AUTONOMIC CONTROL OF THE EMMETROPIZATION PROCESS IN THE CHICK

A thesis presented to the graduate faculty of
New England College of Optometry in partial fulfillment
of the requirements for the degree of Master of Science

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Abstract

Purpose: Emmetropization has been found to be sensitive to changes in temporal and spatial changes in light (luminance and color contrast). However, the role of parasympathetic and sympathetic nervous systems effectors in the color and luminance emmetropization pathways is poorly understood. This study analyzed the color and luminance emmetropization response in chicks treated with the non-selective parasympathetic antagonist atropine and the non-selective sympatholytic drug timolol in chicks exposed to illumination conditions that stimulate the color and luminance pathways.

Methods: Chicks were binocularly exposed eight hours each day, for four days, to one of three illumination conditions: 2 Hz sinusoidal luminance flicker (LUM), 2 Hz sinusoidal color flicker (B/Y), or steady light. Mean illuminance was 680 lux. In the atropine experiment, one eye of each chick received daily injections of either 20 µl atropine (18 nmol) or 20 µl phosphate-buffered saline (N = 8) (N = 8). In the timolol experiment, one eye of each chick received either 2 drops of 0.5% timolol (N = 8) or 2 drops of distilled H_2O (N = 8). Measurements of the axial dimensions of ocular components and refraction were performed using A-scan ultrasonography and a Hartinger refractometer. Data are expressed as mean ± standard error. Within each illumination condition, the change in the fellow eye

(ΔN) was subtracted from the change in the treated eye (ΔX) in both the drug and saline/H_2O conditions. The mean change in the saline/H_2O treated eyes was then subtracted from the change in each of the drug treated eyes [Drug (ΔX-ΔN) – Mean Saline/H_2O (ΔX-ΔN)]. This analysis allowed the effect of the drug to be separated from the effect of the injection/drop treatment and the effect of the lighting condition.

Results: Atropine caused a reduction in eye growth with LUM and B/Y (LUM: -0.08 ± 0.02 mm, p = 0.01; B/Y: -0.05 ± 0.02 mm, p = 0.01), and a reduction in vitreous chamber depth (LUM: -0.10 ± 0.02 mm, p = 0.004). These changes evoked a hyperopic shift in refraction with LUM (3.40 ± 1.77 D, p = 0.20) that was counteracted by an increase in lens thickness (0.14 ± 0.05 mm, p = 0.004). B/Y flicker caused choroidal thinning (-0.05 ± 0.02 mm, p = 0.03).

Timolol produced an increase in eye length with LUM (0.045 ± 0.030 mm) leading to a significant myopic shift in refraction (-4.07 ± 0.92 D, p = 0.001) while color flicker and steady light caused a significant decrease in eye length (B/Y: -0.05 ± 0.018 mm, p = 0.02; Steady: 0.047 ± 0.018 mm, p = 0.01). No significant change in vitreous chamber depth for color flicker was observed due to choroidal changes (-0.052 ± 0.015 mm, p = 0.01).

Conclusions: Luminance flicker with atropine demonstrated opposite effects on eye growth and refraction with LUM compared to timolol. The opposing effects of atropine and timolol suggest a luminance-dependent balancing mechanism between the parasympathetic and sympathetic system in order to achieve emmetropization.

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Introduction

Causes of Myopia Progression

Myopia or nearsightedness first occurs in school-age children and progresses until early adulthood (Myrowitz, 2012; Wu, Yang, and Fang, 2011). It is a prevalent vision condition affecting nearly 30% of the U.S. population (Chen, Schmid, and Brown, 2003). Furthermore, the myopia progression rate in East Asian children is especially high (nearly -1 D per year) and approximately 24% of this subpopulation becomes highly myopic as adults (Aller, 2014; Lin, Shih, Hsiao, Chen, Lee, and Hung, 2001). The increasing occurrence of myopia presents an important public health issue, due to an association with increased risk of ocular diseases including cataract, glaucoma, retinal detachment, and blindness (Saw, Gazzard, Shih-Yen, and Chua, 2005). Both genetic and environmental factors are thought to contribute to onset and progression of myopia (Myrowitz, 2012). Fortunately, there exist promising optical and pharmacological interventions, such as atropine, for preventing or slowing the development of myopia progression (Morgan, Ohno-Matsui, and Saw, 2012).

Until recently, a popular belief held that regulation of eye size during the process of emmetropization represented an entirely non-visual, genetically-determined process. However, it was soon discovered that myopia could be recreated in the lab either by inducing blur through form-deprivation or by use of negative lenses. Both of these methods can lead to myopia, however, with potentially different underlying mechanisms. With form deprivation (McBrien and Norton, 1992; Tejedor and de la Villa, 2003; Troilo, Gottlieb, and Wallman, 1987; Wiesel and Raviola, 1977), the images on the retina are obscured by lid suture or translucent diffusers that prevent both spatial and temporal transients and result in ocular

elongation and high myopia. Since no images are brought into focus, the eye continues to grow as long as the vision is obscured. This "open loop" condition is believed to be a consequence of an absence in error signal and visual feedback (Wallman and Winawer, 2004). "Lens-induced" myopia, on the other hand, creates a "closed-loop" condition in which the eye attempts to keep an image sharply focused on the retina. Excess growth terminates when the growth stimuli have been neutralized (Morgan, Ashby, and Nickla, 2013; Walline, Lindsley, Vedula, Cotter, Mutti, and Twelker, 2011).

The Eye Determines the Sign of Defocus

"Lens-induced" myopia experiments demonstrated that the eye could determine the sign of defocus, by either inhibiting or increasing eye growth in order to focus the image onto the retina (Wallman and Winawer, 2004). For example, if negative lenses are worn, light is focused behind the retina and causes a myopic shift. Positive lenses, on the other hand, focus the light in front of the eye, inhibiting eye growth and causing a hyperopic shift. In a study by Schaeffel, Glasser, and Howland (1988), the authors utilized negative lenses to create a hyperopic defocus, and positive lenses to create a myopic defocus. They reported that cyclopleged chicks could accurately respond to both signs of defocus. Chick eyes with positive lenses became hyperopic with decreased eye growth, while those fitted with negative lenses became myopic with increased eye growth. Furthermore, studies have shown that animal eyes wearing astigmatic lenses became myopic; however it was not simply due to increased blur (Irving, Sivak, and Callender, 1992; McLean and Wallman, 2003). For example, in a study by McLean and Wallman (2003), chick eyes fitted with both astigmatic

and spherical lenses compensated as completely for the spherical lens power (+6 D, +3 D, -3 D or -6 D) as eyes wearing spherical lenses alone. Therefore, the eye was still able to determine the sign of defocus and control growth.

In addition to compensatory changes in eye length, compensatory modifications to lens-induced defocus occurred in other parts of the eye, such as in choroidal thickness, anterior chamber depth, lens thickness and corneal curvature (Howlett and McFadden, 2006; Rucker and Wallman, 2012; Wallman, Wildsoet, Xu, Gottlieb, and Nickla, 1995; Wildsoet and Wallman, 1995; Zhu and Wallman, 2009). In chicks, the choroid plays an active role in emmetropization by changing its thickness in response to retinal defocus, thickening to move the retina toward the image plane to compensate for myopic defocus and thinning to move it back in case of hyperopic defocus. Alterations to the choroid occur prior to changes in eye growth (Wallman et al., 1995, Wildsoet and Wallman, 1995). When the choroid thins in form-deprived myopic eyes, the lymphatic vessels found on the scleral side of the choroid, as well as major choroidal blood vessels, are constricted (Liang, Crewther, Crewther, and Junghans, 2004; Rymer and Wildsoet, 2005). Contrastingly, when the choroid thickens in response to recovery from form deprivation or myopic defocus, the choroidal lymphatic vessels dilate (Liang et al., 2004; Wallman et al., 1995). Whether eye growth and choroidal changes are a part of the same or separate mechanisms is still actively debated.

However, the question prevails: What is it about vision that enables the eye to detect the sign of defocus? One possible answer is that the eye uses changes in the retinal image produced by the effects of defocus combined with chromatic aberrations in order to identify the refractive error.

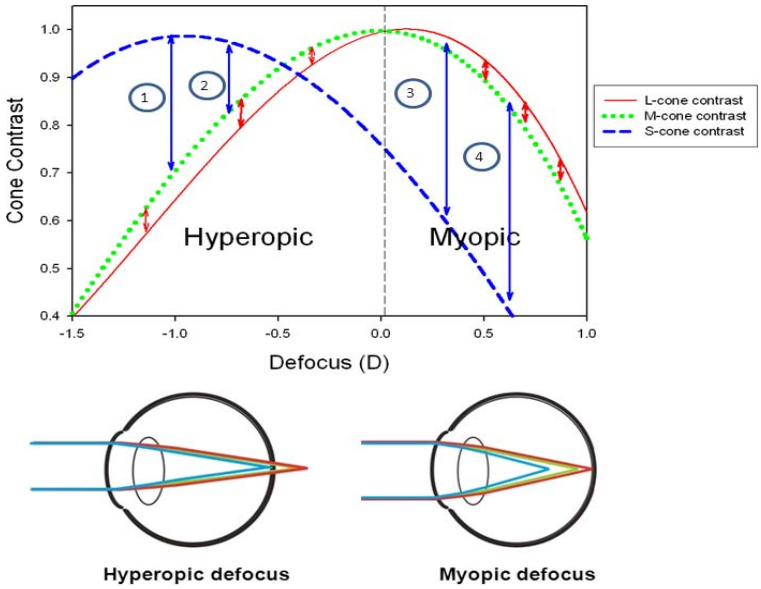


Figure 1. (Top) With hyperopic defocus, the ratio of S-cone contrast to L- or M-cone contrast (as shown by blue arrows 1 and 2) changes with the degree of defocus, creating chromatic change. With myopic defocus, this ratio, as seen with arrows 3 and 4, is constant creating primarily a change in luminance contrast. **(Bottom)** Longitudinal chromatic aberration produces dispersion that causes short wavelengths to be focused closer to retina with hyperopic defocus and long wavelengths focused closer to the retina with myopic defocus.

This theory was first introduced by Fincham in 1951. With longitudinal chromatic aberration (LCA), short-wavelength blue light has a shorter focal length than red light. As a result, it is focused closer to the lens than long-wavelength light. These differences in focus of the different wavelengths produce changes in color contrast with defocus. During hyperopic defocus, shorter wavelengths are focused closer to the retina, and therefore appear clearer than the longer wavelengths. However, during myopic defocus, shorter wavelengths are focused further in front of the retina and consequently appear more blurred than the longer wavelengths [Figure 1 (Top); Rucker and Wallman, 2012].

The difference in focus produced by the different wavelengths is reflected in the contrast detected by the retinal cones [Figure 1 (Top)]. Figure 1 shows the change in retinal image contrast of a black and white, 3 c/d, sine-wave grating, when viewed through a 3 mm pupil. In a hyperopically defocused eye, blue light is focused more accurately. As a result, the cone contrast of short-wavelength-sensitive cones (S-cones) is higher than that of the long-wavelength-sensitive cones (L- and M- cones). In a myopically defocused eye, red light is focused more accurately and therefore the L- and M- cones display higher contrast than S-cones. Furthermore, with hyperopic defocus, the ratio of the S-cone contrast to L- or M-cone contrast changes with the degree of defocus, creating a chromatic change (as represented by the difference in the length of blue arrows 1 and 2). Alternatively, with myopic defocus, this ratio (as represented by the length of arrows 3 and 4) remains relatively constant with the degree of defocus creating primarily a change in luminance contrast.

Luminance and Color Neural Pathways for Vision

As mentioned earlier, if the eye experiences hyperopic defocus, blue light would be focused closer to the retina, while red and green would be focused further behind the retina, and therefore appear relatively more blurred. Thus, with a repetitive sign-wave pattern with black and white bars, the blue component would be clear while the blurred red and green components would form a blurred, reddish fringe (Figure 2). This effect would cause the eye to elongate in order to focus the image on the retina (Rucker and Wallman, 2009). If the eye is myopically defocused, the longer wavelengths are focused closer to the retina, while the

shorter wavelengths are out of focus, thus creating blurred, bluish fringe. The eye will compensate in turn by becoming hyperopic.

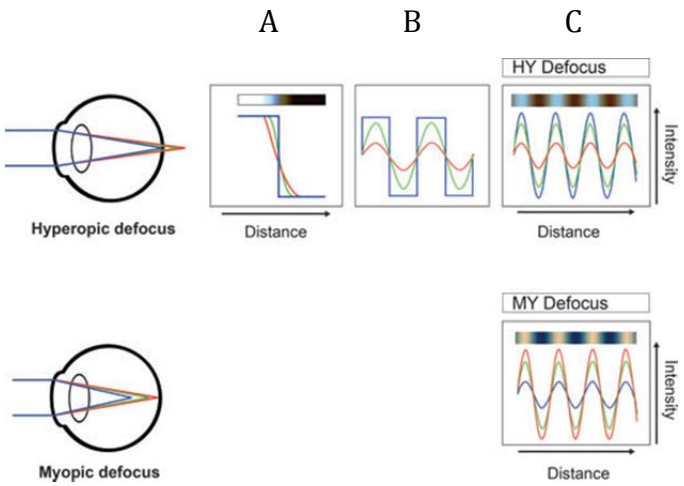


Figure 2. Effect of LCA on defocus and contrast. **(Top)** If the eye is focused on short wavelengths, longer wavelengths would be focused behind the retina and would produce a blurred retinal image. (A) a black/white edge will therefore be better defined by the short wavelengths; (B) if the pattern is a square wave, then the longer wavelengths may never reach maximum or minimum intensity; (C) if the pattern is a sine wave, in which the only change with defocus is a change in contrast, with hyperopic defocus the short-wavelength component will have a higher contrast than the longer-wavelength components (HY defocus). **(Bottom)** The reverse is true for myopic defocus (MY defocus).

If a color signal from the effects of LCA is used to determine the sign of defocus then the first criterion must be that it be large enough to trigger the emmetropization system. This theory is supported by several studies (Kröger and Wagner, 1996; Rucker and Wallman, 2008; Seidemann and Schaeffel, 2002). In these experiments, chicks and fish became more myopic in red than in blue light. Interestingly, Rucker and Wallman (2008) found that lens compensation in dim red (620 nm) and blue (460 nm) light produced different choroidal and ocular length compensatory responses. Blue light lens compensation was mainly due to

changes in eye length, whereas red light compensation was predominantly due to changes in choroidal thickness. With negative lenses, blue light caused an increase in eye length, while red light resulted in a decrease in choroidal thickness. The opposite effects occurred with positive lenses. These studies show that the eye can detect the difference in focus between red and blue light produced by LCA.

Do Color or Luminance Signals Control Emmetropization?

Rucker and Wallman (2012) tested the hypothesis that changes in a blue color signal cue hyperopic defocus, thus leading to a myopic shift in refraction, whereas changes in a red, or luminance, contrast signal cue myopic defocus, thereby causing a hyperopic shift in refraction. The authors exposed chicks to 2 Hz, 100% contrast, sinusoidal changes in either luminance or color contrast for three days. They discovered that luminance changes produced a hyperopic shift (2.01 D), while color flicker generated a myopic shift in refraction. Both refractive shifts occurred primarily due to changes in eye length. These results confirmed that the eye utilizes changes in luminance and color contrast to determine the emmetropization response.

Parasympathetic and Sympathetic Control of Color and Luminance Pathways

The parasympathetic system provides dominant innervation to the ciliary muscle (nearly 99%; Rosenfield and Gilmartin, 1998), which is mediated by acetylcholine (ACh) on muscarinic receptors (Gupta, Drance, McAllister, Prasad, Rootman, and Cynader, 1994; Pang, Matsumoto, Tamm, and DeSantis, 1994). ACh is released from autonomic axon terminals on

vascular smooth muscle, iris and ciliary muscles, as well as from intrinsic interneurons that form synapses within the retina. Two specific ACh receptors have been identified to which the molecule can bind: 1) ionotropic receptors that are selectively activated by nicotine-like ligands (nAChR) and 2) metabotropic receptors that are selectively activated by muscarinic-like ligands (muscarinic ACh receptors; mAChRs). Cholinergic autonomic innervation extrinsic to the eye originates from a population of post-ganglionic parasympathetic neurons originating from the ciliary ganglion and project to smooth muscles such as iris and ciliary muscles, to vessels of the choroid, and the intrinsic interneurons. Parasympathetic activity in the eye results in dilation of choroidal blood vessels, constriction of the pupil, and contraction of ciliary muscle (Millar, Ishimoto, Chubb, Epstein, Johnson, and Morgan, 1987; Millar, Ishimoto, Johnson, Epstein, Chubb, and Morgan, 1985). Five muscarinic receptor subtypes have been identified in humans, four of which are found in the eye. M1 through M4 are located in the ciliary muscle and iris sphincter. M2 and M3 are also found in the trabecular meshwork. Stimulation of M1 and M4 lead to a contraction of the ciliary muscle and therefore an increase in accommodation. Furthermore, stimulation of these receptors causes contraction of the iris sphincter leading to miosis (Bonner, Buckley, Young, and Brann, 1987; Gil, Krauss, Bogardus, and WoldeMussie, 1997; Peralta, Ashkenazi, Winslow, Smith, Ramachandran, and Capon, 1987). mACh receptor sites are also situated in various layers of the retina, such as the inner nuclear layer and among the amacrine cells located in the ganglion cell layer (Fischer, McKinnon, Nathanson, and Stell, 1998). In chicks, four avian mAChR subtypes have been characterized (Tietje and Nathanson, 1991). Eight years later,

Fisher et al. (1998) reported localization of mAChRs subtypes in the retina, choroid, ciliary body, retina pigment epithelium (RPE) and ciliary ganglion of the chick.

The sympathetic system innervates the ciliary muscle, iris dilator muscle and smooth muscle controlling vasoconstriction, through the action of the neurotransmitter noradrenaline. Noradrenaline is the hormone and neurotransmitter responsible for vigilant concentration, in contrast to its most chemically similar hormone, dopamine, which primarily controls cognitive alertness. Noradrenaline exerts its effect by increasing vascular tone (tension of vascular smooth muscle) through α-adrenergic receptor activation (Gilmartin, Bullimore, Rosenfield, Winn, and Owens, 1992). α-adrenoreceptors regulate contraction of the iris dilator muscle (mydriasis) as well as vascular changes that lead to ciliary-body volume changes (slight decrease in accommodation). Another subtype, β-adrenoceptors, consists of two further subtypes, β-1 and β-2. β-1 receptors are mainly found in cardiac tissues, leading to increases in heart rate and blood pressure. β-2 receptors induce smooth muscle relaxation in the lungs, gastrointestinal tract, and ciliary muscle. This relaxation of the ciliary muscle in turn causes a decrease in accommodation. β-agonists, such as isoprenaline, induce hyperopic shifts in accommodation, whereas β-antagonists, such as timolol, produce myopic shifts in accommodation (Kern, 1971; van Alphen, 1976).

The sympathetic innervation to the ciliary muscle plays a role in refractive changes (Gilmartin et al., 1992). Evidence of sympathetic inputs into the control of accommodation has largely been discovered from nerve stimulation in animal models. For example, Törnqvist (1967) demonstrated that cervical sympathetic nerve stimulation in monkeys led to hyperopic changes in their distance refraction. In agreement with Törnqvist (1967), Hubbard,

Robinson, Schmidt, Rohen Tamm, and Kaufman (1999) reported a 0.8 D myopic shift in distance refraction in monkeys with superior cervical ganglionectomy. This shift in refraction was independent of vascular changes and a direct consequence of β-adrenoceptors present in ciliary muscle. It is well established in the literature that dual parasympathetic and sympathetic innervation to the ciliary muscle occurs, consequently triggering the control of accommodation (Toates, 1972). During the course of a near task, the parasympathetic and sympathetic input act in a state of equilibrium in order to maintain sustained single, clear binocular vision without excessive fatigue (Gilmartin, 1986; Gilmartin and Bullimore, 1987). Therefore, several investigators (Ciuffreda and Lee, 2002; Ciuffreda and Wallis, 1998; Culhane and Winn, 1999; Gilmartin and Bullimore, 1987; Gilmartin and Hogan, 1985; McBrien and Millodot, 1986; Woung, Ukai, Tsuchiya, and Ishikawa, 1993) have proposed that an imbalance of the autonomic system may give rise to an anomalous accommodation response during or following sustained near work, thus resulting in myopia.

Molecular Pathways for the Color and Luminance Emmetropization Mechanisms

Since it has been established that the eye responds to environmental visual signals via sensitivity in change to luminance and color contrast, one of the major challenges remaining is to identify the biochemical mechanisms that translate retinal visual responses into molecular signals that regulate eye growth. One way in which this can be investigated is by blocking the parasympathetic system and sympathetic system via atropine and timolol, respectively.

Parasympathetic Blocking Agents

Atropine, a non-selective muscarinic antagonist, has been used historically in clinical treatments to slow myopia progression (Shih, Chen, Chou, Ho, Lin, and Hung, 1999; Yen, Liu, Kao, and Shiao, 1989). Atropine induces mydriasis by blocking contraction of the circular pupillary sphincter muscle, which is normally stimulated by acetylcholine release. Lack of acetylcholine allows the radial pupillary dilator muscle to contract and in turn dilate the pupil. Atropine induces cycloplegia by paralyzing the ciliary muscles and thereby inhibiting accommodation (Yen et al., 1989). Atropine has been shown to reduce myopia progression even in low dosages (0.05-0.1%) (Kennedy, Dyer, Kennedy, Parulkar, Kurland, Herman, McIntire, Jacobs, and Luepker, 2000; Lee, Fang, Yang, Chen, Lin, Lin, Kuo, and Wu, 2006; Shih et al., 1999). Although effective clinical dosages of atropine in slowing myopia progression have been determined, the molecular pathways driving these changes are still undefined (Walline et al., 2011).

Atropine's Mechanism of Action

Accommodation

Atropine's anti-myopiagenic effects were initially thought to work via cycloplegic action. It has been reported that an association exists between school-age myopia and near-work demands secondary to excessive accommodation (Fan, Lam, Chan, Fan, Cheung, and Rao, 2007; Hepsen, Evereklioglu, and Bayramlar, 2001; Saw, Chua, Hong, Wu, Chan, Chia, Stone, and Tan, 2002; Tan, Ng, Lim, Ong, Snodgrass, and Saw, 2000, Wallman and Winawer, 2004). This theory has been refuted, however, by animal studies showing that atropine was

effective in preventing form-deprivation myopia in chicks via a non-accommodative mechanism (Luft, Ming, and Stell, 2003; McBrien, Moghaddam, and Reeder, 1993). With optic nerve sectioning, lesioned chicks continued to emmetropize in response to myopic and hyperopic defocus (Troilo, 1990; Wallman and Adams, 1987). Lastly, Wildsoet (2003) observed that the eye was still able to emmetropize to negative lenses (-5 or -15 D) following ciliary nerve section while Schaeffel and Diether (1999) reported that chicks compensated for positive lenses via eye growth even though the lenses matched the accommodative plane. Therefore, it is now widely accepted that atropine does not exert its effect via accommodative changes.

Dopamine

In a study by Schwahn, Kaymak, and Schaeffel (2000) the authors hypothesized that atropine suppresses myopia by increasing dopamine (DA) release (a neuroretinal transmitter). They found that atropine-injected eyes exhibited an increase in the release of DA from retina and a concurrent reduction in eye growth. This supports the theory that atropine inhibits eye growth via dopamine release. Dopamine is thought to be one of the retinal neurotransmitters involved in a signaling cascade that controls eye growth by vision (Stone, Lin, Laties, and Iuvone, 1989). Multiple animal studies have shown that an inverse relationship between dopamine release and axial elongation exists. When dopamine levels increase, eye growth is inhibited (Dong, Zhi, Pan, Xie, Qin, Lu, Mao, Chen, Willcox, Qu, and Zhou, 2011; McBrien, Cottriall, and Annies, 2001; Papastergiou, Schmid, Laties, Pendrak, Lin, and Stone, 1998; Stone et al., 1989; Stone, Sugimoto, Capehart, Pendrak, and Lin, 2006). Furthermore, luminance flicker has been shown to increase dopamine release, thereby inhibiting eye

growth. In a study by Rohrer et al. (1995), the authors illustrated that form deprivation myopia can be prevented with 10 Hz stroboscopic flicker, presumably through the release of dopamine.

In summary, evidence has been presented that dopamine plays an important role in the development of experimentally-induced myopia. Although the mechanism by which this occurs is still unknown, the fact that atropine and luminance flicker causes an increase in dopamine release, along with eye growth inhibition, could provide a key piece of the puzzle in understanding the mechanism behind myopia progression.

The Role of Atropine in Animal Models of Myopia Development

Several studies have investigated the role of atropine in emmetropization. McBrien et al. (1993) determined that monocularly form-deprived chicks, injected with atropine, exhibited a nearly seven-fold reduction in myopia compared to saline injections (-2.8 D vs. -20.8 D, respectively) mainly due to a decrease in axial length. This observation is consistent with other reports stating that both atropine and other anti-muscarinic drugs inhibit form-deprivation myopia in chicks (Cottriall, McBrien, Annies, and Leech, 1999; Leech, Cottriall, and McBrien, 1995; Schmid and Wildsoet, 2004; Schwahn et al., 2000; Stone, Lin, and Laties, 1991). Luft et al. (2003) obtained similar results when injecting form-deprived chick eyes with atropine (an eight-fold reduction in refractive error). Furthermore, chicks injected with atropine and wearing negative lenses (hyperopic defocus) exhibited less myopia in conjunction with transient choroidal thickening (Nickla, Zhu, and Wallman, 2013). In combination, the data suggest that atropine inhibits myopia progression by slowing eye growth and reducing axial elongation.

Sympathetic Blocking Agents

Timolol and Myopia

Timolol, a non-selective beta-adrenoceptor antagonist, has been utilized as a hypotensive drug for several decades (Schwartz, Takamoto, and Lavin, 1995; Vogel, Crick, Mills, Reynolds, Sass, Clineschmidt, and Tipping, 1992). Many studies have reported that IOP readings are higher in human myopes than emmetropes (David, Zangwill, Tessler, and Yassur, 1985; Quinn, Berlin, Young, Ziylan, and Stone, 1995; Tomlinson and Phillips, 1972), although the differences are small (2 mm Hg). It has been suggested that excessive eye enlargement could lead to myopia if the scleral walls of such eyes were equally or more susceptible than emmetropic eyes to stretch under the influence of IOP increase. Therefore, it is logical to postulate that IOP-lowering drugs should reduce or prevent eye enlargement and thus myopia development and/or progression. One such study by Goldschmidt, Jensen, Marushak, and Ostergaard (1985) discovered a correlation between reductions in IOP and decrease in the rate of myopia progression. In contrast, Jensen (1991) and Hosaka (1988) found no effect of timolol on myopia progression even though timolol was reported by Jensen (1991) to be effective at lowering the IOP (3 mm Hg reduction). In a more recent study by Schmid, Abbott, Humphries, Pyne, and Wildsoet (2000), the authors observed no change in form-deprivation or lens-induced myopia in chicks, even with significant IOP reduction. The inconsistent results of these studies beg further study of the mechanism of action of timolol on eye development.

Innervation of the Choroid

The choroid is comprised of the choriocapillaries and the larger blood vessels and lacunae (Wallman et al., 1995). The main function of the choroid is to provide nourishment and oxygen to the outer portion of the retina including the photoreceptors and the RPE. The choroid also plays a role in regulating intra-ocular pressure through the uveoscleral pathway (De Stefano and Mugnaini, 1997). Tissue fluids can be filtered out of the capillary endothelium or reabsorbed into the capillaries themselves, depending on changes of the hydrostatic pressure gradient between the choroidal tissues and blood vessels. The choroid receives autonomic input from ciliary, superior cervical, and pterygopalatine ganglia as well as from trigeminal, facial and oculomotor nerves (Mann, Riva, Stone, Barnes, and Cranstoun, 1995). Parasympathetic innervation leads to vasodilation of the choroidal vessels while sympathetic innervation leads to vasoconstriction in the avian choroid (Bill, 1995).

Neural Pathways for Myopia Development

In order to elucidate the mechanism behind the effect of muscarinic antagonists, Nickla and Schroedl (2012) performed a double pterygopalatine plus ciliary ganglionectomy (PPG/CGX), which severs the main parasympathetic input to the retina and choroid, on week-old chicks. Their eyes were either form-deprived or fitted with +10 D or -10 D lenses for four days. The results showed that complete parasympathetic blockade (PPG/CGX) prevented eyes from developing myopia in response to form deprivation by inhibiting eye growth, yet had no effect on chicks wearing negative lenses. The lesions also prevented the normal choroidal-thickening response to myopic defocus. PPGX alone, without ciliary

ganglionectomy, had no effect on the development of myopia or axial elongation but did prevent choroidal thickening.

In contrast to PPG/CGX, sectioning of the superior cervical ganglion (SCG) in conjunction with the pterygopalatine ganglion (PPG) prevented negative-lens compensation, but not positive-lens compensation (Nickla and Schroedl, 2012). The SCG is the major ganglion for the sympathetic nerve supply to the head and eye, and provides adrenergic input to the choroid. Lastly, both PPGX and PPG/SCGX inhibited choroidal thickening in response to myopic defocus (positive lens). Lesions of the SGC alone did not induce this change. As the loss of PPG occurred in all three groups, a role for pterygopalatine ganglia in choroidal changes is plausible (Nickla and Schroedl, 2012).

In summary, total parasympathetic blockade (PPG/CGX) prevents form deprivation myopia and positive lens compensation, but not negative lens compensation. PPGX alone prevents choroidal thickening. Sympathetic blockade (SCGX) prevents negative lens compensation but not positive lens compensation. These results suggest that the molecular mechanisms underlying the responses to form deprivation, negative lenses and positive lenses are fundamentally different.

Purpose of Study

In this study, we analyzed the role of the parasympathetic and sympathetic nervous systems on the color and luminance emmetropization pathways. We determined the response to the non-selective parasympathetic antagonist, atropine, and the non-selective sympatholytic drug, timolol, in chicks exposed to illumination conditions that selectively stimulate the color and luminance pathways. Our experimental design represents an effective model system for investigating myopia progression under controlled illumination conditions. The results allow us to gain a better understanding of the molecular substrates involved in controlling the color and luminance emmetropization responses, thereby providing new insights into the biochemical pathways involved in myopia progression.

We hypothesize that the atropine-induced reduction in eye growth will be enhanced by luminance flicker due to the additional release of dopamine, whereas timolol will tend to counteract the effect of luminance flicker, resulting in a myopic shift.

Materials and Methods

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 hatching, the chicks were housed in 12 hour light/12 hour 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 ARVO Resolution for the Care and Use of Animal Research.

Experimental Design

Illuminance Conditions

Chicks were free-roaming in a 32 x 20 inch wire cage that was illuminated with a computer-controlled, sinusoidally-modulated light source (mean 680 lux) using light-emitting diodes (LED) 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 degrees). Three illumination conditions were used: steady light, luminance flicker or blue/yellow color flicker. 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 BuckPucks (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) LEDS. 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 $\mu W/cm^2$ for red, green and blue. Intensity was controlled with neural density filters to maintain a mean illuminance (equivalent to 680 human lux, Center Lightmeter 337).

Procedure (Atropine and Timolol Experiments)

Chicks were exposed (both eyes) to their assigned illuminance condition for four days, eight hours a 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 and Wallman, 1998). Measurements were taken prior to and immediately following the experimental period (after four days). Pupil reactions, accommodation fluctuations, and refraction were observed with an infrared photorefractor according to Schaeffel, Farkas, and Howland (1987) and 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.

Experimental Groups

For both the atropine and timolol experiments, chicks were divided into seven groups; each group containing eight chicks chosen at random. In the atropine experiment, chicks in each experimental group received either 20 µl atropine (18 nmol) or 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. Chicks with even-numbered tags received injections in the right eye, while chicks with odd-numbered tags were injected in their left eye (chicks were tagged at random). In the timolol experiment, chicks in each experimental group received either two drops of 0.5% timolol or 2 drops of distilled H_2O in one eye mid-morning under anesthesia (isofluorane 1.5-2%) using an eye dropper (based on Schmid et al., 2000). The following experimental conditions were employed:

Experiment 1: Atropine Experiment

Group 1 (Atropine + LUM flicker): Chicks received 20 µl intravitreal injections of atropine and exposed to luminance flicker (N = 8).

Group 2 (Atropine + Color Flicker): Chicks were injected as in Group 1 but were exposed to color flicker (N = 8).

Group 3 (Saline + LUM Flicker): Chicks received intravitreal injections of 20 µl phosphate-buffered saline and exposed to luminance flicker (N = 8).

Group 4 (Saline + Color Flicker): Chicks received intravitreal injections of 20 µl phosphate-buffered saline and exposed to color flicker (N = 8).

Group 5 (Atropine + Steady Light): Chicks were injected intravitreally with atropine but without flicker exposure (N = 8).

Group 6 (Saline + Steady Light): Chicks were injected with saline, as in Group 4, without flicker exposure (N = 8).

Experiment 2: Timolol Experiment

Protocols for timolol concentrations and administrations followed those of Schmid et al., (2000). The illumination conditions are listed below:

Group 1 (Timolol + LUM flicker): Two drops of 0.5% timolol were applied topically twice a day at 10 am and 4 pm throughout the treatment period. Chicks were exposed to luminance flicker (N = 8).

Group 2 (Timolol + Color Flicker): Chicks received 0.5% timolol drops as in Group 1 but were exposed to color flicker (N = 8).

Group 3 (Water + LUM Flicker): Two drops of distilled water (control) were applied topically twice a day at 10 am and 4 pm throughout the treatment period. Chicks were exposed to luminance flicker (N = 8).

Group 4 (Water + Color Flicker): Two drops of distilled water were applied as in Group 3. Chicks were exposed to color flicker (N = 8).

Group 5 (Timolol + Steady Light): Two drops of 0.5% timolol were applied. Chicks were exposed to steady light (N = 8).

Group 6 (Water + Steady Light): Two drops of distilled water were applied. Chicks were exposed to steady light (N = 8).

Experiment 3: Transient Choroid Experiment

For the transient choroid experiment, chicks were either exposed to luminance or color flicker with no-injection or exposed to steady light with atropine-injections for a three-hour period. Choroidal measurements were taken using A-scan ultrasonography before (11: 00 am) and again after the experimental period (2:00 pm). Eight birds were used in each illumination condition and exposed to the same living conditions as in the atropine and timolol experiments.

Data Analysis

Variations between the drug (atropine or timolol) and control (saline or water) conditions were determined. Within each illumination condition, the change in the fellow eye (ΔN) was subtracted from the change in the treated eye (ΔX) in both the drug and saline/H_2O conditions. The mean change in the saline/H_2O treated eyes was then subtracted from the change in each of the drug-treated eyes [Drug (ΔX-ΔN) – Mean Saline/H_2O (ΔX-ΔN)] (Figure 3). In previous experiments (Nickla et al., 2013) a single saline control had been used for comparison with drug-injected eyes. In this experiment, data for a saline control has been collected for each illumination condition. By subtracting the change in the saline control from the change in the experimental animal, it is possible to compare the effect of the drug across all illumination conditions.

In order to examine the effects within each drug or illumination condition, the changes in ocular components between the experimental and fellow eyes were compared by two-way ANOVA in Datadesk after correction for saline injection/H_2O drop effects. The interaction of

the drug and illumination condition was subsequently examined by comparing the effect of the drug on the injected and fellow eyes using two-way ANOVA. *t*-tests were performed if the F value was found to be significant.

	LUM	Color	Steady
Δ Drug 5 – 10 days old			
—	(ΔX – ΔN)	(ΔX – ΔN)	(ΔX – ΔN)
Δ Saline/H_2O 5 – 10 days old			
	Mean(ΔX – ΔN)	Mean(ΔX – ΔN)	Mean(ΔX – ΔN)
=	Δ Drug – Δ Mean Saline/H_2O		

Figure 3. Graphical representation of data analysis.

Results

Effects of Atropine

Anterior Eye

Atropine exerted one of its biggest effects on inter-ocular lens changes. Our data demonstrate that lens thickness was strongly influenced by an interaction between the drug and lighting condition [Figure 4 (Top)]. With luminance flicker, chicks with atropine injections displayed significant thickening of the lens in experimental eyes compared to fellow eyes (0.140 ± 0.049 mm, $p = 0.02$), which corresponded with a decrease in the anterior-chamber depth (-0.063 ± 0.029 mm, $p = 0.1$). In contrast, steady and color flicker demonstrated a trend of increased anterior-chamber length (steady: 0.051 ± 0.040 mm, $p = 0.25$; color: 0.057 ± 0.029 mm, $p = 0.8$) along with a decrease in lens thickness (steady: -0.170 ± 0.076 mm, $p = 0.59$; color: -0.085 ± 0.049 mm, $p = 0.11$) (Table 1).

Posterior Eye

Atropine induced a hyperopic shift in refraction through a reduction in eye growth. Eyes exposed to luminance flicker were three times more hyperopic (3.4 D) compared to eyes exposed to color flicker (0.85 D) and steady light (1.23 D) ($p < 0.05$). Atropine-injected eyes under luminance conditions exhibited a significant decrease in both the vitreous (-0.095 ± 0.023 mm, $p = 0.005$) and eye length (-0.078 ± 0.021 mm, $p = 0.007$). Atropine-injected eyes under color flicker conditions also exhibited a decrease in eye growth (Color: -0.054 ± 0.017 mm, $p = 0.01$), but not vitreal or refraction changes, partially due to choroidal compensation [Figure 4 (Top)] (Table 1). Color flicker with atropine was the only illumination condition to show a significant decrease in choroidal thickness. With color flicker choroids thinned by -

0.048 ± 0.019 mm (p = 0.03), slightly more than with luminance flicker (-0.034 ± 0.016 mm, p = 0.067) and steady light (0.024 ± 0.017 mm, p = 0.20) (Table 1).

Effects of Timolol

Anterior Eye

Timolol treatment demonstrated no significant change of the anterior segment of the eye for any of the illumination conditions. Anterior chamber depth increased slightly in luminance flicker (0.030 ± 0.031 mm, p = 0.35) and steady light (0.043 ± 0.037 mm, p = 0.28), but there was little change with color flicker (-0.009 ± 0.04 mm, p = 0.83).

Posterior Eye

When eyes were exposed to luminance flicker, timolol had the opposite effect on refraction and eye growth compared to color flicker and steady light. A significant myopic shift in refraction was seen with LUM (-4.07 ± 0.92 D, p = 0.001), which coincided with a small increase in eye length (0.045 ± 0.030 mm, p = 0.17). 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: -0.051± 0.018 mm, p = 0.02; Steady: -0.047 ± 0.018 mm, p = 0.01) [Figure 5 (Top)]. This is due to the fact that no significant changes in vitreous-chamber depth took place, partially because the choroid thinned in the color condition 0.04 ± 0.034 mm, p = 0.27) (Table 2).

Interactive Effects of Drugs and Lighting

Refraction and eye length changes revealed an interaction between the lighting and drug conditions (ANOVA: Rx: $p = 0.0133$, AL: $p = 0.04$ and Vit: $p = 0.0504$). Firstly, the drug effects were predominantly seen with luminance flicker, with the combination of luminance flicker and atropine producing a significantly shorter eye than luminance flicker and timolol. When chicks were exposed to luminance flicker, timolol rendered the eyes more myopic than atropine (Atropine: 3.40 ± 1.77 D; Timolol: -4.07 ± 0.92 D; Diff: -7.47 ± 1.49 D, $p < 0.001$), with less growth (Atropine: -0.078 ± 0.021 mm, $p = 0.067$; Timolol: 0.045 ± 0.030 mm, $p = 0.17$; Diff: 0.123 ± 0.026 mm, $p = 0.024$) and a greater reduction in vitreous chamber depth (Atropine: -0.095 ± 0.023 mm, $p = 0.005$; Timolol: 0.045 ± 0.031 mm, $p = 0.18$; Diff: 0.14 ± 0.027, $p = 0.04$) in atropine-injected eyes.

Secondly, the type of lighting condition, specifically luminance flicker, proved important in producing the changes in eye length, choroidal thickness and lens thickness. Timolol-treated eyes grew more with luminance flicker than with color flicker (LUM: 0.045 ± 0.030, $p = 0.17$; Color: -0.05 ± 0.02, $p = 0.02$) making the eye more myopic (LUM: -4.07 ± 0.92 D, $p = 0.001$; Color: -0.57 ± 0.84 D, $p = 0.51$; Diff: -3.49 ± 1.36 D, $p = 0.045$). Atropine-treated eyes showed more lens thickening with luminance flicker than color flicker (ANOVA: $p = 0.0024$; Diff: 0.14 ± 0.05 mm, $p = 0.0046$) or steady light (Diff: -0.19 ± 0.05; $p = 0.027$).

Color flicker induced choroidal thinning in both atropine and timolol treated eyes (ANOVA: $p = 0.007$) (Atropine: -0.048 ± 0.019, $p = 0.03$; Timolol: -0.05 ± 0.0, $p = 0.01$) (Tables 1 and 2).

Saline Injections (Atropine Experiment)

Saline injections produced a minimal effect on all ocular components in all three illumination conditions [Figure 4 (Bottom)]. However, injected eyes exposed to the LUM condition became more myopic compared to fellow eyes (-2.68 ± 0.96 D, $p = 0.02$) without an increase in eye length. In other conditions, the injected eye was smaller than the fellow eye, particularly in eyes exposed to color flicker (AL: -0.139 ± 0.025 mm, $p < 0.001$; Vit: -0.075 ± 0.019 mm, $p = 0.002$). Since both eyes were exposed to the same illuminant, the relative myopic shift in LUM, and the relative decrease in eye size in color, can likely be attributed to a combined injection effect and illumination effect (Table 3).

Distilled Water Drops (Timolol Experiment)

Treatment with water drops also led to minimal effects on the ocular components. In steady light conditions, the vitreous of the experimental eye was longer than the control eye (0.06 ± 0.026 mm, $p = 0.045$) probably as a result of the small increase in eye length combined with the small decrease in choroidal thickness [Figure 5 (Bottom)]. No injection was given in this experiment and there is no obvious explanation why without flicker, in the steady light condition, greater than normal eye growth was observed [Figure 5 (Bottom)] (Table 4).

Transient Choroid Experiment

There was no significant change in transient choroid thickness measured over a three-hour period in chicks without injection under luminance or color flicker conditions. (LUM:

0.012 ± 0.018, p = 0.50; Color: -0.016 ± 0.027 mm, p = 0.562), as well as chicks with atropine-injected eyes under steady light conditions (0.007 ± 0.046 mm, p = 0.852).

Correlation Between Choroid and Eye Length

No correlation was found between choroid and axial length for LUM and color flicker in 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 (Figure 6).

Correlation Between Refractive Error and Eye Length

No correlation was found between refractive error and axial length for LUM and color flicker in 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 (Figure 7).

Tables of Ocular Component Values

Atropine

	CAC	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
Steady	0.051 ± 0.040 p = 0.25	-0.170 ± 0.076 p = 0.59	0.022 ± 0.072 p = 0.77	0.024 ± 0.017 p = 0.20	-0.062 ± 0.039 p =0.16	0.85 ± 1.14 p = 0.48
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

Table 1. P-values of measured ocular components in atropine-injected chick eyes under LUM, steady and color-flicker conditions. Significant values are indicated in red.

Timolol

	CAC	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
Steady	0.043 ± 0.037 p = 0.28	-0.055 ± 0.037 p = 0.18	-0.036 ± 0.017 p = 0.07	0.00 ± 0.016 p = 0.98	**-0.047 ± 0.013** p = 0.01	-0.94 ± 1.03 p = 0.39
Color	-0.009 ± 0.04 p = 0.83	-0.05 ± 0.06 p = 0.39	0.04 ± 0.034 p = 0.27	**-0.05 ± 0.01** p = 0.01	**-0.05 ± 0.02** p = 0.02	-0.57 ± 0.84 p = 0.51

Table 2. P-values of measured ocular components in timolol-injected chick eyes under LUM, steady and color-flicker conditions. Significant values are indicated in red.

Saline

	CAC	Lens	Vit	Choroid	AL	RE
LUM	-0.034 ± 0.080 p = 0.67	-0.046 ± 0.092 p = 0.61	-0.019 ± 0.044 p = 0.67	0.015 ± 0.017 p = 0.40	-0.06 ± 0.04 p = 0.15	**-2.68** **± 0.96** **p = 0.02**
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
Color	-0.041 ± 0.026 p = 0.132	-0.014 ± 0.042 p = 0.742	**-0.075** **± 0.019** **p = 0.002**	-0.006 ± 0.018 p = 0.75	**-0.139** **± 0.025** **p = 0.000**	-0.65 ±1.07 p = 0.56

Table 3. P-values of measured ocular components in saline-injected chick eyes under LUM, steady and color-flicker conditions. Significant values are indicated in red.

Distilled Water

	CAC	Lens	Vit	Choroid	AL	RE
LUM	-0.008 ± 0.045 p = 0.86	-0.041 ± 0.060 p = 0.49	-0.024 ± 0.040 p = 0.54	0.010 ± 0.020 p = 0.60	-0.046 ± 0.037 p = 0.24	2.57 ± 1.94 p = 0.21
Steady	0.050 ± 0.06 p = 0.39	-0.045 ± 0.063 p = 0.47	**0.06** **± 0.026** p = 0.045	-0.023 ± 0.034 p = 0.77	0.032 ± 0.07 p = 0.24	-2.13 ± 2.03 p = 0.30
Color	-0.050 ± 0.065 p = 0.443	0.06 ± 0.082 p = 0.47	-0.03 ± 0.05 p = 0.50	0.032 ± 0.017 p = 0.08	0.003 ± 0.028 p = 0.91	-0.327 ± 2.69 p = 0.90

Table 4. P-values of measured ocular components in distilled water-treated chick eyes under LUM, steady and color-flicker conditions. Significant values are indicated in red.

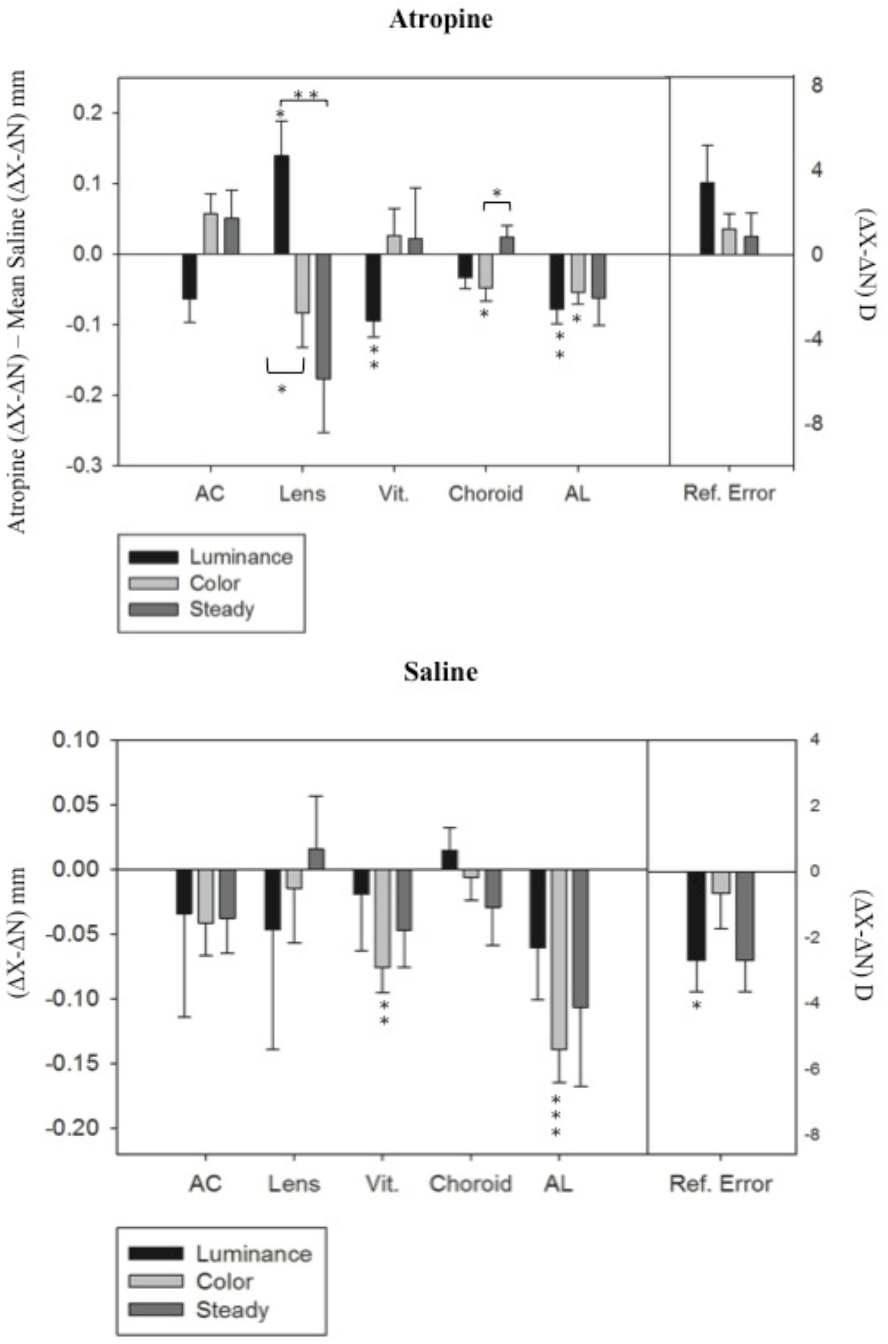


Figure 4. (Top) Mean change in ocular components with atropine over a four-day period. Eyes injected with atropine showed a significant reduction in axial length as well as vitreal chamber depth, resulting in a trend towards a hyperopic shift. An increase in lens thickness was also seen with LUM flicker. Color flicker produced a reduction in eye length and significant choroidal thinning, preventing a change in vitreous-chamber depth. *$p < 0.05$, **$p < 0.01$. **(Bottom)** Mean change in ocular components with saline over a four-day period. Saline-injected eyes demonstrated a reduction in eye growth, though the injected-eyes showed a myopic shift in refraction, particularly with LUM flicker. These results can be attributed to an injection effect in the particular illumination condition. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

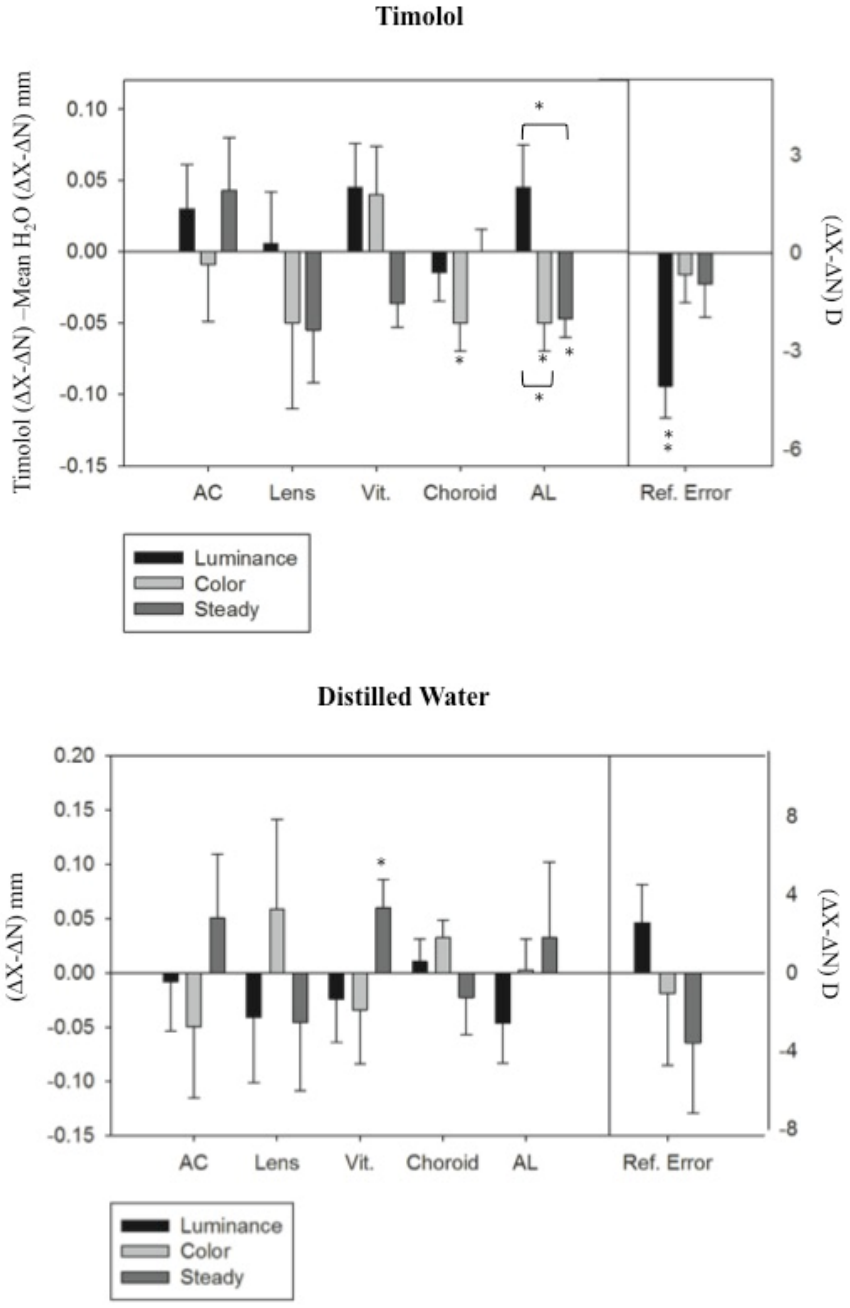


Figure 5. (Top) Mean change in ocular components with timolol over a four-day period. Timolol-injected eyes exposed to LUM flicker produced an increase in axial length and myopic shift in refraction. Color flicker and steady light caused a reduction in eye length that was compensated for by choroidal thinning in the color condition. *$p < 0.05$, **$p < 0.01$. **(Bottom)** Mean change in ocular components with distilled water over a four-day period. Eyes injected with distilled water had very little effect on ocular components. In steady light, a small increase in eye length combined with choroidal thinning produced a significant increase in vitreous chamber depth. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

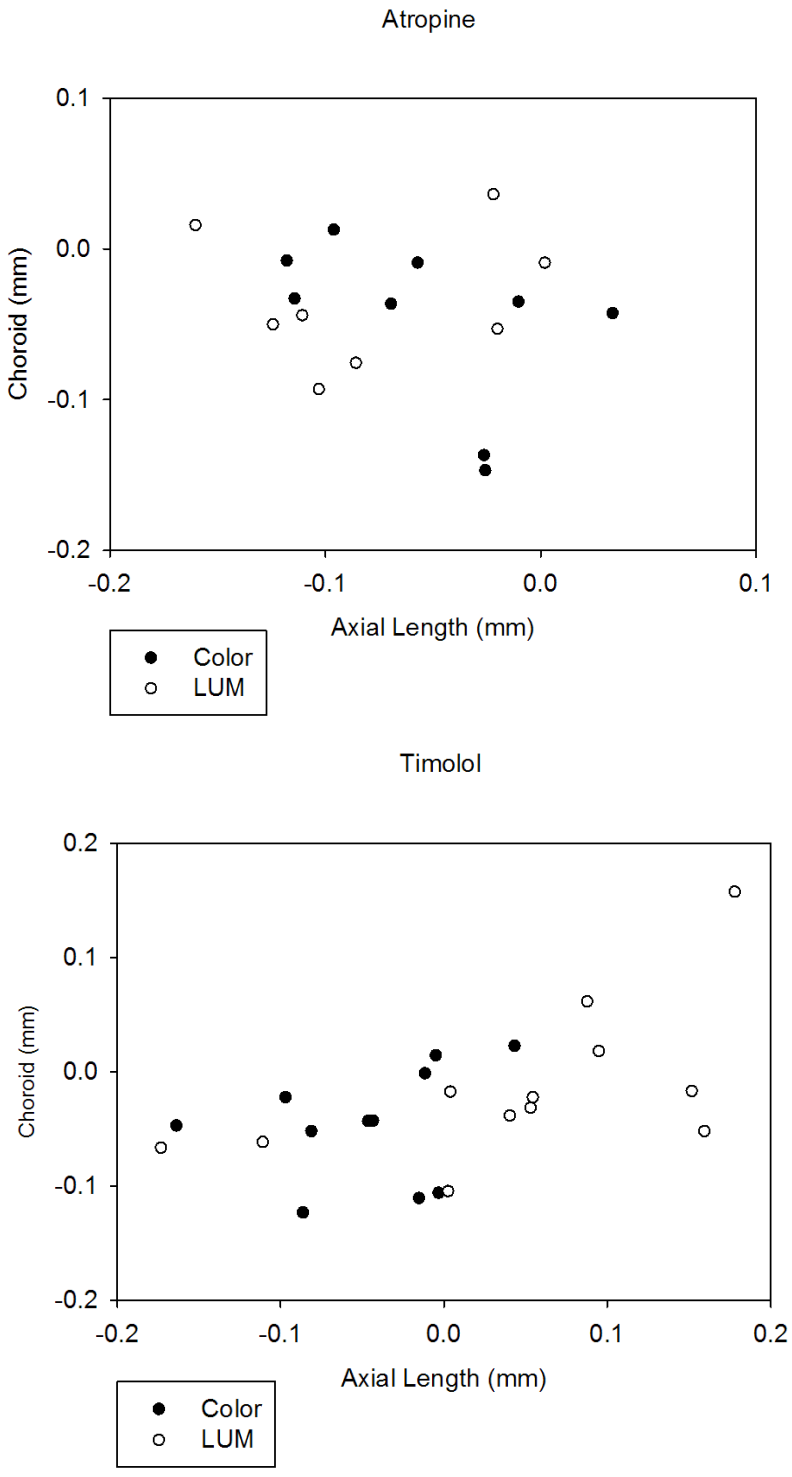
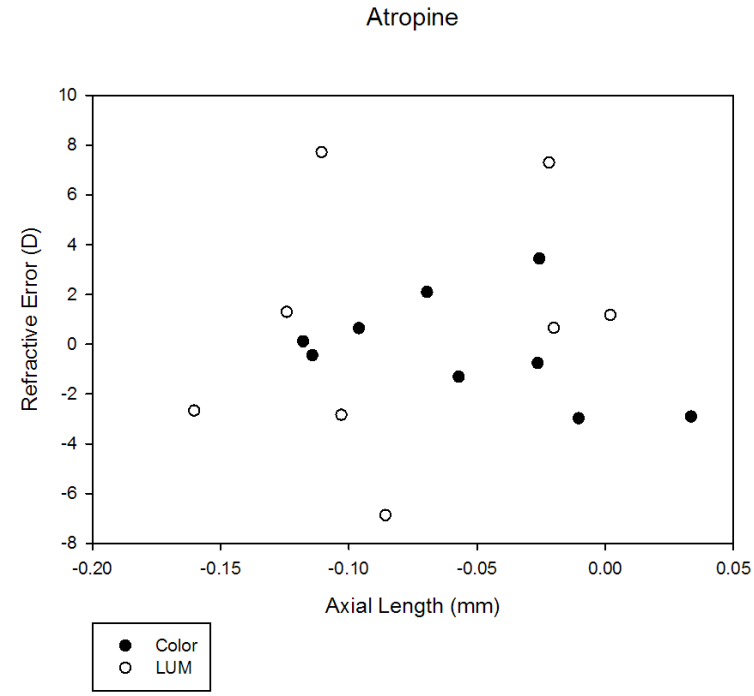


Figure 6. Correlation between choroid and axial length for LUM and color flicker for atropine and timolol experiments. For all conditions, choroid and axial length demonstrated no significant correlation.



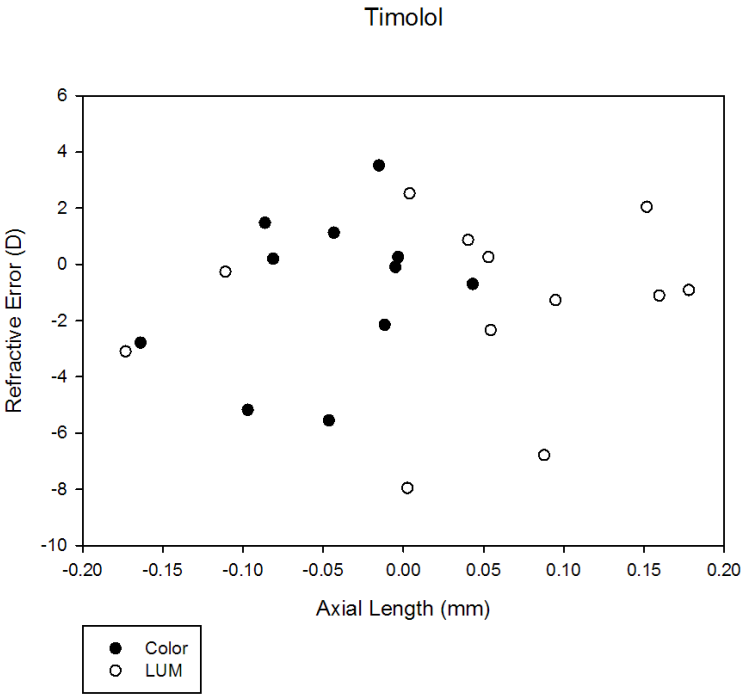


Figure 7. Correlation between refractive error and axial length for LUM and color flicker for atropine and timolol experiments. For all conditions, refractive error and axial length demonstrated no significant correlation.

Discussion

Atropine has been used as a way to help prevent myopia progression for decades. Past studies have shown that atropine is effective in reducing myopia progression in young children (Chia, Chua, Cheung, Wong, Lingham, Fong, and Tan, 2012; Wu et al., 2011). Our data support this hypothesis, since atropine-injected eyes showed a reduction in eye growth and a hyperopic shift compared to the fellow eyes, with both color and luminance changes. On the other hand, timolol caused an increase in growth with luminance flicker and more myopic refractions, but a decrease in growth with color flicker. Therefore, these findings suggest that while the environmental stimulation from changes in luminance contrast can enhance the protective effect of atropine, they can also increase the risk of myopia with timolol. Color changes are protective with both drugs. Thus the risk of myopia is affected by the activity of the parasympathetic and sympathetic β receptors when the eye is stimulated with changes in luminance contrast. Interestingly, with steady light only timolol caused a reduction in eye length suggesting a benefit in increasing retinal stimulation when prescribing atropine.

Why would luminance flicker enhance the effect of atropine? Luminance flicker in the form of stroboscopic illumination upregulates the expression of the *c-fos* gene in the retina. This gene promotes expression of tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis (Rohrer, Iuvone, and Stell, 1995). The association of dopamine with myopia has been forged as a result of the finding that form-deprivation myopia (FDM) coincides with a reduction in retinal dopamine levels and an increase in ocular elongation (Stone et al., 1989). FDM can also be prevented with 10 Hz stroboscopic flicker (Rohrer et

al., 1995) presumably through the release of dopamine. Indeed, apomorphine, a dopamine agonist, has also been shown to inhibit myopia progression (Feldkaemper and Schaeffel, 2013; Chuang 2014). Although only limited information exists on how atropine achieves its anti-myopia effects in the eye, it has been proposed that atropine inhibits eye growth via stimulation of retinal dopamine release (Schwahn et al., 2000) and/or by action on the sclera (Lind, Chew, Marzani, and Wallman, 1998; Tigges, Iuvone, Fernandes, Sugrue, Mallorga, Laties, and Stone, 1999). The combined release of dopamine from the actions of atropine and luminance flicker would be expected to have a greater inhibitory effect on eye growth.

As discussed in the Introduction, past studies have agreed with the current results in that they have linked atropine's anti-myopia effect with a reduction in eye length and changes in anterior-chamber depth. Atropine-injected eyes showed a significant increase in lens thickness under luminance conditions compared to fellow eyes, as well as a decrease in anterior chamber depth. 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. It remains to be determined whether the lens thickening preceded the changes in eye length, or whether they occurred in response to the relaxation of the lens zonules in a smaller eye.

In our study, we found no significant correlation between axial length and refractive error. This may be due to high variability in refractions or changes in corneal curvature and movement of lens thickness, which were not measured.

Choroidal Changes

Two general theories exist regarding the mechanisms behind the defocus-induced changes in choroidal thickness. One possibility is that signaling proceeds with paracrine molecule messengers (Morgan, 2003; Rymer and Wildsoet, 2005; Wallman and Winawer, 2004) such as nitric oxide (NO) via the parasympathetic system, and neuroadrenaline via the sympathetic system (Fischer and Stell, 1999; Fujikado, Kawasaki, Fujii, Taniguchi, Okada, Suzuki, Ohmi, and Tano, 1997; Nickla and Wildsoet, 2004; Nickla, Wilken, Lytle, Yom, and Mertz, 2006), causing vasodilation and vasoconstriction, respectively. A second possibility suggests that light-stimulus-driven changes in the ionic (potassium, sodium chloride, and calcium) environment around the photoreceptors alter the distribution of ions across the retina, choroid and sclera. These events directly control the rate and direction of transretinal fluid flow and thus choroidal thickness (Crewther, Barutchu, Murphy, and Crewther, 2006; Crewther, Murphy, and Crewther, 2008; Goodyear, Crewther, and Junghans, 2009; Goodyear, Junghans, Giummarra, Murphy, Crewther, and Crewther, 2008). In support of the first theory, Lovasik, Kergoat, and Wajszilber (2000) found that blue flicker, which stimulated rod activity, led to an attenuated subfoveal choroidal blood flow, via vasoconstriction of choroidal blood vessels. Although not stated specifically in this study, it is well known that vasoconstriction of choroidal blood vessels is due to stimulation of sympathetic system via release of the neurotransmitter, noradrenaline (Gilmartin et al., 1992). In support of the latter, Liang et al. (2004) discovered a significant difference in relative concentrations of Na and Cl ions in the outer retina, RPE and choroid between FD myopic eyes and fellow non-deprived eyes. It is still unclear if one or both mechanisms are involved in choroidal changes.

Studies have explored the effects of atropine on choroidal thickness. Nickla et al. (2013) reported that atropine injections caused axial-length inhibition along with transient choroidal thickening, with normal choroidal thinning after three hours of injection. Our experiments, however, found no evidence of transient choroidal thickening with atropine, but instead a significant choroidal thinning with color flicker.

These types of observations have led to a noteworthy debate in recent years as to whether eye growth and choroidal thinning are controlled by the same mechanism. Past studies have suggested an association between choroidal thickening and ocular growth inhibition (Nickla and Schroedl, 2012; Nickla and Wallman, 2010). However, other experiments have shown a dissociation of the choroidal- and axial-length changes under specific visual conditions (McLean & Wallman, 2003; Park, Winawer, and Wallman, 2003; Rucker and Wallman, 2012; Wildsoet, 2003; Winawer & Wallman, 2002). Rucker and Wallman (2012) exposed chicks to 2 Hz luminance flicker and showed a decrease in axial length compared to steady light, yet demonstrated an increase in choroidal thinning. The current study supports the latter theory that axial length and choroidal thinning are part of two separate mechanisms (Figure 6).

Lastly, β-2 adrenergic receptors have been demonstrated in the human optic nerve (Dawidek and Robinson, 1993) as well as in choroidal and retinal blood vessels (Grajewski, Ferrari-Dileo, Feuer, and Anderson, 1991). Blockade of these receptors can cause vasodilation **(**van Buskirk, Bacon, and Fahrenbach, 1999). Past studies have shown the β-adrenergic blockers, such as betaxolol and levobunolol, exert vasodilatory effects on retinal vessels and increase pulsatile ocular blood flow in ocular hypertensive patients (Krakow,

1992; Langham, 1987; Morsman, Bosem, Lusky, and Weinreb, 1995; Silver, Farrell, Langham, O'Brien, and Schilder, 1989). In regards to timolol, however, findings are inconsistent. While some researchers note increases in retinal vein diameter (Schwartz et al., 1995) most studies have found that timolol treatment causes a decrease in retinal vessel diameter (vasoconstriction) (Martin and Rabineau, 1989; Yoshida, Feke, Ogasawara, Goger, Murray, and McMeel, 1991) as well as reduction in choroidal blood flow (Yoshida et al., 1991).

We propose that both drugs cause release of dopamine. With timolol, color flicker causes a reduction in eye length and choroidal thinning. Similar changes were also seen with luminance flicker in both atropine-injected and apomorphine-injected eyes (Chuang, 2014). This combination of eye length reduction and choroidal thinning would thus be characteristic of a dopamine-related response. These results therefore suggest an alternative treatment mechanism through which color stimulation of the retina combined with timolol application can also protect against myopia.

Timolol–Induced Changes

Timolol induces opposite effects on eye growth with luminance flicker as compared to atropine. However, contrary to atropine, these changes were seen in only the posterior portion of the eye. Luminance flicker combined with timolol resulted in a myopic shift in refraction due to an increase in eye length. We postulate that the blockage of the sympathetic β receptors also blocks the dopamine response, collectively resulting in an increase in eye growth and concurrent myopic shift. In support of this theory, Chuang's study (2014)

illustrated a significant increase in eye length in chick eyes injected with spiperone, a dopamine antagonist, and exposed to luminance flicker.

Conclusions

Our findings suggest that a fine balance between the parasympathetic and sympathetic nervous system influences the emmetropization process though a luminance-sensitive neural pathway. The eye demonstrates a bi-modal response under luminance flicker; blocking of the parasympathetic system via atropine causes a decrease in eye length and a hyperopic shift, while inhibition of the β receptors of the sympathetic nervous system leads to an increase in eye length and a myopic shift. These effects are likely the result of an increase in dopamine release with atropine and an inhibition of dopamine release with timolol.

Color flicker also plays a role in controlling eye growth with both parasympathetic and sympathetic blockers causing a reduction in eye growth via dopaminergic mechanisms and choroidal thinning through vasoconstriction.

Clinically, the results of these experiments present evidence that the effects of atropine on refraction can be enhanced by changes in luminance, potentially increasing the protective effect of atropine alone on myopia development. However, since luminance flicker also enhances eye growth with timolol, the results suggest that an imbalance of autonomic stimulation may also increase the risk of myopia. Since the eye is exposed to luminance changes every time the eye changes fixation in the natural environment, the role of the autonomic nervous system in the development of myopia needs to be further investigated.

Although atropine has been found to reduce myopia progression in children, its use is not without severe side effects. Therefore, it would be beneficial to test whether luminance contrast, either individually or possibly in combination with reduced atropine treatment, could demonstrate more effective anti-myopia effects. To test this hypothesis, perhaps a future study could be conducted in which children are asked to read black and white print to simulate luminance contrast versus colored paper (color contrast) to determine if these conditions cause variations in myopia progression.

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