EVIDENCE FOR THE INVOLVEMENT OF CHOROIDAL DOPAMINE IN THE OCULAR RESPONSES TO EVENING BLUE LIGHT

Short Title:
BLUE EVENING LIGHT AND THE CHOROID AS A DOPAMINE SOURCE

A thesis presented to the graduate faculty of
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Arthur Watson

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This manuscript has been read and accepted by the Thesis Committee in satisfaction of the thesis requirement for the degree of Master of Science.

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ABSTRACT

Purpose: To assess if the growth stimulation in blue evening light is related to tyrosinase in the choroid of chickens, and if dopaminergic agonists can inhibit the growth.

Methods: Four experiments are described in this thesis involving young chickens. The lighting used was either always white (white light) or changed to blue light four hours before lights-off (blue evening light). The first experiment was an evaluation for the enzymes tyrosinase and tyrosine hydroxylase in the choroid of chicks raised in white light by western blotting. The second experiment was an evaluation of tyrosinase concentration in the choroid of chicks raised in white light and blue evening light with ELISA. The third experiment was to evaluate the effects of intravitreal administration of dopaminergic agonists on eye growth in blue evening light. The final experiment was to evaluate the effects of topical dopamine on eye growth in blue evening light. For the intravitreal and topical growth experiments, drug or control was delivered to the experimental eye daily and ocular measurements were taken on the first and last day.

Results: The choroid western blot results showed very equivocal if any support for tyrosine hydroxylase, but indicate tyrosinase presence. ELISA evaluation of choroidal

tyrosinase showed enzyme levels in blue evening light were significantly lower compared to white lights for most of the times assessed, and there was time of day differences in both groups. Intravitreal apomorphine was found to cause a significant decrease in growth rate and interocular rate difference compared to the saline control. Intravitreal dopamine did not have this effect. Topical dopamine had no significant effects on eye growth.

Conclusion: Tyrosinase is present in the choroid of chicks. Blue evening light causes a decrease in choroidal tyrosinase and likely a subsequent decrease in ocular dopamine. We suggest that decreased ocular dopamine due to decreased tyrosinase explains the eye growth stimulation mediated by blue evening light. Intravitreal apomorphine inhibits growth in blue evening light while dopamine alone does not. Topical dopamine has no effect on eye growth in blue evening light.

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INTRODUCTION

Myopia

The incidence of myopia has been increasing at an alarming rate, projected to affect 50% of the global population by the year 2050 (Holden, 2016). Despite the incidence increasing, the prevalence is not uniform across the globe. Occurrence in East Asia is very frequent with one study showing adolescent myopia in Hong Kong and Taiwan at a prevalence near 85% (Arumugam et al., 2021). Contrastingly, a systematic review of myopia in 19 African countries shows a prevalence of childhood myopia to be about 5% (Kobia-Acquah et al., 2022). Even with this lower prevalence in the African study, this value is still double what was seen ten years prior. These observations suggest that myopia is related to our local environment.

There are two main types of myopia that exist: refractive, in which an eye of normal size has a focal length that is abnormally short; and axial, where there is a normal focal length in an abnormally long eye. While a combination of both is likely to be present in most, the axial component seems to predominate (Flitcroft et al., 2019). Axial elongation and degree of myopia are associated with ocular pathologies such as retinal detachment, myopic macular degeneration, and open angle glaucoma; all of which can cause considerable vision loss (Haarman et al. 2020).

For these and other reasons, understanding the cause of myopia development has been a key proponent in eye growth research for years. Emmetropization is the process

power. Refractive errors such as myopia occur when eyes fail to emmetropize. This process

is controlled in a closed-loop, negative feedback fashion and utilizes alterations of the retinal image as its error signal (Shaeffel 2024). The question that arises with a negative feedback paradigm is why do refractive errors still abound?

General Hypotheses of Myopia Development

Many hypotheses have been presented as to what leads to myopia development, and what about the world today is precipitating the increased prevalence. Many of these are closely related and potentially working in tandem. There has been considerable work concerning near-work as a potential instigating factor. The association between level of education obtained and increasing degree of myopia aligns with this hypothesis (Nickels et al., 2019). This is related given the considerable reading and other near tasks undertaken

the reading distance has been identified as a protective factor by researchers in China (Jiang et al., 2022). The concept of increased near work has multiple facets involved as a near working distance is accompanied by concomitant accommodation and most near tasks occur indoors.

With a closer working distance, the accommodative demand increases. In a perfect optical system, light from an object would be refracted such that it lands exactly on the imaging surface. In the eye, if there is too much accommodation the image lands anterior to the retina, and if too little the image lands posterior to the retina; theses errors are termed a lead or a lag of accommodation, respectively. A small lag of accommodation is normal in the general population (Scheiman and Wick, 2019). It has been shown that the lag of accommodation is significantly larger in those who are myopic (Kaphle et al., 2022).

Given that near work tasks are typically indoors, decreased time outdoors has been proposed as a potential catalyst of myopia. There have been many human studies where most support the protective effect of increased time outdoors on myopia (Tariq et al., 2023). Time outdoors is compelling enough that there have been some government instituted policies in attempts to decrease myopia, which have yielded positive results (Nischal, 2025). What it is about the outdoor environment leading to this effect is a question that has motivated many scientific queries.

Possibly the most notable mention as a potential cause for the inhibiting effects outdoor exposure is sunlight. The lighting environment experienced outside is greatly altered from that provided by incandescent, fluorescent, or LED lighting. Sunlight is broadband, meaning that the electromagnetic radiation contains unique wavelengths spread across the entirety of the visual spectrum. Additionally, sunlight has nonvisible ultraviolet and infrared rays. Typical indoor lighting emits a fraction of this spectral diversity (Biswas & Kim, 2020). In addition to the decrease in breadth of wavelengths, there is also a profound drop in spectral power (Dhakal, 2023).

Different components of these hypotheses of myopia development are manipulated in laboratory settings to elicit or inhibit eye growth in animal models. Most interventions to elicit myopia development alter some aspect of the visual signals reaching the retina. Some conditions that can elicit eye growth are subjecting the retina to hyperopic defocus with application of minus lenses, depriving the eye of sharp vision buy using a diffusing lens or lid suture, and altering lighting conditions (Flitcroft, 2012).

Animal Models and Myopia

Many animal models have been utilized for research and have taught us much about eye growth. Common models are the chicken, guinea pig, tree shrew, and primates like the marmoset or rhesus monkey (Schaeffel and Feldkaemper, 2015). Other animals have been used but are less common. There are aspects of each model that make them uniquely desirable or difficult to study. The chicken model has been a mainstay in myopia research since early findings of eye growth with occlusion (Wallman et al., 1978).

Deprivation myopia (DM), also known as form deprivation, has become a foundational learning model for understanding myopia development. It has been accomplished in monkeys using lid sutures or causing corneal opacities (Wiesel and Raviola 1977 & 1979). Commonly, occluding or diffusing lenses are used. Chickens respond to this visual manipulation with myopia development (Wallman et al., 1978; Stone et al., 1989). Many species including guinea pigs, mice, tree shrews, and others also respond the same (Zheng et al., 2024).

Another common visual manipulation is the use of lenses to affect eye growth. Minus lenses simulate hyperopic defocus which some consider a potential optical signal for the eye to grow. This defocus stimulates a thinning response of the choroid and an axial elongation of the eye, leaving the eye myopic when the lens is removed (Wildsoet and Wallman, 1995). This is called lens-induced myopia (LIM). LIM has been shown to be effective in many species as well (Schaeffel and Feldkaemper, 2015). Conversely, the use of positive lenses can be used to simulate myopic defocus causing choroidal thickening and axial shortening and in turn lead to lens-induced hyperopia (Wallman and Winawer, 2004).

Findings have shown that emmetropization is controlled locally within the eye. The eye growth stimulated by lens-induced retinal defocus have been shown to be local when partial lenses are applied, causing elongation and growth only in the area experiencing the defocus (Diether & Schaeffel, 1997). These regional effects are also present with partial occlusion (Wallman et al., 1987). These results are consistent in primates (Smith et al., 2013). Local control is further confirmed with the findings that myopia is still inducible if the eye is disconnected from the central nervous system.

DM and LIM are possible even following optic nerve transection (Choh et al., 2006). The physiologic side effects of the surgical intervention alone are not what caused the myopia development because pharmacological signal blocking had similar effects. Intravitreal administration of tetrodotoxin blocks the action potentials of the ganglion cells leaving the eye through the optic nerve, but even with this intervention experimental myopia was still inducible (McBrien et al.,1995). Despite these findings, there does seem to be some modulating effect of the brain on normal emmetropization; eyes that underwent optic nerve transection or administration of tetrodotoxin without any optical intervention were found to become smaller and hyperopic (Trolio et al., 1987; McBrien et al.,1995). Others have reported similar findings in species like the guinea pigs and rhesus macaque monkeys (McFadden & Wildsoet, 2020; Raviola & Wiesel, 1990).

The model used in this thesis is the chicken. The chicken eye differs in some ways from that of a human. The chicken cornea is involved in accommodation in tandem with the lens (Schaeffel and Howland, 1987). The ciliary muscle is striated in chickens, whereas it is smooth muscle in humans; this makes the use of tropicamide and cyclopentolate as

cycloplegics futile (Zheng et al., 2024). The retina in chickens has a differing photoreceptor array. Chickens have rods, four types of single cones, and the presence of double cones. The single cones are sensitive to ultraviolet, short, middle, and long wavelengths which likely been suggested to be involved in processing luminance, not color (Osorio et al., 1999). The chicken retina lacks a fovea, but has a comparable region devoid of rods termed the area centralis (Gisbert and Schaeffel, 2018). As opposed to retinal blood vessels in humans, the retinal oxygen supply in chicks comes from diffusion through the vitreous from a highly vascularized pigmented structure called the pecten, or diffusion from the underlying choroid (Brach, 1977). The scleral matrix of all vertebrates is fibrous in nature, but chicks and some other nonmammalian species have a cartilaginous component (Franz-Odendaal, 2023). Avian species have scleral ossicles which are absent in mammals (Wisely et al., 2017).

Despite its differences, the chicken model has been successful because their eyes are relatively large, grow rapidly, they have good optics and visual acuity, relatively easy husbandry, and a reasonably docile nature (Schaeffel and Feldkaemper, 2015). Each model gives only a single perspective of emmetropization which must be considered when attempting to extrapolate findings to humans. Evaluating multiple species and their responses to differing stimuli can help identify commonalities.

Dopamine, the Choroid, and the Potential Role in Eye Growth

Dopamine is a well-known neurotransmitter that appears to be involved in emmetropization. DA is a catecholamine synthesized from the amino acid tyrosine. It is created directly from the precursor dihydroxyphenylalanine (DOPA) by the enzyme DOPA

decarboxylase (Zahoor, 2018). The creation of DOPA from tyrosine is the rate-limiting step in DA creation and is carried out majorly by the enzyme tyrosine hydroxylase (TH), and alternatively tyrosinase (TYR) (Meiser et al., 2013). Ocular dopamine levels are highest in the daytime and lowest at night which persists even in conditions devoid of light, but normal light exposure drastically increases the peak amplitude (Feldkaemper & Schaeffel, 2013).

Many experimental findings support that the dopaminergic system is involved in eye growth regulation. Early research identified that light increases dopamine output from the retina (Iuvone, 1978). Bright light, which is protective against some myopia-inducing interventions, further elicits an increase in dopamine (Karouta et al., 2025). Additionally, early work found DA levels are decreased during DM in chicks and rhesus monkeys, but only in daytime (Stone et al., 1989; Iuvone et al., 1989). This daytime only decrease was the first line of evidence for involvement of the circadian system in eye growth regulation. In general, eyes often have an associated decrease in dopamine in response to myopia development (Wang et al., 2021).

When dopaminergic agonists are used, myopia development is typically inhibited (Stone et al., 1989; Rohrer et al., 1993; Schmid & Wildsoet, 2004; Nickla et al., 2010; Feldkaemper & Schaeffel, 2013). A dopamine
with coadministration of an antagonist (Stone et al., 1989; Thomson et al., 2020). The dopaminergic antagonist spiperone inhibits the protective effects of bright light (Ashby & Schaeffel, 2010; Karouta et al., 2025).

These findings support the role of the dopaminergic system, but the simple

levels are highest in the daytime and lowest at night, but axial elongation occurs during the day and stops at night (Weiss & Schaeffel, 1993). The finding of axial growth during the time when ocular dopamine levels are highest seemingly contradict the simple idea of increased dopamine inhibiting eye growth. Perhaps the released dopamine has a delayed effect on eye growth rather than immediate. Additionally puzzling are the results seen with total suppression of the dopaminergic system. Destruction of dopaminergic cells with the neurotoxin 6-hydroxydopamine completely inhibits the development of DM, seemingly confounding the importance of DA in inhibiting myopia (Weiss & Schaeffel, 1993). Though, this compound could have varying toxic effects which alters the interpretation of this reported finding. Though these results must be considered, ample studies suggest that there is a relationship between dopamine and eye growth, albeit complicated.

Retinal DA comes from a subtype of amacrine cells which use TH (Witkovsky, 2004). Though much of the ocular dopamine comes from the retina, there is interest in the

pigment epithelium (RPE) have the potential to produce dopamine by a TYR-dependent mechanism. In addition to being a potential secondary dopamine source, the choroid has been shown to be active in eye growth and emmetropization.

There is an alteration of choroid thickness in response to different experimental conditions. A thinning is seen in response to form deprivation and negative lens wear, and a reciprocal thickening is seen if these interventions are terminated (Nickla, 2006). Positive lens wear and bright light exposure both cause an associated choroidal thickening (Wallman and Winawer, 2004; Lan et al., 2013). Administration of dopaminergic agonists are followed

by a significant transient choroidal thickening seen just after administration. Intriguingly, the agonists that are more effective at inhibiting growth are the ones that elicited more notable thickening (Nickla et al., 2010).

permeate the ocular structures. The signal must make its way from the retina, across the underlying choroid, and arrive at the sclera to enact changes that affect axial length. Due to

likely involvement and potential as an additional source of dopamine, research in this area is warranted.

Circadian Rhythms

Living organisms exhibit regular changes in behavior and physiology. These alterations occur with a regular rhythm of roughly 24-hours. They are termed circadian, the

term commonly used is diurnal, which means daily. Some diurnal rhythms occur due to response to outside environment changes, but for a rhythm to be truly circadian it must persist in constant environmental conditions, although many do not hold these definitions to such scrutiny as these terms are often used synonymously (Vitaterna et al., 2001).

The ability of circadian rhythms to persist without outside stimulus is due to the presence of an internal biological clock or circadian pacemaker. In mammalian species, the pacemaker is a region of the hypothalamus called the suprachiasmatic nucleus (SCN) (Vitaterna et al., 2001). In avian species, the location of the pacemaker is the pineal gland, which then uses the SCN as its output system (Yu and Li, 2023). The SCN modulates

circadian rhythms via DNA transcription of regulatory sites termed clock genes (Wahl et al., 2019).

The main input signal to the SCN is light. Specifically, there are intrinsically photosensitive retinal ganglion cells (ipRGC) which do not contribute to visual perception, but depolarize to light and communicate directly to the SCN (Starnes and Jones, 2023). These ipRGCs contain the photopigment melanopsin which has peak sensitivity for 480nm blue light (Hoseini-Yazdi, 2024). Despite being able to persist without it, light helps entrain

Light and Color

The retina senses light and sends this information to the brain to compose a visual perception. As discussed concerning outdoor lighting, not all light is equal. In the laboratory, altering lighting conditions can and does affect eye growth. Additionally, some lighting conditions have confounding results depending on the animal model used.

Animals can have their refractive states altered by changing the chromaticity of the lighting they are reared in. In chickens, mice, and guinea pigs, rearing in long-wavelength light induces myopia while rearing in short-wavelength light induces hyperopia. Rearing in either long or short wavelength light elicits the opposite effect in tree shrews and rhesus monkeys (Chen et al., 2024; Rucker, 2019; Foulds et al., 2013). In chicks, the hyperopic effect of lights-on blue light can transiently negate LIM (Riddell et al., 2021).

It is essential to point out that these findings are from studies assessing the effects of rearing chicks in an essentially monochromatic environment for all lights-on hours. Interestingly, the effects of transient light exposure are not the same. When chicks are

transiently exposed to blue light in the evening, blue light stimulates eye growth (Nicka et al, 2022). Four evening illuminances were used in this experiment, and all illuminances evaluated except the highest intensity caused this effect. Blue evening light has also been shown to enhance LIM (Nickla et al., 2023).

When the intensity of experimental lighting is increased, there is an inhibitory effect against myopia. High intensity light sways emmetropization so chicks stay hyperopic while low intensity light makes chicks becomes myopic (Cohen et al., 2011). This is in harmony with the reported finding that the myopic effects of blue evening light were not present when the highest intensity was used (Nickla et al., 2022). High intensity lighting can also inhibit DM but was seen to only slow LIM (Ashby et al., 2009; Ashby & Schaeffel, 2010).

Another consideration for light and colors is longitudinal chromatic aberration (LCA). This is a physical phenomenon observed where shorter wavelength light is refracted more intensely than longer wavelengths through the same medium. This causes a spread of white light into its spectral components. Light undergoes LCA through the ocular media and is marginally separated. This makes the focal points for blue light more anterior than that of green and red light. Is has been seen that the retina can discern the sign of defocus of an object, whether hyperopic or myopic, and LCA may be what makes this possible (Rucker, 2013; Rucker et al., 2020).

LCA causes the long/medium or short wavelength cone contrasts to be high depending if the retinal defocus is myopic or hyperopic, respectively (Zhang and Zhu, 2022). It is suggested that if the blue components of an ideally distanced object are imaged sharply on the retina, the short wavelength cone contrast is high while medium and long wavelength

cones experience hyperopic defocus with lower contrast. This would be a signal for elongation essentially telling the eye that the retina should be placed closer to the focal lengths of longer wavelength light to yield higher contrast for medium and long wavelength cones (Rucker & Wallman, 2012; Rucker et al., 2020). In the chicken model, it has been shown that increased short-wavelength cone contrast induced axial elongation and a myopic refractive shift; interestingly, this effect is opposite if the light stimulus frequency was low (Rucker et al., 2020).

Thesis Purpose

Emmetropization is complex and studying it involves many considerations. We want to further explore the effects of blue evening light in the process of myopia development and assess how the choroid is involved. Does the hypothesized dopaminergic system present in the choroid of some other animals also play a role in chickens? If dopamine is a key molecule in growth regulation, will application of dopaminergic agonists inhibit the effects of blue evening light? Answering these questions will further elucidate the role of the choroid in emmetropization and assess how evening blue light may be involved in myopia development in general.

MATERIALS AND METHODS

Animals

White leghorn chicks (*Gallus gallus domesticus*, Cornell K strain; Cornell University, Ithaca, NY) that were hatched on site were used in all experiments. After hatching and before experimentation, chicks were kept in a brooder that was on a 12-hour-on, 12-hour-off light-dark cycle. The brooder was 98x67x38 cm (length x width x height), contained a heating block (Cozy Products, Model CL-COOP-R, 2022), and had an illuminance of approximately 350 lux at the center. Food and water were supplied ad libitum. All experiments were carried out after the chicks had aged about two weeks old; experimental groups are described below. All animal care and experimental interventions were in line with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the NECO Institutional Animal Care and Use Committee.

Lighting Conditions

After reaching the desired age, chicks were placed in an 80x61x26 cm (length x width x height) cage and raised for eight days while the differing experimental interventions were carried out. The cage and lighting set up has been used previously in other research work (Nickla et al. 2022). Eight strips of RGB LEDs (Super Bright LEDs Inc., NFLS-RGBX2, 2022) were mounted to white cardboard that was situated from above, immediately in contact with the top of the cage. Each strip of LEDs was 50 cm long, 1 cm wide, with each individual LED spaced 1.5 centimeters apart. Two strips of LEDs were paired up separated by 1cm and each pair of strips were separated by 10 centimeters. LED color and intensity were controlled via a Raspberry Pi running custom software written in Python (Taylor, 2022).

Chicks were placed in the experimental cage and raised in 12-hour-on, 12-hour-off light-dark cycle. Zeitgeber time (ZT) is used with lights-on being ZT00 and lights-off being ZT12. The main experimental lighting used was white light which changed to narrow band blue light four hours before lights off; this condition is referred to as blue evening light throughout this work. The white light had an illuminance of 588 lux and the blue light (460nm) had an illuminance of 600 lux at the center of the cage. For one experiment, we also had some groups of chicks which were raised in the white light without any chromatic changes before lights off. While in the experimental cage, chicks were supplied food and water ad libitum, and a heating block was placed outside of the cage for the duration of all experiments.

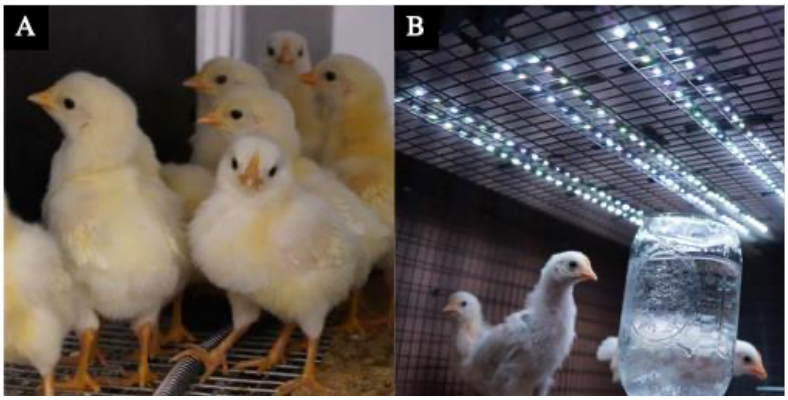

Figure 1: Chicks and Lighting

Ocular Biometry

Ocular measurements were done using high frequency a-scan ultrasonography (Nickla et al. 1998). After chicks had been gently restrained in a paper towel, a respirator was placed over their beak and they were supplied a gas mixture of 2.5% vaporized isoflurane in

oxygen. After adequately anesthetized, a speculum was used to retract the lids and expose the eye. The chick was aligned such that the pupillary axis of the eye and the ultrasonographic probe were as coaxial as possible. Ultrasound transmission gel was then introduced to fill the space between the corneal apex and the probe face. The probe was then finely manipulated along the x, y, and z axes to yield the best possible scan with acceptable peaks.

About 25 scans were saved, then evaluated with a custom software (Nickla et al. 1998). In this software, peaks were set marking the surface of the anterior cornea, anterior and posterior lens, retina, choroid, and scleral interface. The software used computes the standard error of the mean (SEM) for each measure of selected scans. About 5-10 of the available scans were selected for use in the program that yielded a SEM less than 0.001 for all variables.

Baseline measurements were always taken before any experimental drug interventions. This eliminated the chance of erroneous measures due to a preceding injection. It was also for this same reason that the chicks did not receive any drug administration on day eight because post-treatment biometry was already complete.

Out of these values, we were most interested in four measures: anterior segment depth, vitreous chamber depth, choroidal thickness, and axial length. Anterior segment depth was taken as the distance from the anterior corneal surface to the posterior lens. Vitreous chamber depth was taken as the distance from the posterior lens to the retinal surface. Choroidal thickness was taken as the distance from anterior choroidal surface to the scleral interface. Axial length was determined from the anterior corneal surface to the scleral interface.

Tissue Collection and Sample Preparation

After the chicks had been exposed to the experimental lighting intervention for the desired time, the choroidal tissue was harvested for further analysis. Tissue extraction was performed immediately after euthanasia. All chicks destined for tissue evaluation were euthanized by means of decapitation. Immediately following this, enucleation of the eye was performed. The globe was bisected at the best approximation of the ora serrata, separating the eye into the anterior segment and the posterior eye cup. After the vitreous body was removed and discarded, the posterior eye cup was placed into distilled water.

Then, under surgical microscope, the neurosensory retina and retinal pigment epithelium (RPE) was removed. Often when removing these tissues, some residual RPE remained adherent to the choroid. Care was taken to debride as much RPE from the face of the choroid as possible by gentle scraping with a blunt surgical probe. Using fine forceps, the choroidal tissue was peeled away from the sclera and from around the pecten of the posterior eye cup. After harvesting the tissue, the sample was immediately placed in a 1mL test tube with solution on ice.

Depending on expected analysis, samples were stored in solutions of either phosphate buffered saline (PBS) or radioimmunoprecipitation assay (RIPA) buffer. PBS was used for tissues that would be analyzed with enzyme-linked immunosorbent assay (ELISA), and RIPA buffer was used for the western blotting protocol. Tissues destined for ELISA were always placed in 220µL of PBS. The volume of RIPA buffer used with tissues destined for western blotting varied and is specifically outlined below in the unique primary antibody study descriptions. Each test tube also contained a proteinase inhibitor cocktail at a dilution

of 1:100 in relation to buffer solution used. Samples were stored at -80°C until time of analysis.

Before analysis, tissue samples underwent two freeze-thaw cycles, and then were homogenized with an ultrasonic tissue processor. Homogenization was performed for 30 seconds, the samples then rested on ice approximately one minute, and finally sonicated briefly again for 10 seconds. The homogenate was then briefly centrifuged at 5000 x g relative centrifugal force. Sample aliquots were taken from the supernatant after centrifugation.

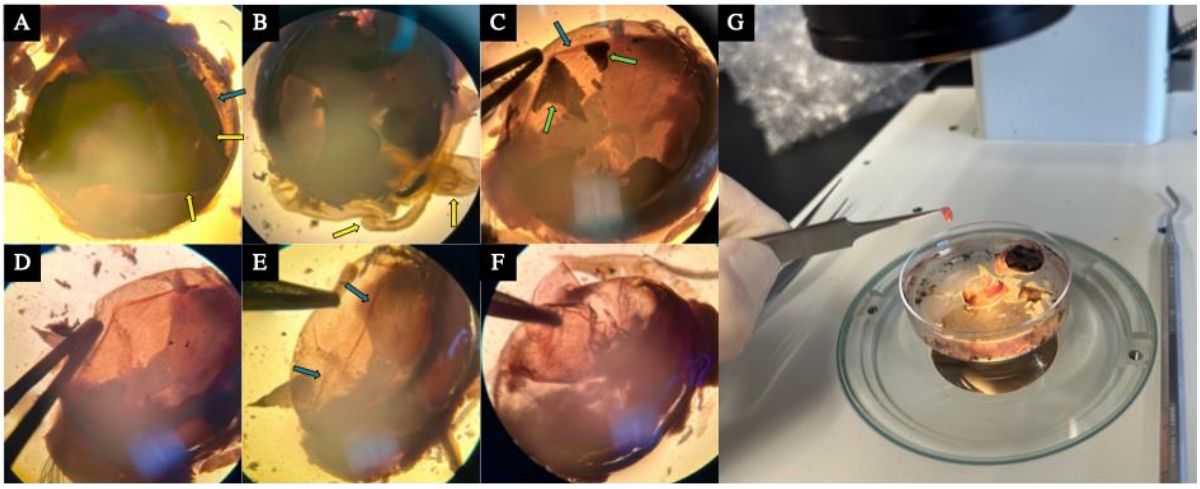

Figure 2: Collection of Choroidal Tissue Samples

EXPERIMENT I: Western Blot for Tyrosine Hydroxylase and Tyrosinase in Choroid

Western blotting was performed to serve as a qualitative evaluation for the presence of the enzymes tyrosine hydroxylase and tyrosinase. Tyrosine hydroxylase is the enzyme

responsible for catalyzing the rate limiting step in dopamine production. It is a homotetrameric protein with a subunit molecular weight of approximately 56 kilodaltons (kDa) (UniProt: Q9PU40, 2023). Tyrosinase is the enzyme involved in the rate limiting step of melanin production, but alternatively can perform the rate limiting step in dopamine production too. It is a monomeric transmembrane protein with an approximate molecular weight of 60 kDa (UniProt: P55024, 2023).

Gel electrophoresis was done with a precast, 10 well (50µL), 10% polyacrylamide gel cassette (Bio-Rad Laboratories, Catalog 4561094, 2022). When first opened, the wells were rinsed three times and then filled with tris/glycine/sodium dodecyl sulfate running buffer solution (Bio-Rad Laboratories, Catalog 1610732, 2022) diluted to 1x concentration with distilled water. An aliquot of was taken from a homogenized sample and mixed with a sample buffer (Bio-Rad Laboratories, Catalog 1610747, 2022) in 1mL test tubes at a buffer to sample ratio of 1:3. This sample buffer was constituted by adding beta-mercaptoethanol (BME) at a buffer to BME ratio of 9:1. The samples were then heated at 95°C for 10 minutes followed by a brief centrifugation. A 50µL portion of each sample and buffer solution was then loaded into the cassette wells while saving two wells for 20µL of a protein ladder reference (Bio-Rad Laboratories, Catalog 1610374, 2022).

The gel cassette was placed into the electrotransfer tank adequately filled with the running buffer, and the electrophoresis was performed at 120 volts for one hour. After running, the cassette was carefully opened; the top comb end as well as the inferior most segment of the polyacrylamide gel, beyond the extent of the protein ladder, were carefully cut away with a razor blade. Protein transfer from the gel to a nitrocellulose membrane was

then performed. A transfer stack was assembled, assuring the gel was closer to the negative electrode, and then placed into the tank adequately filled with tris/glycine transferring buffer solution (Bio-Rad Laboratories, Catalog 1610734, 2022) that had been diluted to 1x concentration with methanol and distilled water as the packaging indicates. The electrotransfer was then run at 100 volts for one hour.

The membrane was briefly rinsed with distilled water, then methanol, and then was blocked with a 5% dry milk solution reconstituted with 1x tris-buffered saline (TBS) for one hour. After blocking, the membrane was exposed to the primary antibody of choice diluted with more of the milk solution in a shallow dish. At the same time, either the entire membrane or a portion of it was exposed to the control antibody diluted in milk solution in a shallow dish. The membrane was exposed to the primary antibody and the control at -4°C with gentle agitation overnight. The shallow dishes used allowed complete and continuous contact between the primary antibody solution and the membranes during agitation.

The next morning, the membrane was removed and briefly rinsed with distilled water. We then washed the membrane in TBS three times, 15 minutes each time by utilizing the shallow dish and gentle agitation. All fluid was removed between washes. The membrane was then exposed to the needed secondary antibody diluted in milk solution for 1 hour with gentle agitation. During and after administration of the secondary antibody or control antibody, care was taken to protect the membrane from light exposure. This was achieved by covering the dish containing the membrane with foil. After this step, the membrane was rinsed and washed again in the same fashion as after primary antibody exposure, submersed

in methanol, and then allowed to dry. The dry membrane was transported from the NECO campus laboratory and analyzed at the Boston University Medical Campus.

We performed western blot evaluations utilizing multiple sample preparations, and various types and dilutions of primary and secondary antibodies in attempts to obtain protein banding. These experiments were pilots designed to test for the presence of enzyme, but the procedure preparations had never been done in this lab. All tissue used in sample preparations came from chickens that had been raised in the white light condition for two weeks. The various antibody studies and types used are explicitly mentioned below. The control used in all our western blot analyses was a mouse antibody sensitive for the protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Invitrogen, Catalog MA5-15738-A647, 2022), a ubiquitous enzyme of glycolysis, at a dilution of 1:500. GAPDH has a molecular weight of approximately 36 kDa (UniProt: P00356, 2024).

Assay I

For our first study, our choroid sample was processed in 300µL of RIPA buffer. The primary antibody used was a rabbit antibody sensitive for tyrosinase (Lifespan Biosciences Inc, Catalog LS-C40464, 2022). The membrane was separated into three unequal pieces which allowed evaluation of an equal number of columns for both antibody dilutions. We evaluated this primary antibody at dilutions of 1:500 and 1:800. Our secondary antibody was a goat antibody sensitive for rabbit immunoglobulins at a dilution of 0.2µg/mL (Invitrogen, Catalog A32731, 2022).

Assay II

In our second study, we pooled two choroids per test tube and homogenized using 100µL of RIPA buffer to increase protein concentration. Prior to primary antibody exposure step, we cut the membrane in half and treat half with the protein specific antibody and the other half with the control anti-GAPDH antibody. We used the same rabbit anti-tyrosinase primary antibody but only utilized the dilution of 1:500. The goat anti-rabbit secondary antibody was used again, but at a higher concentration of 0.4µg/mL.

Assay III

Our third study also utilized samples with two choroids per test tube with 100µL of RIPA buffer. We again cut the membrane in half and only one half was treated with the control anti-GAPDH antibody. In this study, we used a mouse anti-tyrosine hydroxylase primary antibody (Immunostar, Catalog 22941, 2022). We evaluated both 1:800 and 1:500 dilutions. Our secondary antibody was a goat anti-mouse immunoglobulin that was diluted to 0.4µg/mL (Invitrogen, Catalog A32723, 2022).

Assay IV

In our fourth study, we pooled many choroids together to further increase the protein concentration. Samples were prepared with eight choroids and 80µL of RIPA buffer. During this study we also ran two western blots at the same time. Both membranes were cut in half. The first membrane was treated with mouse anti-tyrosine hydroxylase primary antibody at a dilution of 1:500 for one half, and 1:800 for the other half. The second membrane had one half treated with rabbit anti-tyrosinase primary antibody at a dilution of 1:500, and the other half was treated with the anti-GAPDH control again at a dilution of 1:500. We used the same

0.4µg/mL dilution for the secondary antibodies, which were the goat anti-mouse immunoglobulin, and anti-rabbit immunoglobulin antibodies, respectively.

Assay V

Our fifth study was run with samples containing two choroids per test tube with 100µL of RIPA buffer. We again ran two western blots synchronously. Both membranes ran were cut in half unequally. This allowed for us to be able to have roughly equal assayed wells across three groups when treating with antibodies. In this study we investigated two new primary antibodies.

Two membrane sections were treated with a rabbit antibody selective for tyrosine hydroxylase phosphorylated at amino acid serine-40 at a 1:200 dilution (Cell Signaling Technology, Catalog 2791S, 2022). Phosphorylation is a biochemical means of enzyme regulation. TH has four serine phosphorylation sites, and Ser40 has the largest effect on activity (Dunkley and Dickson, 2019). Phosphorylation of serine at this site increases enzyme activity. A western blot study has shown that, in response to light, Ser40 phosphorylated TH greatly increased in the rat retina (Witkovsky, 2000). Additionally, this same antibody has been shown successful in guinea pig studies (Jiang et al., 2018)

Another membrane section was treated with a different mouse anti-tyrosine hydroxylase antibody at a 1:200 dilution (MilliporeSigma, Catalog MAB318, 2023). The final membrane section was treated with the anti-GAPDH control at a dilution of 1:500. We, again, used the same 0.4µg/mL dilution for the secondary antibodies, which were the goat anti-rabbit immunoglobulin and anti-mouse immunoglobulin antibodies, respectively.

EXPERIMENT II: ELISA for Choroidal Tyrosinase at Differing Times of Day

We evaluated tyrosinase concentrations across four time points in two different lighting conditions. We evaluated white light at ZT00 (n=6), ZT06 (n=7), ZT12 (n=6), and ZT16 (n=7). We also evaluated blue evening light at ZT00 (n=8), ZT06 (n=7), ZT12 (n=8), and ZT16 (n=7). For these tissue experiments, both eyes were used, and each choroid was taken as separate samples.

ELISAs were performed to assess concentrations of tyrosinase in the choroid. Written protocol for the chicken tyrosinase ELISA kit (MyBioSource, Catalog MBS289229, 2022) was followed. First, serial dilution of a stock standard was done and added to their specific wells. Individual samples were then added to their own wells. In the 96-well plate, the standard and each sample were run in replicate. A volume of 100µL was used in each well. The plate was then covered and incubated at 37°C for two hours.

Following incubation, the fluid was then removed, and 100µL of the first reagent was added to each well. The plate was re-covered and incubated again for one hour. The wells were then emptied and thoroughly washed three times. After the final wash, the fluid was carefully aspirated with mechanical pipet to ensure full removal and the plate was gently blotted upside down on a clean paper towel. Following the wash, 100µL of the second reagent was then added to each well. The plate was then re-covered and incubated for another hour. The wells were then emptied and thoroughly washed five times followed by aspiration and blotting on a paper towel. Following this, 90µL of substrate solution was then added to each well, it was re-covered, and then incubated a final time for 20 minutes. Then, 50µL of the stop solution was added to each well and the plate was gently tapped on the work surface

to ensure even mixing. The wells then underwent photometric analysis in a 96-well plate reader set for a wavelength of 450nm. A standard curve of absorbance was plotted using the average of the replicate columns for the known standard concentrations. Each individual

replicate absorbance and the standard curve.

Total protein evaluation was done to evaluate tyrosinase in comparison to total choroidal protein concentration. Written protocol for the Pierce 660nm protein assay kit (Pierce Biotechnology, Catalog 22662, 2022) was followed. Pre-diluted solutions were used to create the standard curve. 8µL of either known standard or unknown sample were placed into their respective tubes. Then, 120µL of protein assay reagent was added. These volumes maintained the 1:15 sample to reagent ratio which was outlined in the protocol.

These tubes were then vortexed, covered, and incubated at room temperature for 5 minutes. The samples were then transferred to cuvettes and analyzed by a spectrophotometer set to a wavelength of 660nm. A standard curve was plotted for the known concentrations and then the unknown sample total protein concentrations were calculated using linear

Finally, the data is expressed as tyrosinase in nanograms over total protein concentration in micrograms.

EXPERIMENT III: Effects of Exogenous Dopamine on Blue Evening Light Growth Stimulation

To further evaluate the mechanisms of blue evening light, we exposed chicks in blue evening light to different pharmacologic agents to assess their effects on the stimulated growth. All chicks were exposed to white light that changed to blue four hours before lights-

off, as outlined above. We administered the drugs via intravitreal injections or ophthalmic drops.

Chicks received daily administration of dopamine (n=26), apomorphine (n=26), or saline (n=24) via intravitreal injections. All injections were delivered into only the left eye for each injection group. Baseline biometry was taken on day one prior to any injections. Over the eight-day experimental course, chicks were given a total of seven injections and then final eye measurements were taken on day eight.

Chicks received daily administration of dopamine (n=14) or saline (n=14) via topical application to the ocular surface. All applications were delivered to only the right eye in each ophthalmic drop group. Baseline biometry was taken on day one prior to any drop application. Over the eight-day experimental course, chicks were given a total of fourteen drops and the final measurements were taken on day eight.

Pharmacologic Preparation and Administration

All solutions used for both intravitreal and topical administration were compounded on site at the college. Avian saline (0.75% NaCl) was used as the control for both interventions. All apomorphine and dopamine solutions were created by dissolving the compound in distilled water (Invitrogen, Catalog 10977015, 2019) at the desired concentration. All administrations were carried out at approximately ZT05. The timing of administration was kept consistent across all groups as well as across drug administration modalities.

Intravitreal Injections

To explore the mechanisms of blue evening induced axial elongation, we evaluated the effects of intravitreal administration of two different pharmacologic agents in comparison to saline control. We evaluated apomorphine and dopamine. Based on previous research showing apomorphine administration combating LIM, we used a concentration of 1mg/10mL and a volume of 20µL for the apomorphine group (Nickla et al., 2010). This administration yields a delivery of approximately 7.5nmols per day.

Based on previous research that showed effectivity of dopamine administration combating FDM, we used a concentration of 2.85mg/mL at a volume of 10µL for the dopamine group (Thomson et al., 2020). This administration yields a delivery of approximately 0.15µmols per day.

The procedure was identical across all the solutions used. Chickens were gently restrained with a simple swaddle in a disposable paper towel. A respirator was then placed over their beak, and they were supplied a gas mixture of 2.5% vaporized isoflurane in oxygen. Using 70% isopropyl alcohol prep wipes, the feathers and tissue of the superior left eyelid were cleaned. Using a disposable pipet, a 12.5mm 30-gauge needle was first filled with the desired solution before being applied to a fine microliter syringe (Hamilton Company, Model 1705 LT, 2011). This was done to limit the residual air within the syringe. The appropriate amount of the desired solution was then drawn into the syringe. Once the

chickens were anesthetized to a level deep enough to be unresponsive to stimulation with a tweezer, the injections were administered.

The needle was inserted through the upper eyelid, through the superior aspect of the eye, and approximately 5mm into the vitreous chamber. Care was taken to keep the needle's maintaining this position, the syringe plunger was then depressed at a slow but reasonable speed. After approximately 10 seconds of wait time following full evacuation of solution from the syringe, fine point tweezers were used to hold the overlying tissue surrounding the needle. The needle was slowly withdrawn from the globe and the overlying skin was firmly pinched with the forceps for approximately 10 seconds. During the procedure, care was taken to not angle the needle in any way to prevent undesirable secondary damage to the lens, retina, or any other internal structure. Additionally, care was taken to not put pressure onto the eye while pinching the overlying tissue to avoid researcher induced reflux of the experimental drug or intraocular contents.

Ophthalmic Drops

In an effort of probing clinical applicability, we desired to know if topical administration of dopamine could inhibit blue evening light growth stimulation. After proper formulation, the solution was placed into plastic dropper bottles (Nalgene, Catalog 2750-9025, 2010). When measured in the lab, each drop of solution from this dropper was approximately 55μL. This is within the range of what other sources often define the volume of an ophthalmic drop as (Van Santvliet, 2004).

We decided to use just over a tenfold concentration relative to that which was used intravitreally: 29mg/mL. This concentration provided a drug administration level consistent with prior findings that have seen topical dopamine to be effective against FDM (Thomson et al., 2020). Each chick received two drops each day in the right eye. This dosage yields delivery of approximately 16.9µmols per day.

The procedure was identical across both solutions used. The dropper with solution was stored at -4ºC, and prior to each use the solution was vortexed for ten seconds to ensure the drug was fully dissolved and without precipitate. The chickens were gently restrained by being swaddled in a disposable paper towel. After being restrained, they were placed on their side and their eyelids were retracted by the gentle digital pressure of the experimenter. One drop was then applied to the ocular surface. The experimenter maintained gentle hand-held

the loss of solution from the surface of the eye that would occur if the chicken immediately adopted an upright posture or shook its head. Chicks were then released from the paper towel for the space of five minutes. We chose this intermission time as it is the recommended time to wait between ophthalmic drop instillation (Gudgel, 2023). After waiting, the chicks were again gently restrained and the second drop was applied in the same manner as before.

Figure 3: Ophthalmic Drop Administration

Statistics

All statistical analyses were completed using the R statistical computing and graphing language (R Core Team, 2024). All coding written and run for statistical analysis and graphical creations was done using the RStudio platform (Postit Team, 2024). All data collected and used for analyses and all code written for the statistics and figures created are readily available on GitHub (Watson, 2024). A p-value less than 0.05 was considered significant for all analyses.

To assess for outliers, all data were evaluated with preliminary graphic representations by plotting the data with the box plot geometry in R. The package utilized in R to identify these potential outliers evaluates the data using the common interquartile range (IQR) method and singles out any values that 1.5 times the IQR smaller than the first quartile, or 1.5 times the IQR larger than the third quartile (Wickham, 2016). Any values identified by this method to be outliers were further assessed. Any values then found to be

greater than two standard deviations from the mean were removed from the data. Using this methodology, a total of seven values were removed from the entirety of the data evaluated. The specifics of which groups and experiments from which these outliers were removed are explicitly outlined in Tables 1, 2, and 3. Following the removal of any potential outliers, the data was evaluated for normalcy using the Shapiro-Wilk test (Tables 4, 5, and 6).

If groups were found to be parametric, a two-sample t-test was used for data with two groups, and analysis of variance (ANOVA) was used for data with more than two groups.

Difference (HSD) post-hoc testing to evaluate the comparisons between groups. If groups were found to be non-parametric, a Mann-Whitney test (MW) was used for data with two groups, and a Kruskal-Wallis test (KW) was used for data with more than two groups. Any

-hoc testing (DT) to evaluate the comparisons between groups with adjusted p-values using the Bonferroni method.

Our paired statistics concerning the eye growth assessment were directional when possible. Both the t-test & MW test were one-tailed with the assumption that the experimental eye or intervention would have a decreased growth compared to the fellow eye or control.

Finally, post-hoc analysis of effect size was carried out on our experiments that involved ocular growth assessment with pharmacologic intervention. We assess effect using

(d) for t-tests, rank biserial correlation (r) for MW, and omega squared (^{2}) for ANOVA. These findings are detailed in tables 7 and 8.

RESULTS

EXPERIMENT I: Western Blots

The western blot protein assay separates proteins based on molecular weight. It does this by utilizing an electric field that influences the zwitterionic proteins to move through the electrophoresis gel. Proteins of a larger molecular weight move through the gel slower than those which are smaller. After the proteins have been transferred from the gel to the blotting membrane, we use antibodies with a certain affinity for our proteins of interest. By using the reference protein ladder with known molecular weights, we can compare the protein sample bandings obtained to verify them to the molecular weight of the protein of interest. Our two proteins of interest and their molecular weights were tyrosinase (TYR): 60 kDa and tyrosine hydroxylase (TH): 56 kDa. The control protein assessed was GAPDH: 36 kDa.

Assay I Tyrosinase

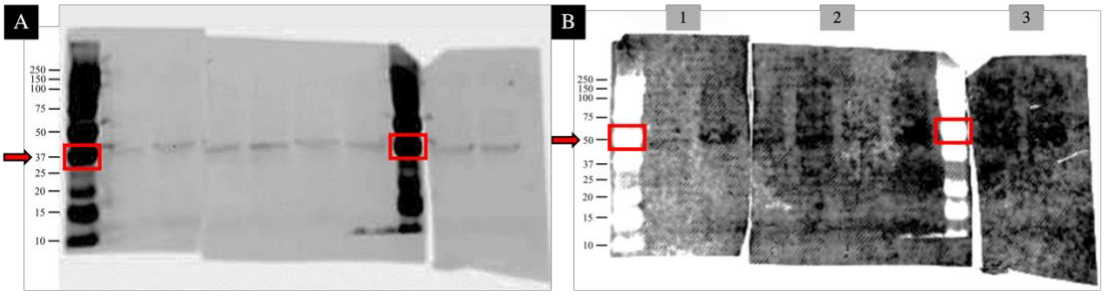

Figure 4: Assay I for TYR

Our control for GAPDH showed consistent banding across all columns in this assay at the proper protein ladder reference (Figure 4). However, for tyrosinase, our banding results

were not as neat. There are definite bands at the 50 kDa reference of the protein ladder which matches well with the molecular weight of TYR. The bands obtained were broad and mildly weak in signal, so some photo elements had to be altered to best visualize these bands.

These findings suggest that the presence of tyrosinase in the choroid of chicks. The faint banding obtained is possibly due to the paucity of protein present in the choroidal tissues sampled, which may be compounded by tissue processing with potentially too much fluid.

Assay II Tyrosinase

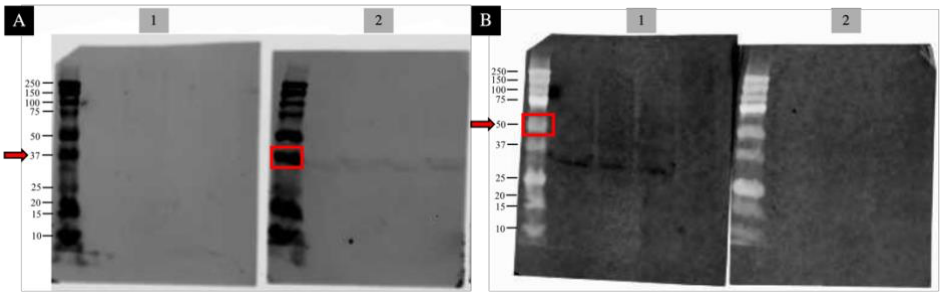

Figure 5: Assay II for TYR

In assay II we altered the sample preparation by using two choroids rather than one in hopes of increasing protein concentration. The GAPDH control here is faint but apparent on all tested columns at the proper protein ladder reference level (Figure 5). The columns evaluating for TYR have banding across three of the four treated. In reference to the protein ladder, these bands are at the level of approximately 30 kDa. This is half the expected size of our protein of interest.

These results again show a lack of banding at the proper level to be indicative of tyrosinase. Despite this, presence of somewhat broad, but definite banding is interesting. These findings could be an example of prominent non-specific binding with other proteins containing similar epitopes.

Assay III Tyrosine Hydroxylase

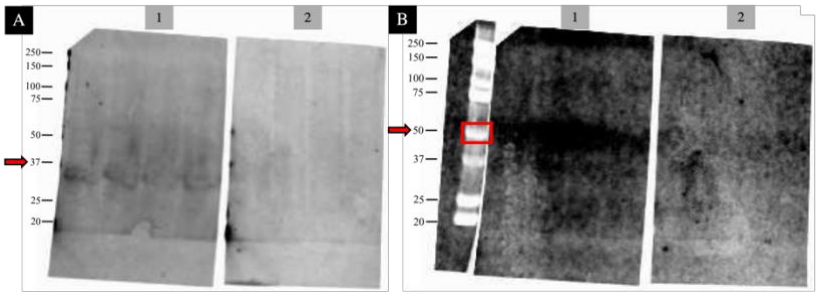

Figure 6: Assay III for TH

After difficulty obtaining sharp TYR bands, we decided to evaluate for TH with this assay. The GAPDH control bands are faint and broad in the treated half of the membrane (Figure 6). Though this is the case, the bands obtained are also close to the expected level in comparison to the reference ladder. Two primary antibody dilutions were tested to evaluate for TH, and neither produced sharp bands. Both concentrations yielded notably broad bands that span between columns. It is notable that the banding is much fainter in the membrane treated with the more dilute concentration. These bands for TH are at the expected level in reference to the protein ladder.

These faint and broad bands for TH are weakly positive, if at all. If the bands are true, the notably decreased banding across different primary antibody dilutions suggest us that a 1:800 dilution was likely far to dilute for our purposes. The broad and faint banding made us consider that perhaps the tissue concentration we were using needed to be increased for future experiments.

Assay IV Tyrosinase and Tyrosine Hydroxylase

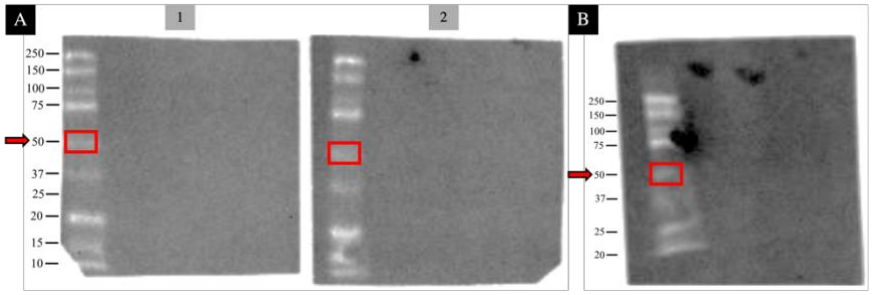

Figure 7: Assay IV for TYR and TH

In this assay, we pooled eight choroids together and decreased the fluid used when homogenizing the samples. We wanted to evaluate the previously used antibodies for both TYR and TH on this increased protein concentration. Despite this attempt to obtain more definitive banding, this experiment was entirely fruitless. Only the protein ladder control was seen on evaluation along with scattered splotching (Figure 7).

Due to the reasonably run protein ladder control and lack of any GAPDH control banding, the error in this procedure was likely not the electrophoresis aspect of this experiment. There were no atypical deviations in sample preparation, experimental

procedure, or antibody preparations when running this assay that obviously explain these results. Our images of the membranes treated for GAPDH in this assay were not saved but, similar to those in Figure 7, showed no banding.

Assay V Tyrosinase and Tyrosine Hydroxylase

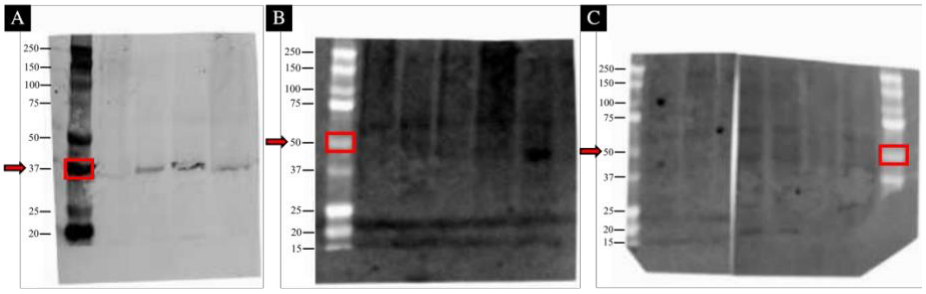

Figure 8: Assay V for TYR and TH

We decided to change the antibodies used in attempt to obtain improved results for both TYR and TH. We maintained the same GAPDH control. For this assay, GAPDH showed proper banding in three columns evaluated, while the fourth column was essentially devoid of banding (Figure 8).

The assay for TYR showed four perceivable separate bands across all columns as well as high background signals. Two of these bands are adjacent to the expected TYR reference level, but the other two are adjacent to the 20 kDa reference near the bottom of the columns. These two lower bands are quite dense and even appear to extend between columns.

The assay for the serine phosphorylated TH also showed multiple perceivable bands across all columns tested. Most of these columns have two bands that are adjacent to where the reference for TH should be. The other bands that can be seen in some columns appear much at levels much smaller than would be expected for TH. Additionally, there are some splotching artifacts seen in this membrane.

The numerous levels of banding obtained for both antibodies tested is likely from nonspecific binding. This type of error is often associated with high antibody concentrations (Gavini, 2023). We used a primary antibody concentration of 1:200, but this preparation proved much too concentrated.

In summary, Assay I was the most convincing with banding indicating TYR presence in chicken choroid, but the bands were faint. Assay III presents very weak evidence for TH presence in chicken choroid. The results of the other western blot assays failed to identify TYR or TH because they had issues such as absence of banding or the presence of multiple bands which make the results inconclusive.

EXPERIMENT II: ELISA for Choroidal Tyrosinase at Differing Times of Day

We evaluated choroidal tyrosinase concentrations across four time points: ZT00, ZT06, ZT12, and ZT16. We did this evaluation for both white and blue evening light conditions. Across the four timepoints, we found significant diurnal variations in TYR concentration in both white light control (KW, $p=5.40E-5$) and blue light experimental (KW, $p=3.01E-10$) groups. These variations are expressed in supplementary Figure 18.

Our results also show that the blue evening group had a statistically lower concentration of TYR than the white light group at ZT00 (t-test, $p=8.11E-5$), ZT06 (t-test,

p=0.031), and ZT12 (t-test, p=7.20E-5); the difference at ZT16 was insignificant (MW, p=0.069).

These findings are indicative of time-of-day alterations in the TYR expression patterns of the choroid. It also appears blue evening light exposure alters the expression pattern and magnitude. The significant depression in TYR concentration seen in blue evening light for most of the time points tested suggests that this paradigm attenuates the TYR production in the choroid.

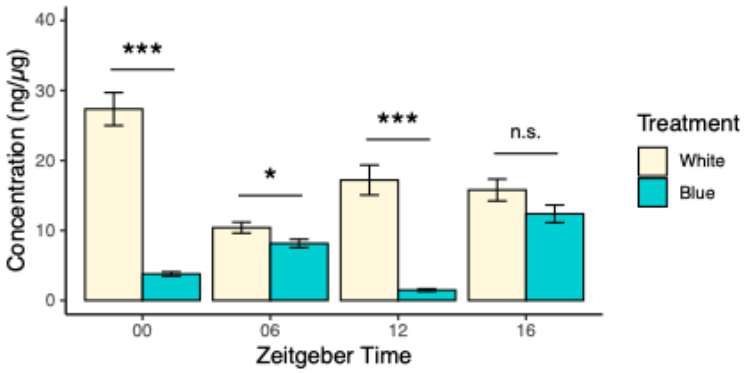

Figure 9: Tyrosinase Concentrations Changes Between Treatment

EXPERIMENT III: Exogenous Dopamine and Blue Evening Light

We evaluated ocular biometry changes in response to intravitreal as well as topical administration of dopaminergic drugs. For the injection group, we used dopamine and apomorphine, a non-specific dopamine agonist; saline injections were the control. For the topical drops, we used dopamine and a saline control. We evaluated the growth rate directly

comparing experimental and fellow eyes, and presented the data as interocular rate difference. The rate was difference between the final measure and the starting measure over the treatment duration. The interocular difference was calculated as treated eye minus the non-treated eye (experimental - fellow). The changes in ocular growth were measured for four ocular components: anterior segment depth (AC+L), vitreous chamber depth (VC), choroidal thickness (CT), and axial length (AL).

Intravitreal Injections

Axial Length

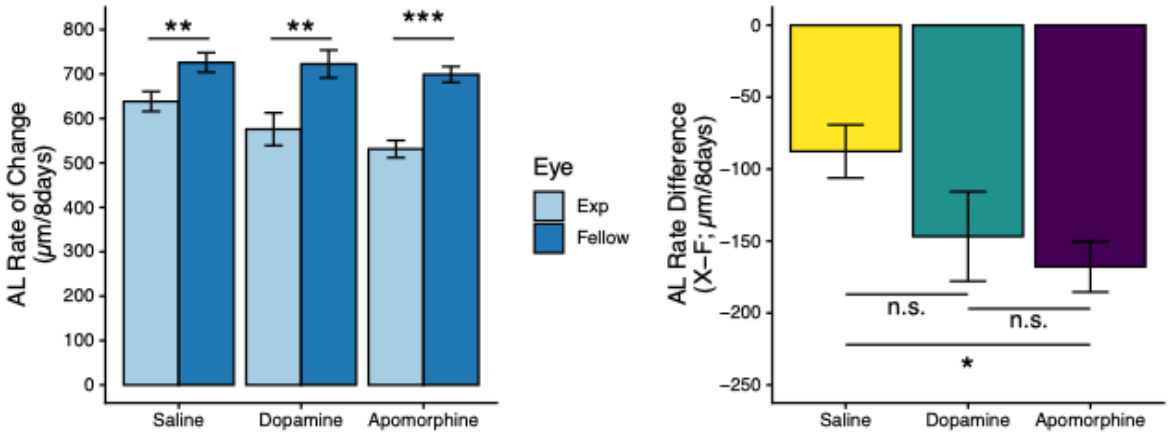

Figure 10: Injections Axial Length

We found that the AL rate was statistically lower for the experimental eye in all three groups: saline (t-test, p=0.005), dopamine (t-test, p=0.005), and apomorphine groups (MW, p=2.88E-06). This significant finding in the saline-injected eyes evinces an injection effect.

The far lower p-value for the apomorphine group is indicative of a true effect apart from simple injection. Additionally, when the interocular differences were examined, we found that there was a significantly greater difference in the apomorphine group relative to that of the saline control.

The AL interocular rate difference had group means which were significantly different (ANOVA, p=0.020). The interocular difference was significantly more negative in the apomorphine group versus the saline group (HSD, p=0.017), suggesting that apomorphine was effective at reducing the blue-light-induced growth stimulation. There was no statistically significant difference between the saline and dopamine (HSD, p=0.178), nor dopamine and apomorphine (HSD, p=0.787) groups. A more negative interocular difference suggests that the . Our findings suggest that intravitreal administration of apomorphine attenuates the growth stimulation caused by blue evening light.

Anterior Segment Depth

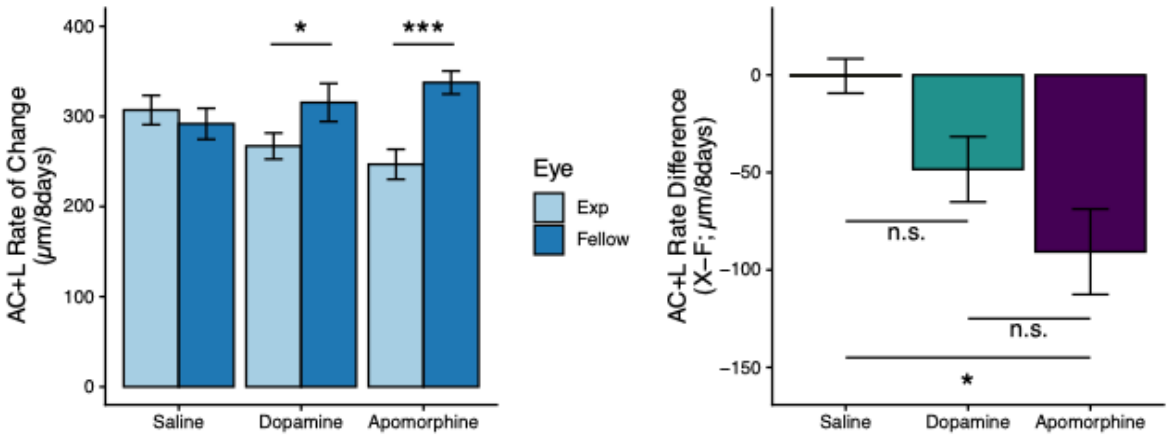

Figure 11: Injections Anterior Segment Depth

We found a significantly decreased the anterior segment depth (AC+L) growth rate relative to fellow eyes for dopamine (t-test, p=0.043) and apomorphine (t-test, p=0.0001). There was no significant change in the AC+L rate between the experimental and fellow eyes of the saline group (t-test, p=0.737). The interocular rate differences for AC+L had a significant difference between the treatment group means (ANOVA, p=0.003). The interocular difference was significantly more negative in the apomorphine group versus the saline group (HSD, p=0.002). There was no statistically significant difference between saline and dopamine (HSD, p=0.224), nor dopamine and apomorphine (HSD, p=0.285) groups. These findings suggest that the axial effect with intravitreal apomorphine involves a reduction in anterior chamber growth.

Vitreous Chamber Depth

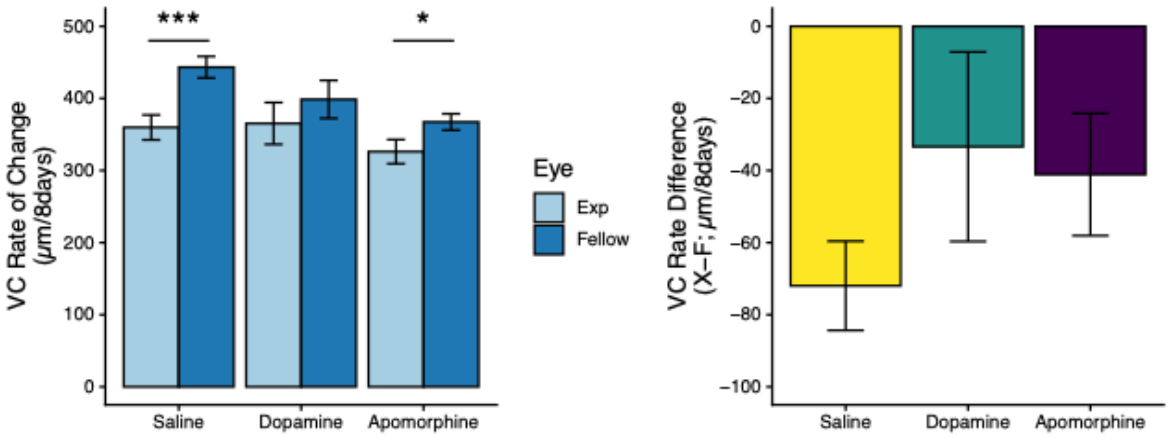

Figure 12: Injections Vitreous Chamber Depth

For vitreous chamber growth, we found that both intravitreal saline (t-test, p=0. 0007) and apomorphine (t-test, p=0.027) caused a significantly decreased VC growth rate relative to the fellow eye. Dopamine (t-test, p=0.205) had no statistically significant difference between the eyes. The significant finding for saline and for apomorphine is indicative of an injection effect. When considering the VC interocular rate difference variable, there was no significant difference between group means (ANOVA, p= 0.276). Therefore, the axial effect with intravitreal apomorphine was predominantly due to an effect on anterior chamber growth.

Choroid Thickness

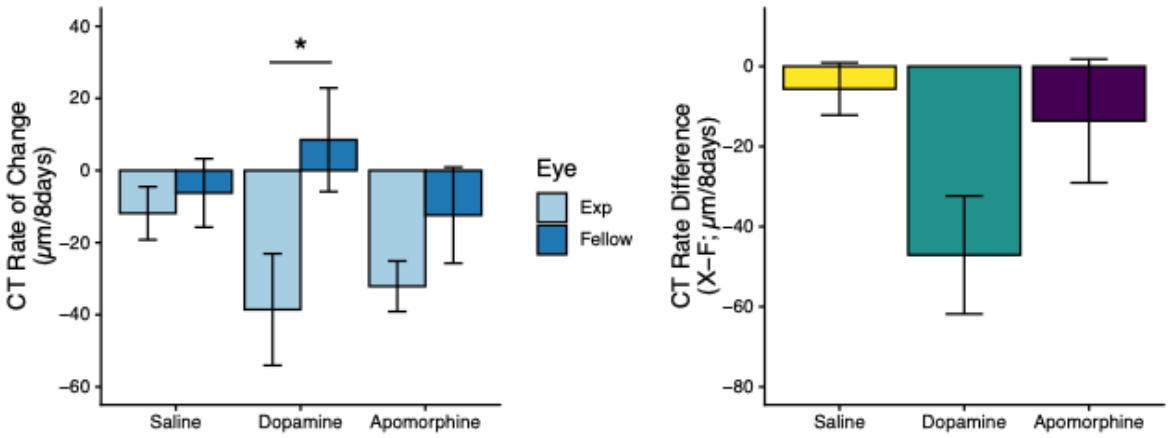

Figure 13: Injections Choroid Thickness

We found significant choroidal thinning with intravitreal dopamine injections (t-test, p=0.023). However, neither saline (t-test, p=0.322) nor apomorphine (t-test, p=0.103) had any significant changes. When considering the CT interocular rate difference variable, there was no significant difference between group means (ANOVA, p=0.125).

Ophthalmic Drops

In general, topical ophthalmic drops had no significant effects on any ocular parameter.

Axial Length

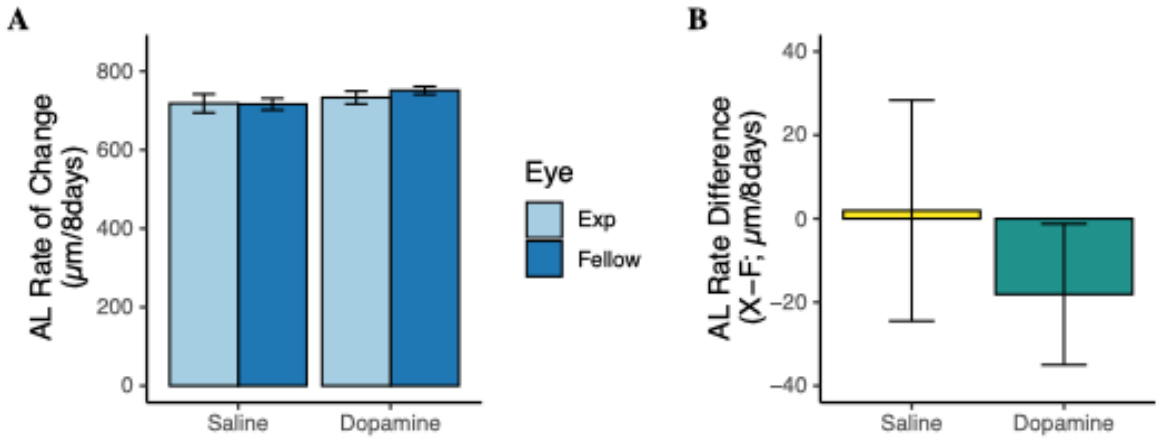

Figure 14: Drops Axial Length

We found no statistically significant difference in the axial growth rate of eyes that were administered saline (MW, p=0.402) or dopamine (t-test, p=0.185). Additionally, the interocular AL growth rate difference was also not statistically different between saline and dopamine (t-test, p=0.537).

Anterior Segment Depth

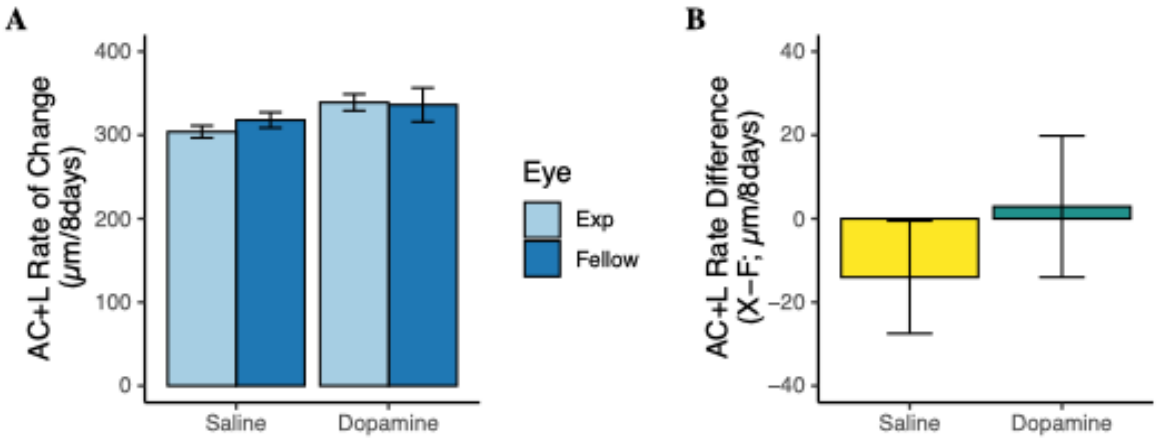

Figure 15: Drops Anterior Segment Depth

There was no statistically significant difference in AC+L growth rate between the eyes for saline (t-test, p=0.13) or dopamine (t-test, p=0.550). The interocular AC+L growth rate difference similarly showed no statistical difference between saline and dopamine (t-test, p=0.450).

Vitreous Chamber Depth

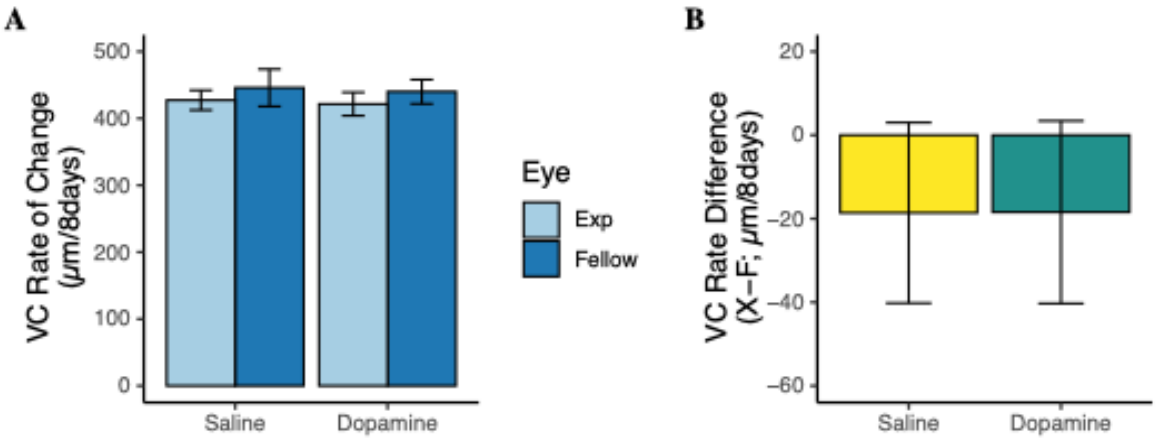

Figure 16: Drops Vitreous Chamber Depth

The VC growth rate showed no statistically significant difference between the eyes for saline (t-test, p=0.285) or dopamine (t-test, p=0.239). The VC growth rate difference showed no statistical significance between saline and dopamine (t-test, p=0.996).

Choroid Thickness

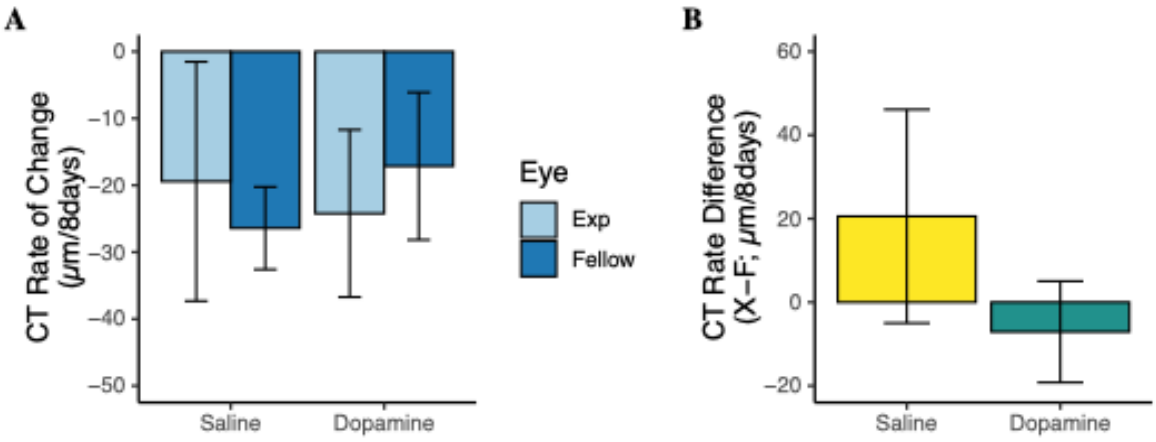

Figure 17: Drops Choroid Thickness

We found no statistically significant difference in CT growth rate between the eyes for saline (t-test, p=0.639) or dopamine (t-test, p=0.340). There also was no statistical significance in interocular CT growth rate difference between saline and dopamine (t-test, p=0.355).

DISCUSSION

This thesis provides evidence supportive of the hypothesis that blue evening light stimulates eye growth by a mechanism involving decreased dopamine, likely of choroidal origin. Our choroid sample western blots showed no evidence of TH, the retinal dopamine synthetic enzyme, but we found TYR. This was additionally confirmed using ELISA. The choroids of eyes exposed to blue evening light showed reduced TYR. Finally, daily intravitreal injections of the nonspecific dopaminergic agonist apomorphine inhibited the growth stimulation usually seen with blue evening light. Together these findings suggest that the chicken choroid likely performs TYR-based dopamine synthesis, and blue evening light decreases this choroidal dopamine pool which subsequently leads to axial elongation.

Dopamine and Myopia

We report that intravitreal injections of apomorphine, a non-selective dopamine agonist, inhibited the growth stimulation caused by evening blue light. However, dopamine alone was not effective when administered intravitreally or topically. The intravitreal DA results had a trend for an effect; AL & AC+L interocular rate difference show no significance between dopamine and either the saline control or the apomorphine group. The apomorphine group was significantly decreased compared to saline for both of these parameters. This trend should be considered but was still not statistically significant.

Our post-hoc assessment of effect size on AC+L shows a small saline effect (d=0.28) and a large effect for intravitreal dopamine (d=-1.16) and apomorphine (d=-1.82). Effect size on AL was large for saline (d=-1.19), dopamine (d=-1.79), and apomorphine (r=-0.93). This

Others have reported the ability of intravitreal and topical dopamine to suppress eye growth, specifically in DM (Thomson et al., 2020). The µmol/day dose in our experiment is within the range of what these researchers found effective. This may suggest that blue evening growth stimulation is less sensitive to inhibition by topical dopamine than DM. More likely, this may be the result of insufficient dosage or, in the case of topically administration, physical barriers to site of action. Apomorphine is a nonspecific agonist of the dopaminergic system with a greater affinity for the D2 receptor relative to dopamine (Durdagi et al., 2016). The D2 receptor has been shown to be more involved in growth inhibition in chicks (Feldkaemper and Schaeffel, 2013). Perhaps this inequality of receptor affinity also played a role in apomorphines effects relative to dopamine.

There are five types of dopaminergic receptors that functionally fall into two overarching classes: D1-like or D2-like dopamine receptors (Witkovsky, 2004). Considering our findings and apomorphines effect, an evaluation of more agonists or antagonists specific for the different receptor types would be beneficial. A D2-related pathway for inhibiting myopia has been suggested for DM, but not other experimental myopias like LIM (Rohrer et al., 1993; Morgan et al., 2013). Following similar prior experimental paradigms with administration of apomorphine with co-administration of a D2 antagonist like spiperone in blue evening light would be a beneficial study to elucidate the possible preference for a dopaminergic receptor pathway for preventing blue evening myopia.

The Choroid and Dopamine

Because of its likely role in emmetropization and the fact that the signal cascade from retina to sclera must cross choroid, there is interest in the possibility that the choroid is a

source of dopamine apart from the retina. Our results are the first in chicks showing the presence of TYR in choroid, supporting that hypothesis. We also found a significant TYR reduction in eyes that had been exposed to evening blue light, strongly suggesting the likelihood that dopamine is involved in that effect.

Prior research has evaluated and suggested a role for choroidal tyrosinase in guinea pigs (Jiang et al., 2018). Albino guinea pigs, which have nonfunctional tyrosinase but normal tyrosine hydroxylase, consistently develop myopia while normally pigmented guinea pigs are hyperopic. When treated with peribulbar kojic acid, a tyrosinase inhibitor, normally pigmented guinea pigs develop increased axial length and significant myopia comparable to the albino strain. Furthermore, the DA levels in the retina of normally pigmented and albino guinea pigs were similar, while the choroidal DA levels were lower only in the albino group. These findings support the choroid as a protective dopamine source in this animal model. Choroidal TYR may function directly in the DA signaling pathway or as a production buffer to modulate retinal dopamine output. Our findings of decreased TYR in blue evening light support the hypothesis that DA from choroidal TYR is protective against eye growth.

Given that TYR in the choroid and TH in the retina can both synthesize L-DOPA, the precursor of dopamine, TYR likely directly contributes to the dopamine pool (Meiser et al., 2013). In mice, it has been shown that peripheral dopamine production occurs in a tyrosinase-dependent manner when young, which this goes away in adulthood (Eisenhofer et al., 2003). Increased TYR concentration has been associated with increased cellular dopamine in cultivated neural tissue (Hasegawa, 2010). Also, in a review on skin and

neurodegenerative disorders, increased TYR activity has been suggested to possibly cause high dopamine production (Jin et al., 2024).

In chickens, RNA sequencing data show that the expression for TH in the choroid was essentially absent while TYR was present (Stone et al., 2024, Supplementary Data; See figure 19). Immunohistochemical studies have shown TH presence in nerves and around blood vessels in the choroid of chickens (Mathis et al., 2023). This suggests that though TH gene expression was low, the protein is still present. Very recent results from an immunohistochemical evaluation of human choroids verified the presence of DA and two related proteins, TH and dopamine beta hydroxylase, in choroidal neurons (Konwar et al., 2025). Interestingly, there were neurons identified in this study that had the presence of DA without colocalization with TH, thus supporting our stance that TH is not the sole producer of DA in the choroid.

Our findings of decreased TYR concentrations in response to blue evening light provide a potential mechanism for the growth stimulation in this visual condition. We

suggest blue evening light exposure decreases TYR levels which causes a drop in dopaminergic stimulation, precipitating axial elongation. Very recent work also proposes a differing potential alternate pathway to dopamine synthesis in the choroid of humans (Schroedl et al., 2025). The proposed pathway involves the enzyme cytochrome P450, well known for metabolizing many drugs, which was seen to colocalize with DA within intrinsic choroidal neurons. This is a new development that should be considered going forward, in addition to TYR.

The choroid is an exciting area in myopia research. The growth signal sensed by the retina must be transmitted through this intermediary to elicit or inhibit scleral changes. The physical characteristics of the RPE and choroid make simple diffusion of the molecular signal from the retina and across the choroid unlikely; a sequential cascade is more plausible (Wallman & Winawer, 2004). As in the guinea pig studies mentioned, we suggest in this work that nase-based mechanism and likely plays a role in the signal cascade (Jiang et al. 2018).

Changes in choroidal thickness have been associated with different experimental interventions. In general, the choroid typically thins during myopia development. In response to interventions that combat myopia or during recovery from experimental myopia, the choroid has been seen to transiently thicken (Nickla et al., 2010; Fitzgerald et al., 2001). Furthermore, prior work in chickens shows decreased choroidal blood perfusion associated with increased myopia (Reiner et al., 1995). Research in the guinea pig concerning choroidal perfusion has shown similar findings, and that increasing choroidal perfusion is protective against FD (Zhou et al., 2021 & 2020). Zhou et al. propose that scleral hypoxia seen with decreased choroidal blood perfusion elicits biochemical changes to the sclera that allow for axial elongation.

There have been reported findings of decreased choroidal thickness and perfusion

myopia regulation (Hao, 2024). I propose the possibility that choroidal thickening with increased perfusion may increase dopamine production via TH or TYR, given that the increased oxygen supply gives more reactant to the biochemical change of tyrosine to DOPA

(Meiser et al., 2013). Perhaps this, in concert with inhibiting hypoxia-induced changes of the sclera, explains the protective effects of increased perfusion.

The choroidal blood perfusion studies cited in chicks utilized surgically invasive laser doppler flowmetry. Given the advent and substantial improvement of optical coherence tomography angiography (OCT-A), which was used in the guinea pig research, using OCT-A in chicks would be a beneficial change for future research endeavors. Though our results

and assessing if experimentally increasing perfusion may be protective against myopia development in this setting would be valuable.

Albino chicks have been proposed as a novel model to understand eye growth, and (Rymer et al. 2007). Rymer et al. report albino chicks developing myopia and longer vitreous chamber depths. Others have also reported similar findings and also thinner choroids in albino chicks (Wildsoet et al. 2002). Albinism is due to a mutation in TYR. Evaluating albino chickens to understand myopia development in albino versus normally pigmented chicks would be valuable in further elucidating the effect tyrosinase functionality plays in emmetropization. Following the guinea pig paradigm, comparing eye growth between normally pigmented chickens treated with kojic acid and albino chickens would be beneficial in further assessing the role of choroidal tyrosinase in this animal model.

Blue Light and Circadian Rhythms

Rearing in blue light induces hyperopia in some species (Foulds et al., 2013). However, time of day exposure is a crucial variable as brief morning or evening exposures

cause growth stimulation. Circadian rhythms are modulated by light and can be affected by altering time of exposure to light. There are other signals that facilitate circadian entrainment, but light is paramount (Vitaterna et al., 2001).

Uniquely, blue light is the most potent modulator of circadian rhythms (Wahl et al., 2019). Blue light stimulates ipRGCs which project to the SCN which then modulates diurnal

gradually increase to a maximal point at midday, then gradually decrease as the sun sets. In the modern day developed world with indoor lighting and screens, this cyclic pattern of light exposure is increasingly rare. Many components of modern light exposure are worthy of consideration concerning circadian rhythms. Indoor lighting has a fraction of the wavelengths and intensity that sunlight offers (Biswas & Kim, 2020; Dhakal, 2023). Common screens have a spectral output with the largest component in the blue spectrum (Wahl et al., 2019). Blue light before or at bedtime can delay normal rhythms; the outcome of which has various physiological effects.

Alterations of circadian cycles from jet lag or shift work have been associated with multiple deleterious health outcomes (Wahl et al., 2019; Vaghefi et al., 2024). It is reasonable to consider that at least a portion of the breakdown in emmetropization currently prevalent today may be related to altered circadian rhythms. In a point-counterpoint article concerning outdoor environment and its protective effects on myopia, Flitcroft comments that while light exposure is modulating on eye growth, it does not explain the closed loop regulation of emmetropization; he suggests that other aspects of the outdoor environment likely additionally confer protective effects (Ngo et al., 2013). Likewise, a closed-loop

.

Different components of the eye change throughout the day. There is the opposite relationship of axial length and DA variations which have been discussed. The choroid also varies in that there is thinning near midday and thickening near midnight. Axial length and choroid thickness have an approximate antiphase relationship normally which shifts to being in-phase when eyes are slowing their growth (Nickla et al., 2006). When exposed to blue evening light, both axial and choroidal rhythms showed significant alterations (Nickla et al., 2022). Our findings support a TYR rhythm in the choroid and that blue evening light alters this rhythm which further highlights the importance of regular circadian cycles.

There is still much to be understood concerning the growth stimulation seen in blue evening light, the choroid as a source of dopamine, as well as the involvement of the choroid

ly say that

exposure to evening blue light alone causes the findings because we evaluated only one color and one time of exposure. Morning blue light exposure also caused axial elongation when the intensity was low (Nickla et al., 2023). Evening exposures of both yellow and red have also stimulated eye growth (Nagpaul et al., 2024). Further exploration with morning dim blue light exposure, and differing chromaticity with the same paradigm of short duration of exposure in evening or morning is needed. Evaluation of TYR levels and eye growth in these settings would better facilitate understanding if the drop in TYR levels we saw is unique to blue evening light, or if it is some other process related to altered lighting conditions in general during lights-on or lights-off transition times.

A review of screen use and sleep in the adolescent population reported screen use being associated with shorter sleep duration, among other poor sleep outcomes (Silva et al., 2022). Screentime at night when lying down is associated with delayed sleep onset (Yoshimura et al., 2017). Light exposure at night, especially blue light, can suppress and delay the normal melatonin rhythm which regulates sleep (Tahkamo et al., 2019). Current evidence is mixed but suggests a potential association between poor sleep and childhood myopia (Liu et al., 2023). Considering that most humans today are not experiencing a twelve-hour-on, twelve-hour-off lighting environment due to screen use and other artificial sources, altering experimental conditions may better show how the effects of these changes may impact emmetropization. Exploring the effects of extending the window of lights-on for control white light and blue evening exposures beyond the typical 12 hours lights-on would be an interesting paradigm to evaluate this.

Limitations

There are some limitations to consider in this thesis and the results reported. Firstly, our western blot results identified TYR in only one assay. Equivocal banding was obtained for both TYR and TH despite multiple experimental alterations in the remaining assays. Considering all experiments performed, we utilized a choroid:µL RIPA buffer ratio sample preparations of 1:300, 1:50, and 1:10. Most of our assays struggled with faint banding and nonspecific binding. Faint bands can be caused by paucity of antigen or antibody, or also masking from milk proteins used in the blocking step (Mahmood and Yang, 2012). Our problems were likely due to low sample protein concentrations, so we suggest for future experiments to utilize a sample preparation ratio between 1:50 and 1:10. Choosing to block

the membrane with bovine serum albumin (BSA) or decreasing the amount of milk utilized have also been suggested for the shortcomings that were seen with our westerns and could be considered for future experiments (Mahmood and Yang, 2012).

Titrating the concentrations of the primary antibodies used are also likely necessary for best results in future experiments. We trialed primary antibody concentrations of 1:800, 1:500, and 1:200. For future experiments using the primary antibodies for TYR and TH utilized in assays I-IV, we suggest trialing primary antibody dilutions between 1:400 and 1:300 (Lifespan Biosciences Inc, Catalog LS-C40464, 2022; Immunostar, Catalog 22941, 2022). For the antibodies evaluated in assay V, due to the notable amount of nonspecific binding, the dilution of 1:200 was likely much too concentrated. We would recommend a dilution between 1:500 and 1:300 for these antibodies (Cell Signaling Technology, Catalog 2791S, 2022; MilliporeSigma, Catalog MAB318, 2023). We trialed secondary antibody concentrations of 0.2µg/mL and 0.4µg/mL. The higher concentration was utilized for four out of the five assays performed. This is the high end of the concentrations recommended for western blotting in the datasheet for both products (Invitrogen, Catalog A32731, 2022; Invitrogen, Catalog A32723, 2022). For that reason, we recommend first making the above alterations for primary antibodies before trialing a different secondary antibody concentration of 0.4µg/mL.

Another limitation in this study is

have a topical apomorphine group. Evaluating topical apomorphine would be very interesting especially given our findings that the axial changes in the apomorphine injections group in part were due to reduction in anterior chamber growth. Given this, perhaps topical

apomorphine could have elicited an effect due to potentially less distance to travel to a potential site of action. Secondly, we only evaluated one concentration of dopamine. The effect of dopamine may be underrepresented given the diffusion barriers with topical administration.

Conclusion

We conclude that blue evening light causes a drop in choroidal tyrosinase and propose this leads to a decrease in dopamine output. This decrease causes a dysregulation of emmetropization and the subsequent axial elongation. Increasingly frequent use of screens creates a common evening exposure to blue light and raises questions on the potential etiology of increasing myopia prevalence. There seems to be a complex interplay between light composition, time of day exposure, and the retinal and choroidal dopaminergic systems which require further research to fully elucidate their roles in eye growth.

SUPPLEMENTARY TABLES AND GRAPHS

ELISA TYR Concentration Number of Outliers Discarded				
	ZT00	**ZT06**	**ZT12**	**ZT16**
White	0	1	0	0
Blue	0	1	1	0

Table 1: Values Removed from ELISA Data

Experimental & Fellow Rate of Change Number of Outliers Discarded					
	AC+L	**VC**	**CT**	**AL**	**Eye**
Injections					
Saline	0	0	0	0	Experimental
Dopamine	0	0	0	0	Experimental
Apomorphine	0	0	1	0	Experimental
Saline	0	1	0	0	Fellow
Dopamine	0	0	0	0	Fellow
Apomorphine	0	0	0	0	Fellow
Drops					
Saline	0	0	0	0	Experimental
Dopamine	0	0	0	0	Experimental
Saline	0	0	1	0	Fellow
Dopamine	0	0	0	0	Fellow

Table 2: Values Removed from Growth Rate Data

Interocular Rate of Change Difference Number of Outliers Discarded				
	AC+L	**VC**	**CT**	**AL**
Injections				
Saline	1	0	0	0
Dopamine	0	0	0	0
Apomorphine	0	0	0	0
Drops				
Saline	0	0	0	0
Dopamine	0	0	0	0

Table 3: Values Removed from Growth Rate Difference Data

ELISA Concentration Choroid Samples				
	ZT00	**ZT06**	**ZT12**	**ZT16**
White	0.674	0.138	0.973	0.009
Blue	0.607	0.142	0.144	0.010

Table 4: Evaluation of Normalcy for ELISA Data

Pharmacologic Intervention Experimental & Fellow Rate of Change					
	AC+L	**VC**	**CT**	**AL**	**Eye**
Drops					
Saline	0.724	0.797	0.932	0.047	Experimental
Dopamine	0.056	0.591	0.988	0.994	Experimental
Saline	0.886	0.658	0.540	0.615	Fellow
Dopamine	0.532	0.498	0.841	0.928	Fellow
Injections					
Saline	0.280	0.200	0.783	0.996	Experimental
Dopamine	0.071	0.384	0.742	0.091	Experimental
Apomorphine	0.174	0.853	0.456	0.753	Experimental
Saline	0.221	0.068	0.077	0.079	Fellow
Dopamine	0.667	0.379	0.548	0.263	Fellow
Apomorphine	0.996	0.793	0.210	0.032	Fellow

Table 5: Evaluation of Normalcy for Growth Rate Data

Pharmacologic Intervention Rate of Change Difference				
	AC+L	**VC**	**CT**	**AL**
Drops				
Saline	0.339	0.625	0.921	0.445
Dopamine	0.180	0.142	0.356	0.247
Injections				
Saline	0.579	0.947	0.936	0.124
Dopamine	0.982	0.235	0.790	0.467
Apomorphine	0.098	0.993	0.099	0.899

Table 6: Evaluation of Normalcy for Growth Rate Difference Data

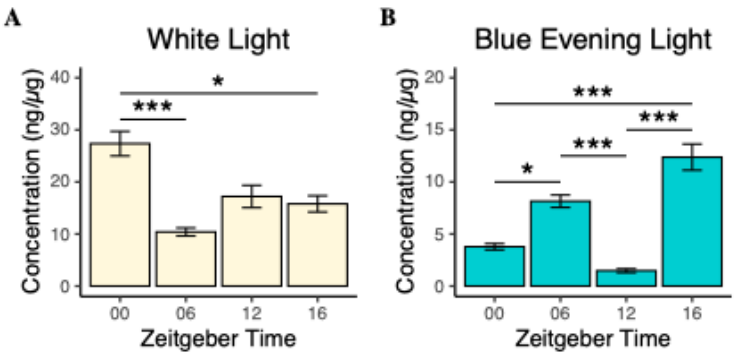

Figure 18: Tyrosinase Concentration Variations with Time

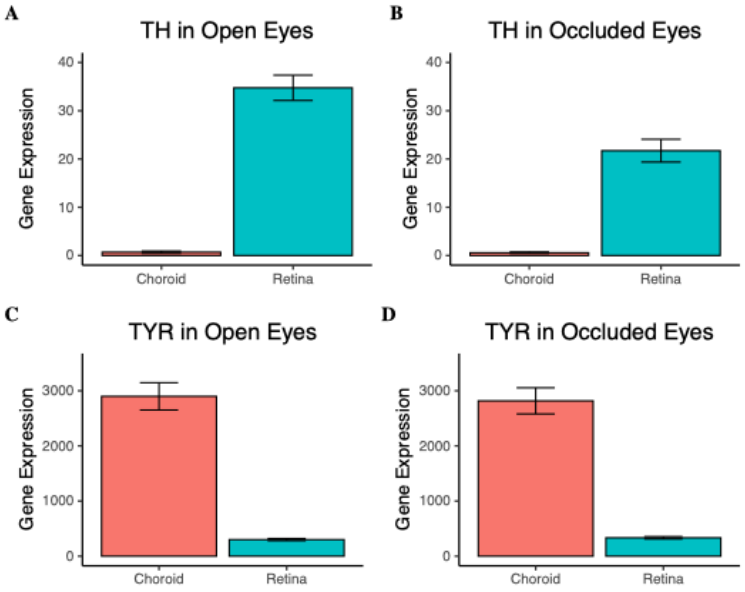

Figure 19: Gene Expression of Tyrosinase and Tyrosine Hydroxylase

Effect Size of Eye Growth Experiments Intravitreal Injections				
	Eye Measure	**Effect Size**	**Statistic**	**Interpretation**
Growth Rate				
Saline	AC+L	0.28	Cohen's d	Small
Saline	VC	-1.60	Cohen's d	Large
Saline	CT	-0.21	Cohen's d	Small
Saline	AL	-1.19	Cohen's d	Large
Dopamine	AC+L	-1.16	Cohen's d	Large
Dopamine	VC	-0.49	Cohen's d	Small
Dopamine	CT	-1.29	Cohen's d	Large
Dopamine	AL	-1.78	Cohen's d	Large
Apomorphine	AC+L	-1.82	Cohen's d	Large
Apomorphine	VC	-0.89	Cohen's d	Large
Apomorphine	CT	-0.62	Cohen's d	Medium
Apomorphine	AL	-0.93	Rank Biserial	Large
Interocular Difference				
NA	AC+L	0.28	ω^2	Large
NA	VC	0.02	ω^2	Small
NA	CT	0.07	ω^2	Medium
NA	AL	0.18	ω^2	Large

Table 7: Effect Size in Intravitreal Growth Experiments

Effect Size of Eye Growth Experiments Ophthalmic Drops				
	Eye Measure	**Effect Size**	**Statistic**	**Interpretation**
Growth Rate				
Saline	AC+L	-0.70	Cohen's d	Medium
Saline	VC	-0.39	Cohen's d	Small
Saline	CT	0.27	Cohen's d	Small
Saline	AL	-0.10	Rank Biserial	Small
Dopamine	AC+L	0.09	Cohen's d	Small
Dopamine	VC	-0.42	Cohen's d	Small
Dopamine	CT	-0.25	Cohen's d	Small
Dopamine	AL	-0.59	Cohen's d	Medium
Interocular Difference				
NA	AC+L	0.46	Cohen's d	Small
NA	VC	0.003	Cohen's d	Small
NA	CT	-0.67	Cohen's d	Medium
NA	AL	-0.40	Cohen's d	Small

Table 8: Effect Size in Topical Growth Experiments

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