MUSCARINIC MECHANISMS IN EMMETROPIZATION IN THE CHICK: EVIDENCE FOR DIFFERENCES IN FORM DEPRIVATION VERSUS NEGATIVE LENS-WEAR

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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New England College of Optometry, 2013

Abstract

Purpose: Antimuscarinic agents such as atropine and pirenzepine have been proven to be effective in preventing the development of myopia. However, uncertainty remains as to which of the muscarinic receptors mediate this effect. Previous work, in the avian and mammalian models of myopia, implicates the M4 receptor as a potential target. The current study uses a highly selective M4 receptor antagonist, MT3, to determine whether MT3 inhibits the development of myopia in two different myopiagenic paradigms, and if so, if it also results in choroidal thickening similar to other effective growth inhibitors. We also examine the role of two untested muscarinic agonists, arecaidine and pilocarpine, on the choroidal and ocular growth response.

Methods: Ocular components were measured using high frequency A-scan ultrasonography at various intervals. Refractions were measured with a Hartinger's refractometer. Pilocarpine: Normal eyes received either daily injections (n = 5) or on day 1 and day 3 of experimentation (n = 4) of drug or saline (10 µl; n = 4). IOP was measured at various intervals in a separate experiment after either a daily injection (n = 4) or alternating days of injection was

administered (*n* = 4) when compared to saline (*n* = 8). Arecaidine: Normal eyes received a single injection of drug or saline (*n* = 4, 4). IOP was measured at frequent intervals in a separate experiment when chicks were given a single injection of drug or saline (*n* = 4, 4). *$PGF_{2\alpha}$:* Normal eyes received a single injection of drug and IOP was measured at frequent intervals (*n* = 4). *MT3:* (1) Normal eyes received a single injection of drug (*n* = 7) or saline (*n* = 7). (2) Eyes wore either -10D lenses (*n* = 17, 17), -15D lenses (*n* = 12, 13) or diffusers (*n* = 13, 11) for 6 days and were injected with drug on alternating days.

Results: None of the tested agonists had any effect on ocular growth. Arecaidine, however, resulted in a significant choroidal thinning at 24 hrs (-46 vs 3 µm; $p < 0.05$). It also decreased IOP at 3 hrs (-3.00 mm Hg vs 0.3 mm Hg; $p<0.05$); IOP returned to baseline by 24 hrs (-0.5 vs. 1.75 mm Hg, $p < 0.05$). Pilocarpine resulted in choroidal thickening at 3 hrs (66 vs 11 µm; $p<0.05$), which returned to baseline by 24 hrs. It also decreased IOP by 3 hrs (-3.9 vs 0.3 mm Hg; $p<0.05$) which was maintained at 24 hrs (-3.63 vs. 1.75 mm Hg, $p < 0.05$). $PGF_{2\alpha}$ decreased IOP within 3 hrs (drug vs. saline: –4.4 vs 0.3 mm Hg; $p < 0.05$) which lasted for 24 hrs. Surprisingly, MT3 resulted in rapid (3hrs: -45 vs 16 µm; $p<0.05$) and long-lasting (72 hrs) choroidal thinning in normal eyes, the opposite of the effect of other antagonists; it did not affect ocular growth (250 vs 280 µm/3d). As expected (McBrien et al., 2011), it inhibited deprivation myopia (-3.2 vs -7.4 D; $p = 0.0001$) by inhibiting growth (610 vs 827 µm; $p=0.009$), however, it had <u>no</u> effect on lens-induced myopia in neither the -10 D lenses (ANOVA: $p <0.001$; drug vs saline: -1.3 vs -2.5 D; $p=0.23$; axial growth: 585 vs 600 µm; $p = 0.78$) or in the -15 D lenses (ANOVA: $p <0.001$; drug vs saline: -4.0 vs -4.9; $p <0.01$; axial growth: 747 vs 748 µm; $p= 0.99$).

Conclusions: These results suggest that acetylcholine plays a role in the thinning of the choroid in response to hyperopic defocus or form deprivation. The growth inhibition effected by MT3 in form-deprived eyes was not associated with choroidal thickening, suggesting that choroidal thickening is not required for growth inhibition. Furthermore, while MT3 inhibited form-deprivation myopia by inhibiting growth, it had no effect on lens induced myopia, suggesting different underlying mechanisms.

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Introduction

Background

Myopia can be defined as an ocular condition in which distant objects appear blurry because the image formed by these objects is focused in front of the retina. As a result, the image appears out of focus when looking at a distant object and in focus when looking at a near object. In recent decades, myopia has become one of the most frequent ocular disorders, affecting as many as 30% of the world's population (Morgan and Rose, 2005). Single vision spectacles and contact lenses are commonly prescribed as forms of treatment. While these treatments correct the myopic refractive error, they do not retard the physiological changes associated with excessive axial elongation (Gwiazda, 2009). The high prevalence of myopia emphasizes the importance of gaining an increased understanding of the mechanisms involved in eye growth and of finding effective treatments that slow progression and axial elongation.

Our visual image quality influences the ocular growth and refraction of the developing eye. Poor image quality or an inability to compensate for induced defocus may lead to either insufficient or excessive axial elongation, causing hyperopia or myopia, respectively (Wallman et al., 1978; O' Leary et al., 1979; Hoyt et al., 1981). It has been demonstrated in several animal studies that monocular deprivation of pattern vision in neonates produces a breakdown of the emmetropization process in the deprived eye. This results in an increase in the axial elongation of the eye resulting in high levels of myopia (Wiesel & Raviola, 1977; Hodos & Kuenzel, 1984). It has also been observed that deprivation of pattern vision in human infants due to ptosis, corneal opacification or hemangioma of the lid causes axial elongation in the deprived eye, resulting in high degrees of myopia (Robb, 1977; O' Leary et al., 1979; Hoyt et al., 1981).

Several studies have suggested that ocular accommodation may be a causative factor in both human and experimental myopia in animal models. Epidemiological studies have reported an association between school age myopia and near work demands secondary to excessive accommodation. Accommodation may be a likely candidate for the control of eye growth and refraction secondary to its ability to sense the refractive state of an eye and subsequently respond in order to maintain a clear retinal image (Young, 1977). Using muscarinic antagonists, studies have attempted to pharmacologically block the accommodative function of the eye in order to directly test the role of accommodation in eye growth. The muscarinic antagonists atropine, a non-selective antagonist, and pirenzepine, a mammalian M_1 receptor blocker (Tan et al., 2005), have been proven to decrease the development of form deprivation myopia not only in chicks (Stone et al., 1991) but also in humans (Shih et al., 2001; Siatkowski et al., 2004; Chau et al., 2006). It was originally thought that when a cycloplegic drug such as atropine was used, the accommodative function of the eye was blocked because the smooth ciliary muscles became paralyzed (McBrien et al., 1993). The relative success of atropine administration in preventing myopia development has given support to the initial proposal that accommodation is a causative factor in myopic development.

However, in recent years, several studies have examined other possible underlying mechanisms which may be responsible for altering ocular growth and which question the importance of accommodation in myopic development. Studies have demonstrated that not only can experimental myopia be induced in a species that does not have a functional accommodative system (McBrien et al., 1990) but also that experimental myopia can be induced in species in which the accommodative system has been blocked by a bilateral lesion to the Edinger – Westphal nucleus (Troilo, 1989; Schaeffel et al., 1990). Despite the loss of accommodation,

chicks can still compensate for the effects of defocusing spectacle lenses (Schaeffel et al., 1990) and can recover from induced myopia (Troilo, 1989). It has also been demonstrated that a disruption to the communication between the eye and the brain, through a blockage to the ciliary ganglion (McBrien et al., 1995) or through optic nerve section in chicks (Troilo et al., 1987; Wildsoet & Pettigrew, 1988), does not prevent visual regulation of eye growth nor does it prevent visual deprivation myopia. Lastly, the use of atropine reduces experimental myopia and eye enlargement in chicks (McBrien et al., 1993). Like all birds, the iris and ciliary muscles of the chick are striated and contract through activation of the nicotinic receptors (Pilar et al., 1987). Therefore, the mechanism of atropine and other cycloplegic drugs does not involve an accommodative mechanism because accommodation relies on the muscarinic receptors in the ciliary muscle (McBrien et al., 1993). Thus, the mechanism responsible for inhibiting eye growth and preventing myopic progression occurs at the level of the retina, the choroid, the sclera and/or the RPE layer. This paper examines some possible candidate molecules, specifically muscarinic agonists and antagonists, in the molecular mechanism underlying the changes in choroidal thickness and ocular growth.

The Role of the Choroid

The choroid is comprised of the choriocapillaris and the main portion of the choroid, which consists of large blood vessels and lacunae (Wallman et al., 1995). The choriocapillaris is the inner layer of the choroid which sits posterior to the retinal pigment epithelium and is made up of a network of fenestrated capillaries. The main part of the choroid, the suprachoroid and the choroidal stroma, is situated behind the choriocapillaris. In the chick model, the lacunae are lined with thin endothelial walls which serve as a fluid reservoir for the eye. Even though these lacunae make up the majority of the volume of the choroid, they are free of blood cells (De

Stefano & Mugnaini, 1997). The structures of the choroid are suspended into a trabecular support system of extracellular matrix, smooth muscle fibers, fibroblasts, and pigmented cells (Meriney & Pilar, 1987).

The choroid is responsible for providing nourishment and oxygen to the outer portion of the retina including the photoreceptors and the RPE. It has been suggested that the choroid may function as a heat sink for the eye due to its involvement in the dissipation of heat caused by light stimulation to the photoreceptors (Auker et al., 1982; Bill, 1985). The choroid also plays a role in the maintenance of intraocular pressure, as well as playing a role in the filtering and absorption processes. Depending on the changes of the hydrostatic pressure gradient, tissue fluids can be either filtered from the capillary endothelium or it can be reabsorbed into the capillaries themselves (De Stefano & Mugnaini, 1997). The diverse pattern of neurological innervations to the choroid suggests that the choroid has additional functions (De Stefano & Mugnaini, 1997). The choroid receives autonomic input from the ciliary, superior cervical, and pterygopalatine ganglia as well as from the trigeminal, facial, and oculomotor nerves (Mann et al., 1995). Parasympathetic innervation leads to vasodilatation while sympathetic innervation leads to vasoconstriction in the avian choroid (Bill, 1985).

The choroid plays a role in emmetropization by thinning in response to hyperopic defocus and thickening in response to myopic defocus (Wallman et al., 1995; Wildsoet &Wallman, 1995). Under hyperopic defocus, the image forms behind the retina. The eye will compensate for this hyperopic defocus by increasing the rate of elongation and by thinning the choroid resulting in the retina being pulled back toward the image plane. In myopic defocus, the image forms in front of the retina. The eye compensates for this myopic defocus by slowing its rate of elongation and by thickening the choroid resulting in the retina being pushed toward the

image plane (Wallman et al., 1995; Wildsoet, 1997; Wallman & Winawer, 2004). Thicker choroids are found in eyes with inhibited growth rate while thinner choroids are associated with elongated axial lengths suggesting that choroidal thickness per se may influence eye growth (Wildsoet &Wallman, 1995; Nickla, 2007).

Ultra-structural changes in both the superficial and in the deep choroidal vasculature have been described in experimentally manipulated eyes. Blood flow and the biochemical changes involved have also been incorporated into these studies. Typically, when the eye results in choroidal thinning in form – deprived myopic eyes, the lymphatic vessels found on the sclera side of the choroid, as well as the major choroidal blood vessels, are constricted (Liang et al., 2004; Rymer & Wildsoet, 2005). The choriocapillaris is also affected by becoming less dense than normal. There are fewer capillaries and fewer fenestrations found per unit area in the choriocapillaris (Hirata & Negi, 1998). On the contrary, when choroidal thickening occurs in response to recovery from form deprivation myopia or due to myopic defocus, the choroidal lymphatic vessels dilate (Wallman et al., 1995). These dilated vessels result in an increase in fluid movement since both the number of fenestrations found in the endothelial cell lining increases as well as the junctions between these cells opening up (Junghans et al., 1999). The choroids undergoing choroidal thickening have also shown an increase in plasma proteins indicating that vascular leakage plays a role in thickening (Pendrak et al., 2000). Furthermore, these choroids have also shown increases in hydration indicating that biochemical and possible fluid changes play a role in choroidal thickness changes (Wildsoet, 1997; Pendrak et al., 2000).

There are several hypotheses that have been developed to explain the underlying mechanism for choroidal expansion. One possibility states that there is an increase in the degree of fenestration to the capillaries of the choriocapillaris. This increase in fenestrations may

increase the entry of large osmotically active molecules into the extracellular space (Korte, Burns & Bellhorn, 1989; Wallman et al., 1995; Nickla et al., 1997). Thus, choroidal thickness could be determined by the retinal pigment epithelium by controlling the concentration of these molecules (Korte, Burns & Bellhorn, 1989; Wallman et al., 1995). Another possibility is that an increase in the amount of proteoglycans in the choroidal extracellular matrix may thicken the choroid. As water enters the choroid, the choroid swells resulting in an increase in thickness (Wallman et al., 1995). This is supported by Nickla et al. who found that choroids that thickened in response to myopic defocus synthesized more glycosaminoglycans (GAGs) than normal eyes, while eyes under the manipulation of hyperopic defocus synthesized fewer GAGs than normal eyes (1997). Lastly, the degree of contraction of the non-vascular smooth muscles of the chick choroid may play a role in changes in choroidal thickness (Wallman et al., 1995).

Emmetropization in the Chick

Emmetropization describes the disappearance of neonatal refractive errors during early development. Through visual feedback, the avian eyes can control their refractive states. Young chicks emmetropize over a period of six weeks (Wildsoet, 1997). When emmetropization is disrupted by depriving them of form vision either through lid suture (Yinon & Koslowe, 1986) or through translucent goggles placed over the eye (Wallman et al., 1978), the eyes, as a result, become highly myopic (Wildsoet, 1997). If the diffusers are removed, recovery to emmetropization occurs (Wallman & Adams, 1987). When spectacle lenses are worn, there is a bi-directional growth response. When positive lenses are worn, the image plane for distance objects is shifted forward. This myopic defocus will induce hyperopia. The converse is true for negative lenses where the image plane for distance objects is shifted behind the retina. This hyperopic defocus will induce myopia (Schaeffel et al., 1988). During the process of

emmetropization, changes in axial length, the choroid, and the vitreous chamber depth together combined with the progressive flattening of the cornea result in the restoration of emmetropia (Troilo & Wallman, 1991; Wildsoet, 1997).

Muscarinic Mechanisms in Emmetropization

Previous studies have demonstrated that muscarinic antagonists, which inhibit the ocular growth of the eye, induce transient choroidal thickening (Nickla et al., 2013). Research on muscarinic receptor antagonists such as atropine and pirenzepine have previously shown that they are effective in preventing form deprivation myopia (Luft et al., 2003). However, there remains some uncertainty as to which of the five muscarinic receptor subtypes are responsible for this effect. Previous work on the avian model has demonstrated that muscarinic antagonists such as the M1/M4 muscarinic antagonist pirenzepine prevents the progression of myopia potentially by the M4 receptor pathway since chicks do not possess a functional M1 muscarinic receptor (Yin et al., 2004; McBrien et al., 2011).

It has also been demonstrated that muscarinic antagonists which result in choroidal thickening may be associated with ocular growth inhibition (Nickla et al., 2013). As previously mentioned, muscarinic antagonists inhibit the development of myopia in animal models by a non-accommodative mechanism. Therefore, if muscarinic antagonists result in choroidal thickening, then muscarinic agonists should result in a stimulation of eye growth and choroidal thinning. Previous studies supporting this hypothesis have demonstrated that muscarinic agonists such as carbachol and oxotremorine cause significant choroidal thinning and are associated with an increase in ocular growth (Nickla et al., ARVO, 2010).

Lens - Induced Myopia vs. Form Deprivation

Previous studies have demonstrated that the growth response to negative lenses occurs more rapidly than that to form deprivation (Kee et al., 2001). Several lines of evidence indicate that different underlying mechanisms may exist between form deprivation and negative lens wear. In lens induced myopia, the homeostatic control of the eye tries to keep an image sharply focused on the retina. When the eye length increases slower than the focal length, the focal length will fall behind the retina, creating hyperopic defocus on the retina (Wallman & Winawer, 2004). In order to regain sharp focus, the retina needs to be displaced backwards by having the eye increase its rate of growth by remodeling the sclera at the posterior pole of the eye (Nickla et al., 1997; Gentle & McBrien, 1999). Recent evidence has shown that in experimentally induced myopia, there is an increase in the synthesis of scleral protein, DNA and proteoglycans (McBrien et al., 1991). This results in an increase in total scleral collagen content, indicating that there is active growth in the sclera (McBrien et al., 1991). The hyperopic defocus induced by negative lenses causes ocular elongation and choroidal thinning, which both act to move the retina toward the image plane. This thinning occurs within hours of the lens being applied, and so can prevent overshooting of axial elongation which occurs after a delay (Wallman & Winawer, 2004). This axial growth and choroidal thinning reduces the hyperopic defocus imposed by the lens on the eye, thus creating a “closed loop condition” (Morgan et al., 2013). In this condition, the excessive growth terminates when the growth stimuli have been neutralized.

By contrast, in form deprivation, the images on the retina are obscured by lid suture or translucent diffusers which prevent both spatial and temporal transients and result in ocular elongation and high myopia. Because no images are brought into focus by the excessive ocular elongation, the eye continues to grow as long as the vision is obscured (Wallman & Winawer,

2004). This “open loop” condition is believed to be a result of an absence of an error signal and visual feedback. The excessive ocular elongation results in vitreous chamber elongation by as much as 25% (Wallman & Adams, 1987). When the diffusers are removed, the visual system experiences myopic defocus, which causes the choroid to thicken, the rate of ocular elongation to slow down, and the refractions to return to normal (Wallman & Winawer, 2004). Even though both form deprivation myopia and negative lens induced myopia produce a thinned choroid with a longer axial length and myopic refractive error, studies have demonstrated that they differ in their mechanisms. Part of this thesis will address this issue.

Purpose of this Paper

While muscarinic receptor antagonists have been proven to be effective in preventing the development of myopia, there remains uncertainty whether anti-muscarinic compounds work through a receptor mediated mechanism and, if so, which of the five muscarinic receptor subtypes mediate this effect. Research carried out in different animal models, as well as in humans, has confirmed that the most effective anti-muscarinic agent in the control of myopia is the broad band muscarinic antagonist, atropine. As previously stated, despite the initial theories that atropine worked by blocking ocular accommodation during near work (Young 1977), it has been demonstrated that muscarinic antagonists inhibit eye growth and prevent myopic progression through their site of action at the level of the retina, the choroid, the sclera and/or the RPE layer. While several studies have demonstrated the effectiveness of atropine in controlling progression of childhood myopia, atropine is problematic because of its adverse side effects including loss of accommodation, pupil dilation and light sensitivity. Furthermore, studies have demonstrated that once atropine treatment is ceased, the previously treated eye proceeds to become myopic (Tong et al., 2009), indicating that any treatment regime would need to be

continued throughout adolescence and until cessation of juvenile onset myopia (Fledelius, 1995). Such findings have driven the search for more selective anti-muscarinic agents, which ideally have a prolonged clinical effect with minimal side effects.

To elucidate the possible muscarinic receptor involved in myopia prevention, we investigated the effects of muscarinic toxin 3, a highly selective M4 receptor antagonist, on the choroidal and ocular growth response. It has been previously shown by McBrien et al. that MT3 inhibits the development of form deprivation myopia (2011). However, it is unclear whether MT3 has the same effect on lens-induced myopia. The purpose of this paper is to further clarify the effect of MT3 on form deprivation myopia versus negative lens wear.

We also asked whether muscarinic agonists resulted in stimulation to eye growth and choroidal thinning. We examined the role of two muscarinic agonists, pilocarpine and arecaidine, on the choroidal and ocular growth response and on the intraocular pressure. Finally, we looked at the effect of prostaglandin $PGF_{2\alpha}$ on IOP in an attempt to answer the question if increases in choroidal thickness by pilocarpine may be due to changes in fluid dynamics.

Methods

Subjects

White Leghorn chicks (Gallus gallus domesticus) were hatched in an incubator and raised from day one in temperature – controlled brooders under normal visual conditions. The light cycle was 12L/12D (8:30 am to 8:30 pm). Food and water were supplied *ad libitum.* Care and use of the animals conformed to the ARVO Resolution for the Care and Use of Animals in Research.

At 2 weeks of age, for those experiments using form deprivation or spectacle lenses, the right eye served as the treated experimental eye and the left eye served as the untreated control.

Using collodion glue, lenses or diffusers were attached to a Velcro ring and the matching ring was glued to the feathers around the eye. The lenses were either -10 D or -15 D. The diffusers were white half sphere shaped plastic translucent occluders, which allowed light to pass through while diffusing the image. This set up allowed for the removal and replacing of the rings in order for experimental manipulations and multiple measurements. The eyes wore either occluders or lenses for 5 – 7 days (allowing form deprivation or lens induced myopia to occur). The occluders/lenses were removed at various time intervals over the course of the experiments for injections and measurements. During each experiment, eyes were measured using high frequency A-scan ultrasonography (for details see below) and all chicks were measured for refractive error using a Hartinger's coincidence refractometer under isoflurane inhalation anesthesia (1% in oxygen).

Injections and Equipment:

All eyes were injected with an intravitreal injection using a Hamilton syringe with a 30G needle. All drugs were purchased and the proper dosages were prepared in lab. The concentrations of the drugs and selectivities are shown in Table 1. All injections were performed under isoflurane inhalation anesthesia. Injections were made by puncturing the sclera at approximately a 45 degree angle from the superior temporal side of the eye after the removal of feathers and cleaning the skin. The needle remained in place for approximately 30 seconds before being slowly withdrawn. The skin around the site was held tightly together using a small forceps in order to minimize leakage. In the case of multiple injections, care was taken to use the same injection site. The chick's recovery from the anesthesia generally occurred within two to three minutes.

Ocular dimensions were measured at various times using a high frequency A-scan (30 MHz) ultrasound system. Ultrasound gel was applied to the chick's eye before an ultrasound probe was aligned with the pupillary axis. Once the eye was properly aligned, approximately 8 - 10 traces were captured, averaged and analyzed. The axial length was defined as the distance from the anterior cornea to the anterior sclera. The vitreous chamber was defined as being the distance from the posterior lens to the anterior retina. Choroidal thickness was also determined and was defined as the distance from the posterior retina to the anterior sclera.

Refractive errors were measured using a Hartinger's coincidence refractometer. This instrument measures the refraction of a small pupillary area (2 – 3 mm in size). The instrument requires manual adjustment of two sets of three vertical vernier lines and two horizontal vernier lines. The vernier lines are reflected off the retina. The visibility of the vernier lines depends on the optical quality of the eye. Four measures were recorded at the end of experimentation for the treated experimental eye and two measures were recorded for the untreated control eye. Each measurement was recorded along the vertical and the horizontal meridian with the refractometer being repositioned after each measurement. These measurements were then transferred into an Excel spreadsheet which converted the Hartinger measure into an appropriate refractive error using the equation (2.09 x Hartinger measure) x 20. The average of these measures represents the refractive error of the eye.

IOP was measured using a Medtronic Solan Tono-pen XL application tonometer. When the eyelids of the chick were retracted using a lid retractor, the Tono-pen was positioned onto the central cornea. To obtain measurements, the Tono-pen was pressed gently and quickly onto the cornea and then withdrawn. The procedure was repeated until four independent readings were

averaged and displaced on the LCD screen. Three to four LCD measures were obtained and averaged for a final IOP measure.

Category	Drugs	Affinities	Amount of intravitreal injection	Total dosage
Muscarinic Agonists	Arecaidine	M2	20 µl	220 nmol
	Pilocarpine	M3	10 µl	200 nmol
Muscarinic Antagonist	MT3	M4	10 µl	90 nmol
Prostaglandin	$PGF_{2\alpha}$		20 µl	80 ng

Table 1 - Receptor selectivity and dosages of muscarinic agents and prostaglandin PGF2α. The dosage used for arecaidine was determined by the effectiveness of this dosage for oxotremorine and carbachol as noted by Nickla et al. (2013). The dosage used for pilocarpine was approximated from Schwahn et al. (2000). Our dose is about 10x higher than theirs. MT3's dose was the same dosage used by McBrien et al. (2011). 90 nmol is the dose in the 10 µl. The dosage used for PGF2α was approximated from Jin & Stjernschantz (2000).

I: Muscarinic Agonists: Effects on ocular growth and IOP

I.1:1 Pilocarpine

Ocular Growth: Chicks were injected daily (n = 5; aged 14 – 16 days) or on day 1 and day 3 of the experiment (n = 4; aged 6 – 9 days) with a 10 µl intravitreal injection of pilocarpine hydrochloride (200 nmol) into the right eye, around mid-morning, under isoflurane inhalation anesthesia (1.0% in oxygen). In addition, four additional chicks, aged 6 – 9 days, which served as a control, were injected with a 10 µl intravitreal injection of 75% saline avian solution on day 1 and day 3 of the experiment. All left eyes were left untreated. Under light anesthesia, using an A-scan ultrasound machine, measures were taken pre and post injection. Post injection measures were taken after one hour, three hours, 24 hours, 48 hours and 72 hours after the initial injection.

IOP: Chicks were given either a daily injection (n = 4; aged 14 – 15 days old) or were injected on day 1 and day 3 of experimentation (n = 4; aged 6 – 9 days old) with a 10 µl intravitreal injection of pilocarpine hydrochloride (200 nmol) into the right eye, around mid-morning, under isoflurane inhalation anesthesia. IOP measures were taken before and after

injection. Three to four measurements were taken for each eye and an average for each eye was calculated. IOP measures were recorded using an application tonometer at 1 hr, 3 hrs and 24 hrs post injection. IOP measures were also taken at the indicted times for chicks injected with 10 µl of 75% saline avian solution (*n* = 8).

I.2.1 Arecaidine

Chicks, aged 12 – 15 days old, were given a single injection (20 µl) of arecaidine but-2-ynyl ester tosylate (220 nmol; *n* = 4). In addition, four additional chicks, which served as a control, were injected with a single injection of 20 µl of 75% saline avian solution. All right eyes were injected. IOP measures and ocular dimensions were measured before and at 1hr, 3hrs and 24hrs post injection.

II: Muscarinic m4 Antagonist: Muscarinic Toxin 3

A. Normal Eyes. Normal eyes received a one-time injection of 10 µl of MT3 (90 nmol; *n* = 7) dissolved in 75% saline solution (*n* = 7). Ocular dimensions were measured repeatedly before and 3hrs, 6hrs, 24hrs, 48hrs and 72hrs post injection.

B. Spectacle Lenses or Form Deprivation. Chicks (9-15 days old) wore either -10D lenses, -15D lenses or translucent diffusers on the right eye for 6 days. Experimental animals were divided into three groups: one eye wore a -10 D lens (*n* = 17); one eye wore a -15 D lens (*n* = 12); one eye wore a diffuser (form deprived eye) (*n* = 13). Saline injections were given as controls in all groups: -10 D lenses (*n* = 17), -15 D lenses (*n* = 13) and form deprived eyes (*n* = 11). All experimental eyes were given injections of 10 µl of MT3 (90 nmol) on alternate days. Control chicks received injections of 10 µl of 75% saline avian solution. The injections were administrated on day 2, 4, and 6 of experimentation. Ocular dimensions were measured at

baseline and on day 7. All comparisons are between drug and saline injected eyes using 2-sample T tests.

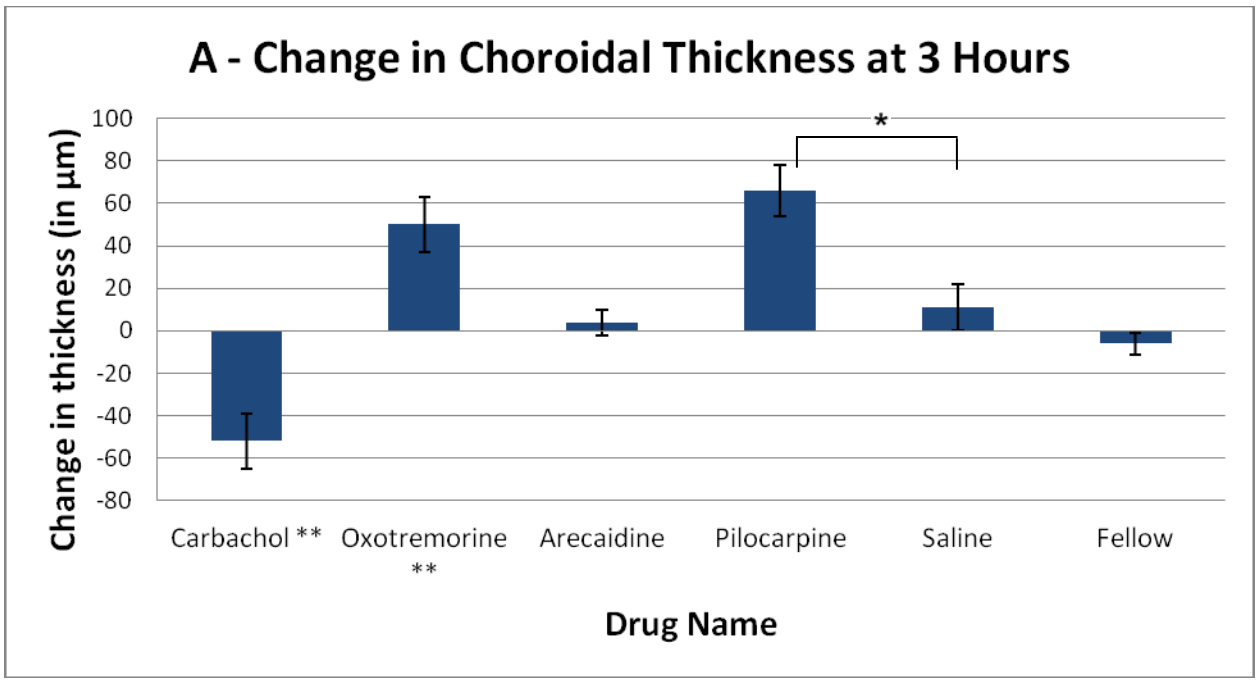
III: Prostaglandin $PGF_{2\alpha}$ and IOP

In 13 - 14 day-old chicks, normal eyes were given a one-time injection of 20 µl of prostaglandin $PGF_{2\alpha}$ (80 ng, $n = 4$). IOP levels were measured at baseline and at 1hr, 3hrs, and 24hrs post injection.

Results

Effect of Muscarinic Agonists on the Choroid

Work in this lab has shown that both carbachol and oxotremorine result in choroidal thinning by 24 hours (drug vs saline, respectively: -35 and -25 µm vs 3 µm; $p<0.05$); for carbachol the thinning occurs rapidly, by 3 hours (drug vs saline: -52 vs 11 µm; $p<0.05$) while oxotremorine causes choroidal thickening at 3 hrs (50 vs 11 µm; $p<0.05$) (Nickla et al., 2013) (Figure 1A-1B). I examined the effects of two other agonists: arecaidine and pilocarpine. Arecaidine had no effect on the choroid at 3 hours (3.6 vs 11 µm; n.s.) but resulted in a significant choroidal thinning at 24 hrs (-46 vs 3 µm; $p<0.05$). Pilocarpine, on the other hand, resulted in a significant choroidal *thickening* at 3hrs (66 vs 11 µm; $p<0.05$), and returned to baseline by 24hrs (3 µm).



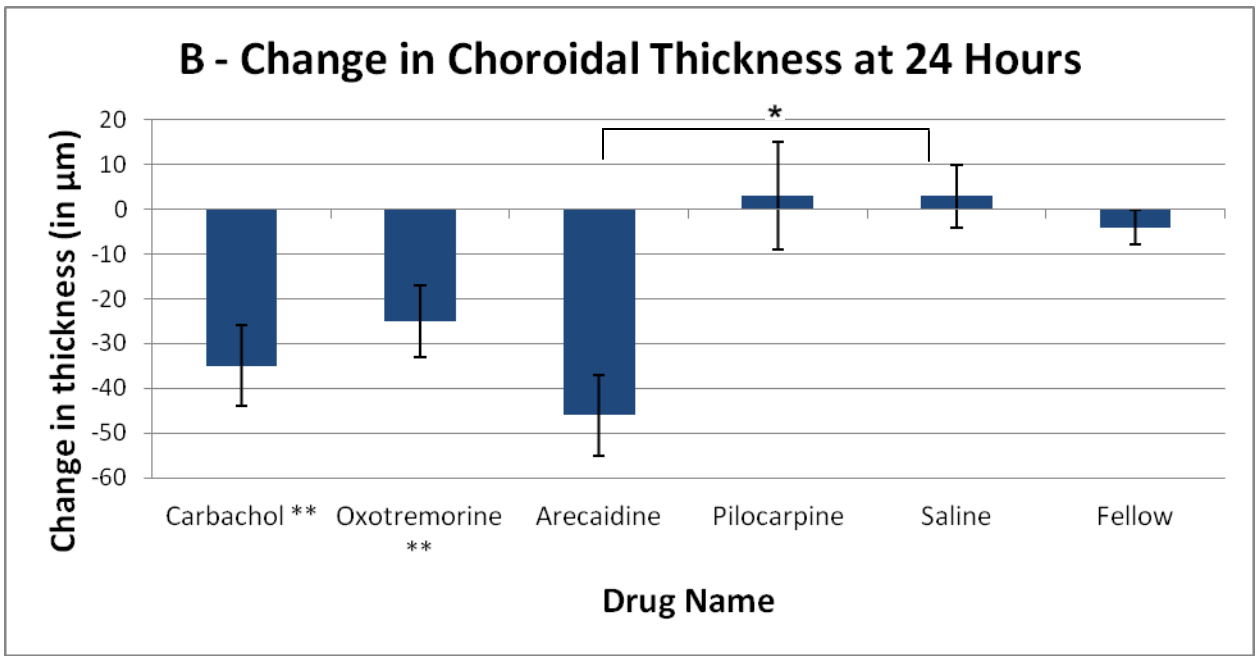


Figure 1: A - Change in choroidal thickness after 3 hours (in µm). B - Change in choroidal thickness after 24 hours (in µm). Note that the data used for carbachol and oxotremorine (indicated by **) was examined previously by Nickla et al., 2013. This data is used as a means of comparsion with the two additional muscarinic agonists tested: arecaidine and pilocarpine. "Fellow" denotes all untreated fellow eyes from all groups in all graphs. The error bars are standard errors of the mean in all.

Figure 2 shows the data re-plotted as a function of time over a 3 day period. Pilocarpine caused the choroid to thicken within the first 3 hrs and then it reached normal baseline at 24 hrs and remained at baseline thereafter. Arecaidine thinned the choroid by 24 hrs, which returned to baseline thickness by 72hrs. Saline has no effect on choroidal thickness.

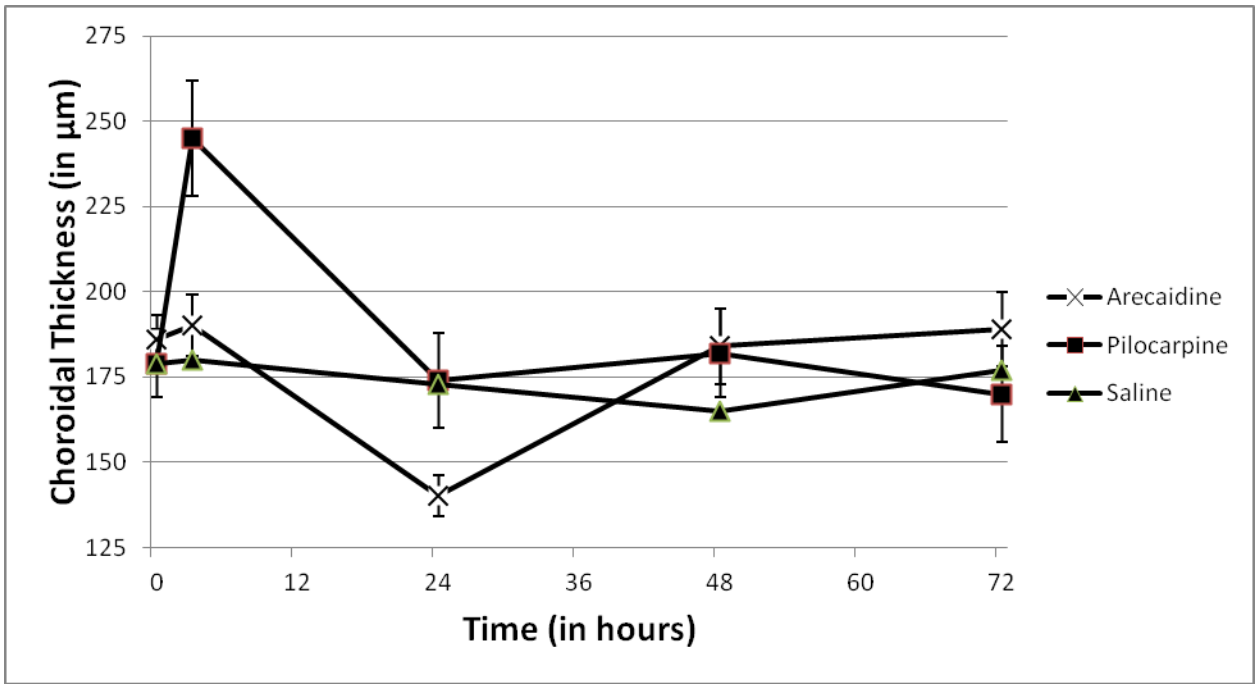


Figure 2 - Change in choroidal thickness over a three day period. Error bars are standard errors of the mean in all. Note the initial increase in choroidal thickness of pilocarpine at 3 hrs which returns to baseline by 24 hrs. Arecaidine, on the other hand, thins the choroid by 24 hrs. Saline has no effect on choroidal thickness.

Neither pilocarpine (drug vs. saline controls: 180 vs. 180 µm/3days, p=0.98) nor arecaidine (273 vs. 276 µm/3days, p = 0.94) had any effect on axial elongation or on refractive error.

Effect of arecaidine, pilocarpine and $PGF_{2\alpha}$ on IOP

In order to test whether the initial choroidal thickening observed with pilocarpine was perhaps due to an increase in the aqueous flow, we examined the effect of prostaglandin $PGF_{2\alpha}$ on the intraocular pressure (IOP). Prostaglandin $PGF_{2\alpha}$ is known to increase outflow and decrease IOP in humans (Lee et al., 1988), so perhaps pilocarpine is having a similar effect, and

resulting in choroidal thickening at 3 hours. Prostaglandin $PGF_{2\alpha}$ resulted in a decrease in IOP within the first 3 hrs (drug vs saline: –4.4 vs 0.3 mm Hg; $p < 0.05$). Both pilocarpine and arecaidine also decreased IOP by 3 hours (Figure 3: respectively vs saline controls: -3.9 and -3.00 mm Hg vs 0.3 mm Hg; $p<0.05$ for both). At 24 hours, prostaglandin $PGF_{2\alpha}$ maintained the decrease in IOP seen at 3 hrs (drug vs. saline controls: –2.5 vs. 1.75 mm Hg, $p < 0.05$). In the pilocarpine –injected eyes as well, IOP remained lower at 24 hrs (-3.63 vs. 1.75 mm Hg, $p < 0.05$). Arecaidine-injected eyes, on the other hand, started to return back to its baseline IOP by 24 hrs (-0.5 vs. 1.75 mm Hg, $p < 0.05$).

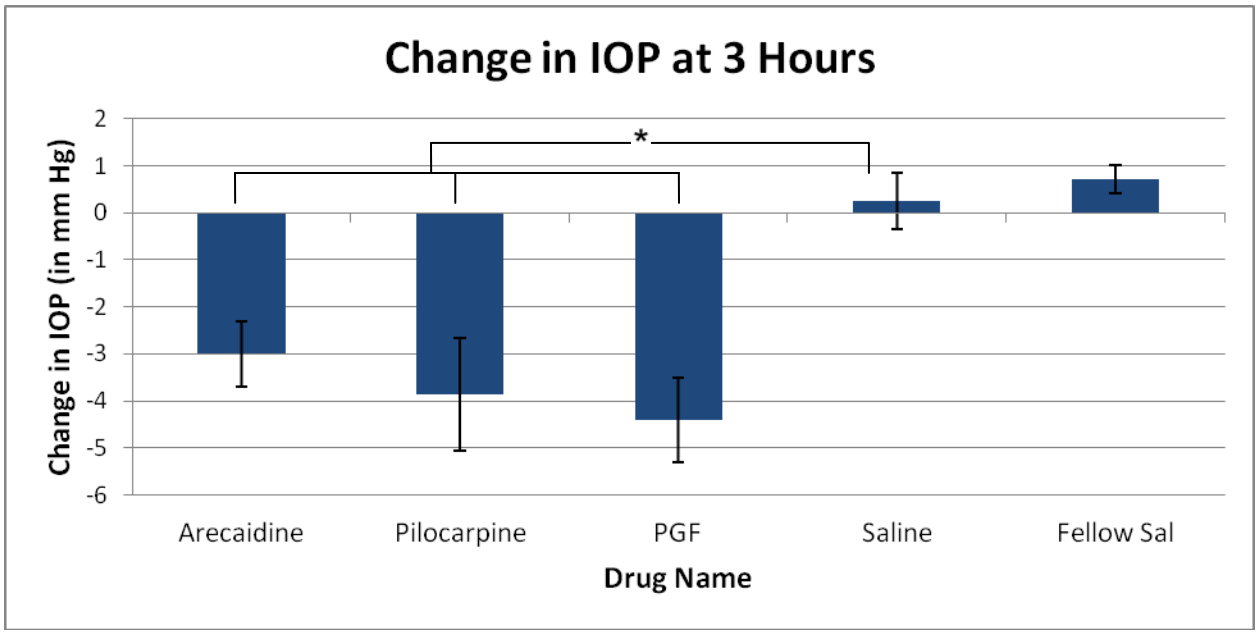


Figure 3 - Change in IOP at the 3 hour time point. Error bars are standard errors of the mean. Note how arecaidine, pilocarpine and PGF2α all lower IOP at the 3 hour time point and are statically different from saline.

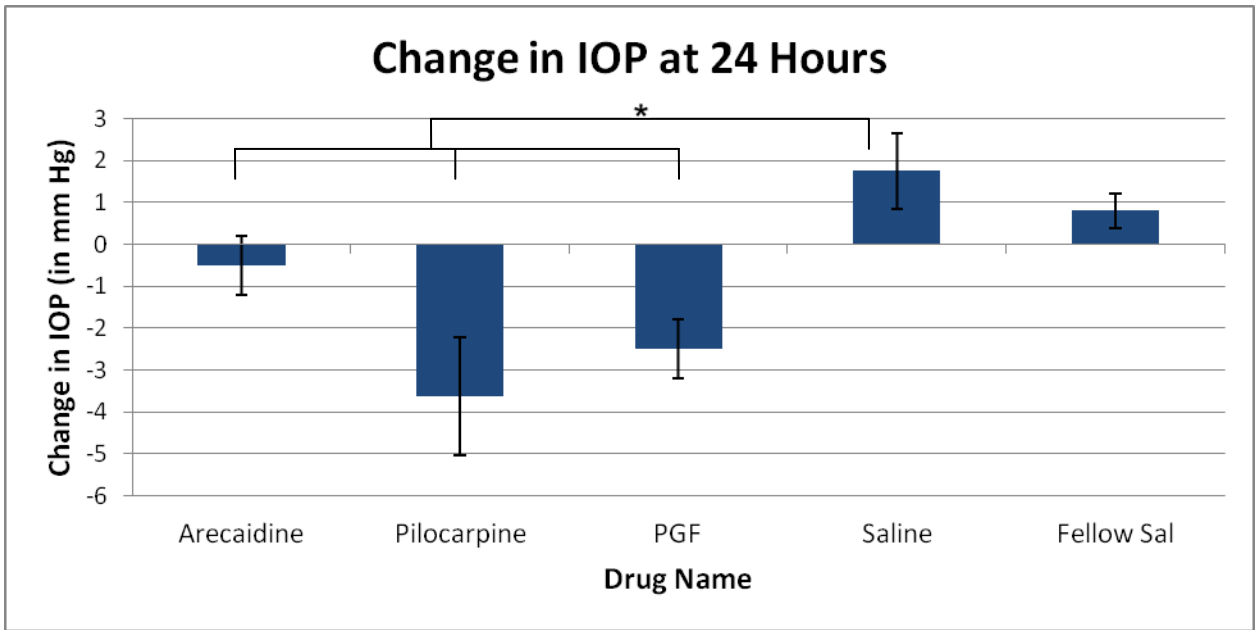


Figure 4 - Change in IOP (in mm Hg) at the 24 hour time point. Error bars are standard errors of the mean. Note that at 24 hours, PGF2α and pilocarpine injected eyes maintained the decrease in IOP as seen at 3 hours. Arecaidine injected eyes, on the other hand, started to return back to baseline IOP.

The Effect of Muscarinic Toxin 3 on Normal Eyes

Unlike other muscarinic antagonists which cause a choroidal thickening, MT3 caused a rapid choroidal thinning at 3 hrs (drug vs. saline controls: -45 vs. 16 µm; $p < 0.05$). This choroidal thinning was still present at 24 hrs (-65 vs. 2.4 µm; $p < 0.05$) and at 72 hours (-67 vs. 1.7 µm; $p < 0.05$) (Figure 5A). There was no effect on axial elongation (250 vs. 280 µm /3d) (Figure 5B) or on refractive error after 72 hrs (-0.4 vs. 1.1 D, $p=0.3$) (Figure 6). Note however, the large variability in the refractive errors for the drug-injected eyes, so this data needs to be interpreted with caution.

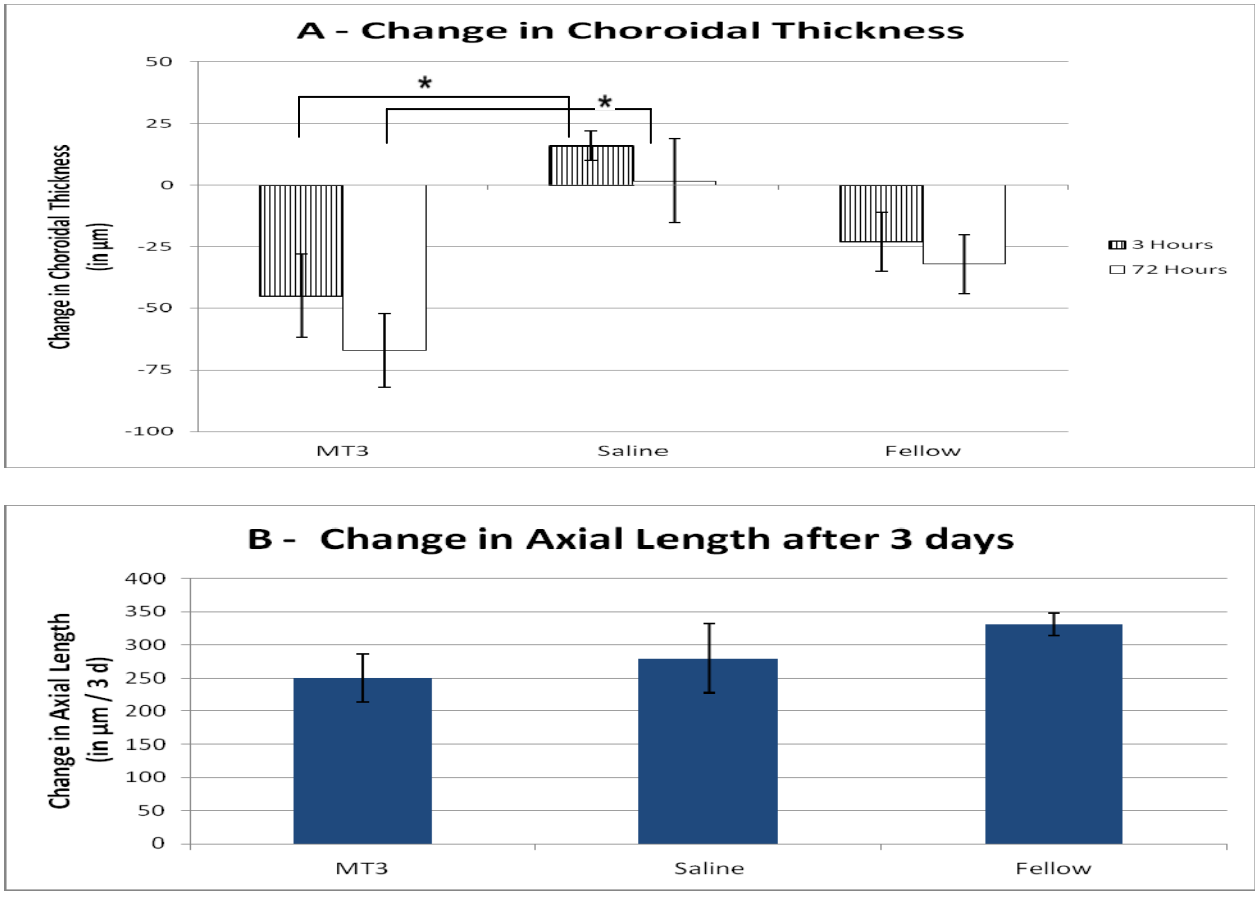


Figure 5 - A: Change in choroidal thickness (in µm) after three days of experimentation in normal eyes. Controls are chicks injected with saline. Note how MT3 causes a rapid choroidal thinning at the 3 hour time point which is maintained at 72 hours. B: Change in axial length (in µm) after a 3 day course period of the same groups. Note how there is no effect on axial elongation in MT3 injected eyes when compared to saline injected eyes. Error bars are standard errors of the mean in all.

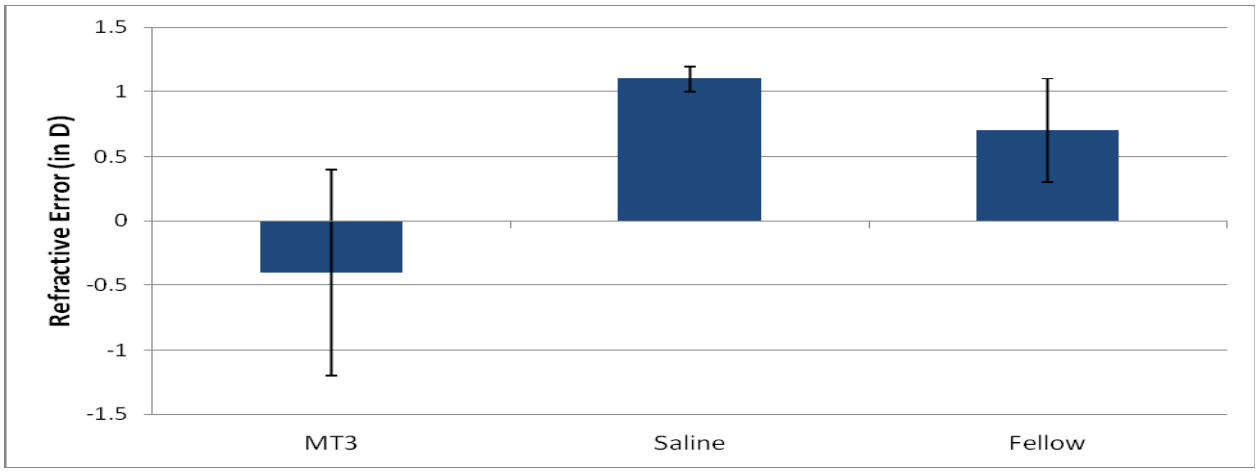


Figure 6 - Final refractive error (in D) after 3 days of experimentation in normal eyes injected with MT3 or saline. Note that there is no effect of MT3 on refractive error when compared to saline injected eyes. Error bars are standard errors of the mean in all.

The effect of MT-3 on form-deprived eyes

In form-deprived eyes, MT3 caused a significant inhibition in myopia compared to saline controls (-3.2 D vs. -7.4 D; p = 0.0001; Figure 7). The refractive errors for both experimental groups were significantly more myopic than that of fellow control eyes (vs 1.2 D; p <0.00001 for both comparisons). This reduction in the myopia was due to an inhibition in eye growth. For vitreous chamber depth growth, drug-injected eyes grew significantly slower than saline-injected eyes (446 µm vs 646 µm/6d; p=0.004; Figure 8) and significantly faster than fellow control eyes (446 vs 194 µm/6d; p<0.0001). For axial length (front of cornea to sclera), a similar inhibition was found in the drug-injected eyes compared to saline controls (610 µm vs. 827 µm/6d; p = 0.009; Figure 9). However, in this parameter, axial elongation in drug-injected eyes did not differ from fellow controls (610 vs 552 µm/6d; n.s.), while the more myopic saline injected eyes grew significantly faster than fellow controls (827 vs 552 µm; p<0.0001).

The discrepancy between axial length and vitreous chamber depth in these two groups can be explained by a significant inhibition in the growth of the anterior chamber in both injection groups (drug, saline vs fellow: 227 and 246 µm vs 385 µm; p<0.05 for both

comparisons; Figure 10), which is a common occurrence in intravitreal injections (McBrien et al., 2011). This smaller change in the anterior chamber offset the axial length in drug-injected eyes, so that axial length was similar, despite a significant difference between the amounts of vitreous chamber growth. In summary, the growth of the posterior eye was significantly greater in both saline and drug-injected eyes than fellow controls, which is consistent with them both being myopic, with the amount of myopia (and growth) being inhibited by the drugs.

Finally, there was no effect of the drug on the choroidal thinning response to the form deprivation: choroids thinned compared to fellow eyes (-83 µm vs -16 µm/6d; p = 0.002), and the effect was similar to those of saline controls (-83 µm vs -68 µm/6d; p = 0.26; Figure 11).

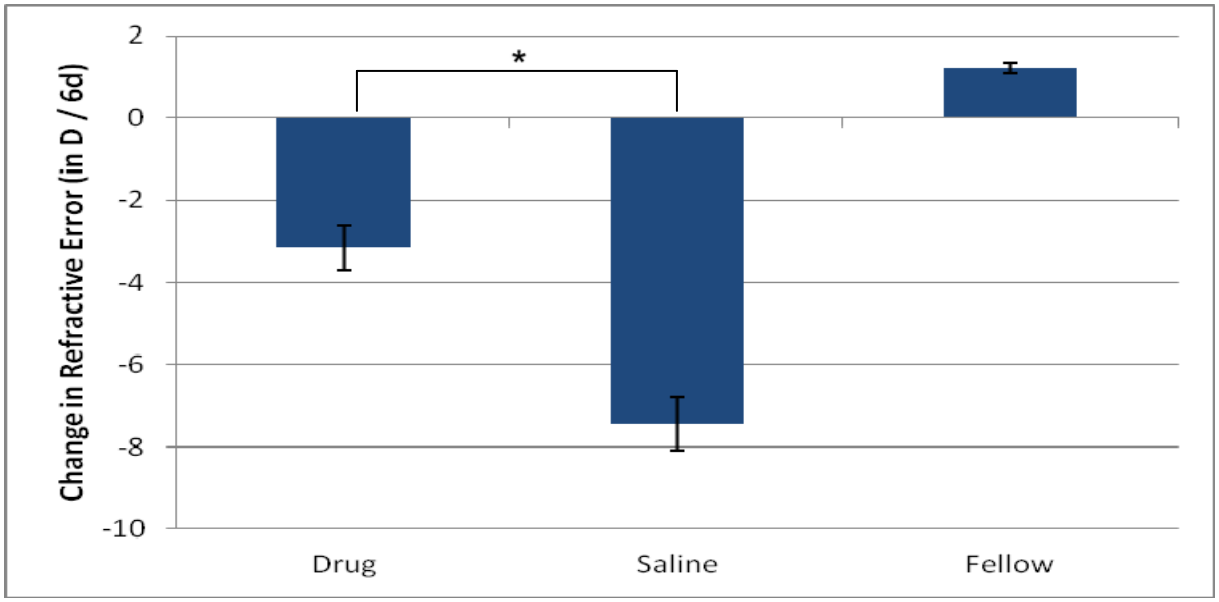


Figure 7 - Refractive error after 6 days of form deprivation. Error bars are standard errors of the means in all. Note the inhibition in myopia of MT3 when compared to saline.

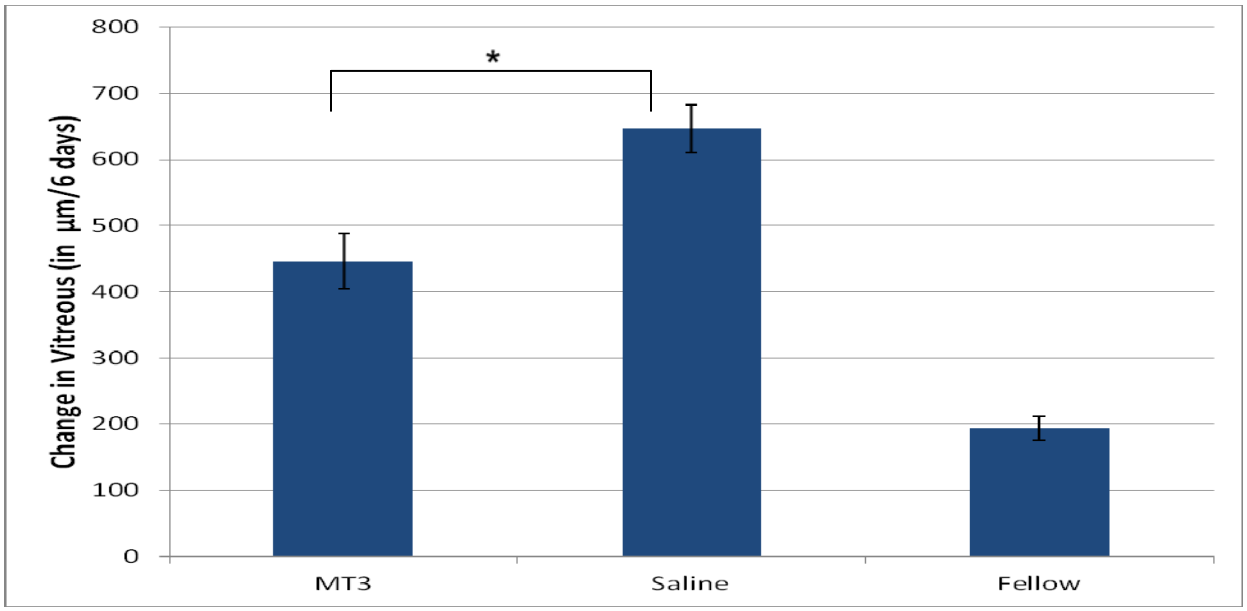


Figure 8 - Change in vitreous (in µm) after 6 days of form deprivation. Error bars are standard errors of the means in all. Note that in drug injected eyes, the vitreous chamber grew significantly slower than in saline injected eyes; however, there is a significant growth in MT3 when compared to fellow controls. Both drug injected eyes and saline injected eyes grew significantly faster than fellow controls.

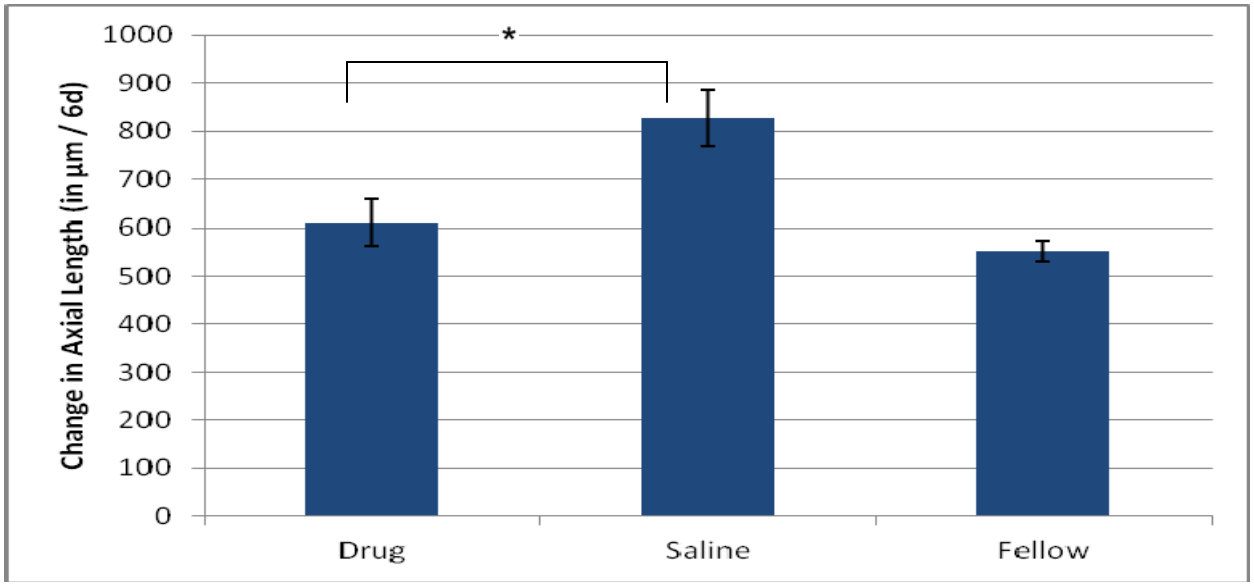


Figure 9 - Change in axial length (in µm) after 6 days of form deprivation. Error bars are the standard error bars of the mean in all. Note how drug injected eyes grew slower than saline injected eyes but did not differ from fellow controls.

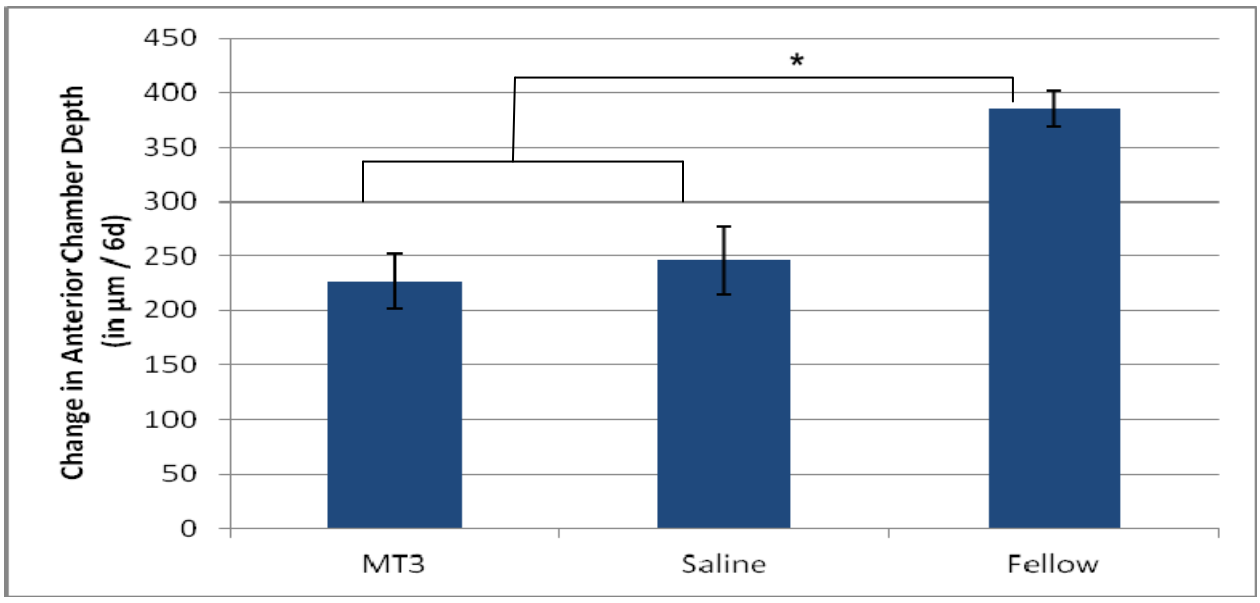


Figure 10 - Change in anterior chamber depth (in µm) after 6 days of form deprivation. Error bars are standard errors of the means in all. Note the significant inhibition in the growth of the anterior chamber in both the drug and saline groups when compared to the fellow controls.

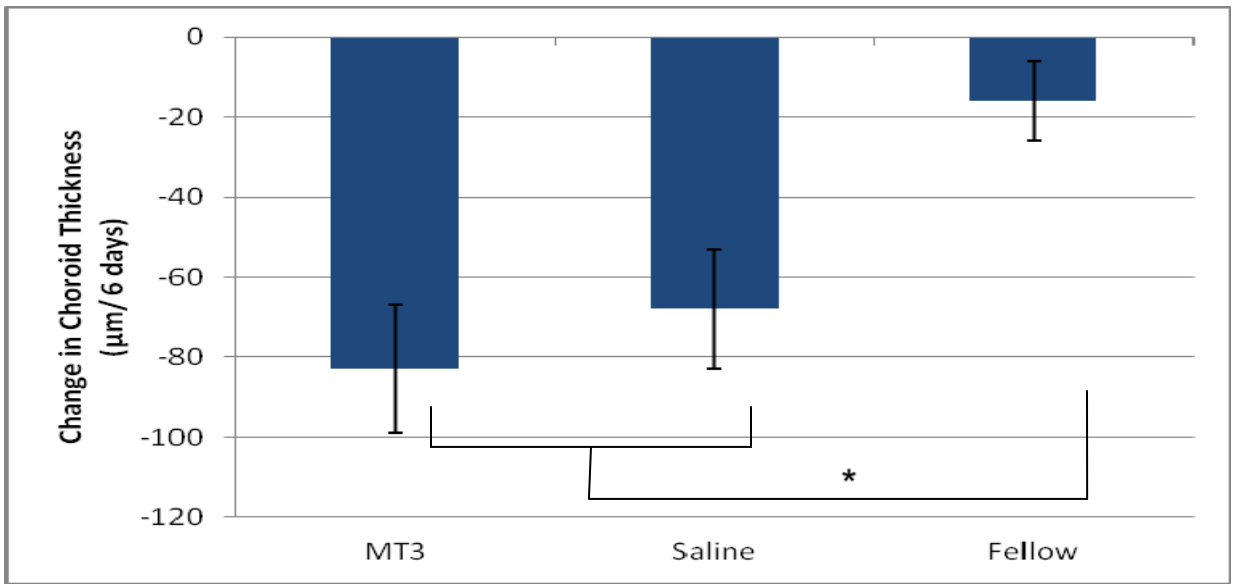


Figure 11 - Change in choroid thickness (in µm) after 6 days of form deprivation. Error bars are standard errors of the means in all. Note that there is no effect of the drug on the choroidal thinning response to form deprivation. The choroids thinned in both MT3 and in saline eyes when compared to fellow eyes.

The effect of MT-3 on negative lens-wearing eyes

We tested the effect of MT-3 on two powers of negative lenses, -10 D and -15 D. In eyes wearing -10 D lenses, there was no significant difference in refractive error between the drug-injected and saline-injected eyes (ANOVA: $p<0.001$; drug vs saline: -1.3 vs. -2.5 D; $p = 0.23$; Figure 12), although both were significantly more myopic than fellow controls (0.71 D; respectively: $p=0.008$, $p=0.0002$). If we increased the lens power to -15 D, we got a larger refractive effect (drug and saline vs fellow controls; ANOVA, $p<0.001$; -4.0 D and -4.9 D vs 0.4 D; $p<0.01$ for both), and, similar to the -10 D group, there was no difference between the drug- and saline-injected eyes (-4.0 vs. -4.87 D; $p = 0.338$).

The lack of a refractive effect on the drug-injected eyes in both lens groups is consistent with there being no significant differences between the two in axial elongation (drug vs saline: -10 D: 585 vs. 600 µm, $p=0.78$; -15 D: 747 vs. 748, $p=0.99$; Figure 13). In the -10 D group, an unusual result was that the saline-injected eyes did not elongate more than the fellow controls (600 µm vs 539 µm/6d). In the -15 D group, both drug and saline-injected eyes grew the same amount (747 µm vs 748 µm/6d), and both were significantly faster than fellow controls (vs 588 µm/6d; $p=0.008$). However, if we examine vitreous chamber elongation, we find that, as expected, for both groups, the drug- and saline-injected eyes elongated more than fellow controls (drug and saline vs fellow: -10 D: 446 and 460 µm vs 236 µm/6d, $p=0.0005$; -15 D: 524 and 531 µm vs 309 µm/6d; $p<0.00002$; Figure 14). The lack of an effect on axial length in the saline injected eyes in the -10 D group can be explained by the finding that there was a significant inhibition in the anterior chamber depth in both the saline and drug injected eyes that “masked” a difference in axial elongation, similar to the finding in form deprived eyes (ANOVA $p<0.001$: drug and saline vs fellow; 191 and 200 µm vs 312 µm; $p<0.005$ for both comparisons; Figure

15). Strangely, there was no injection effect on anterior chamber depth in eyes wearing -15 D lenses (199 and 142 µm vs 111 µm). To conclude, MT-3 had no effect on the myopia produced by either power lens. This effect was on eye growth; there was no difference in either lens group between drug and saline injected eyes. For both lens groups, the vitreous chamber growth was significantly greater than fellow eyes, as expected, but there was an inhibition in anterior chamber growth in both injected groups, that masked some of the axial effect. Similar to form deprived eyes, there was no effect on the choroidal thinning by MT-3 in lens-wearing eyes. Choroids thinned similar to saline controls in both groups (-10 D: -51 vs. -26 µm; $p = 0.19$; -15 D: -57 vs. -64 µm; $p = 0.62$; Figure 16).

To summarize, MT-3 inhibits the development of myopia induced by form deprivation, but NOT that induced by negative lenses. There are no drug-induced effects on the choroidal responses in either form deprived or lens-wearing eyes: choroids thinned as expected in all 3 paradigms.

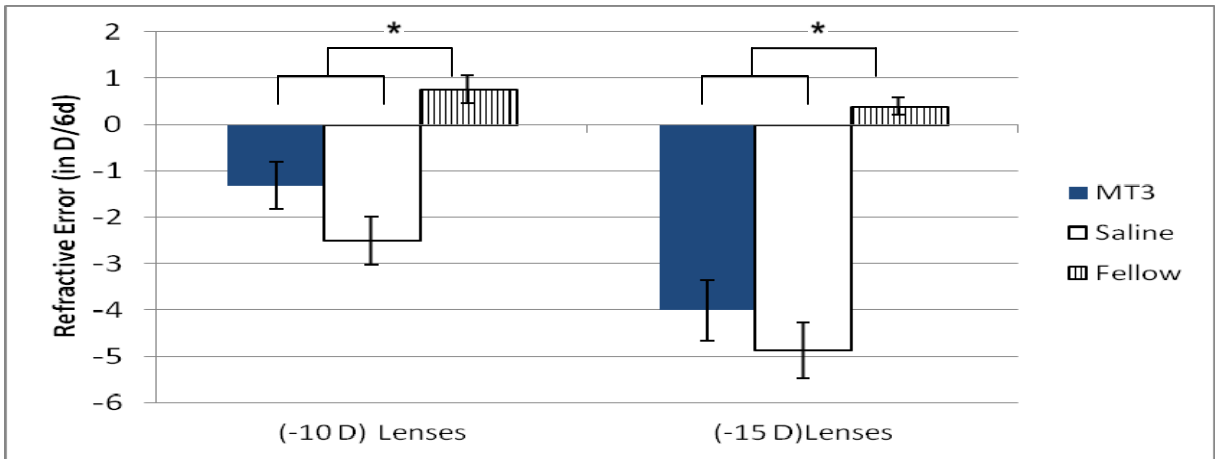


Figure 12 - Refractive error after 6 days of wearing either -10 D or -15 D lenses. Error bars are standard errors of the means in all. Note the amount of myopia in both lens paradigms when compared to the fellow controls. There is no significant difference in refractive error between drug and saline injected eyes in eyes wearing either -10 D or -15 D lenses.

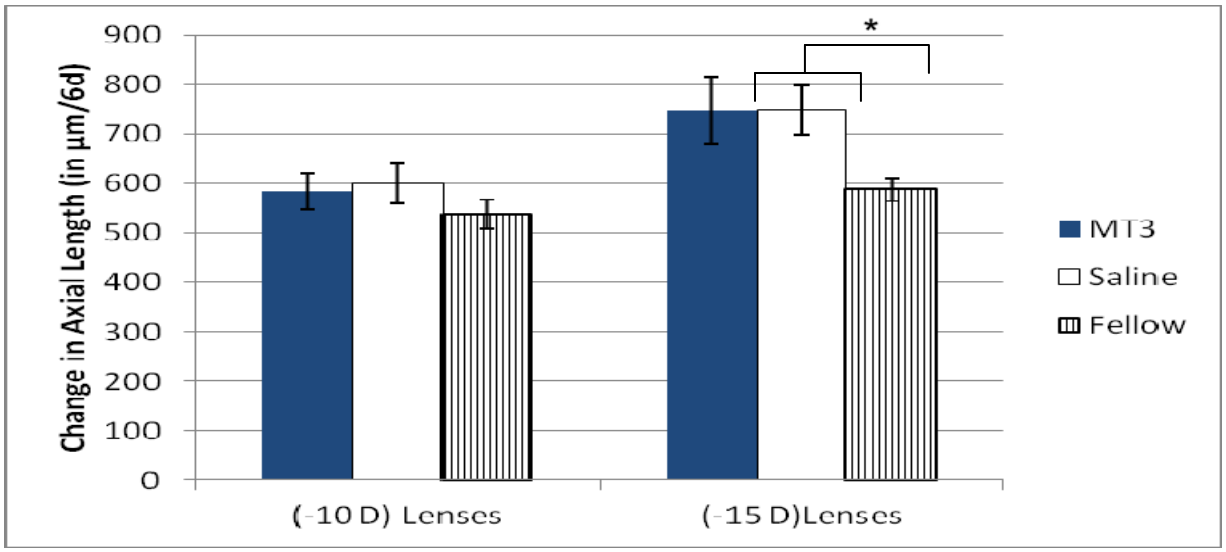


Figure 13 - Change in axial length (in µm) after 6 days of wearing either -10 D or -15 D lenses. Error bars are the standard error bars of the mean in all. Note that there is no significant difference between the drug and saline injected eyes in both lens paradigms. There is also no difference in axial elongation in both drug and saline injected eyes when compared to fellows in eyes wearing - 10D lenses. However, in the -15 D lenses both the drug and saline injected eyes grew significantly faster than fellow controls.

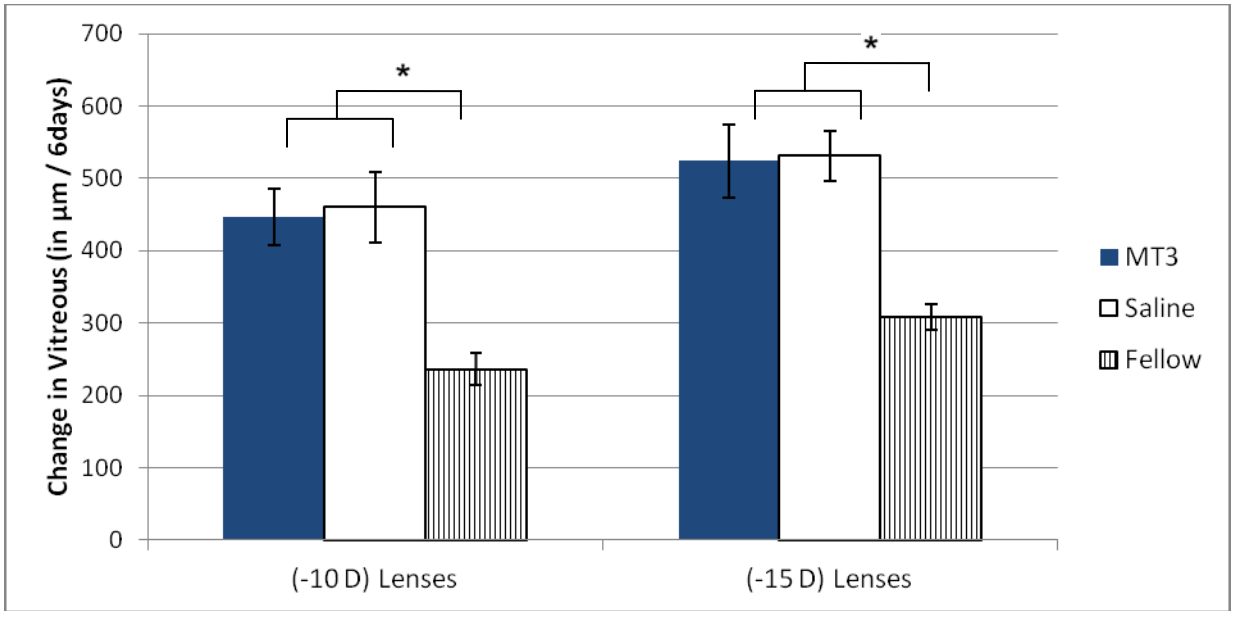


Figure 14 - Change in vitreous (in µm) after 6 days of wearing either -10 D or -15 D lenses. Error bars are standard errors of the means in all. Note that in both lens paradigms, the drug and saline injected eyes had a significant longer vitreous when compared to fellow controls.

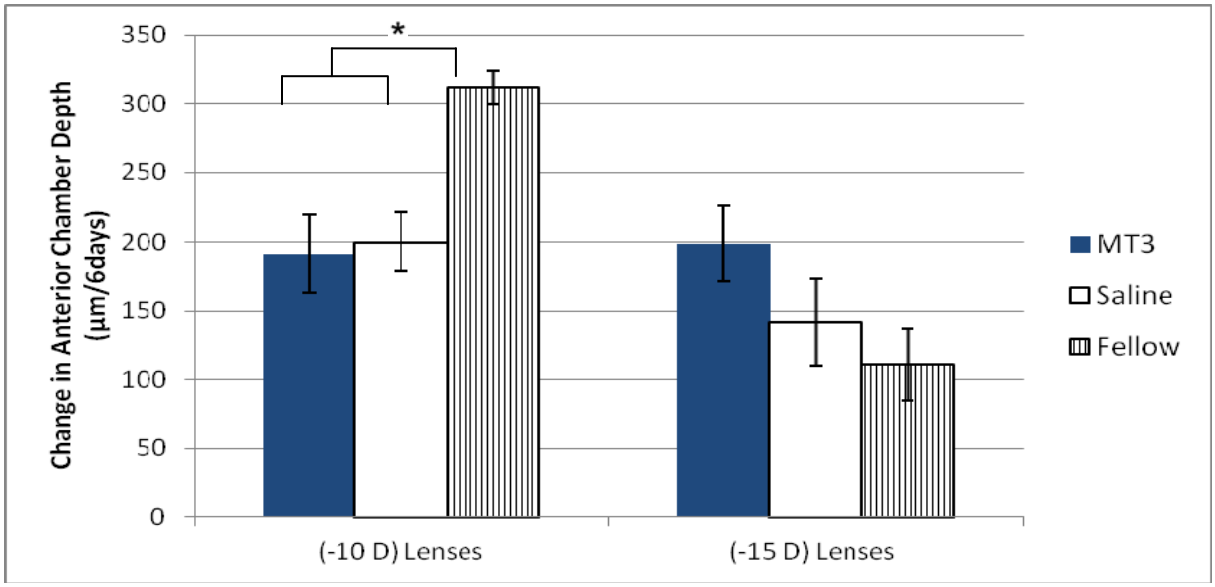


Figure 15 – Change in anterior chamber depth after 6 days of experimentation. Error bars are standard errors of the means in all. Note the significant inhibition in the anterior chamber depth in both the drug and saline injected eyes when compared to fellow controls in the -10D lens group. Strangely, there is no significant difference in anterior chamber depth between drug, saline and fellow controls in the -15 D lens group.

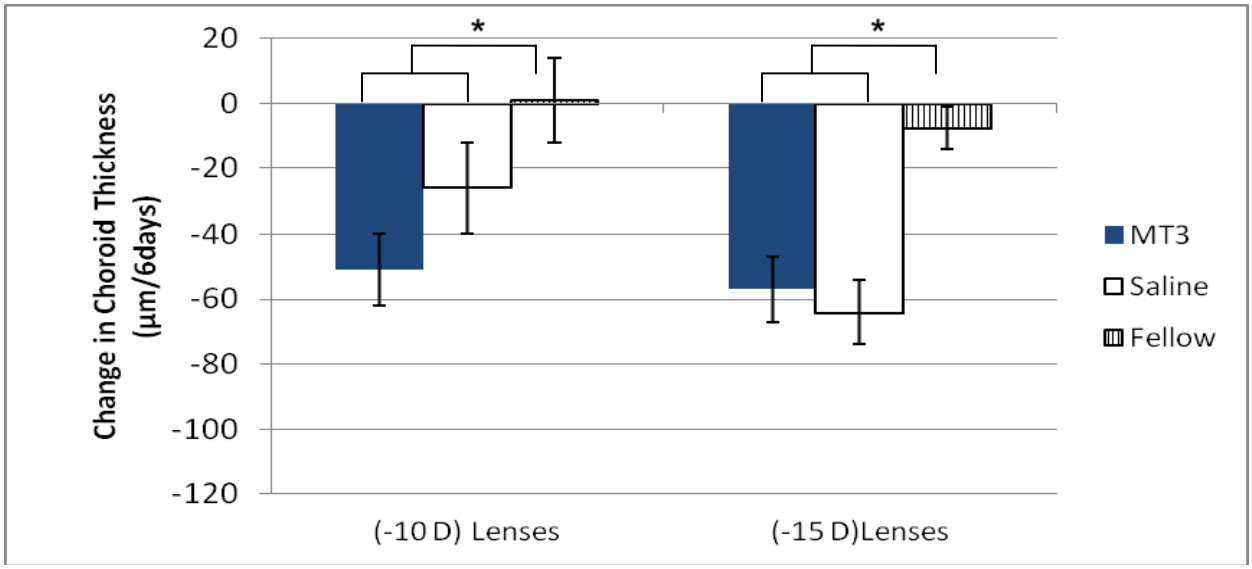


Figure 16 - Choroid thickness changes (in µm) after 6 days of either -10 D or -15 D lens induced myopia. Error bars are the standard error bars of the mean in all. Note that there is no effect on the choroidal thinning by MT3 in lens wearing eyes. Choroids thinned similarly to saline controls in both lens paradigms.

Discussion:

Choroidal thinning may be mediated by a muscarinic mechanism

Of the two muscarinic agonists tested, arecaidine produced choroidal thinning by 24 hours and showed an initial decrease in IOP. This finding is consistent with Nickla and colleagues who have demonstrated that arecaidine causes choroidal thinning in intact eyes, as well as in eyecups without retinas. These findings support a role for a muscarinic cholinergic mechanism in the visually induced choroidal thinning that occurs in response to negative lenses and/or form derivation (Nickla et al., 2013), with acetylcholine as the key transmitter in the transduction pathway (Luft et al., 2003). Findings from Nickla's and colleagues' lab have demonstrated that of the four muscarinic agonists tested, all four caused choroidal thinning in eyecups without retinas, further supporting the role of a muscarinic mode of action on either the choroid or the RPE (Nickla et al., 2013).

It has been proposed that arecaidine and pilocarpine, as well as carbachol and oxotremorine, stimulate the non-vascular smooth muscle cells (NVSMC) that are located in the stroma of the choroid in birds (Guglielmone & Cantino, 1982; Meriney & Pilar 1987) and primates (Krebs & Krebs, 1991; May, 2005). NVSMC in birds is found in the suprachoroidal and vascular layers and especially behind the fovea, the contraction which may thin the choroid, thereby opposing the thickening that may be caused by the expansion of the lacunae (Wallman et al., 1995; Nickla & Wallman, 2010). Currently there are no studies on the type of receptors expressed on choroidal NVSMC, but studies have shown that bird choroids contain cm2 and cm3 receptors (Fischer et al., 1998), which are present on mammalian smooth muscle (Levey, 1993; Caufield, 1993), and cm3 receptors are found in bird ileal smooth muscle (Darroch et al., 2000). Indirect evidence for cholinergic activation has been demonstrated by Meriney & Pilar who

demonstrated that stimulation of post ganglionics of the ciliary ganglion caused contraction of choroids that was blocked by atropine (1987). We propose that choroidal thinning is mediated by a cholinergic muscarinic mechanism in the choroidal response to form deprivation or hyperopic defocus. However, further research is necessary to examine if a muscarinic mechanism is involved directly on the choroid or indirectly via the RPE, through experimentations on eyecups without an RPE (Nickla et al., 2013).

Pilocarpine, even though is a muscarinic agonist, caused a transient choroidal thickening at 3 hours before returning to baseline at 24 hours. This finding is inconsistent with the finding that in eyecups, pilocarpine resulted in choroidal thinning within 3 hours (Nickla et al., 2013). In order to test whether the initial choroidal thickening observed with pilocarpine was perhaps due to an increase in the uveoscleral flow, we examined the effect of prostaglandin $PGF_{2\alpha}$ on the intraocular pressure. Topically applied prostaglandin $PGF_{2\alpha}$ has been shown to decrease IOP in cats (Stern & Bito, 1982), monkeys (Camras and Bito, 1981), and in glaucomatous humans (Villumsen & Alm, 1987). It has been suggested that the mechanism at play is due to an increase in the uveoscleral outflow pathway (Gabelt & Kaufman, 1989). When the extracellular matrix is modified (Lindsey et al., 1997) and the ciliary muscle relaxes, there is an increase in the uveoscleral drainage of aqueous humor (Toris et al., 1997). Nickla et al. has shown that prostaglandin $PGF_{2\alpha}$ results in a significant choroidal thickening response which may be attributed to the increase in uveoscleral outflow. In other words, when fluid from the anterior chamber moves into the choroid, the choroid thickens. Since prostaglandin $PGF_{2\alpha}$ is known to increase outflow and decrease the intraocular pressure of the eye, we can predict that drugs that induce choroidal thickening may do so by an increase in the aqueous flow.

We found that prostaglandin $PGF_{2\alpha}$, resulted in a decrease in IOP at both the 3 hour and at the 24 hour time point. This finding is consistent with previous studies that have demonstrated $PGF_{2\alpha}$ causing a significant IOP reduction in cynomolgus monkeys (Gabelt & Kaufman, 1989), cats (Stern & Bito, 1982), and in human subjects (Lee et al., 1988, Toris et al., 2001). This decrease in IOP has been attributed to an increase in the uveoscleral outflow (Crawford & Kaufman, 1987; Nilsson et al., 1989). We propose that this choroidal thickening may be a result of prostaglandins increasing outflow.

Ocular growth stimulation and choroidal thinning may be likely via separate pathways

Previous work by Luft et al. has demonstrated that atropine, pirenzepine and oxyphenonium are all effective in eliciting ocular growth inhibition in form deprived chick eyes (2003). Ocular growth inhibition by these antagonists is associated with a transient choroidal thickening (Nickla et al., 2013). If a muscarinic system is involved in both the choroidal and ocular growth response, then muscarinic agonists should thin the choroid and stimulate ocular growth. However, we found that neither agonists, pilocarpine and arecaidine, had any effect on axial elongation indicating that the inhibition caused by the antagonists may not be via a muscarinic receptor mediated mechanism (Nickla et al., 2013). This finding is supported by work on other muscarinic agonists which have resulted in choroidal thinning but have *not* caused an increase in ocular growth (Nickla et al., 2013). In fact of the four muscarinic agonists tested, only oxotremorine caused ocular growth stimulation (Totonelly et al., ARVO, 2011; Nickla et al., 2013). These findings imply that choroidal thinning and stimulation of growth are likely via two separate pathways.

Muscarinic toxin 3

The results of the present study demonstrate that the highly selective M4 muscarinic receptor antagonist MT3 is effective in inhibiting deprivation myopia, by inhibiting ocular growth in chicks. This is consistent with McBrien et al. who found that MT3 produced a dose dependent inhibition of myopia in form deprived chicks (2011). Our results also demonstrated that MT3 had no effect on lens induced myopia. This result is slightly different from that of Diether et al., who reported that MT3 only partially inhibited lens-induced myopia in chickens (ARVO, 2005). In their experiment, Diether and colleagues examined the effect of daily injections of different dosages of MT3 on eyes wearing -7 D lenses. After 4 days of experimentation, refractive error was measured by infrared photoretinoscopy. MT3 was only partially effective at inhibiting lens induced myopia amongst the four different dosages (Control: 0 μg: -6.5 D vs. MT3 of 0.1 μg: - 4.2 D, $p < 0.05$; 1 μg: -3.9 D, $p < 0.01$; 10 μg: -2.0 D, $p < 0.001$; 25 μg: -2.8, $p < 0.001$). We, on the other hand, noted that MT3 had no effect on lens-induced myopia with -10 D lenses. To control for the possibility that the lack of an effect was a difference in time course between form deprivation and negative lenses, we used a higher powered lens of -15 D. However, the response to -15 D lenses was still similar to the result obtained with the lesser lens power. This result further demonstrates a clear difference in ocular growth response between the two paradigms since in form deprived eyes, MT3 significantly inhibited deprivation myopia.

The lesser effect of MT3 found on lens induced myopia by Diether and colleagues when compared to our results may be attributed to procedural differences and the type of ultrasound used. The A-scan ultrasound used by Diether et al. is a modified low frequency 10 MHz powered biometer, while Nickla's lab uses a 30 MHz, high frequency ultrasound machine. Our study also

used a different dosage, alternating injection days, had a larger sample size and used two different lens powers. These differences may explain the difference between these two findings. Interestingly, in their abstract, Diether et al. further reported that in addition to MT3, muscarinic toxin 7, an M1 muscarinic receptor antagonist, was partially effective in inhibiting myopia development. This is contrary to studies which have demonstrated that chicks do not possess a functional M1 mACHR (Yin et al., 2004; McBrien et al., 2011). Both of their findings, however, failed to provide evidence of a structural change and as a result must be interpreted with caution.

Unlike other muscarinic antagonists in which growth inhibition was associated with a transient choroidal thickening, MT3 resulted in a rapid and long-lasting choroidal thinning in normal eyes, but had no effect on ocular growth. Furthermore, the growth inhibition effected by MT3 in form deprived eyes was also associated with choroidal thinning. The finding that growth inhibition is not associated with thickening and that choroidal thinning is not associated with growth stimulation further support the hypothesis of separate pathways for the choroidal and axial responses.

Form deprivation and lens – induced myopia are likely via separate mechanisms

The fact that MT3 inhibited the development of form deprivation myopia but had no effect at preventing the compensation to negative lenses may imply separate mechanisms of action. Previous studies have demonstrated that the eye uses different mechanisms when responding to diffusers and lenses. Biochemical studies and the effects of cutting the ciliary nerve (McBrien et al., 1995) or the optic nerve (Troilo et al., 1987; Wildsoet & Pettigrew, 1988) have suggested that different underlying mechanisms exist between form deprivation and lens induced myopia.

Evidence for different underlying mechanisms in form deprivation vs. negative lens wear

I: FDM vs. LIM: Environmental light differences

Perhaps the most compelling supporting evidence for differences between the two paradigms are the dissimilarities found under various environmental light manipulations (Morgan et al., 2013). Seminal work on animal myopias induced in the chick model has demonstrated that constant light and darkness cause excessive axial growth and myopia (review: Lauber, 1991), suggesting that emmetropization is disrupted in the absence of environmental time cues (Morgan et al., 2013). Therefore, in form deprivation where the absence of visual cues occurs, we expect that the outcome to growth has the same consequences as in constant light conditions, while in negative lenses; we would not expect an alteration to the circadian process secondary to the defocused image and closed loop feedback system that occurs (Morgan et al., 2013). Therefore, we would expect the two paradigms to have different mechanisms of action under various environmental light conditions. In the following four experiments, three of the four experiments affect form deprivation only, further supporting the hypothesis of different mechanisms of action between the two paradigms.

I.1:1 – Constant Light

In the first experiment, Bartman et al. has reported that chickens raised in constant light for 8 days resulted in a reduction to the development of deprivation myopia and after 13 days resulted in entire blocking of myopic development. In contrast, in eyes wearing negative lenses, changes in axial eye growth induced by lenses were unaffected by constant light in neither the 6 nor the 11 day experiments (1994). It should be noted, however, that the effects of constant light on negative lens-wear by Padmanabhan et al. were contradictory (2007). Padmanabhan and colleagues noted that chicks reared in constant light for two weeks while wearing negative

lenses, resulted in a small hyperopic shift in refractive error at the beginning of the second week, rather than having no effect on the compensation to negative lenses as noted by Bartman et al.

I.1:2 – Stroboscopic & Fluorescent Light

Another study supporting differences between the two paradigms examined the effect of stroboscopic light on the development of myopia (Kee et al., 2001). Birds reared with either negative lenses or diffusers, received daily stroboscopic light of thirty minute intervals at dawn and dusk. The effect of this light manipulation resulted in a significant attenuation of refractive error in form deprived eyes and coincidently resulted in an inhibition to form deprivation. In contrast, stroboscopic light had no effect on the compensation to negative lenses (2001). However, this effect was reversed with the compensation to negative lenses being inhibited and having no effect on form deprived eyes when chicks received 5 minutes of fluorescent light every 20 minutes during the night for a total of 10 hours (Kee et al., 2001).

I.1:3 – Bright Light

The last experiment demonstrating differences between the two paradigms, examined the effect of bright light on the compensation to imposed optical defocus (Ashby and Schaeffel, 2010). In their study, Ashby and Schaeffel reared chicks with either diffusers or negative lenses, for a period of either 4 or 6 days, respectively. During this treatment period, chicks were exposed to high luminance levels for 5 hours per day. This light manipulation significantly slowed the compensation to negative lenses while it inhibited the development of form deprivation myopia by roughly 60% (2010). This study further favors the existence of different underlying mechanisms between the two paradigms.

II: FDM vs. LIM: Retinal and Choroidal Differences

II.1:1 – Parasympathetic Inputs

The hypothesis that form deprivation and lens induced myopia are likely mediated by two separate signaling pathways has been demonstrated by a number of studies. When influences of the parasympathetic inputs to the choroid were tested, Nickla and Schroedel examined the effect of a lesion to the ciliary nerve pathways and the pterygopalatine ganglia (sectioning N VII) on the choroidal and axial responses in eyes wearing either negative lenses or diffusers (2012). They noted that in form deprived eyes; ocular growth inhibition and emmetropia resulted, while parasympathectomy had no effect on the compensation to negative lenses and coincidently resulted in myopia. These results further argue for different underlying mechanisms for the growth responses to form deprivation versus negative lens wear.

II.1:2 – Electroretinography

Other studies have reported different retinal effects between the two paradigms. When flash electroretinography was used to measure the retinal function in chick eyes after 6 days of experimentation with either negative lenses or with form deprivation, Fujikado et al. noted that there were differences in the oscillatory potentials between the two myopiagenic paradigms. While the a- and b- wave amplitudes were similar between the two paradigms, the oscillatory potentials of the electroretinogram was reduced only in form deprived eyes, suggesting different effects on the inner retina (1997).

II. 1:3 - Optic Nerve Section

Other studies that have examined the effect of optic nerve section on eyes wearing either negative lenses or diffusers, have shown that optic nerve section reduces vitreous chamber elongation of eyes in lens induced myopia while having no effect in form deprived eyes

(Wildsoet, 2000). Similarly, when the ocular growth responses of chicks with optic nerve section were examined in eyes wearing either negative lenses or with diffusers, eyes with diffusers exaggerated the optical axial length changes (Choh et al., 2006). While both groups resulted in increases in axial length and enhanced vitreous chamber growth, the axial responses to form deprivation was enhanced by approximately 50% more than the response of eyes wearing -15 D lenses, despite the loss of accommodation which should have reduced the disparity between the degree of imposed spatial blur between the two paradigms (Morgan et al., 2013).

II. 1:4 - Genetics

The last two studies which noted fundamental retinal dissimilarities between form deprivation and negative lens wear was a study which analyzed the retinal transcriptome in chicks with form deprived eyes (McGlinn et al., 2007) or in eyes wearing negative spectacle lenses (Stone et al., 2011). Using microchip technology, alterations in gene expression was identified in both groups after 6 hours and 3 days of either goggle or negative lens wear. In eyes undergoing form deprivation there were only 15 altered retina/RPE genes at 6 hours and 280 altered genes at 3 days of goggle wear (McGlinn et al., 2007). In contrast, in negative lenses there were 1015 altered genes after 6 hours and 608 altered genes at 3 days in eyes wearing negative lenses (Stone et al., 2011). Despite the growth and refractive similarities between the two paradigms, the retina/RPE transcriptome response to negative lens wear differed considerably from the response to goggle wear, further supporting the notion of different underlying mechanisms between form deprivation and negative lens wear.

III: FDM vs. LIM: Time course and signal integration differences

III. 1:1 – Normal Lighting Conditions

While some studies have reported similarities between the two paradigms, the time course it takes for inhibition of myopic development to occur is notably different. In a study which examined differences in time course of ocular responses to lenses and diffusers, Kee et al. exposed chickens to normal lighting conditions with eyes wearing either negative lenses or diffusers (2001). Kee and colleagues reported that eyes wearing negative lenses elongated faster and were significantly longer than their fellow eyes by 25 hours, while eyes wearing diffusers did not differ from fellows until 71 hours. By 48 hours, the rate of elongation in eyes wearing negative lenses relative to the fellow eye was five times greater than in eyes wearing diffusers. By 71 hours, the eyes wearing -15 D lenses still had a greater rate of elongation than in eyes wearing diffusers.

III. 1:2 – Scleral Proteoglycan Synthesis

In a separate experiment, Kee et al. examined the time course of sclera and choroidal proteoglycan synthesis after brief periods of eyes wearing either negative lenses or diffusers. They found that the time course for the increase in scleral proteoglycans synthesis to occur between the two paradigms significantly differed. In negative lens wear, by 8 hours, scleral proteoglycan synthesis was higher than normal and by 11 hours the rate of scleral proteoglycan synthesis nearly doubled relative to untreated fellow eyes. In form deprived eyes, however, it took 27 hours for the difference to reach significance. These results indicate that although both negative lenses and diffusers cause similar increases in the rate of ocular elongation, the responses differ in time course.

III. 1:3 – Switch Paradigm

To further test the hypothesis of different mechanisms mediating form deprivation and lens induced myopia, young chicks were exposed to a switch paradigm where negative lenses and diffusers were interchanged every 3 hours across each 12 hour day for 4 days (Choh et al., 2006). These "switched" eyes grew significantly slower and were significantly smaller than those in the diffuser group; however, they were not significantly smaller than those of the lens group. The observation that switching between the two treatments resulted in slowed growth suggests different signal integration mechanisms between defocus induced- and form deprivation myopia.

IV - FDM vs. LIM: Pharmacological differences

IV. 1.1 – Atropine & Apomorphine

Aside from our finding of MT3 having different modes of action on the two myopiagenic paradigms, another study performed by Schmid and Wildsoet further suggest pharmacological differences for cholinergic molecules. In order to test whether apomorphine and atropine act sequentially via the same control pathway or via different parallel pathways, Schmid and Wildsoet examined the effect of a combination of both drugs on lens-induced and form deprivation myopia (2004). They found that atropine had a greater inhibitory effect and resulted in greater form-deprivation-induced changes (interocular difference in refractive error: 63%) than the combination of both drugs (49%) and apomorphine alone (27%) on the development of myopia. In negative lens wearing eyes, however, it was noted that the combination of both drugs (59%) was slightly more effective at reducing lens-induced changes than either atropine (53%) or apomorphine (32%). This study further suggests different modes of action for cholinergic molecules.

IV. 1.2 – Dopamine

Another result demonstrating pharmacological differences between form deprivation versus negative lens wear examined the effect of spiperone, a D2 /D4 antagonist, on the ocular growth response in both myopiagenic paradigms. In this experimental design by Nickla and Totonelly, chickens wore either monocular diffusers or -10 D lenses. Each day for 4 days, chicks were injected daily with spiperone and the diffusers or lenses were removed immediately after for 2 hours (2011). Nickla and Totonelly noted that spiperone prevented the ocular growth inhibition normally effected by brief periods of vision in form deprived eyes. However, spiperone did not have an effect on negative lens wearing eyes given similar unrestricted vision. These results indicate that the dopaminergic mechanisms mediating the protective effects of brief periods of unrestricted vision differ for form deprivation versus negative lens wear (Nickla and Totonelly, 2011). It has been proposed that the difference in these paradigms may be a result of D2 prevailing in the absence of visual feedback as occurs in form deprivation, but not in responses to hyperopic defocus caused by negative lenses (Nickla and Totonelly, 2011; Morgan et al., 2013).

In summary, the results reported here support the notion that different underlying mechanisms regulate form deprivation and lens induced myopia. This does not, in any way, advocate the elimination of form deprivation as a paradigm for the study of emmetropization (Morgan et al., 2013). Rather, it urges caution when extrapolating results from one paradigm to the broader picture.

Mechanism and tissue site of action of MT3

It has been demonstrated through radiolabelled drug administration that in order for the inhibition of myopia to occur, sufficient levels of muscarinic antagonist must reach the retina,

choroid and/or sclera (McBrien et al., 2011). Therefore, we can assume that MT3 has a direct action on M4 receptors in the ocular tissues. McBrien et al. has demonstrated, through physiologically relevant nanomolar doses, that there is a strong implication of a retinal site of action for M4 receptor inhibition of myopia in form deprived eyes. Furthermore, it has been demonstrated that muscarinic antagonists may exert their effect through stimulation of the transcription factor ZENK (Bitzer et al., 2006). ZENK is a member of the immediate early gene family of transcriptional regulators, and is the avian ortholog of the immediate early gene *Egr-1* (Ashby et al., 2010). ZENK has been implicated in the modulation of eye growth due to its bi-directional response to opposing growth stimuli (Fischer et al., 1999); resulting in an up-regulation of glucagon immunoreactive amacrine cells in ocular growth inhibition and in a down regulation of ZENK in ocular growth elongation (Bitzer et al, 2006). It has been demonstrated that MT3 is capable of stimulating retinal glucagonergic amacrine cells which produce *Egr-1* (Bitzer et al., 2006), an established marker for suppression of eye growth and myopia progression (McBrien et al., 2011). This finding further supports a retinal site of action for MT3 in inhibiting myopia.

Another component of emmetropization is the choroid, which can change its thickness in response to different visually induced manipulations. In their study, McBrien et al. noted that MT3 prevents the choroidal thinning response induced by form deprivation myopia (2011). Our study, on the other hand, noted that MT3 caused choroidal thinning in not only normal eyes but also in the two different myopiagenic paradigms. In other words, MT3 did not prevent the choroidal thinning response induced by form deprivation and lens induced myopia. The fact that choroidal thinning occurred in all three paradigms makes us believe that this is a real result. The difference in results between ours and McBrien et al. may be attributed to the difference in the

ultrasound's frequency used between the two labs. The A-scan ultrasound used by McBrien et al. is a 10 MHz powered biometer, while Nickla's lab uses a 30 MHz, high frequency ultrasound machine. Our ultrasound has better resolution and can be used to detect details at the posterior pole better than lower frequency ultrasounds. The finding that MT3 does not prevent the choroidal thinning caused by form deprivation and negative lens wear, implicates that the MT3's mechanism of action may also involve the M4 receptors in the choroid as well. We can rule out a scleral route of action because of the strong implication of MT3 at the level of the retina, the fact that MT3 plays a role on the choroid and the fact that the concentration of a muscarinic antagonist reaching the level of the sclera is one-fourth the amount reaching the retina and one-half the amount reaching the choroid (Cottriall et al., 1999; McBrien et al., 2011).

Conclusion / Future Work:

The major finding of this study has indicated that while being a highly specific m4 antagonist, MT3 inhibited deprivation myopia by inhibiting growth; however, it had no effect on lens induced myopia. The fact that there was no effect of MT3 on lens-induced myopia supports different underlying growth mechanisms for deprivation and negative lens-induced myopia. This study also supports the theory that muscarinic antagonists exert their inhibitory effect on myopia in chick via intraocular M4 receptors most likely located in the retina and choroid. Future work in animal models will be necessary to confirm the importance of M4 muscarinic receptors in myopia inhibition and to determine if the M_1 specific muscarinic antagonist is also involved in regulating myopic ocular growth in both form-deprivation and lens induced myopia, prior to advancing to clinical trials in humans.

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