timolol-induced changes

The most remarkable result from this experiment was that timolol produced an increase in eye length when the eyes were exposed to luminance flicker, an opposite effect to atropine, and a decrease in eye length when eyes were exposed to color flicker. One possible explanation is that luminance flicker stimulates the sympathetic nervous system and induces the release of dopamine from dopaminergic amacrine cells (Rohrer et al., 1995), an effect that is blocked by timolol. In support of this hypothesis, it was shown that similar reductions in eye growth and refraction were seen with luminance flicker in both atropine-injected and apomorphine-injected eyes (Chuang & Rucker, 2013; Schmid & Wildsoet, 2004). A correlation for the action of a β-adrenergic blocker on dopamine release is seen in the role of dopamine in cognitive flexibility; propanolol (a β-adrenergic blocker) improves cognitive flexibility under stress (Zabelina, Colzato, Beeman, & Hommel, 2016). On the other hand, when the eye is exposed to color flicker or steady light, timolol application reduced eye growth. These results suggest that timolol is causing a small decrease in eye growth potentially through an IOP-reducing mechanism.

It has been suggested that increased intraocular pressure could lead to myopia if the scleral walls of such eyes were equal to or more susceptible than emmetropic eyes to stretch under the influence of IOP increase. Therefore, it follows that IOP-lowering drugs, such as timolol, should reduce or prevent eye enlargement and thus myopia development and/or progression. However, significant IOP reductions have been shown to have little effect on the development of form deprivation or lens-induced myopia in chicks (Schmid et al., 2000). The reduction in eye growth seen in this experiment in the color and steady-light conditions may be apparent because of the smaller eye. A form deprived eye is grossly over extended and the tissues are likely to be less susceptible to contraction under the influence of small IOP reductions.

4.4. Choroidal changes with atropine and timolol

In this study choroidal thinning was found with exposure to color flicker, with both drug types, but not with exposure to steady light or luminance flicker. Two general theories exist regarding the mechanisms behind the defocus-induced choroidal changes. One possibility is that signaling proceeds with paracrine molecule messengers (Morgan, 2003; Rymer & Wildsoet, 2005; Wallman & Winawer, 2004) such as nitric oxide via the parasympathetic system and noradrenaline via the sympathetic system (Fischer, McGuire, Schaeffel, & Stell, 1999; Fischer & Stell, 1999; Fujikado et al., 1997; Nickla & Wildsoet, 2004; Nickla, Wilken, Lytle, Yom, & Mertz, 2006), causing vasodilation and vasoconstriction, respectively. A second possibility is that light-stimulus-driven changes in the ionic (potassium, sodium, chloride, and calcium) environment alter the distribution of ions across the retina, choroid, and sclera. The movement of these ions directly controls the rate and direction of transretinal fluid flow through changes in osmotic pressure and thus choroidal thickness (Crewther, Liang, Junghans, & Crewther, 2006; Crewther, Murphy, & Crewther, 2008; Goodyear, Crewther, & Junghans, 2009; Goodyear et al., 2008). In support of the first theory relating to vascular changes, Lovasik, Kergoat, and Wajszilber (2005) found that blue flicker, which stimulated rod activity, led to an attenuated sub-foveal choroidal blood flow via vasoconstriction of choroidal blood vessels. In support of the latter, Liang, Crewther, Crewther, and Junghans (2004) demonstrated a significant difference in relative concentrations of Na^+ and Cl^- ions in the outer retina, retinal pigment epithelium, and choroid between form-deprived myopic eyes and fellow non-deprived eyes. It remains unclear whether one or both mechanisms are involved in the choroidal thinning observed in this experiment with color flicker.

With respect to timolol, β2-adrenergic receptors have been identified in choroidal and retinal blood vessels (Grajewski, Ferrari-Dileo, Feuer, & Anderson, 1991) and blockade of these receptors can cause vasodilation (Van Buskirk, Bacon, & Fahrenbach, 1990). Past studies have shown that β-adrenergic blockers such as betaxolol and levobunolol exert vasodilatory effects on retinal vessels and increase pulsatile ocular blood flow in ocular hypertensive patients (Krakau, 1992; Langham, 1987; Morsman, Bosem, Lusky, & Weinreb, 1995; Silver, Farrell, Langham, O'Brien, & Schilder, 1989). In regard to timolol, however, findings are inconsistent. Although some researchers noted increases in retinal vein diameter (Schwartz, Takamoto, & Lavin, 1995), most studies have found that timolol treatment causes a decrease in retinal vessel diameter (vasoconstriction) Martin & Rabineau, 1989; Yoshida et al., 1991 as well as a reduction in choroidal blood flow (Schwartz et al., 1995) that we would expect to lead to choroidal thinning.

4.5. Clinical relevance

Clinically, the results of these experiments present evidence that the effects of atropine on refraction can be enhanced by changes in visual stimulation with luminance contrast, induced with myopic defocus and fixation changes, potentially increasing the protective effect of atropine alone on myopia development. However, because luminance contrast also enhances axial length with timolol, the results suggest that an imbalance of autonomic stimulation may increase the risk of myopia, as previously suggested (Charman, 1982; Gilmartin & Bullimore, 1987; McBrien & Millodot, 1986). On the other hand, protective effects of timolol on refraction can be enhanced by exposure to changes in color contrast or steady light, possibly through a reduction in IOP.

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