Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Erica G. Landis Date

The role of ambient light on dopamine signaling and myopia susceptibility

By

Erica G. Landis
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Neuroscience

Machelle T. Pardue, PhD Advisor	Scott Lambert, MD Committee Member
Ellen Hess, PhD Committee Member	Gary Miller, PhD Committee Member
P. Michael Iuvone, PhD Committee Member	Sam Sober, PhD Committee Member

Accepted:

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

Date

The role of ambient light on dopamine signaling and myopia susceptibility

By

Erica G. Landis
B.S., Pacific Lutheran University, 2009

Advisor: Machelle T. Pardue, PhD

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in
Graduate Division of Biological and Biomedical Sciences
Neuroscience

2018

ABSTRACT

The role of ambient light on dopamine signaling and myopia susceptibility

By Erica G. Landis

Myopia, or nearsightedness, results in a blurred image of objects at a distance caused by an elongated eye. In recent decades rates of myopia prevalence have risen dramatically. The increases in myopia are likely due to environmental factors during childhood. Research into the growing myopia prevalence has led to new discoveries of how visual experience influences refractive development and myopia. Evidence in both clinical studies and animal models of myopia have indicated that bright light exposure during time outdoors can prevent myopic eye growth. However, the effect of a broad range of ambient light on myopia susceptibility had not been investigated. By housing mice in dim, intermediate, and bright light with and without lens defocus, I was able to test the effect of a wide range of ambient lighting to determine the role each plays on myopia susceptibility in the mouse model. My novel findings show that dim light, in addition to bright, is protective against myopia. To determine the retinal signaling mechanisms behind this protection, dopamine, which had previously been implicated as a "stop signal" in myopic eye growth, and proteins related to dopamine synthesis, packaging, uptake, and degradation were measured in myopic and control mice from each light level. My results show that dopamine dynamics are dependent on an interaction between ambient light and lens defocus. To determine the potential for dopamine to prevent myopia, I measured myopia susceptibility after either pharmacological or transgenic approaches to increasing endogenous dopamine. L-DOPA, a dopamine precursor, completely prevented form deprivation myopia in mice. The clinical applicability of these findings was investigated by analyzing light exposure data from a cohort of children. I showed that non-myopic children spend as much time in dim light as in bright light, supporting the potential of dim light to be used as a preventive therapy for myopia. Together, these findings reveal a more complex effect of ambient light and visual defocus on dopamine signaling and refractive eye growth. Furthermore, these data show that a broad range of ambient light is important for healthy ocular development.

The role of ambient light on dopamine signaling and myopia susceptibility

By

Erica G. Landis
B.S., Pacific Lutheran University, 2009

Advisor: Machelle T. Pardue, PhD

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in
Graduate Division of Biological and Biomedical Sciences
Neuroscience

2018

ACKNOWLEDGEMENTS

The work I present in this dissertation has been supported by my mentors, peers, and friends and family and would not have been possible without them.

First, I would like to thank my advisor Machelle T. Pardue. Dr. Pardue has taught me how to do quality research and be a good scientist, how to share my work and contribute to the field, and how to build a successful career. Dr. Pardue has consistently made time to help me and discuss my science with me. She has inspired and motivated me to enjoy my work. She has supported my efforts to explore and pursue skills outside of the lab while in graduate school. Dr. Pardue is a fantastic mentor and I know that my success in graduate school is due in large part to a good choice I made by joining the Pardue Lab five years ago.

I would also like to thank my dissertation committee members Dr.s Ellen Hess, Michael Iuvone, Scott Lambert, Gary Miller, and Sam Sober. By advising my research and the preparation of this document, my committee members have positively guided my graduate work and have been excellent mentors. I would like to thank Dr. Hess for her particular support of my professional development and for sharing laboratory resources and expertise. Dr. Iuvone has, from the beginning, made time to advise my experimental design, help interpret scientific findings, and has generously shared materials and services which are described in this dissertation. Finally, I would like to particularly thank Dr. Miller for inviting me into his lab to learn from his students, try new experiments, and for sharing resources with me.

The funding and institutions which have supported this work include Emory University, the Atlanta Veteran's Medical Center, Georgia Institute of Technology, and the National Eye Institute and National Institutes of Health.

Many members of the Pardue Lab, past and present, have helped me complete my graduate work and have made the lab a fun and creative place. I would like to especially thank Dr. Ranjay Chakraborty, Han na Park, Ryan Strickland, Victoria Yang, and Dillon Brown. I would also like to thank Dr. Rachael Allen who dedicated a significant amount of time to helping me prepare this dissertation.

Finally, to my friends and family. I could not have finished this work without the love and support of my wife Rebekah, who cooked and cleaned for me when I was busy, listened to my ramblings about everything from graphs to presentation outfits, and consistently helped and encouraged me through all of the challenges of graduate school. I would like to thank my parents and my brother for their support. And of course, I would like to thank my all of my friends in the Neuroscience Program, especially Elle, Maria, Phil, and Brendan; I couldn't have asked for better friends!

TABLE OF CONTENTS

FIGURES

TABLES

CHAPTER 3

CHAPTER 5

ABBREVIATIONS USED IN THIS DISSERTATION

Listed in alphabetical order,

- AA — ascorbic acid
- AADC — amino acid decarboxylase
- aCSF — artificial cerebral spinal fluid
- BAC — bacterial artificial chromosome
- BCA — bicinchoninic acid assay
- BME — ß-mercaptoethanol
- BSA — bovine serum albumin
- cDNA — complementary DNA
- CT — circadian time
- D — diopter
- D2R — dopamine receptor 2
- DA — dopamine
- DACs — dopaminergic amacrine cells
- DAT — dopamine transporter
- ddPCR — droplet digital PCR
- DOPAC — 3,4-dihydroxyphenylacetic acid
- dUTP — deoxyuridine triphosphate
- FAM — fluorescein amidite
- FD — form deprivation
- FDM — form deprivation myopia
- HEX — hexachloro-fluorescein
- HPLC — high performance liquid chromotography
- HPRT — hypoxanthine phosphoribosyltransferase
- HRP — horseradish peroxidase
- HVA — homovanillic acid
- ipRGCs — intrinsically photosensitive retinal ganglion cells
- L-DOPA — L-,3,4-dihydroxyphenylalanine
- L:D — light:dark cycle
- LIM — lens induced myopia
- MAO — monoamine oxidase
- MPTP — 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
- OD — *oculus dextrus*
- OS — *oculus sinister*
- OMR — optomotor response
- P — post-natal day
- PCR — polymerase chain reaction
- pTH^{Ser40} — tyrosine hydroxylase phosphorylated at amino acid site Serine-40
- *rd10* — retinal degeneration
- RGCs — retinal ganglion cells
- ROAM — Role of Outdoor Activity in Myopia

- SD-OCT — spectral domain optical coherence tomography
- SER — spherical equivalent subjective refractive error
- TH — tyrosine hydroxylase
- VMAT2 — vesicular monoamine transporter 2
- VMAT2 HI — transgenic mice with increased expression of VMAT2
- VMAT2 LO — transgenic mice with decreased expression of VMAT2
- WT — wild type
- ZT — zeitgeber time

CHAPTER 1: Introduction

1.1 Anatomy and Physiology of the Eye and Retina

1.1.1 Brief summary of ocular anatomy and visual processing

All vision relies on the eye to capture light and transmit information to the brain for interpretation. In this way, the structure of the eye must be suited to the properties of environmental light. Two of these properties, reflection and refraction, are critical for vision. Light which is reflected off an object in our environment carries information about the object's location, color, and texture. Refraction, the bending of light as it travels through a material, allows our eyes to focus light rays onto the neural retina for processing. The structure of our eyes allows us to capture light such that most of what we see is light reflected off objects, refracted into our eyes, and converted into signals which our brain interprets. The eye is a perfect example of the fundamental physiology principle of structure supporting function as the collecting and processing of light information is dependent on the shapes of individual structures of the eye.

When viewing the ocular globe of the human eye from the front, we usually notice the color of the iris around the black pupil and the surrounding white sclera (**Figure 1.1A**). Covering the iris and pupil is the transparent cornea. The cornea, which has a higher refractive index than the air, is the most optically powerful part of the eye. Therefore, it has the greatest contribution to refracting light. As light from the environment passes through the curved cornea, it converges before passing through the aqueous humor, and then converges again, though less dramatically, by the transparent, crystalline lens (**Figure 1.1B**). This lens and the ciliary body are specialized for accommodation, the process by which the lens changes shape for additional optical power to focus at different distances. Light from objects in the distance travels in parallel lines compared to light reflected from objects in our foreground, therefore, the cornea which bends all light

equally has no ability to compensate for object distance and the shape of the lens provides the ability to refract near light onto the retina, as needed. The lens is manipulated by a ring of ciliary muscles to induce accommodation.

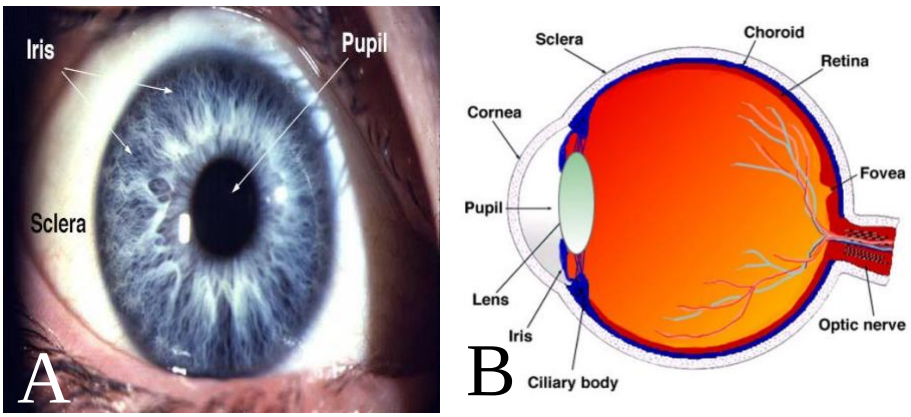

Figure 1.1. The human eye. Ocular structures important to image formation are highlighted here. (A) Visible structures of the eye include the white collagenous sclera, the colored iris, and the pupil where light is focused through the cornea and lens to the back of the eye. (B) The cornea and lens generate most of the optical power of the eye. The retina at the posterior of the eye is neural, light sensing tissue which contains many cell types to process and transmit information about light from the environment to the brain through the optic nerve of retinal ganglion cell axons. Behind the retina are the vascular choroid and collagen sclera. Images with permission from http://webvision.med.utah.edu/.

1.1.2 The neural retina

By refracting and converging incoming light, the eye focuses light rays onto the neural retina at the back of the eye. The retina which converts light to electrical and chemical signals is made up of layers in a laminar organization such that each layer is characterized by particular cell types (**Figure 1.2**). In vertebrates, the retina seems inverted because the photoreceptors, the cells primarily responsible for detecting light, are at the back of the retina and light passing through the eye must travel through several layers of other retinal neurons before being detected. The photoreceptors convert light energy into electrical signals that then travel through the other layers of neurons, back toward the front of the eye until it reaches the retinal ganglion cells (RGCs). The axons of RGCs form the optic nerve which leaves the eye for the brain. In order to avoid light being diffused and scattered by the other retinal layers before being captured by photoreceptors, the human eye has a fovea, an area rich with cone, high acuity, photoreceptors where other cell types are pushed aside to allow light to reach the photoreceptors directly. The fovea is the area of the retina with the highest acuity vision.

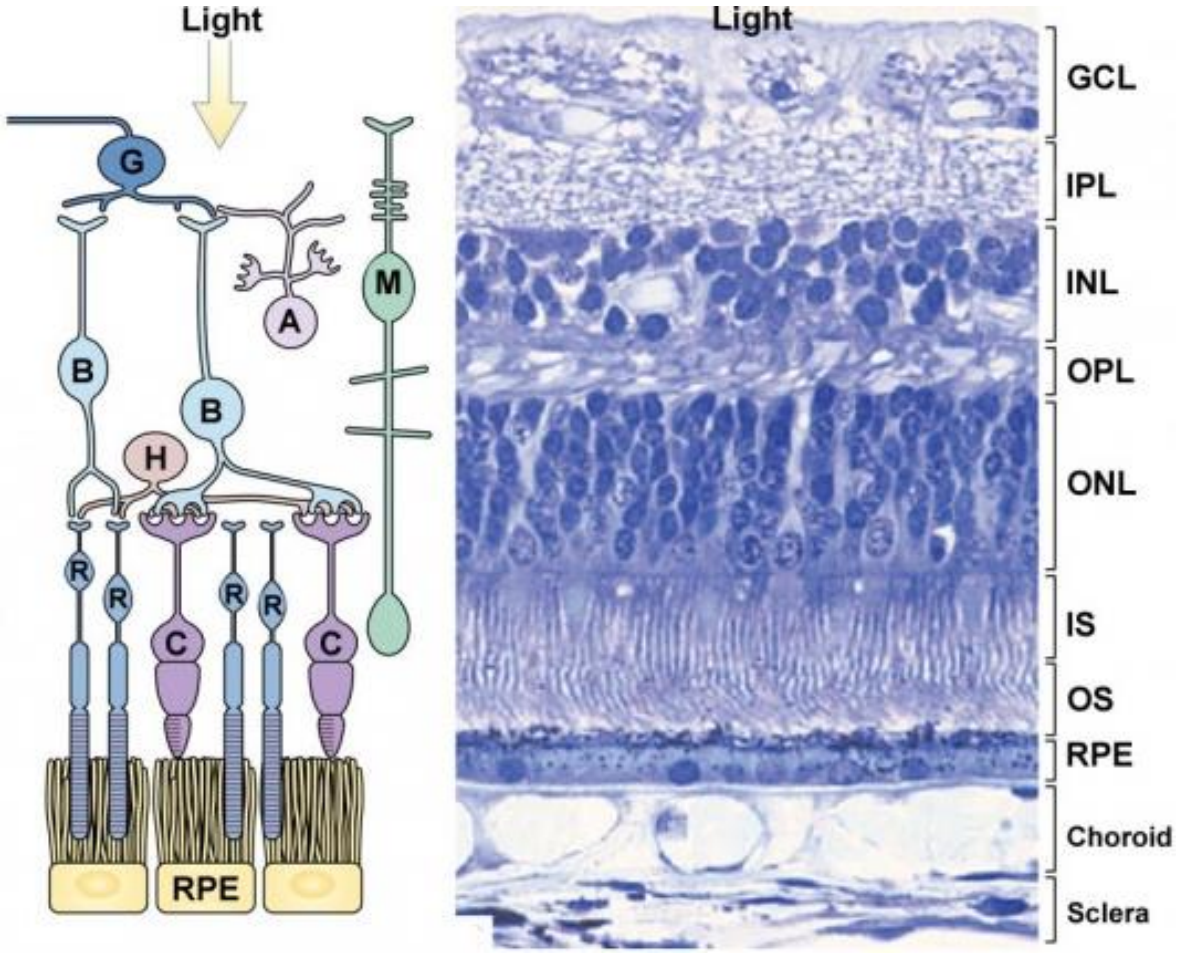

Figure 1.2. Retina structure with laminar organization. The retina is organized into cellular layers with distinct morphological and functional characteristics. Diagram depicting the cellular components and structure of the retina, left (A = amacrine cell; B = bipolar cell; C = cone photoreceptor; G = ganglion cell; H = horizontal cell; M = Müller cell; R = rod photoreceptor; RPE = retinal pigment epithelium). Between each layer, multiple cell types transmit chemical and electrical signals to integrate and modulate responses to light. The pigment epithelium, not technically a part of the retina, is important for maintaining photoreceptor health. Histological image of a transverse section the human retina, right (GCL = ganglion cell layer; INL = inner nuclear layer; IPL = inner plexiform layer; IS = inner segment of the photoreceptor; ONL = outer nuclear layer; OPL = outer plexiform layer; OS = outer segment of the photoreceptor). Image obtained with permission from (Sung and Chuang, 2010).

Light sensing photoreceptors are also specialized. Three types of photosensitive cells are in the retina. Traditional photoreceptors, rods and cones, are present in the outer retina. Rods express the photopigment rhodopsin and are highly sensitive to light, capable of hyperpolarizing to single photons. Rods are primarily responsible for vision under dim light, such as starlight. Creating retinal sensitivity across a broad spectrum of light, cones are responsible for detecting high levels of light and are therefore specialized for high acuity vision, at the fovea, and for color vision. Subtypes of cones express different opsin proteins specific to various wavelengths of light. Cones express one of three photopigments in humans which hyperpolarize in response to red, green, or blue wavelengths of light. Outside of the fovea, the density of cones drops off quickly and the retina is dominated by rods. The number of rod photoreceptor also decreases with distance from the fovea and into the periphery. While we primarily rely on cones for vision in bright environments, rods have traditionally been shown to respond to dim light, saturating in bright light. However, recent studies have shown rods to play a role across a wide range of illumination from starlight to sunlight (Tikidji-Hamburyan et al., 2017) -- more on this work later. The third type of photosensitive cell is the recently discovered intrinsically photosensitive retinal ganglion cell (ipRGC) which express a melanopsin photopigment (Berson et al., 2002; Hattar et al., 2002). Five subtypes of ipRGCs have been found in the retina; most of them are responsible for non-image forming vision which controls mechanisms like pupil constriction and circadian rhythms (Guler et al., 2008; Lall et al., 2010; Ksendzovsky et al., 2017; Lazzerini Ospri et al., 2017). Recently however, some evidence of image forming function in the M1 and M4 ipRGC subtypes have been discovered (Dacey et al., 2005; Ecker et al., 2010; Sonoda et al., 2018). The ipRGCs are not as well-known as rods and cones, and their potential to influence other light sensing retinal pathways is still being uncovered.

Photoreceptors convert light energy to neural activity through phototransduction. Phototransduction begins with a conformational change to an opsin molecule which starts a cascade of molecular signals resulting in hyperpolarization of rods and cones or depolarization in ipRGCs. Through the retinal layers, these signals are modified and organized first by ON and OFF bipolar cells, then RGCs. The integration pattern of signals through the retina is highly dependent on the intensity and spatial properties of the light. The properties of the light detected dictates which retinal pathways are activated. As signals are passed to higher order cells, lateral crosstalk occurs through horizontal cells at the level of the photoreceptors and from amacrine cells at the level of bipolar cells. As RGCs integrate visual signals across the retina, basic information such as light intensity and edges, are combined to form perception of movement, direction, and orientation. The integration of these light signals through the retina results in a semi-processed image being sent to the brain which further integrates and decodes signals from both eyes, including inverting the image to its correct orientation, to create the perceived visual image.

1.1.3 Neurotransmitters in the retina

To generate and integrate neural signals in response to light, the retina uses neurotransmitters and neuromodulators to stimulate, inhibit, and modify the activity of other neurons. With the laminar organization of the retina, each layer is comprised of distinct neuronal cell types with specific functions. Through phototransduction, the electromagnetic energy of light is converted to a chemical signal in photoreceptors, hyperpolarizing the cell and inhibiting the release of glutamate from the synapse at the photoreceptor inner segment. Like the brain, glutamate is the primary excitatory signal in the retina. Glutamate is also the primary neurotransmitter for vertical signaling pathways in the retina. After being released from

photoreceptors, glutamate acts on local bipolar cells in a manner dependent on the classification of that cell. ON bipolar cells express a G-protein coupled receptor, mGluR6, which when activated triggers the hyperpolarization of the cell. Alternatively, OFF bipolar cells express gated ion channels, AMPA or kainate receptors, which lead to a depolarization of the cell. Therefore, both types of bipolar cells respond to the glutamate expressed by photoreceptors and their response is dependent on the onset or offset of the light stimulus.

GABA and glycine are the major inhibitory neurotransmitters in the retina. Each is mainly found in several types of amacrine cells localized along vertical pathways to fulfill specific roles in visual processing. First, GABA is found in the wide-field amacrine cells of the inner plexiform layer, providing lateral inhibition to bipolar cells (Lin and Masland, 2006). The slow release of GABA in response to depolarization of many types of GABAergic amacrine cells and some horizontal cells activated by glutamate plays a role in the formation of receptive fields and the computation of direction selective responses [for review (Popova, 2014)]. Glycine is localized in many different types of narrow-field amacrine cells, indicating involvement in local processing mechanisms (Hsueh et al., 2008). Similar to GABA, glycine acts to inhibit excitatory pathways, acting with GABA in light dependent and specific proportions that modulate the characteristics of bipolar cell activity. Both GABA and glycine receive input from bipolar cells as well as other amacrine cells and some receive input from photoreceptors (Eggers and Lukasiewicz, 2011).

1.1.4 Dopamine dynamics in the retina

The most abundant neuromodulator in the retina is dopamine (DA). Although it is only one of many different neuromodulators in the retina, this dissertation project will focus on DA. As a neuromodulator, DA is responsible for many diverse functions and signaling processes in

the retina. Like GABA and glycine, DA is also synthesized and released from amacrine cells. In the mammalian retina, DA is found in specific DAergic amacrine cells (DACs) and interplexiform cells in some species (Nguyen-Legros et al., 1997; Witkovsky et al., 2008). These DACs sparsely span the mammalian retina with a broad dendritic network that covers the entire retina despite their low density. In the mouse, retina DAergic amacrine cells total only about 700 [(Witkovsky, 2004), **Figure 1.3**]. With excitatory input from bipolar cells and inhibitory input from other amacrine cells, DACs are able to respond to light and act to modulate that signal themselves (Witkovsky, 2004). DACs release DA in response to acute light exposure which creates a circadian rhythm (Iuvone et al., 1978; Doyle et al., 2002b). Tyrosine hydroxylase (TH, **Figure 1.4**), the rate-limiting enzyme responsible for the synthesis of DA is phosphorylated following stimulation of bipolar cells by light exposure (Witkovsky et al., 2004). TH is present throughout the cell bodies and dendrites of DAergic amacrine cells, indicating that DA is released from all regions of the cell where it acts via diffusion on cell types across the retina (Puopolo et al., 2001). In order to be released from the DAergic cell, synthesized DA first has to be packaged into vesicles through the action of vesicular monoamine transporter 2 (VMAT2), a hydrogen antiporter, which actively transports DA and other monoamines across the vesicle membrane. VMAT2 is essential for monoamine neurotransmission and has been shown to impact the volume of transmission in certain brain regions (Lohr et al., 2014). After release, DA diffuses through the intercellular space of the retina, and may even reach other structures of the eye (Witkovsky, 2004).

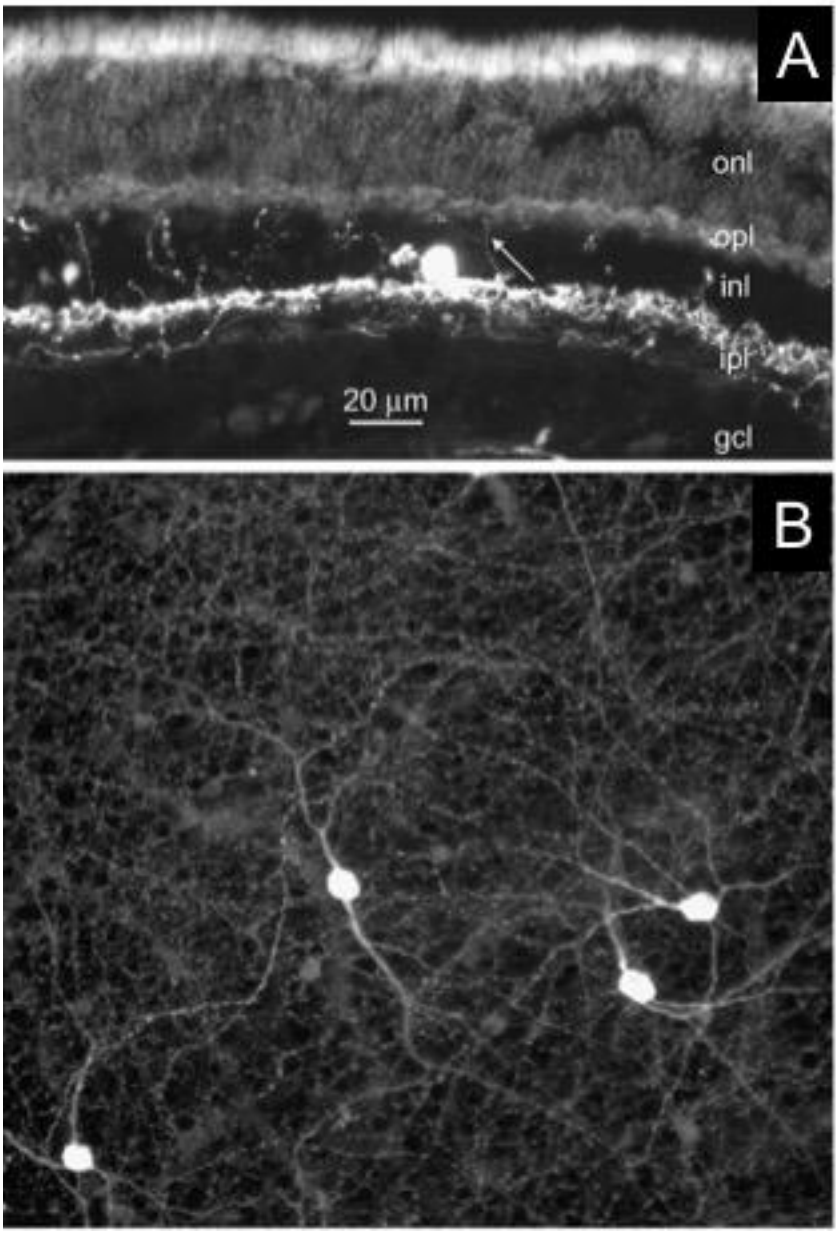

Figure 1.3. Morphology and lamina of the dopaminergic amacrine cells. Dopaminergic amacrine cells (DACs) are the main DA synthesizing cells in the retina. (A) Vertical section of the retina shows they are sparsely located throughout the inner nuclear layer with processes extending throughout the inner plexiform layer. (B) DAC processes which both synthesize and release DA extend out from the soma to form a broad net spread evenly across the entire retina. DACs are labeled by tyrosine hydroxylase (TH) which converts tyrosine to L-DOPA and is the rate limiting protein in the synthesis of DA. The 20 µm marker bar functions for both images; gcl = ganglion cell layer; inl = inner nuclear layer; ipl = inner plexiform layer; onl = outer nuclear layer; opl = outer plexiform layer. Figure used here with permission from (Witkovsky, 2004).

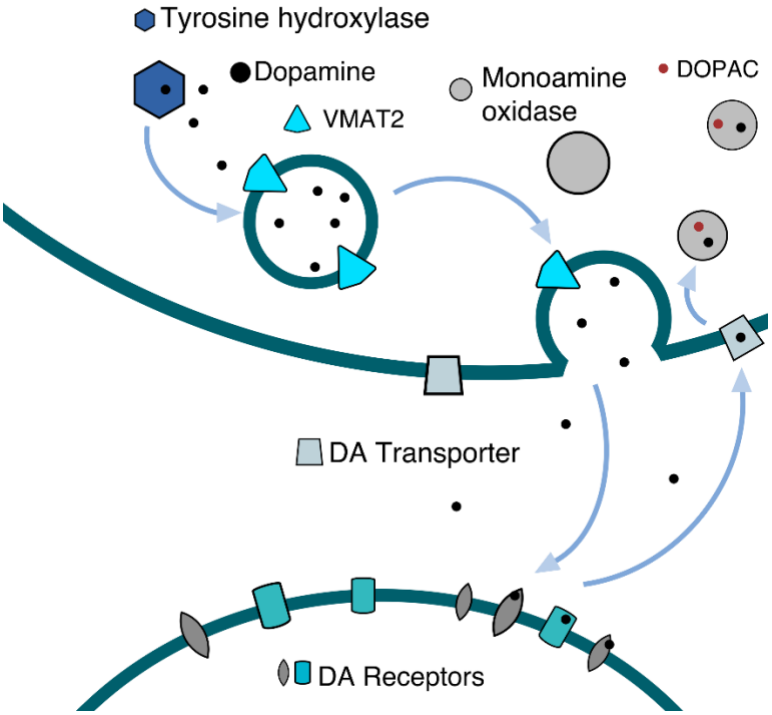

Figure 1.4 Schematic of dopamine dynamics in dopaminergic amacrine cells. DAergic cells (DACs) which synthesize and release DA utilize a large group of proteins to facilitate the synthesis, transport, storage, release, and subsequent degradation of DA. Typically, DA is synthesized by tyrosine hydroxylase (TH) then through active transport moved into vesicles by vesicular monoamine transporter 2 (VMAT2). After vesicles fuse with the cellular membrane and DA is released, it acts through volume transmission on DA receptors found throughout the retina. DA remaining near DAergic amacrine cells is transported back into the cell by the dopamine transporter (DAT) to be either loaded into vesicles again or degraded by monoamine oxidase (MAO). Original image by author.

DA plays many roles in the retina including regulation of circadian rhythms, modulation of light sensitivity, and related functions such as electrical gap junction coupling and melatonin synthesis (Witkovsky, 2004). Which function DA plays at any particular time is dependent on the target cell and DA receptor type. Four of the five known DA receptors have been found and studied in the retina; D1, D2, D4, and D5. These G-protein coupled, transmembrane receptors are fairly similar in structure yet are coupled to several different G-proteins, indicating they activate different downstream signaling cascades (Gingrich and Caron, 1993; Seeman and Van Tol, 1994). Generally, the D1 family of receptors (D1 and D5) are coupled to proteins that activate adenylyl cyclase and D2 family of receptors (D2 and D4) lead to the inhibition of adenylyl cyclase.

Once DA has been released from the receptor, it is removed from the extracellular space by the dopamine transporter (DAT), which acts rapidly to maintain homeostasis of DA levels. DAT utilizes the ion concentration gradient across the plasma membrane to transport DA via a conformation change (Chen and Reith, 2000). Once DA is across the membrane, DAT begins the process again. Due to the cytotoxic nature of free DA, the molecule must be degraded or reused. In the retina, this process has not been fully studied as some of the proteins involved in the uptake and degradation or reuse of DA have not been localized in the mouse retina and have distinct isoforms which we know little about. Therefore, we must utilize knowledge from other central nervous system regions to build an understanding of what is likely occurring in the retina. Cytosolic DA is broken down into 3, 4 – Dihydroxyphenylacetic acid (DOPAC), its primary metabolite, by monoamine oxidase (MAO), then into homovanillic acid (HVA), which is passed through the body as waste.

Through this process, the retina is able to respond to light to control DA release which modifies retinal signaling pathways to set circadian rhythms and regulate light responses downstream of DACs. As I will discuss further later in this chapter, DA also plays a role in the development of refractive error, indicating its effects go beyond regulating retinal function.

1.2 Overview of myopia

1.2.1 Refractive error and myopia as visual disorders

Refractive error, or a discrepancy between the optical power and size of the eye, is the most common visual disorder in the world (Resnikoff et al., 2008). As described earlier, the cornea and lens are important focusing structures in the eye which utilize refraction to bend light through the pupil to the retina. However, an important factor in this process is the position of the retina in relationship to the focal point of incoming light. If the eye is too long or too short there will be a poor match between the optical power of the cornea and lens and the size of the eye resulting in the focal point of light landing either in front of or behind the retina (**Figure 1.5**). Emmetropization is the process by which an eye grows to match the optical power of the cornea and lens so that the focal point of light is on the retina resulting in perfect vision. Therefore, emmetropia would describe an eye with matched optical power and size. Hyperopia describes an eye that is too short and therefore the focal point lands "behind" the retina. Hyperopia, also known as farsightedness, results in a blurred perception of objects close to the viewer (**Figure 1.5c**). When the eye grows too long, the focal point is "in front" of the retina, so the eye is myopic. Myopia, or nearsightedness, creates a blurred perception of objects farther away from the viewer (**Figure 1.5b**).

The process of emmetropization is thought to be highly conserved across species, since experimental myopia has been induced in species from fish to primates (Schaeffel and Feldkaemper, 2015). Both genetic and environmental factors contribute to emmetropization and refractive error development (Saw et al., 2000; Wojciechowski, 2011). While this work will not focus on genetic causes of refractive error, the influence of genetics could alter the effect of environmental factors and the starting point of refractive error at birth (Goldschmidt and Jacobsen, 2014; Chen et al., 2016). Environmental factors, which will be discussed in more detail later, can aid the eye in emmetropization or trigger myopia or hyperopia. Emmetropization can continue until early adulthood when eye growth slows. The exact mechanisms controlling optical power at birth, the effect of environmental factors, and the progression of refractive development are still unknown.

Due to the differences in optical power and eye length, the severity of myopia can vary between individuals. Most individuals have low myopia [<-2.5D of refractive error, (Gwiazda et al., 1993; Group, 2010; Borchert et al., 2011)]. However, if the eye continues to grow, individuals can develop high myopia, also called pathological myopia [>6.0D of myopia by most definitions; (Morgan et al., 2017)]. It has been hypothesized that as the rate of myopia increases in a population, the rate of those individuals with high myopia increases exponentially (Morgan et al., 2017). Patients with high myopia have increased risk for vision loss later in life due to glaucoma, cataracts, retinal detachment, myopic maculopathy, and others. Though the mechanism through which high myopia leads to each of these complications is unknown the risks of these myopia-associated complications have been shown through reports from multiple populations [for review (Verkicharla et al., 2015; Morgan et al., 2017)]. By preventing myopia

and progression of myopia the risk of vision loss later in life is reduced for a large percentage of the world population.

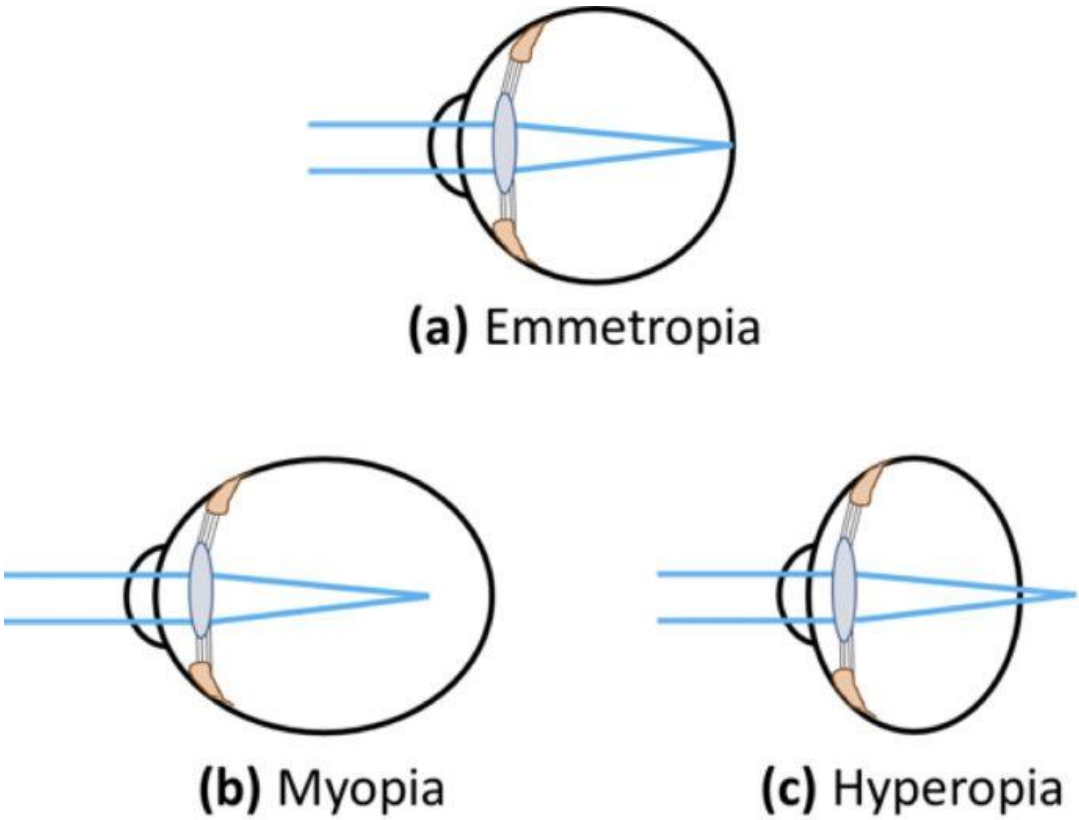

Figure 1.5. Diagram of optical power in common refractive errors. Growth and shape of the eye can alter the image based on optical power of the anterior structures. Rays of light, represented in blue, are refracted through the anterior region of the eye by the cornea and lens into a focal point. (a) When the size of the eye matches its optical power, the eye is emmetropic and has perfect vision. (b) An eye that is elongated with a focal point in front of the retina is myopic. (c) Eyes that do not grow long enough to match their optical power have focal points behind the retina and are hyperopic. Blue lines represent incoming light. Image modified with permission from http://webvision.med.utah.edu/.

1.2.2 Current treatments for refractive errors

The most common and oldest treatments for myopia and hyperopia involve optically correcting the light before it enters the eye by placing powered lenses in front of the eye to either converge or diverge the light rays. In myopia, a diverging lens essentially subtracts some of the optical power of the eye and moves the focal point further back in the eye and onto the retina (**Figure 1.6**). For hyperopia, a converging lens is used to move the focal point onto the retina.

Today, the most common forms of corrective lenses are glasses and contact lenses. The power of these lenses can be customized to match any severity of refractive error, can be individualized to each eye, and are therefore highly accessible compared to care for other disorders where treatments are not so easily customized or do not adequately compensate for the symptoms. With a pair of glasses, almost all individuals with refractive error and no other visual complications have visual acuity nearly matching emmetropic eyes. Despite their ease of use, several problems still exist with corrective lenses. In many places corrective lenses require a prescription including a potentially costly visit to an eye clinic and ordering custom-made lenses, making them more difficult to obtain in rural or underserved areas. Both glasses and contact lenses can be easily lost or damaged and might be cumbersome in emergency situations. Contact lenses are also a main cause of ocular infections because they need to be kept moist and cleaned regularly (Wu et al., 2015b; Cheung et al., 2016). The cost of correcting refractive errors worldwide, including myopia, hyperopia, and presbyopia, is an estimated $28 billion USD (Fricke et al., 2012). Finally, neither glasses nor contact lenses can prevent the progression of refractive error or prevent the long-term complications that occur with severe refractive error.

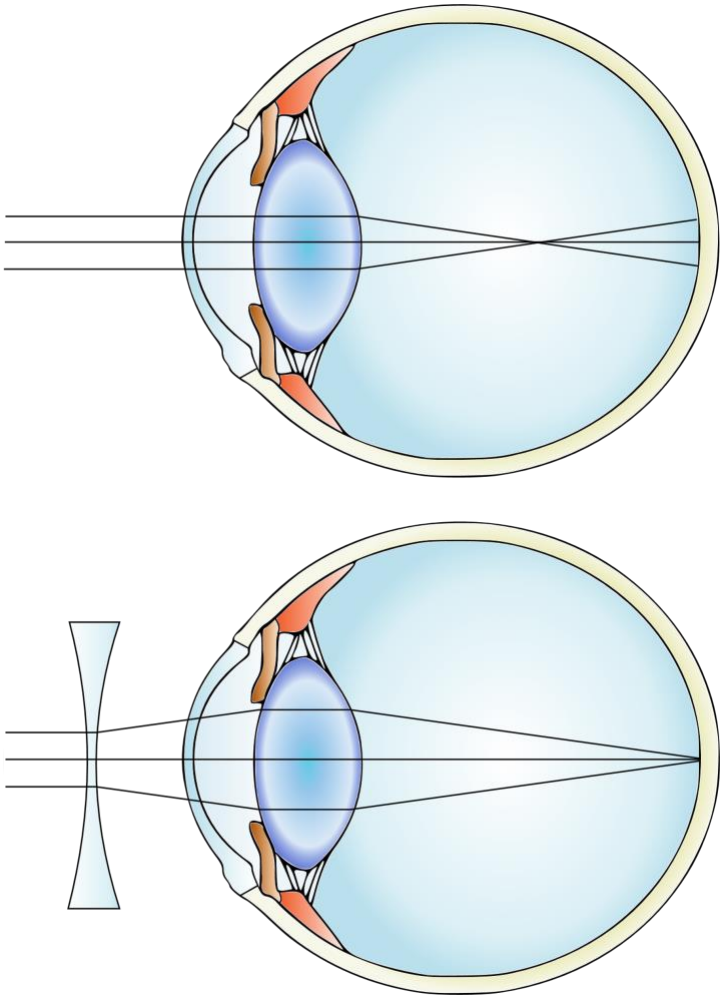

Figure 1.6. Diverging lenses focuses light on the retina in a myopic eye. Myopia (*upper image*) can be corrected by a diverging lens placed in front of the eye (*lower image*) which widens the light rays entering the light such that when combined with the power of the cornea and lens, the focal point is on the retina. Images from https://www.quora.com/How-does-a-concave-lens-cure-myopia used here under Creative Commons license CC BY-SA 4.0.

The second most common treatment for refractive error is to surgically alter the power of the cornea or lens to correct the optical error. There are several forms of this surgery, the most common form is LASIK which uses laser ablation to reshape the inner cornea and alter its optical power (FDA website, https://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/SurgeryandLifeSupport/LASIK/ucm061358.htm). Other forms of refractive surgery use different lasers or surgical tools and can target different areas of the cornea to affect the same result. Finally, the lens can be corrected or replaced with intraocular lenses of a different optical power as well. These lenses are manufactured in a variety of types and are most often used to replace lenses with cataracts. Though refractive surgery is well established, safe, and less cumbersome than corrective lenses, the surgery, like corrective lenses, cannot combat the increased risk of vision threatening conditions. Additionally, the use of refractive surgery in young patients, while myopia is developing and progressing, is not always practical. Unfortunately, no treatment for refractive error prevents the progression of myopia or later in life risks caused by high myopia.

1.2.3 Epidemiological understanding of refractive error and myopia

While refractive errors, specifically myopia, are relatively easy to treat, the numbers of individuals affected by myopia have risen dramatically in recent decades (Resnikoff et al., 2008; Fricke et al., 2018). Today the numbers of myopic individuals in some regions have reached epidemic levels. Within the last few decades researchers have found evidence of increased myopia from around the world with some regions more affected [(Morgan et al., 2018; Resnikoff et al., 2008), **Figure 1.7**]. In many Asia countries including China, Taiwan, South Korea (Jung et al., 2012; Kim et al., 2013), Japan (Ding et al., 2017) and Singapore (Pan et al., 2012a; Pan et al., 2012b; Koh et al., 2014) rates of myopia have increased dramatically; now approximately 80-

90% of the population is affected by myopia [for review (Resnikoff et al., 2008; Morgan et al., 2012)].

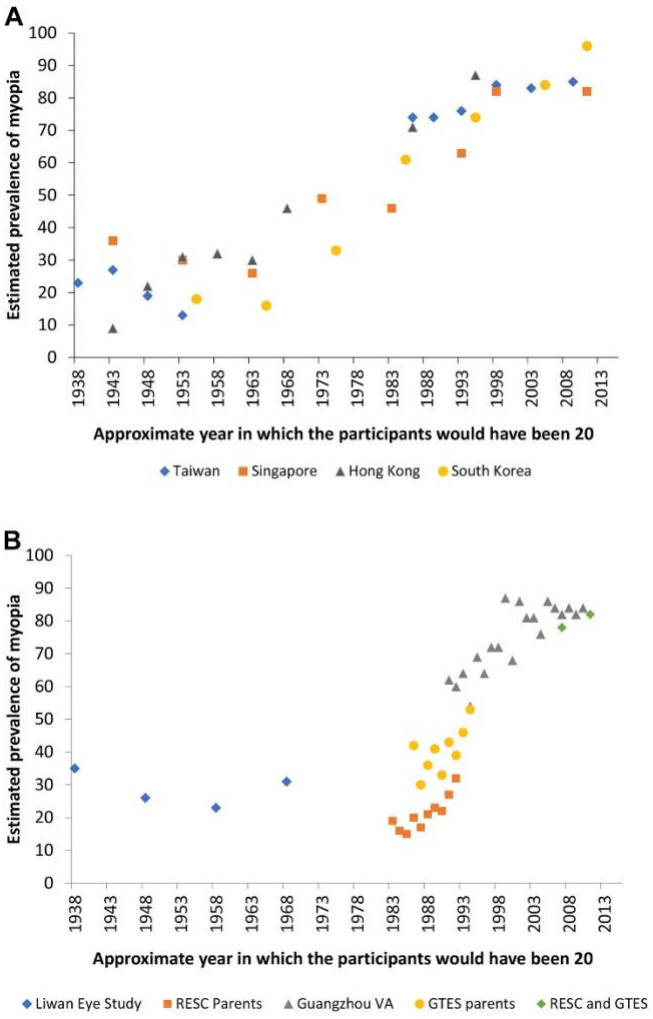

Figure 1.7 Increasing rates of myopia in multiple study populations. (A) The estimated prevalence of myopia aggregated from multiple studies of young adults in South Korea, Singapore, Taiwan, and Hong Kong around the age of 20 years old. (B) Estimated prevalence of myopia in different cohorts of young adults from Guangzhou, China aged around 20. Figures published by and used here with permission from (Morgan et al., 2018).

Outside of Asia, studies of western populations have also found increases in myopia, estimating increases by 20-40% over the last several decades (Vitale et al., 2009; Pan et al., 2012a; Cumberland et al., 2015; Williams et al., 2015). Interestingly, rates of myopia in less developed countries including those in Western Africa, the Middle East, and Latin America have shown increases as low as 5-10% in young people compared to older populations (Pokharel et al., 2000; Dandona et al., 2002; Jimenez et al., 2004; Khandekar and Abdu-Helmi, 2004; Anera et al., 2009; Lindquist et al., 2011; Casson et al., 2012; Gao et al., 2012; Jimenez et al., 2012; Soler et al., 2015). Together, these analyses indicate a role of ethnicity or inheritance in the development of myopia, possibly implying that increases outside of Asia are due to the migration of Asian populations. However, several Asian populations without increases in myopia have been found (Yingyong, 2010; Casson et al., 2012; Gao et al., 2012). Also, studies of ethnic Chinese children raised in Australia have shown no effect of genetic or ethnic background on myopia risk (Rose et al., 2008a). In fact, Chinese children in Australia had refractive errors more similar to Caucasian Australian children compared to Chinese children raised in China. Therefore, rather than a role for ethnicity in myopia, these findings would indicate environmental and perhaps cultural aspects to the development of myopia.

The association of myopia with societal modernization follows previously reported evidence that myopia risk increases with education level (Morgan and Rose, 2005). In fact, recent studies have shown that myopia progression can be avoided by keeping children from educational opportunities (Rosner and Belkin, 1987; Quek et al., 2004; Saw et al., 2007; Ip et al., 2008; French et al., 2013a). However, education level does not explain all non-genetic myopia and another factor, time spent outdoors, which will be discussed later has also been identified by these epidemiological studies as a factor in myopia development. Time outdoors, which is at

times inversely related to academic performance can be considered a cultural dependent variable and fits well with existing epidemiological data on regional trends in myopia.

By quantifying the individuals affected by myopia world-wide, the field has gained an understanding of how and where treatments and preventative measures for myopia are most needed. Researchers have begun to collaborate with local governments, school officials, and opticians to implement preventative strategies like increasing in-school recess time (Morgan et al., 2018). We have also gained an understanding of how myopia increases the risk for vision threatening complications later in life. The importance of preventing further increases in myopia prevalence is clear, however, the stimuli that influence myopia are still being investigated. Analyses of myopia prevalence across populations has led to investigations of environmental factors contributing to myopia.

1.3 The Scientific Problem of Myopia

By studying individuals affected by myopia and the environmental factors that contribute, we ultimately become aware of the scientific problem: the exact mechanisms of how visual experience regulates eye growth are unknown. The environmental factors consistently associated with myopia are visual cues including light intensity, color, and focal distance (near work). While the effect of each of these on retinal processing is somewhat understood, how these effects are impacting the growth of the eye is unknown. Additionally, the differential impacts of specific environmental cues and their interactions with each other and with genetic predispositions is also unclear.

In the field of myopia research today, animal models are used to mimic and isolate the effects of myopiagenic factors. By inducing experimental myopia in animals, the environmental, pharmacological, and genetic aspects of eye growth and emmetropization can be elucidated.

1.3.1 Dopamine as a 'stop signal' in myopic eye growth

To study the mechanism of myopia development, scientists have used experimental animal models of myopia. While animals do not generally become myopic under normal visual conditions, myopia can be induced by placing a diffuser or powered lens over the eye [(Sherman et al., 1977; Wiesel and Raviola, 1977; Wallman et al., 1978), for review (Schaeffel and Feldkaemper, 2015)]. These techniques are called form deprivation myopia (FDM) and lens induced myopia (LIM, described in CHAPTER 2), respectively. The application of a diffuser or negative lens defocus over the eye during early development will induce eye growth, including elongated axial length. Using this model, animals can be exposed to controlled laboratory lighting conditions and the response to FDM or LIM can be studied to make conclusions about how each environmental condition impacts myopia development. In past studies using animal models of myopia, regular animal facility lighting was used [50-300 lux].

Briefly, animal models previously used in myopia have included chick (Wallman et al., 1978; Wallman and Adams, 1987), monkey (rhesus macaque and marmoset (Raviola and Wiesel, 1990; Troilo and Judge, 1993; Smith et al., 1999), tree shrew (Sherman et al., 1977; Norton et al., 2010), guinea pig (Howlett and McFadden, 2006, 2007, 2009), and more recently the mouse (Schaeffel et al., 2004; Faulkner et al., 2007; Tkatchenko et al., 2010; Pardue et al., 2013; Gu et al., 2016; Jiang et al., 2018).

Both FDM (Wallman et al., 1978; Goss and Criswell, 1981; Wallman and Adams, 1987) and LIM (Schaeffel et al., 1988; Sivak et al., 1990) are characterized by increased axial

elongation and/or choroid thinning. Once these myopigenic effects were established, investigators wanted to determine if the mechanisms controlling myopia development were in the brain or originated locally in the eye. Studies used hemispheric diffusers (Wallman et al., 1978; Hodos and Kuenzel, 1984), optic nerve section (Troilo et al., 1987), and pharmacological interventions to show that changes in refractive error and axial elongation were controlled locally, without feedback from the brain (Hodos and Kuenzel, 1984; Norton et al., 1994; McBrien et al., 1995; Norton and Siegwart, 1995; Wildsoet and Wallman, 1995; Diether and Schaeffel, 1997; Wildsoet, 2003; Stone et al., 2006; Smith et al., 2009; Smith et al., 2010). Interestingly, the hemispheric diffusers triggered myopia on only the covered side of the eye suggesting that myopic growth was controlled not only within the eye, but could even be localized to specific regions of the retina (Wallman et al., 1978). Thus, refractive error development appears to be controlled by the presence of one or more local signaling pathways responding to changes in visual stimuli in the retina and acting downstream on the choroid, sclera, and possibly cornea.

There have been many different neurotransmitters, modulators, and peptides suggested as important retinal messengers for refractive development. The most well-studied and proven messenger has been DA [for review (Feldkaemper and Schaeffel, 2013)]. DA was first identified as an important signal in refractive development in 1989, when chicks with FDM were shown to have lower DA activity in the retina than control animals with normal visual input (Stone et al., 1989). This relationship between lower levels of DA and FDM was replicated in rhesus monkeys; this study also showed lower levels of TH activity in FDM retinas, the DA synthesizing protein (Iuvone et al., 1989). Others showed a decrease in the primary DA metabolite, DOPAC, in chicks given LIM (Guo et al., 1995; Ohngemach et al., 1997).

Interestingly, in a separate study this decrease in DOPAC occurred in half of the retina covered by hemispheric diffusers (Stone et al., 2006). Together, these findings have indicated DACs as a regulator in refractive error development and eye growth [for review (Wallman and Winawer, 2004; Feldkaemper and Schaeffel, 2013; Zhou et al., 2017)].

DACs are the main site of release for DA in the retina. The cells release DA through diffusion thereby creating a DA gradient on both sides of the retina (Ohngemach et al., 1997) to act on DAergic receptors throughout the retina as well as in other ocular tissues. While the exact target of DA in myopia is unknown, one possibility is that DA could act directly on the choroid, sclera, or cornea to change the axial length or optical power of the eye. Few studies have measured DA content in these tissues, however, one study found very little DA in the choroid and sclera, and did not observe differences with myopia (Ohngemach et al., 1997). This would suggest that DA may be released in response to the visual stimuli and then a second messenger is necessary for changes to ocular growth. That second messenger has not been identified though some studies suggest a transcription factor, ZENK and its mammalian homologue Egr-1. mRNA of ZENK has been shown to respond directionally to imposed negative or positive defocus and its absence produces myopia in mice (Ashby et al., 2007; Schippert et al., 2007; Ashby et al., 2010). Other messengers which may be involved include nitric oxide, retinoic acid, and acetylcholine (Kennedy, 1995; Nickla et al., 2009; Ashby et al., 2010).

A possible local DA signaling mechanism for refractive eye growth opens an opportunity to study and possibly treat myopia by intervening with DA activity in the retina. This is done more easily in animal models with well-developed genetic methods and pharmaceutical treatment options. In mice, genetic manipulation of DA receptors, and TH, have supported the hypothesis that DA levels and activity impact myopia development (Zhao et al., 2010; Jackson et

al., 2012; Bergen et al., 2016). Additionally, interventions with DA receptor antagonists such as SCH 23390, which acts on D1 type receptors, (Rohrer et al., 1993; Schaeffel et al., 1995; McCarthy et al., 2007; Nickla and Totonelly, 2011) and spiperone (Rohrer et al., 1993; McCarthy et al., 2007; Arumugam and McBrien, 2010) and sulpiride (Schaeffel et al., 1995), which inhibit D2 type receptors, have shown both enhanced effects of induced myopia or prevention of anti-myopia treatments like lens removal. Additionally, the use of 6-OHDA which is toxic to DAergic cells inhibited FDM in treated animals (Li et al., 1992; Wildsoet and Clark, 1993; Schaeffel et al., 1995). Increasing DA activity in the retina has prevented the effects of induced myopia. Treatment with of L-DOPA, the precursor of DA, in guinea pigs resulted in a decrease in the refractive error and axial length changes after FD (Mao et al., 2010; Mao et al., 2011; Mao and Liu, 2017). Apomorphine, which is a non-selective DA receptor agonist, has inhibited both FDM (Stone et al., 1989; Iuvone et al., 1991; Rohrer et al., 1993; Schmid and Wildsoet, 2004; Huang et al., 2018) and LIM (Schmid and Wildsoet, 2004; Nickla et al., 2010). Overall, genetic and pharmacological approaches to alter DA signaling in the retina can impact the outcome of refractive influences such as FDM or LIM.

While the evidence supporting DA as a signal in myopia is stronger and more compelling than other signaling candidates, there have been some studies contradicting this connection. For example, some studies using the same drugs listed above have found opposite or no effects indicating an important role of dosing, time of intervention, and/or species. SCH 22390 treatment in some cases has enhanced (Schaeffel et al., 1995) or had no effect (Cottriall et al., 2001) on FDM and one study of quinpirole, a D2 type receptor agonist, showed it enhanced spontaneous myopia in guinea pigs (Jiang et al., 2014). A recent study of C57BL/6 mice given FD showed no evidence of changes to total retinal DA levels, or DA signaling in the retina (Wu et al., 2015a).

Finally, a study of FDM in tree shrews showed that systemic treatment with water was as effective at preventing refractive error and axial elongation as treatments with DA agonists (Ward et al., 2016). While these are not the only studies to show evidence against a role for DA in myopia, they highlight the complexity of DA activity across treatments and species and call for a more thorough understanding of how DA could act to prevent myopia, a topic of this dissertation.

The synthesizing protein for DA, TH, is activated when it is phosphorylated by the DAC depolarization in response to photoreceptors and bipolar cells (Hokoc and Mariani, 1987; Yazulla and Zucker, 1988; Zhao et al., 2017). Therefore, TH is most active under bright light during the day when light driven signaling from these cells is the highest (Brainard and Morgan, 1987; Doyle et al., 2002a; Witkovsky et al., 2004). This was shown in 1978 by Iuvone et al. who measured TH activity with gas chromatography – mass spectrometry during both the light and the dark phase [(Iuvone et al., 1978) **Figure 1.8**]. This study revealed a dramatic increase in TH activity with the onset of light. Now, it is well accepted that DA is synthesized and released with light, and plays a role in many light driven and light responsive systems in the retina including circadian rhythm control and gap junction coupling [(Kothmann et al., 2009) for review (Witkovsky, 2004)].

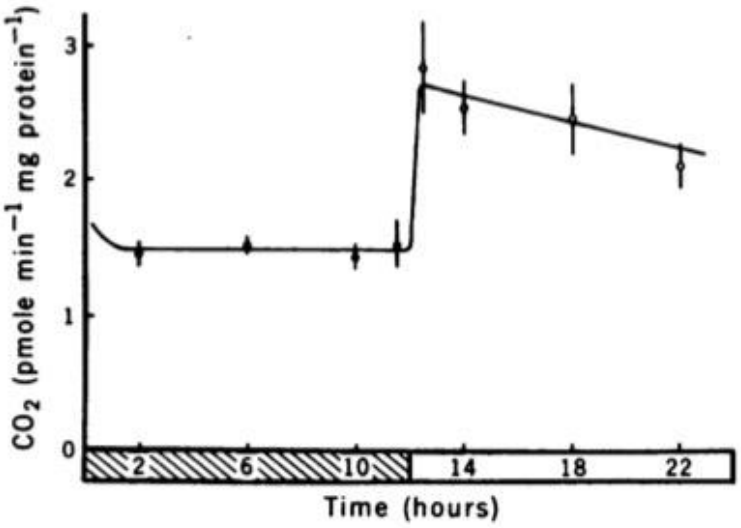

Figure 1.8. Tyrosine hydroxylase activity increased with light onset. A decarboxylase coupled assay was used to measure TH function and activity in the rat retina over 12 hour dark/ light phases. TH activity increased sharply after light onset, indicating a light dependent DA synthesis mechanism in the retina. Originally published in Iuvone PM, Galli CL, Garrison-Gund CK, Neff NH. Light stimulates tyrosine hydroxylase activity and dopamine synthesis in retinal amacrine neurons. Figure used here with permission from (Iuvone et al., 1978).

1.3.2 Light-driven prevention of myopia in animal models

Given the evidence of increased DA activity as a protective mechanism in animal models of myopia, and the increased DA synthesis in the retina in response to light it was hypothesized that bright light exposure could prevent myopia. Using a chick model of myopia, Ashby et al. were able to show that 15 minutes of exposure to bright illumination (15,000 lux) per day could prevent the effect of FDM [(2009), **Figure 1.9A**]. This report was followed by a study showing that bright light exposure [15,000 lux for 5 hours daily] also prevented LIM in chicks (Ashby and Schaeffel, 2010b). This finding has been replicated in tree shrews exposed to bright light [16,000 lux for 7.75 hours daily] (Siegwart Jr et al., 2012) as well as rhesus macaques [25,000 lux light for 6 hours daily] (Smith et al., 2012). Additionally, bright light has been shown to slow myopia progression during LIM in chickens (Ashby and Schaeffel, 2010b) and during FDM in tree shews (Siegwart Jr et al., 2012). However, some studies have reported contradictory results. Bright light did not protect rhesus macaques from LIM (Smith et al., 2013) and a recent study of chicks in natural light, housed outdoors, showed no protective effect (Stone et al., 2016). Further studies have examined the relationship between a range of light levels and timing to optimize preventative conditions for myopia. These experiments have generally shown that increasing the light intensity generates a more dramatic effect and slows myopia progression further [**Figure 1.9B,** (Karouta and Ashby, 2014; Cohen et al., 2008; Cohen et al., 2011; Backhouse et al., 2013; Lan et al., 2014; Chen et al., 2017)]. Other investigators have reported that flickering or non-constant light is more effective at preventing the effects of experimental myopia than constant light (Schwahn and Schaeffel, 1997; Liu et al., 2004; Crewther et al., 2006). Together, these reports in experimental animal models suggest that bright light will be an important part of developing prevention strategies for myopia in humans.

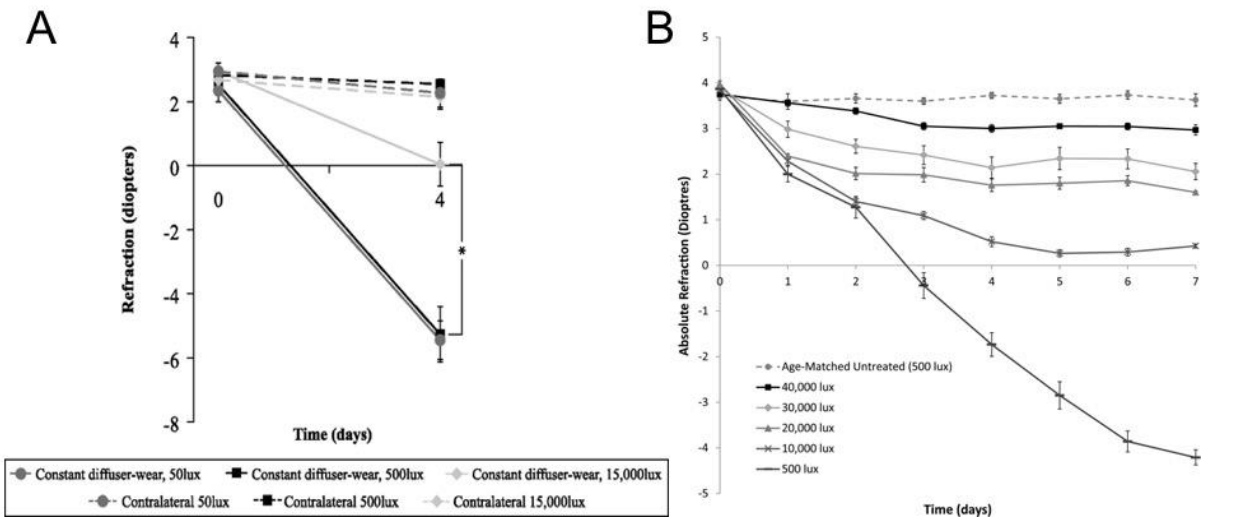

Figure 1.9. Bright light prevention of induced myopia is stronger at greater intensities. (A) Early studies of the effect of bright light on experimental myopia revealed that chicks with FDM showed reduced myopic shifts when given 15 minutes of daily exposure to bright light (15,000 lux) compared to chicks housed in 50 or 500 lux. (B) A follow-up study found that protection from LIM was correlated with the intensity of the light. Chicks with LIM responded less to the lens defocus with exposure to increasing light intensities. (A) Used with permission, originally published in (Ashby et al., 2009). (B) Used with permission, originally published in (Karouta and Ashby, 2014).

Despite the robust evidence supporting bright light protection from myopia, some variability across studies must be recognized. The species of animal used in the study, the type of experimental myopia, and the experimental lighting conditions can alter or prevent bright light protection from myopia. Bright light exposure has been shown to have protective effect for induced myopia across a broad range of animal species, suggesting that the effect may be conserved across species and differences between animals could be exploited to determine the relative role of ocular traits like the presence of a fovea or abundance of rod or cone photoreceptors. Additionally, despite evidence that FDM and LIM have different mechanisms from pharmacological studies, both experimental models have reduced myopic shifts with exposure to bright light. Finally, chromatic properties of the light have been a raised when comparing and drawing conclusions between studies. While most have used white LED lights, the exact wavelengths may vary and this could be playing a role in myopia development [for review (Rucker, 2013)] and should be considered carefully. By studying these differences in experiments, we may be able increase our understanding of the mechanisms behind light-driven protection from myopia.

1.3.3 Connecting light and dopaminergic prevention of myopia

Given the strong evidence supporting a role of DA as a stop signal in myopic eye growth, the knowledge that myopia is prevented under bright light conditions, and that DA synthesis and release is triggered by bright light, a hypothesis emerged that light prevents myopic growth by triggering DA activity in the retina. The initial studies of the impact of bright light on experimental myopia in animals therefore included analyses of DA activity (Ashby et al., 2009; Ashby and Schaeffel, 2010b). Quickly after, studies began to look at a broader range of illuminance levels and found that levels of DOPAC in the vitreous were significantly higher under brighter light, which correlated with less FDM (Cohen et al., 2012). Since this time,

several studies have made similar conclusions and shown that bright light increases DA activity, even in animals with induced myopia, and that this often comes with a lower susceptibility to induced myopia (Karouta and Ashby, 2014). These and other recent studies show that with increasing light, DA driven protection from myopia increases [for review (Norton and Siegwart, 2013), **Figure 1.10**].

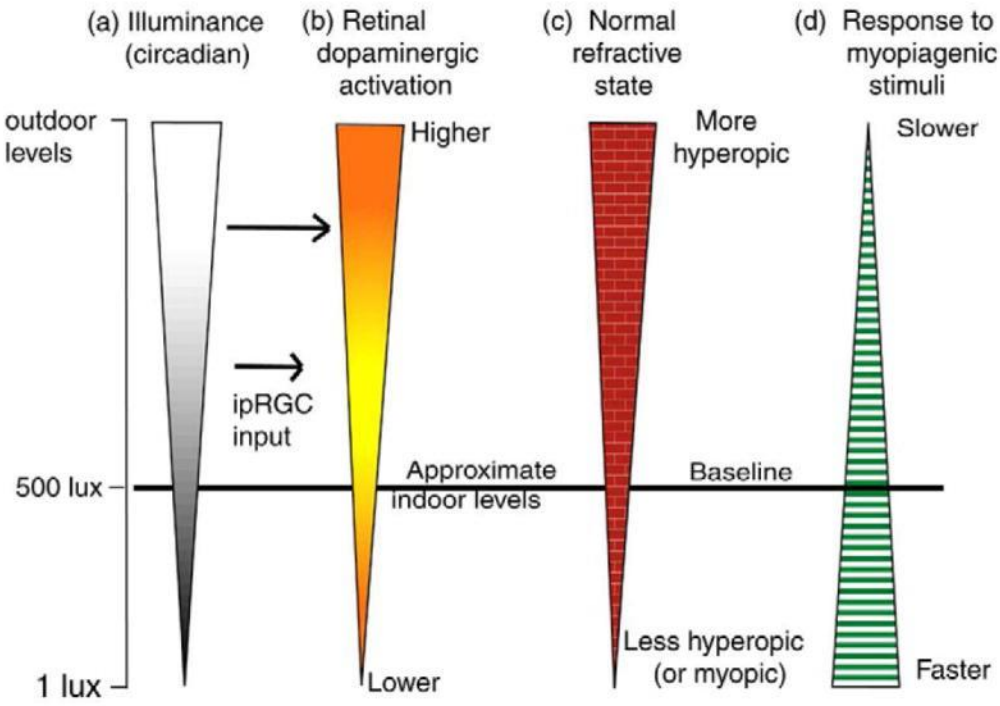

Figure 1.10. Schematic of our current understanding of the relationship between light and myopia. (a) Here, luminance levels are highest in the outdoors. (b) This bright outdoor light leads to increased activation of DACs and increased DA release. (c) Higher levels of DA activity in the retina lead to decreased myopia, and potentially to hyperopia. (d) Finally, in animal models the response to myopiagenic stimuli such as lens defocus or form deprivation is slowed with increased DA activity under bright light. This figure highlights the assumption that the relationships between these aspects of refractive error development are linear. Figure used with permission, originally published in (Norton and Siegwart, 2013).

However, this assumption is based on studies that have investigated the effects of light from 50-20,000 lux, roughly equivalent to a dim indoor space and outdoors on a clear day. It is therefore assumed that this relationship is linear and light less than 50 lux would result in more severe myopia. Unfortunately, this has not been closely studied and recent evidence suggests that rod photoreceptors responsible for dim light detection (Park et al., 2014) are important for refractive development. Furthermore, recent evidence shows that rod pathways that are active under bright light may also be active under dim light (Tikidji-Hamburyan et al., 2017). These data suggest that dim light is a potential important visual signal in refractive development. The experiments described here aim to investigate the role of a broad range of ambient light on lens induced myopia development in the mouse model, the effect of dim light illumination on myopia susceptibility, and the impact of ambient light on DA dynamics in the retina.

1.3.4 The importance of time outdoors and light in human myopia

As the epidemic of myopia worsens in specific regions of the world, epidemiological studies have revealed important information about environmental and behavioral differences between myopes and non-myopes. By understanding these differences, we can begin to study the development of myopia more closely. These studies associated education level with myopia early on. It has been observed by many groups that individuals with higher levels of education are more likely to be myopic and to have more severe myopia than less educated individuals [for review (Rose et al., 2016)]. This was supported by the idea that time spent on near work (reading, using a computer, or other tasks where eyes focus on objects close to the face) was myopigenic (Huang et al., 2015). However, education level has an inverse relationship with time spent outdoors, such that less time outside was also associated with myopia. It was first observed by Parssinen and Lyra in 1993 that a group of Finnish children who spent more time outdoors

had less myopia at follow-up indicating time spent outside was slowing the progression of myopia (Parssinen and Lyyra, 1993). Initially, it was believed that participating in sports, and spending less time on near work, was how time outdoors was mediating myopia protection. This hypothesis was first proposed by the Orinda Longitudinal Study of Myopia and supported by others, which showed that myopic children spent less time playing sports than non-myopic children (Mutti et al., 2002; Jones et al., 2007). Since this time, a number of studies have shown a correlation between increased time outdoors, regardless of activity, and less severe myopic refractions and shorter axial lengths (Saw et al., 2002; Saw et al., 2006; Onal et al., 2007; Rose et al., 2008b; Rose et al., 2008a; Low et al., 2010; Guggenheim et al., 2012; French et al., 2013b).

Several aspects of time spent outdoors have been identified as possible mechanisms for myopia prevention, including a different light spectrum than indoor light, greater light intensity, different focal distances and accommodation, and changes in average pupil diameter. In 2008, the Sydney Myopia Study separated the effects of time outdoors and physical activity by asking parents about time spent on indoor sports, outdoor sports, and other outdoor activities and found that the total time spent outdoors regardless of physical activity was important for myopia prevention (Rose et al., 2008b). Near work was also correlated with myopia in this study, however the relationship was likely confounded by near work being an indoor activity [(Rose et al., 2008b; Mutti and Zadnik, 2009), **Figure 1.11**]. Whether these other potential myopiagenic factors are independent of each other and to what degree is still unclear, however, the Sydney Myopia Study, and many following it, have identified bright light as the major protective factor in time outdoors.

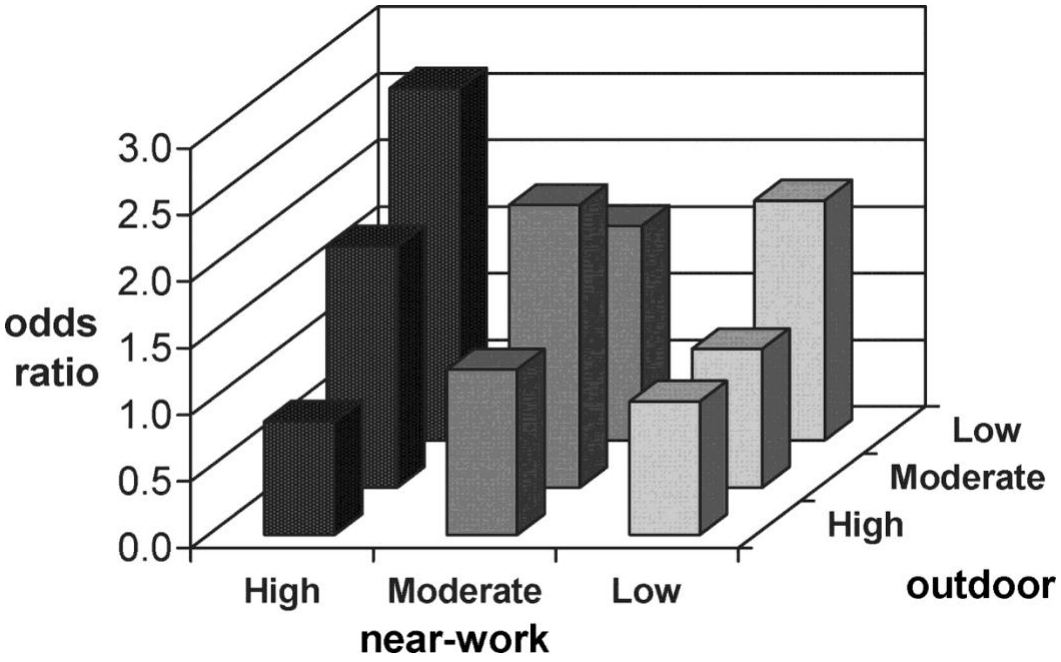

Figure 1.11. Effects of near work are overcome by more time outdoors. Studies have shown the myopiagenic effects of near work can be eliminated by greater time spent outdoors. In this figure, originally published by Rose et al. as a part of the Sydney Myopia Study, the odds ratio of individuals with high near-work, high time outdoors was not statistically different from all individuals with high time outdoors (logistic regression for odds of myopia; p=0.9, 2008). Individuals spending low time outdoors had consistently higher odds of developing myopia than almost all other groups. Figure used with permission, originally published by (Rose et al., 2008b).

The intensity of outdoor light was identified as a major factor in myopia prevention [for review (French et al., 2013a)]. Outdoor light can be several thousand times brighter than indoor light, up to 200,000 lux in sunny places, while indoor light is usually lower than 1,000 lux. Like the Sydney Myopia Study, other analyses of bright light exposure relied on surveys usually taken by the parents or teachers of young study participants to quantify the amount of time spent in bright light. However, as concerns about the accuracy of survey results arose, more accurate measures of light exposure were needed. Soon, commercially available light sensor devices such as the wearable HOBO monitor and wrist-worn Actiwatch were implemented to detect light exposure (Dharani et al., 2012). Other devices made by research groups to be more accurate, worn at eye level, or to also measure time spent on near work, are now being applied to these same questions [unpublished data, International Myopia Conference 2017]. With accurate light sensors available, recent studies have shown more precisely the amount of time myopic and non-myopic children spend in exact light levels and have found subtle differences in behavior which could help prescribe preventative techniques for individuals (Read et al., 2014, 2015; Shah et al., 2017; Verkicharla et al., 2017; Ostrin et al., 2018; Read et al., 2018; Wu et al., 2018). One report of light and myopia, using the Philips Actiwatch 2, showed that non-myopes are consistently exposed to brighter light for longer compared to myopic peers [(Read et al., 2015), **Figure 1.12**].

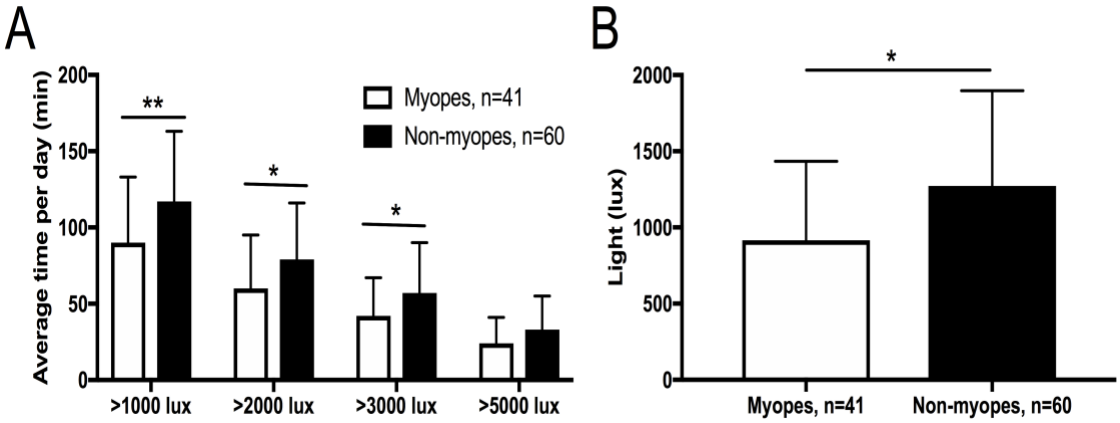

Figure 1.12. Myopes are consistently exposed to less bright light than non-myopes. The Role of Outdoor Activity in Myopia study done in Queensland, Australia used Actiwatches to objectively measure light exposure for myopes and non-myopes. (A) In this study, non-myopes were consistently exposed to more time in each bright light level. (B) The average light intensity across the day was higher in non-myopes than myopes. Data shown are means ± SD. Significance represents results from a repeated measures ANOVA (*$p<0.05$, **$p<0.01$). Figure created with data originally published in a table in (Read et al., 2015).

Recent studies of the effect of light on myopia have probed whether bright light affects not only susceptibility to myopia but its progression as well. By intervening in behavior or environmental experiences, researchers have been able to alter light exposure and monitor myopia progression in their participants. Interventions have included extended recess at school, recess outside versus in a gymnasium, glass walled classrooms, and programs to increase outdoor activities at home (Wu et al., 2013; He et al., 2015; Hua et al., 2015; Wu et al., 2018). While these studies have cleverly tested prevention strategies, the overall conclusions have been mixed. Some have found no relationship between bright light exposure and slower myopia progressions (Jones-Jordan et al., 2012; Saw et al., 2000) and Parssinen and Lyrra found a protective effect on progression only in male participants (1993). With these confusing results, studies in animal models have probed the relationship between light exposure, myopia onset, and progression more thoroughly.

Here, the retinal mechanisms, specifically the role of DA, of light driven protection from myopia are investigated. By using both an animal model of myopia and human behavioral data the role of a broad range of light levels will be investigated for the first time.

1.4 Summary of the Work Described in this Dissertation

In this dissertation, I examine the relationships between ambient light and lens defocus, changes in DA activity which mediate these interactions, and the potential of ambient light as a preventative therapy for myopia. This work focuses on the different retinal signaling pathways that are activated across a broad range of ambient luminance levels which the eye encounters every day. This approach will investigate the effect of all natural light levels, which I hypothesize is key for correct refractive development. The work described in this dissertation

tests the hypothesis that dim light, in addition to bright, prevents myopia through DA mediated retinal signaling. The experiments included here are divided into four main studies.

In the first study (CHAPTER 2), I test the hypothesis that dim light, which triggers responses from rod photoreceptors, prevents LIM. Previous work has shown that both bright light and rod photoreceptors are important in the development of myopia, however, dim light has not been investigated. By exposing mice to dim, intermediate, and bright light, I test their response to LIM as a measure of myopia susceptibility. Refractive error, corneal curvature, axial length, retinal thickness, and other ocular parameters were measured before and after lens defocus treatment to quantify myopia susceptibility.

In the second set of experiments (CHAPTER 3), I test the role of retinal DA in protection from myopia observed under certain ambient light levels. Quantification of DA and the metabolite DOPAC by high performance liquid chromatography was used to determine changes in DA and its metabolism. Changes in DA related proteins, found in DAergic cells in the retina, were measured with both digital droplet PCR and western blot. Finally, a novel assay of light stimulated DA release from in vitro retinas was developed to measure the activity of DACs under light and defocus conditions. These experiments establish the role of DA in the mouse model of myopia.

To test the impact of DA interventions on myopia susceptibility, I conducted a two-part experiment manipulating DA signaling pharmacologically and genetically (CHAPTER 4). First, wild-type mice and transgenic mice with retinal degeneration and low DA activity were treated with L-DOPA or the antioxidant ascorbic acid with simultaneous FDM treatment. An analysis of the refractive error in these mice tests the effects of pharmaceutically increasing DA activity. Second, the transgenic VMAT2 HI mice, with increased expression of VMAT2, were given

FDM to determine how genetically increased DA activity could impact refractive error development. Together, these studies investigate the potential of increased DA activity to prevent myopia.

To determine the clinical relevance of dim light prevention of myopia, I formed a collaboration with the authors of the Role of Outdoor Activity in Myopia study in Queensland, Australia to analyze dim light exposure in a population of myopic and non-myopic children. In this analysis, I investigate light exposure across weekdays and weekends in myopes and non-myopes to test the hypothesis that dim light is an important factor in myopia prevention. Finally, I use the refractive errors of the study participants to correlate the severity of myopia to average amounts of time spent in various ambient light levels.

I have studied a wide range of ambient luminance levels designed to test specific retinal pathways involved in retinal light responses and how DA activity under each light level could be regulating myopia susceptibility. The findings presented here will demonstrate how a wide range of ambient lighting is important in the development of the visual system and extend our knowledge of the impact of environmental stimuli on neurological development more broadly. Additionally, this discovery that the range of ambient lighting impacts refractive development is important for developing preventative approaches to effectively stem the rising epidemiological impact of myopia.

CHAPTER 2: Ambient light alters myopia susceptibility in the mouse model

2.1 Abstract

Both animal models and human epidemiological studies show decreased severity of myopia with exposure to high intensity or outdoor lighting. Knowledge gaps exist as to how light interacts with visual signaling to cause or prevent myopia. Previous work in the mouse retina demonstrated that functional rod photoreceptors are needed to develop experimentally-induced myopia, suggesting a role for rod signaling in refractive development. To determine if dim, rod-dominated luminance levels could also prevent myopia, we housed male C57BL/6J mice under 12:12 light:dark cycles with scotopic (0.005 lux, n=38), mesopic (50 lux, n=36), or photopic (15,000 lux, n=38) lighting from post-natal day (P) 23 until P38. Half the mice received monocular exposure to -10 diopter (D) lens defocus from P28-38. Refractive errors and ocular parameters were measured at baseline and at the final time-point. Mice exposed to either scotopic or photopic lighting developed significantly smaller myopic refractive shifts (lens treated eye minus contralateral naïve eye; -1.807±0.608D and -2.604±0.544D, respectively) than mice exposed to mesopic lighting (-4.741±0.608D; $p<0.005$). This work indicates that scotopic and photopic lighting protect against lens-induced myopia, possibly indicating that a broad range of light levels are important in refractive development.

2.2 Introduction

Myopia has increased at epidemic rates in many countries. Even in the United States, the incidence of myopia has reached 42% in the last three decades (Vitale et al., 2009). While genetic factors are known to contribute to the development of myopia, the magnitude of these recent increases is evidence that myopia development is also influenced by environmental factors. With the intention of finding preventative behaviors to curb increases in myopia prevalence, the field has focused on environmental factors that might be driving myopia.

It was observed that children who spend more time outdoors in bright light are less likely to become myopic or experience a progression of their myopia (Mutti et al., 2002; Jones et al., 2007; Rose et al., 2008b; French et al., 2013a). Therefore, recent research has focused on the potential benefits of bright light on myopia, for instance clinical trials to determine the effectiveness of increasing time outdoors in children (Jones et al., 2007; Rose et al., 2008b; Wu et al., 2013; He et al., 2015). A number of animal studies in tree shrews, chicks, and macaques have confirmed the protective effect of bright ambient luminance on the development of myopia using controlled laboratory conditions (Ashby et al., 2009; Ashby and Schaeffel, 2010a; Siegwart et al., 2012; Smith et al., 2012; Stone et al., 2016). These data have led to a better understanding of how bright light could stop excessive eye growth and what paradigm of bright light might be most beneficial. However, some studies have not reported such benefit from bright light, including monkeys with lens induced myopia and chickens exposed to natural, environmental light (Smith et al., 2012; Stone et al., 2016). In addition, it is unclear if constant uniform light or flickering light is most effective for preventing myopia, as a slow flicker of 1 minute light and 1 minute of darkness at 15,000 lux had the largest protective effect in chickens

(Lan et al., 2014). Thus, it is unknown if bright light is the optimal or only potential environmental light that is beneficial.

Our visual system is optimized to function over a broad range of light conditions. It is remarkable that the same system that can detect a single photon of light through rod pathways can also function under 10^{12} to 10^{15} photons of light using cone pathways (Hunter et al., 2012). Refractive development has often been assumed to be driven by cone pathways which are responsible for high acuity, color vision, and perception in bright light. However, there is a growing body of data that suggests that rod pathways may also provide some input to visually-driven eye growth. Studies in which the fovea was blocked or ablated have shown no significant differences in myopia susceptibility relative to whole retina or rod-dominated peripherally-induced myopia in monkeys (Smith et al., 2007a; Smith et al., 2009). In addition, we recently reported that the disruption of rod function in the *Gnat1-/-* mouse results in abnormal refractive development and the inability to respond to form deprivation [(Park et al., 2014), **Figure 2.1**]. This finding suggests that rod pathways may be important for refractive development. Thus, both rod and cone pathways, stimulated by dim and bright light may be necessary for optimal eye growth. In fact, when considering examples of developing populations that have adopted electrical lighting and experienced large increases in myopia, it may be the absence of both dim and bright light, replaced by the constant exposure to mesopic, indoor lighting that drives myopia development (Norton and Siegwart, 2013).

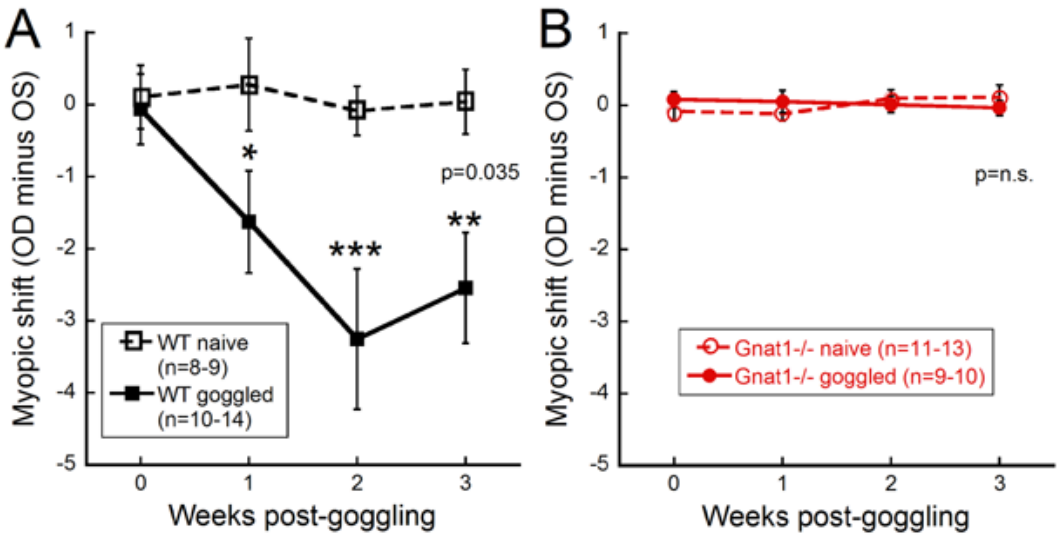

Figure 2.1. Mice with dysfunctional rod photoreceptors show no response to form deprivation myopia. (A) Wild-type mice (Li et al.) showed a significant myopic shift (refraction of treated minus untreated eye), indicating myopia susceptibility, after one week [2-way repeated ANOVA, $F(3,77) = 3.1$, $P = 0.035$]. The effect continued throughout the experiment. Untreated, naïve mice showed no difference between eyes. (B) *Gnat1-/-* mice (*red*) showed no change in refractive error in the treated eyes resulting in no myopic shift changes relative to naive animals. Data shown are mean ± SEM. This figure was originally published in (Park et al., 2014), used here with permission.

In this study, we examined the effect of lens defocus in mice housed in three different environmental luminance levels: scotopic lighting that stimulates only rod pathways, mesopic lighting that stimulates both rod and cone pathways, and photopic lighting that stimulates cone pathways. We hypothesize that both dim and bright light exposure would reduce the lens defocus myopia while mesopic lighting would increase myopic shifts.

These experiments use the mouse model of myopia, a recent animal model to the field of experimental myopia that leverages the mammalian retina with an extensive level of background knowledge on retinal signaling and function. The mouse has been shown to respond to form deprivation and lens defocus in a number of studies (Faulkner et al., 2007; Barathi et al., 2008; Pardue et al., 2008; Huang et al., 2014). In these studies, mice show a myopic refractive shift with treatment due to changes in corneal curvature in some mice (Bergen et al., 2015) or axial elongation in others (Chakraborty et al., 2015b). The mouse has been used extensively to increase our understanding of retinal circuitry and visual processing in humans and other mammals. In the case of refractive development, the mechanisms controlling visually-driven eye growth appear to be well conserved across animal phylum, as a number of animal models have developed myopia in response to form deprivation (Schaeffel and Feldkaemper, 2015). In this study, a mouse model of myopia with lens defocus will be exposed to altered ambient light to determine the role of visual experience on refractive development.

2.3 Methods

2.3.1 Animals and experimental design

This experiment consisted of exposing male wild-type C57BL/6J mice (Jackson Labs, Bar Harbor, ME) to three different ambient lighting levels and measuring the response to lens-

induced myopia. Only male mice were used to minimize cost; pilot data showed no differences between male and female mice. At post-natal day 23 (P23), baseline measurements of refractive error, keratometry, and axial length were obtained. The mice were then placed into scotopic (0.005 lux, n=39), mesopic (50 lux, n=37), or photopic (15,000 lux, n=38) lighting conditions on a 12:12 hour light:dark (LD) cycle to maintain normal circadian rhythms. At P28, refractive and ocular measurements were repeated, and half the mice received a head-mounted goggle to position a -10 diopter (D) lens over the right eye. At P36, the lens was removed, and the refractive and ocular measurements were repeated. Mice were sacrificed at P38, following two additional days of lens defocus.

In the following experiments, animals were housed in individual shoe box cages at the Atlanta Veterans Affairs Medical Center with mouse chow and water available *ad libitum*. Cages were topped with wire lids, and food was provided at the bottom of the cage to prevent shadows. Mice were monitored daily during the experiment. All procedures were approved by the Atlanta VA Institutional Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.3.2 Light exposure

Mice were exposed to the three lighting conditions using a custom-made light-tight box (Actimetrics, Wilmette, IL) which protected the animals from light pollution present in the room. Experimental luminance levels stimulated different photoreceptors, including rod-only scotopic (0.005 lux), mixed rod and cone mesopic (50 lux), and cone-dominated photopic (15,000 lux). The ceiling of the box contained a series of white LEDs which were covered with neutral density filters to obtain the desired lux values for scotopic and mesopic luminances. Photopic lighting was created with white LEDs in a commercial panel (Fancier Studio, Hayward, CA) placed 4-6

inches from the top of the cages. Ambient light levels were measured at the floor of the cage using a light meter (VWR Traceable Dual-Range, Radnor, PA) and spectrophotometer (BWTek Exemplar Spectrophotometer, Newark, DE). Animals were kept in their prescribed treatment luminances for the entire course of the experiment except when measurements were being done, during which cages were covered in dark cloth or kept in the dark.

2.3.3 Refractive and ocular measurements

Refractive and ocular measurements were performed at P23, 28, and 35 as previously described (Cooper et al., 2003; Schaeffel et al., 2004; Park et al., 2013). Pupils were dilated with 1% tropicamide (Bausch + Lomb, Bridgewater, NJ). Refractive errors were measured with a custom-made photorefractor [(Faulkner et al., 2007; Schaeffel, 2008), **Figure 2.2A**] in both awake and anesthetized conditions [ketamine (80 mg/kg)/xylazine (16 mg/kg)]. Corneal curvature was measured with a custom-made keratometer [(Schaeffel, 2008; Bergen et al., 2016), **Figure 2.2B**]. Cross sectional images of the mouse eye were obtained using an optical coherence tomography (OCT) system (Bioptigen Inc., Durham, NC, **Figure 2.2C**). These images were then used to measure axial length, anterior and vitreous chamber depth, and cornea, lens, and retinal thickness (Pardue et al., 2013). Following testing, mice were given yohimbine (2.1mg/kg) to reverse the effects of xylazine and prevent corneal ulcers (Turner and Albassam, 2005) and were then allowed to recover.

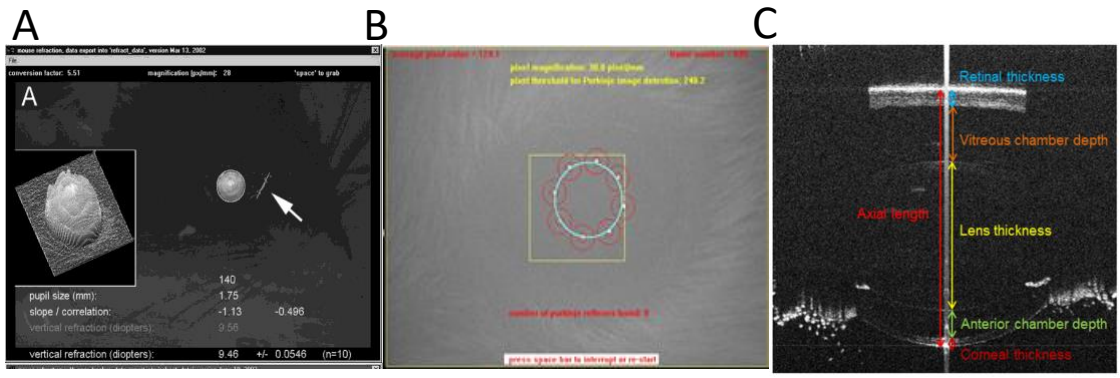

Figure 2.2. Refractive and ocular measurements. (A) The automated photorefractor developed by Schaeffel et al. is custom designed for the mouse eye (Schaeffel et al., 2004). A central camera captures an image of the eye, the pupil imaged as a bright circle, and reflected infrared lights positioned in the bottom half of the camera lens. Using this image, a custom software program calculates the slope of a regression line through pixel brightness values across the vertical axis of the pupil; the slope is then converted to a corresponding refractive error based on a predetermined correlation coefficient. (B) Similar to the photorefractor, the keratometer uses the reflections of a ring of infrared lights around a camera (Schaeffel, 2008). The custom software fits a circle through these reflections and calculates the diameter of that circle which is proportional to the radius of curvature of the cornea. (C) The Envisu R4300 SD-OCT system utilizes light (800nm) to image a cross section of the eye including different brightness levels that represent individual tissue types based on density. These images are analyzed by a blinded observer using a custom MATLAB program to determine the axial length, corneal thickness, anterior chamber depth, lens thickness, vitreous chamber depth, and retinal thickness. Images (A) and (B) are modified from (Schaeffel et al., 2004; Schaeffel, 2008), image (C) is unpublished from the Pardue Laboratory.

2.3.4 Lens-induced myopia

Animals were anesthetized [ketamine (80 mg/kg)/xylazine (16 mg/kg)] and a head-mounted lens holder was surgically attached, as previously described (Faulkner et al., 2007). A clear -10 D lens was held with a custom-made frame over the right eye of a subset of animals from each light level (scotopic: n=21, mesopic: n=20, photopic: n=20 treated with LIM). Frames were "threaded" through a surgically implanted head pedestal and held in place with the tightening of an aluminum cube around the frame (**Figure 2.3**). The cube also held a "balance bar" which rested on the left side of the face (Faulkner et al., 2007; Schaeffel, 2008). Defocus lenses were kept in place until the end of the experiment, checked daily for fit and compliance, and cleaned as needed. The untreated left eye served as a paired control, referred to here as the naïve contralateral eye.

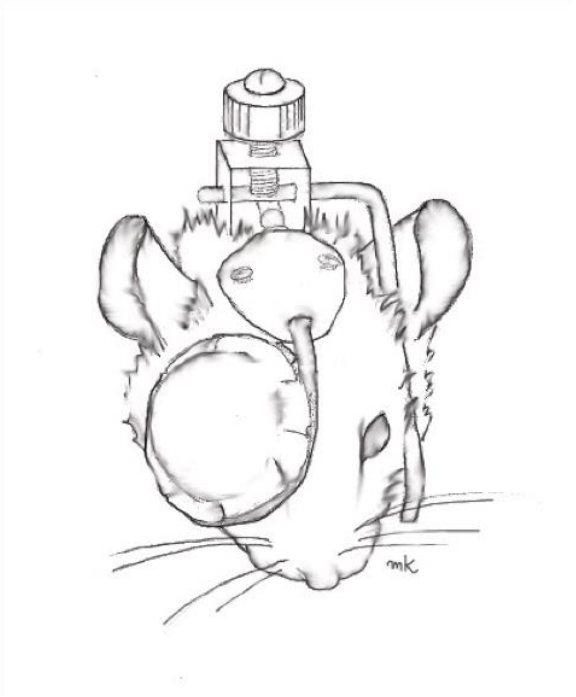

Figure 2.3. Illustration of experimentally induced myopia on mouse. A schematic drawing of the head mounted goggle which is used to experimentally induce myopia in mice. A goggle is placed over the right eye (*oculus dextrus*, OD) and is threaded through a head pedestal surgically attached to the skull. An aluminum cube holds the goggle and balancing bar, on the left side of the face, in place. The entire apparatus is tightened with a thumb screw to prevent movement. Used here with permission this figure was originally published in (Faulkner et al., 2007).

2.3.5 Circadian rhythms in experimental light levels

To determine the ability of the C57BL/6J mice to perceive all three light levels and maintain circadian rhythms under the experimental conditions, running wheel activity was monitored. Male C57BL/6J mice were first exposed to scotopic, mesopic, or photopic light for a 12 hour LD cycle with light onset at 8:00am for one week followed by one week in constant darkness, and one week of the original luminance level of 12 hour LD cycle to determine entrainment ability. Mice were housed individually and given low profile running wheels (Med Associates, Fairfax, VT), which recorded full rotations of the wheel. Counts of wheel spins were converted to distance and binned by hour. Hourly averages across the 24 hour cycle were taken for each of the six mice under each light level and average distances were calculated for all mice under each light level.

2.3.6 Statistical analysis

Experimental and control groups were compared across light levels by determining mean refractive error for control mice (average of both eyes), lens treated eyes, and naïve contralateral eyes. The mean myopic shift (lens defocus treated eye minus naïve contralateral eye) for lens defocus treated mice in each light level was also used as a measure of intra-animal effect. Results of experiments with lens defocus exposed mice were analyzed using two-way repeated measures ANOVAs (SigmaStat, San Jose, CA) and Holm Sidak post hoc comparisons. Representative average running distances from ZT 1 and ZT 13 were compared across light levels using a two-way repeated measures ANOVA with Holm Sidak post hoc comparisons.

2.4 Results

2.4.1 Scotopic and photopic lighting protects against refractive error changes

After one week of exposure to either scotopic, mesopic, or photopic light, a subset of mice were treated with monocular defocus using -10D lenses, which induced myopic shifts in all three experimental groups. Refractive error changes that occurred in the first five days of light exposure (P23-28), prior to lens defocus, were due to normal development and not dependent on light exposure as there were no differences between the three groups. After lens defocus at P28, control mice in all three light levels continue to develop with no significant differences between groups. Lens treated mice however, showed significant differences in refractive error between lens treated eyes and naïve contralateral eyes under all light levels. In scotopic light, lens treated eyes were significantly more myopic (3.21±0.47 D, **Figure 2.4A**) than the naïve contralateral eyes (4.83±0.56 D, p=0.04), and the eyes of control mice [5.59±0.50 D, post-hoc: p=0.004, RM Two-way ANOVA interaction effect $F(4,114) = 5.26$, p=0.001]. Mice housed in mesopic light showed a more severe change in refractive error after lens defocus. Lens defocus and mesopic light treated eyes were significantly more myopic at P35 (0.96±0.59 D, **Figure 2.4B**) than naïve contralateral (4.57±0.56 D, p<0.001), or control eyes [5.14±0.49 D, post-hoc: p<0.001, RM Two-way ANOVA interaction effect $F(4,108) = 13.6$, p<0.001]. Finally, photopic exposed mice, like those in scotopic light, responded mildly to lens defocus such that lens treated eyes (2.16±0.57 D, **Figure 2.4C**) were significantly more myopic than naïve contralateral (3.89±0.62 D, p=0.02) and control eyes (4.71±0.41 D, post-hoc: p<0.001, RM Two-way ANOVA interaction effect $F(4,110) = 5.17$, p<0.001).

To more directly compare the effect of ambient light on the response to lens defocus, the myopic shift is calculated for lens treated eyes in each light level. Lens defocus treated mice

exposed to mesopic light had significantly greater myopic shifts (-3.80±0.57 D) than mice exposed to scotopic (-1.82±0.29 D, p=0.005) or photopic (-1.90±0.38 D, p=0.006) light [One-way ANOVA $F(2,58) = 6.89$, p=0.002; **Figure 2.4D]**. There was no significant difference between the myopic shift of mice exposed to scotopic or photopic light.

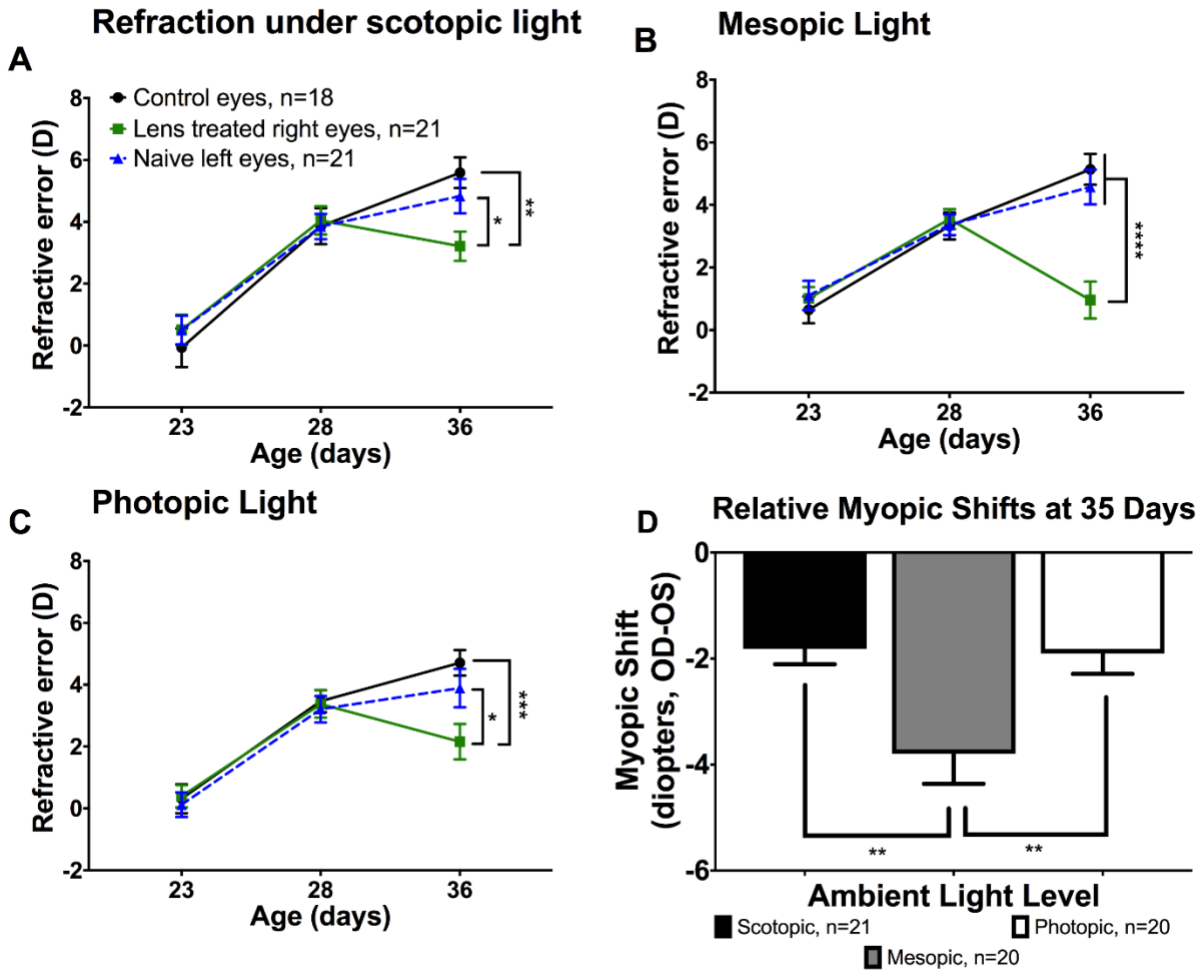

Figure 2.4. Scotopic and photopic light inhibit lens induced myopia in mice. (A) Mice in scotopic light generally became hyperopic with development. After lens defocus at P28 the lens treated eyes (*green*) stop normal development and become relatively myopic compared to their naïve contralateral eyes (*blue*, p=0.04) and control fellows [*black*, p=0.004, RM Two-way ANOVA interaction effect $F(4,114) = 5.26$, $p=0.001$]. (B) Mesopic exposed mice have a similar but more severe response to lens defocus. Lens treated mice exhibit greater myopia [RM Two-way ANOVA interaction effect $F(4,108) = 13.6$, $p<0.001$] than both contralateral and control eyes. (C) Mice in photopic light also responded to lens defocus with a slight myopic refractive error relative to contralateral and control eyes [RM Two-way ANOVA interaction effect $F(4,110) = 5.17$, $p<0.001$]. (D) By comparing the myopic shift of lens treated mice, it is clear that mesopic light exposure results in a greater myopia susceptibility than scotopic or photopic [One-way ANOVA $F(2,58) = 6.89$, $p=0.002$] light exposure. All data shown is mean±SEM, n=17-21/group, post-hoc comparisons indicated by *p<0.05, **p<0.01, ***p<0.001.

2.4.2 Corneal curvature, not other ocular parameters, is altered by lens defocus under mesopic light

To determine the source of refractive error changes, corneal curvature and other ocular parameters were measured. In all light levels and treatment groups, the corneal curvature increased with age (**Figure 2.5A-C**). Overall, mice housed in mesopic lighting showed a significant interaction effect between lens defocus and age on the steepening of corneal curvature (at P35 lens treated eye: 1.433±0.014mm, control eyes 1.452±0.018mm, contralateral naïve eyes: 1.460±0.018mm, [RM Two-way ANOVA, $F(4,108)=3.77$, $p=0.007$) **Figure 2.5B**]. The corneal curvatures of lens defocus-treated eyes in scotopic (at P35 1.431±0.0.017mm) and photopic (at P35 1.425±0.013mm) illuminances were not significantly different than those of their luminance-matched naïve controls (at P35 scotopic: 1.450±0.017mm, photopic: 1.448±0.017mm, **Figure 2.5A,C**). Both scotopic and photopic housed mice showed a main effect of age on corneal curvature regardless of lens treatment [scotopic: RM Two-way ANOVA, $F(2,108)=234.8$, $p<0.001$; photopic: RM Two-way ANOVA, $F(2,106)=195.6$, $p<0.001$]

Other ocular parameters measured included axial length, retinal thickness, lens thickness, corneal thickness, vitreous chamber depth, and anterior chamber depth. No significant differences were found across treatment groups among any of these measures, although all change as the animals age. The parameters which have the most relevance to refractive error, and are thus shown here, are axial length, which commonly increases with myopia, and retinal thickness, which would indicate any damage to the retina caused by bright light exposure. The axial length at P35 in eyes from lens defocus treated mice exposed to scotopic (3.109±0.007mm, **Figure 2.5D**), mesopic (3.114±0.007mm, **Figure 2.5E**), and photopic (3.120±0.011mm, **Figure 2.5F**) lighting were not significantly different from each other and were nearly identical to the

naïve eyes of the same mice and control eyes of mice in the same lighting (**Figure 2.5D-F**). A significant effect of age was found for all light levels indicating a lengthening over time regardless of treatment or light exposure [RM Two-way ANOVA, scotopic: $F(2,94)=472.6$, $p<0.001$, mesopic: $F(2,90)=221.1$, $p<0.001$, photopic: $F(2,90)=690.3$,$p<0.001$]. No significant differences between treatment groups were found for retinal thickness, although a main effect of age was found indicating a general thinning of the retina in all groups [scotopic: $F(2,94)=19.77$, $p<0.001$, mesopic: $F(2,90)=18.22$, $p<0.001$, photopic: $F(2,90)=23.36$,$p<0.001$]. Unlike corneal curvature or axial length, the retinal thickness was more variable across animals, however without differences between groups, it is unlikely that this variability explains the differences in refractive error across light exposure treatments.

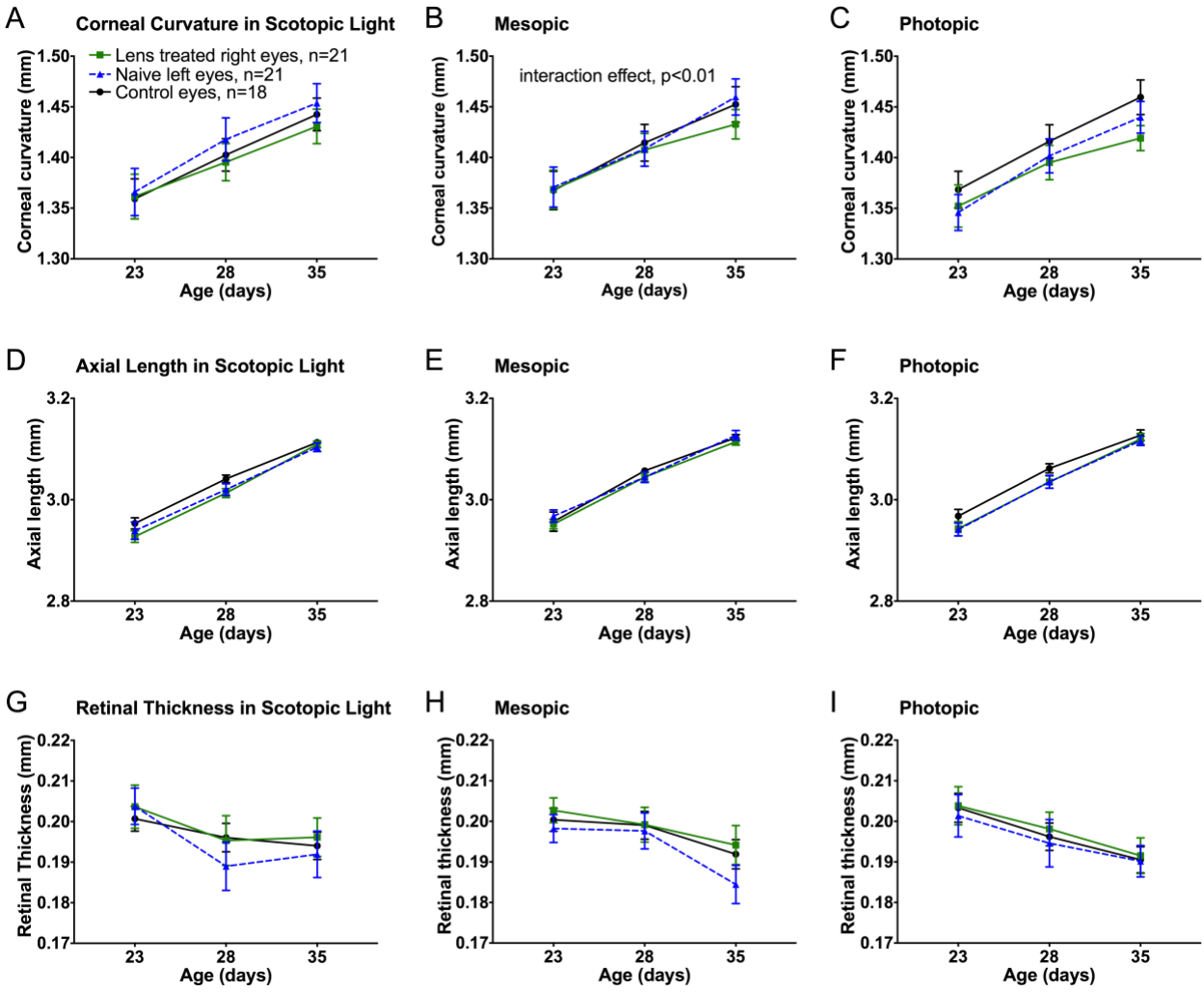

Figure 2.5. Neither light nor lens treatment resulted in changes in corneal curvature, axial length or retinal thickness. The corneal curvature of the anterior cornea strongly impacts the optical power of the eye, however, no differences in corneal curvature with lens defocus were found in this experiment. (A) No differences were observed in animals housed under scotopic light. (B) An interaction effect occurred between treatments and age under mesopic light [RM Two-way ANOVA, $F(4,108)=3.77$, $p=0.007$], however multiple comparisons showed no differences between specific experimental groups. (C) Under photopic light housing, no differences were found between treatment groups. (D-F) The axial lengths of control, lens treated, and naïve contralateral eye did not change significantly across each illuminance level. (G-I) Retinal thickness was measured to test retinal health in bright light. No significant differences were found across groups. All measurements in each light level showed a main effect of age ($p<0.001$ in all cases). Control eyes: *black*, lens defocus eyes: *green*, contralateral, naïve eyes: *blue*. Data are mean ± SEM.

2.4.3 C57BL/6J mice can detect all three experimental luminance levels

To ensure animals could detect the scotopic light, running wheel activity was measured from six animals in each lighting condition to track circadian rhythms. During the first week, under a normal 12 hour LD cycle, mice in all light levels showed a consistent pattern of running activity that fluctuated with light (**Figure 2.6A**). Analysis of average running distance at ZT 1 and ZT 13, one hour after light onset and one hour in to the dark phase, respectively, showed a main effect of time such that all mice at ZT 1 ran significantly less than at ZT 13 [Two-way ANOVA, $F(1,35)=5.54$, main effect of time, $p=0.025$; **Figure 2.6B**]. During the following week of constant darkness, mice maintained running activity peaks during the subjective dark phase but began to increase running during subjective day (**Figure 2.6C**). All mice ran less at circadian time 1 (CT 1) than CT 13 resulting in a main effect of time [Two-way ANOVA, $F(1,35)=18.87$, main effect of time, $p<0.001$; **Figure 2.6D**]. With the light cycle resumed to test entrainment, all mice ran significantly more in the dark than in the light [Two-way ANOVA, $F(1,35)=63.35$, main effect of time, $p<0.001$; **Figure 2.6F**]. Overall, C57BL/6J mice showed the ability to perceive and entrain to scotopic, mesopic, and photopic light used in these experiments.

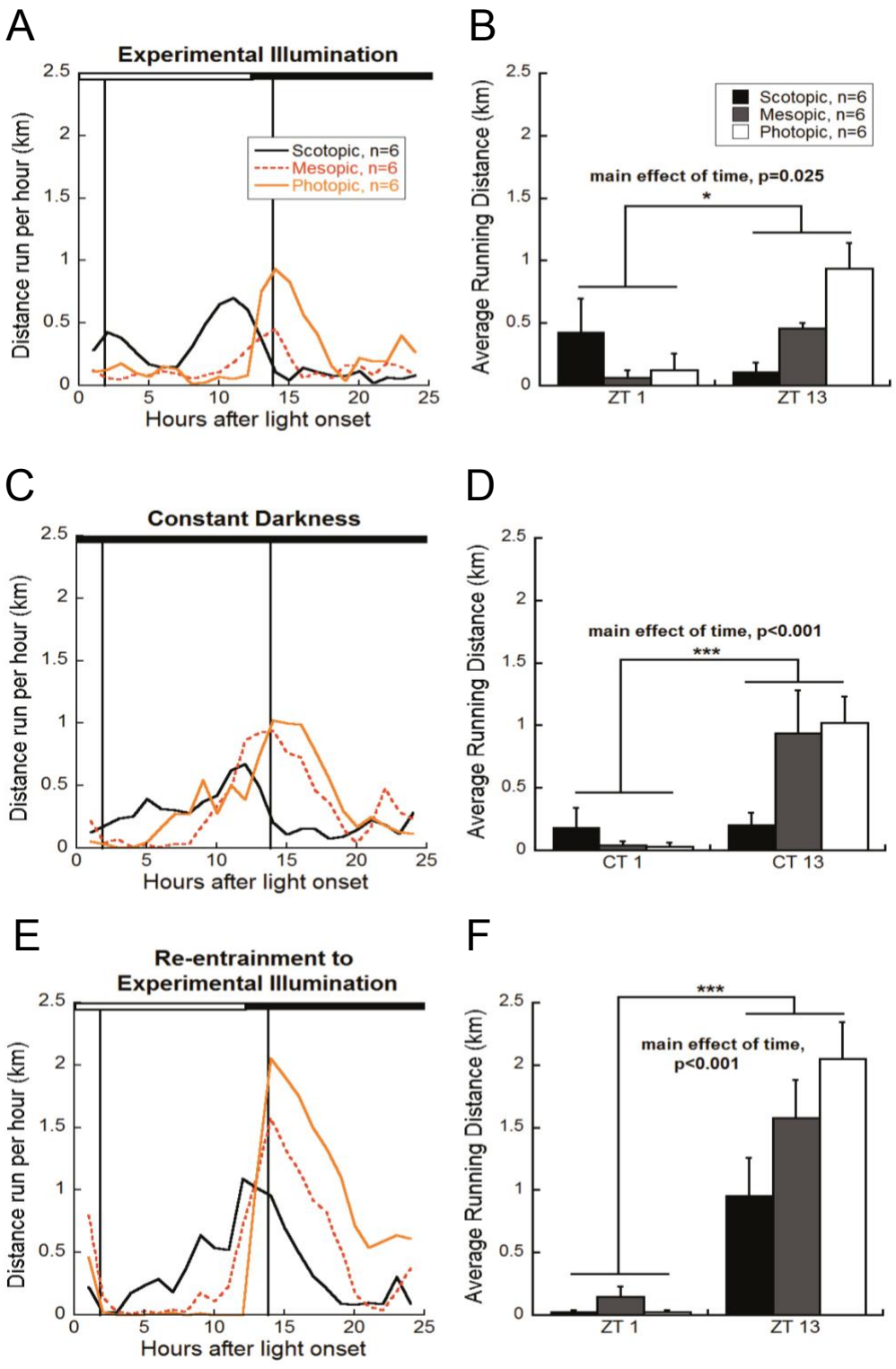

Figure 2.6. All three experimental ambient light levels are detected by C57BL/6J mice. (A) Average distance run per hour for mice exposed to a 12:12 hour LD cycle in one of three luminance levels (scotopic: *black*, mesopic: *orange*, photopic: *yellow*) for one week show

circadian pattern of running behavior with peak activity just before or at the beginning of the dark phase. (B) One hour after light onset at zeitgeber time 1 (ZT 1), mice ran significantly less across all light levels than one hour after darkness (ZT 13, scotopic: *black,* mesopic: *grey,* photopic: *white*) [Two-way ANOVA, $F(1,35)=5.54$, main effect of time $p=0.025$]. (C, D) The same animals followed for one week in constant darkness begin to run more during the light phase but are still running significantly more at ZT 13 than at CT 1 [Two-way ANOVA, $F(1,35)=18.87$, main effect of time $p<0.001$]. (E) Re-entrainment to a light:dark cycle is preserved in all three luminance levels such that running distance peaks with the start of the dark phase. The two early peaks seen in the scotopic exposed group are driven by two individual mice. (F) After re-entrainment, mice run significantly more in the dark (ZT 13) than in light (ZT 1) [Two-way ANOVA, $F(1,35)=63.35$, main effect of time $p<0.001$].

2.5 Discussion

2.5.1 Exposure to scotopic and photopic light prevents lens defocus myopia in the mouse model

Research in multiple animal models has shown bright light exposure to be protective in myopia development [for review (Norton and Siegwart, 2013)], but the effects of a broad range of light levels that reflects natural, environmental light and the sensitivity of the visual system across several log units of illumination is missing. In this study, we show that housing wild-type mice in ambient scotopic and photopic lighting protects against lens induced myopia. In addition to confirming a protective effect of bright light on myopia in mice, these data highlight the potential importance of dim light, which stimulates rod-dominated pathways, in refractive development. Mesopic illumination, similar to indoor lighting, was found to enhance the susceptibility to myopia.

In this experiment, we housed mice in the three different light levels for five days prior to lens defocus, then for one week to allow lens defocus to alter refractive error. Previous studies of wild-type C57Bl/6J mice have shown effects of form deprivation induced myopia within a couple of weeks (**Figure 2.1**). Here, the short-term period of light exposure prior to lens defocus does not affect refractive error across groups, indicating that the light levels only impact the effect of lens defocus, not normal development. It is possible that given a longer exposure period without lens defocus, these light levels would alter normal refractive development.

Myopia is often associated with increased axial length in both humans and animal models (Norton and Siegwart, 1995; Honda et al., 1996; Llorente et al., 2004; Wallman and Winawer, 2004). However, this study, did not find changes in axial length to explain the protective effect of scotopic and photopic light. Our laboratory has reported on several strains and mutant mouse

models that do not show axial elongation with relative myopia (Park et al., 2013; Park et al., 2014; Bergen et al., 2016), while some mutant or treated mice do show the expected axial lengthening with myopia (Huang et al., 2014; Ma et al., 2014a; Chakraborty et al., 2015a). We believe that the signaling mechanisms controlling refractive development and myopia are conserved across species given that a large range of animals respond to form deprivation with a myopic shift [fish, mice, guinea pigs, chickens, tree shrews, primates; for review (Schaeffel and Feldkaemper, 2015)]. In the mouse eye, even very small alterations in ocular parameters have a large impact on refractive power. Thus, our instruments may not have the required sensitivity to detect very small changes. While the ocular parameters measured in these mice did not show changes that would explain the myopic shifts, we anticipate that the underlying mechanisms are the same as those found in other vertebrate species.

2.5.2 Potential signaling mechanisms in the retina for coding light and altering refractive development

While both scotopic and photopic illuminance significantly lessened the response to lens defocus, it is likely that these two light levels trigger different protective signaling mechanisms in the retina. Photopic light driven protection has been carefully and robustly associated with higher levels of dopamine (DA) signaling, which is protective against induced myopia in animal models (Norton and Siegwart, 2013). These data show that scotopic lighting induced signaling pathways also protected the mouse eye from lens induced myopia. Given the different retinal signaling pathways activated across scotopic and photopic light, it is possible that two separate mechanisms are at work. Many other retinal mechanisms have been proposed to signal visually-driven eye growth, including nitric oxide, acetylcholine, Egr-1, GABA, and others (Kennedy, 1995; Nickla et al., 2009; Ashby et al., 2010; Wu et al., 2015a). These

neuromodulators/neurotransmitters have all been shown to be related to DA signaling, suggesting that the potential influence of DA on refractive eye growth could be more complex than is generally thought. Further investigation of DA activity under each of the three experimental light levels used here will likely reveal if DA is playing a role in scotopic light as well as photopic.

Another possible explanation for the protective effect of scotopic lighting is that such dim light does not provide enough visual input during the lens defocus to trigger a response for refractive eye growth. However, our running wheel data show that the mice under scotopic lighting can entrain to the light, suggesting activation of rod pathways (**Figure 2.7**).

Additionally, it is difficult to reconcile that the mouse eye is able to detect the imposed defocus under any lighting condition since the depth of focus of the mouse eye [-28D; (Schmucker et al., 2005)] is much larger than the imposed defocus (-10D). Thus, it is not clear what aspects of the optical defocus are driving myopia development in the mouse. Future studies are needed to investigate the components of the visual environment that drive myopia development.

Our findings that refractive development may require extremes in lighting to appropriately control ocular growth might suggest that circadian rhythms are important in preventing experimentally induced myopia. This is supported by recent reports that circadian genes are altered with form deprivation in chicks (Stone et al., 2013) and that elimination of the master clock gene results in a myopic phenotype in mice (Chakraborty et al., 2015b; Lee et al., 2016).

An alternative interpretation of the results is that mice exposed to mesopic lighting have an increased ability to emmetropize to lens defocus, and exposure to dim or bright lighting

diminishes that ability. In this scenario, optimal refractive regulation in a middle range of luminance levels would be similar to other homeostatic mechanisms in the body that regulate normal function in a moderate range between high and low, such as balancing salt intake, glucose levels in the blood, or blood pressure. However, given the well-established protection of bright light in human myopia, it is unlikely that mesopic light enhances emmetropization.

2.5.3 Implications for clinical treatment of myopia

These results suggest the possibility that spending time in both bright and dim ambient environments during childhood could be more effective for myopia protection than exposure to only bright light. In these experiments, animals were exposed to 12 hours of scotopic lighting during the light phase. While these animals were protected from induced myopia, it would be impractical to advocate for children at risk for myopia to spend significant amounts of time in only scotopic lighting. Future studies are needed to examine the average daily light exposure in children across a range of luminance levels. Similar to the recommendations for children to spend time outdoors in natural sunlight, perhaps children would benefit from more time in all levels of natural light, including dim light such as occurs at dawn, dusk, and nighttime, as opposed to continuous exposure to indoor mesopic lighting.

Our data reveal previously unrecognized protective effects of both dim and bright ambient lighting for experimental myopia in mice and suggest that additional investigation into the contribution of rod pathways on refractive development is needed. This work also suggests that a broad range of light levels, such as found in the natural environment, are important for refractive development. While increased time outdoors is being quickly translated into the clinic to prevent myopia in children (Wu et al., 2013; He et al., 2015) with bright light emphasized as the preventative condition, it is valuable to recognize the importance of the natural luminance

range of light, including dim light, on refractive development and myopia susceptibility. Future studies are needed to define the exact mechanisms that signal refractive eye growth so that optimal environmental and/or pharmacological therapies can be developed to slow or prevent myopia. This study also serves as a reminder of the importance of environmental factors influencing development and maturation of neural systems. Homeostatic mechanisms are constantly at play to maintain internal stability within the body. In this case, dim and bright light may provide important cues for normal visually-driven development.

CHAPTER 3: Dopamine signaling and activity are impacted by ambient light level

3.1 Abstract

Different ambient light environments influence susceptibility to myopia. We previously showed that photopic (15,000 lux) or scotopic (0.005 lux), but not mesopic (50 lux), ambient lighting significantly reduced lens induced myopia (LIM) in mice (ARVO 2015 E-Abstract #2152). This study aims to determine how luminance and lens defocus alter dopamine (DA) synthesis, storage, uptake, and degradation and effect myopia susceptibility in mice. Male C57BL/6J mice were housed in varied luminance (scotopic: 0.005 lux, n=62; mesopic: 50 lux, n=63; and photopic:15,000 lux, n=63) on a 12:12 L:D cycle beginning at post-natal day 23 (P23). A subset of mice received -10D lens placed over the right eye using a head-mounted apparatus at P28 (n=33/light level). At P37, retinas were collected and examined by HPLC to determine changes in DA and 3, 4 – dihydroxyphenylacetic acid (DOPAC, a DA metabolite). Expression of DA related genes were measured with digital droplet PCR. Western blots were used to detect changes in protein levels related to dopamine synthesis (tyrosine hydroxylase, TH) and storage (vesicular monoamine transporter 2). DA levels increased slightly with light intensity in both control and LIM treated mice ($p<0.001$). DOPAC also increased with luminance regardless of lens defocus ($p<0.001$). Expression of *Th* was highest under mesopic light in LIM mice ($p<0.001$). Mice with lens defocus exhibited a trend for lower levels of total TH protein after exposure to photopic or scotopic lighting compared to control mice, while TH levels increased in mesopic light. Phosphorylated TH (pTH^{Ser40}) decreased with higher light intensity ($p<0.01$). A novel technique measuring extracellular DA showed increased DA levels with increasing luminance in control mice, but only modest increases after light exposure in LIM

treated mice. These results suggest that ambient light intensity may interact with lens defocus to alter dopamine signaling.

3.2 Introduction

Myopia, a discrepancy in optical power and eye size which results in a blurred perception of objects at a distance, has been increasing in prevalence over the last several decades. By 2050 is it predicted that myopia will affect half of the world's population (Fricke et al., 2018). Myopia can be genetically inherited, though the recent rise in rates of myopia indicate an environmental factor is also playing a role in myopia development. Understanding how the environment triggers myopia is an important part of developing treatment strategies that can combat the epidemic and slow progression to prevent high myopia. As high myopia increases so does the risk of vision loss through ocular diseases like glaucoma or retinal detachment, methods to slow myopia progression would therefore lower the risk of vision loss. Efforts to determine the environmental factors involved in the increasing prevalence of myopia have identified time spent outdoors, specifically exposure to bright light as a protective factor in myopia [(Parssinen and Lyyra, 1993; Rose et al., 2008b), for review (French et al., 2013a)]. Children who spend more time outdoors in bright sunlight are less likely to be myopic and/or show slower progression of myopia than children who spend more time in intermediate indoor light. Support for these clinical findings has been observed in animal models as well. Intermittent episodes of bright light protected chicks from both form deprivation (FD) and LIM (Ashby et al., 2009; Ashby and Schaeffel, 2010b). Bright light treatment has also shown protective effects in monkeys and tree shrews [(Siegwart Jr et al., 2012; Smith et al., 2012), for review (Norton and Siegwart, 2013)].

The exact mechanisms underlying refractive development and myopia remain elusive. However, retinal dopamine (DA) has been implicated as a stop signal for myopic eye growth as

it decreases with form-deprivation (Iuvone et al., 1989; Stone et al., 1989) or lens-induced myopia (Papastergiou et al., 1998; McBrien et al., 2001; Dong et al., 2011; Feldkaemper and Schaeffel, 2013). DA has also been implicated as the signaling mechanism in the protective effect of bright light. Retinal DA synthesis, as measured by tyrosine hydroxylase (TH) activity, increases with light exposure (Iuvone et al., 1978). Thus, increasing bright light exposure may elevate endogenous DA levels in the retina and prevent myopia (Ashby and Schaeffel, 2010b; Cohen et al., 2012). In addition, studies from several mutant mouse models have shown that the decreased DA turnover at the time of goggling is strongly correlated with increased susceptibility to form deprivation (Park et al., 2013). Thus, environmental light could establish a basal level of DA activity in the retina that influences the response to other visual stimuli during the critical period. However, it is not known how DA signaling is altered with myopia across a large range of luminance levels that predominately stimulate different retinal pathways. Additionally, the exact mechanism by which bright light increases retinal DA levels or DA activity is not clear; DA signaling in the retina is highly compensatory, and it is possible that there are several mechanisms to increase DA signaling and prevent myopia (Cooper et al., 2003).

In the retina, several DA related proteins can control the presence and localization of DA. First, TH which synthesizes DOPA from tyrosine, is the rate-limiting enzyme in the DA synthesis pathway and therefore has the largest effect on DA levels in the retina (Daubner et al., 2011). Vesicular monoamine transporter 2 (VMAT2) is responsible for packaging DA into vesicles for storage and can impact release (Yaffe et al., 2018). VMAT2 prevents DA cytotoxicity by sequestering cytosolic DA into vesicles and is essential for DA release into the extracellular space. Once released, DA is taken back into the cell by the dopamine transporter (DAT) which clears DA from the extracellular space, controlling the downstream signal (Cooper

et al., 2003). Finally, monoamine oxidase A and B (MAO) degrade monoamines including DA after uptake. MAO-A and -B have not been fully studied in the retina and, like the other DA related proteins studied here, have not been fully characterized in myopia.

In this study, we examine the effect of lens defocus in mice housed in three different environmental luminances: scotopic lighting that stimulates only rod pathways, mesopic lighting that stimulates both rod and cone pathways, and photopic that stimulates cone pathways. In order to fully analyze DA dynamics in retinas under varied ambient light and in LIM, several different and novel assays were used to target specific aspects of DA signaling. Retinal DA levels, gene expression and levels of DA related proteins, and extracellular DA after light stimulation were assayed here. We hypothesize that as both dim and bright light exposure have prevented the effects of LIM in mice, DA activity or the presence of DA related proteins will increase in mice housed in scotopic and photopic light compared to mesopic housed mice.

3.3 Methods

3.3.1 Animals, light exposure, and lens defocus

Animals in this experiment were treated with the same light and lens defocus paradigm as has been previously described (CHAPTER 2). Briefly, male C57BL/6J mice (n=188 total) were exposed to one of three experimental light levels for 12 hours during the light phase of the light:dark cycle (scotopic: 0.005 lux, n=62, mesopic: 50 lux, n=63,and photopic:15,000 lux, n=63) beginning at post-natal day 23 (P23). Animals were housed individually, and food was placed on the bottom of the cage to prevent the light from being blocked. Food and water were given *ad libitum* and mouse health was checked daily.

At P28, a subset of animals from each light level were treated with monocular lens defocus to induce myopia. To fit animals with lens defocus goggles over the right eye, animals were anesthetized with ketamine (80 mg/kg)/xylazine (16 mg/kg) and a pedestal was surgically mounted to the head (Faulkner et al., 2007). A clear plastic lens with custom made wire frame was "threaded" through the head pedestal and positioned over the right eye (OD, *oculus dextrus*). The frame and lens were held in place with an aluminum block which also held a wire balance bar on the left side of the face to counter the weight of the frame. The contralateral, left eyes (OS, *oculus sinister*) served as internal controls and are here referred to as contralateral, naïve eyes. Lenses were removed to be cleaned or adjusted as needed. At P35, lenses were removed permanently, and animals were sacrificed by cervical dislocation 4-6 hours after light onset under the particular light level they had been housed in. Eyes were enucleated, and retinas were collected.

3.3.2 Retina collection for HPLC detection of dopamine

Following light and lens defocus treatment animals were sacrificed. eyes were enucleated, and retinas collected (control: n=15/light level, LIM: n=16/light level). Retinas were immediately frozen on dry ice and kept in storage at -80° C.

Collected retinas from both eyes of LIM and control mice were analyzed by high performance liquid chromatography (HPLC) to determine levels of DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC). First, to measure DA and DOPAC, a previously described analysis method was used (Nir, 2000; Pozdeyev et al., 2008). Briefly, retinas were homogenized in 0.1 N perchloric acid, 0.01% sodium metabisulfite with 25 ng/ml 3,4-dihydroxybenzylamine and centrifuged. Supernatant was injected into a Beckman Ultrasphere 5µm ODS column, 250 x 4.6 mm (Fullerton, CA). The mobile phase consisted of 0.1M

phosphoric acid, 0.1mM EDTA, 0.35 mM sodium octyl sulfate and 6% acetonitrile at pH 2.7. Analyzed peaks were identified by retention time and compared to those of external standards quantified by peak area.

Retinas of mice given lens defocus were tested as individual samples (lens defocus and contralateral, naïve) while the right and left eyes of control mice were pooled for analysis. Samples were normalized to total retinal protein content determined by Lowry Assay.

3.3.3 Gene expression of dopamine related proteins

To measure the gene expression of DA related proteins (TH: *Th*, VMAT2: *Slc18a2*, DAT: *Slc6a3*, MAO A: *Maoa*, and MAO-B: *Maob*) retinas frozen in Ribolock (ThermoFisher, Waltham, MA, USA) from control (n=6/light level) and LIM treated (n=8/light level) mice from each ambient light level were homogenized in RLT buffer (Qiagen, Venlo, Netherlands) using a Tissuelyser LT (Qiagen). RNA was extracted using the Qiagen RNAeasy Qiacube Kit (Qiagen). RNA samples were frozen until cDNA was synthesized. A QuantiNova cDNA synthesis kit (Qiagen) was used to make cDNA as per manufacturer's protocol. First, gDNA removal solution was added to RNA to remove genomic DNA from each sample, then cDNA (complementary DNA) was generated using the reverse transcription mastermix, and finally samples were diluted to 5ng/µL.

ddPCR was used to determine relative quantities of transcripts for the genes of interest. FAM labeled hydrolysis probe assays for *Th*, *Slc18a2*, *Slc6a3*, *Maoa*, and *Maob*, and a HEX (hexachloro-fluorescein) labeled probes assay for HPRT (hypoxanthine phosphoribosyltransferase) were purchased from Integrated DNA Technologies or Bio-Rad (**Table 3.1**). ddPCR (digital droplet PCR) was performed using the cDNA generated above as template, a single FAM labeled target assay, HPRT as a normalization control, and ddPCR

Supermix without dUTP (Bio-Rad, Hercules, California, USA). Droplets were generated using an AutoDG droplet generator (Bio-Rad). Then, PCR was performed using a C1000 Touch thermal cycler with deep well block (Bio-Rad). Droplets were read using a QX200 droplet reader (Bio-Rad) and data was analyzed using QuantiSoft analysis software (Bio-Rad) which uses a Poisson distribution model to calculate the number of starting target template molecules in each well from the number of FAM (fluorescein amidite) and HEX positive droplets.

Table 3.1. Probes used in ddPCR analysis of genes related to DA dynamics.

Gene, Protein	Hydrolysis fluor	Company	Catalog number
HPRT	HEX	IDT	Mm.PT.39a322214828
Th, TH	FAM	Bio-Rad	dMmuCPE5121062
Slc18a2, VMAT2	FAM	IDT	Mm.PT.58.42226157
Slc6a3, DAT	FAM	IDT	Mm.PT.58.12888045
Maoa, MAOA	FAM	IDT	Mm.PT.58.8802827
Maob, MAOB	FAM	IDT	Mm.PT.58.33530177

3.3.4 Western blot detection of dopamine related proteins

Western blots were performed to measure levels of DA related proteins TH, pTH^{Ser40} (tyrosine hydroxylase phosphorylated at amino acid site Serine-40), and VMAT2. Retinas (control: n=6-7/light level, LIM: n=7/light level) collected between 4-6 hours after light onset were frozen, then homogenized in RIPA buffer (Teknova, Hollister, CA, USA) with protease inhibitors (Roche, Penzberg, Germany). A BCA assay (bicinchoninic acid assay, ThermoFisher Pierce, Waltham, MA, USA) was used to determine protein concentration in each sample; additional RIPA buffer was added to more concentrated samples. All samples were stored on ice, and only data from control OD and lens defocus OD retinas were used.

A solution of 15µg protein from each sample mixed with ß-mercaptoethanol (BME) and SDS-PAGE dye was loaded into the wells of 10% Criterion™ TGX Stain Free Precast Gels (Bio-Rad) and run at 90-200V. Protein was then transferred to 0.2µm PVDF membranes (Bio-Rad) with the Bio-Rad Trans-Blot Turbo Transfer System. Membranes were blocked with 5% donkey serum (MilliporeSigma, St. Louis, MO, USA) in Phosphate Buffered Saline (Corning, Tewksbury, MA, USA) with 0.05% Tween-20 (PBS-T, MilliporeSigma).

Membranes were treated with primary antibodies (**Table 3.2**) in PBS-T with 5% bovine serum albumin (BSA) at 4° C overnight. Following primary incubation, membranes were washed with Tris buffered saline with 0.1% Tween-20 (Corning, TBS-T) for 10 minutes 3 times, then stained with secondary antibodies and HRP conjugate (horseradish peroxidase, Bio-Rad) in PBS-T with 5% BSA at room temperature for 1 hour and washed again before exposed in HRP substrate (Bio-Rad) and imaged in a Bio-Rad ChemiDoc MP Imaging System. To quantify protein bands, the Bio-Rad computer software was used to detect band intensity and normalize to total protein in each well. Intensity values for each sample were then normalized to those of a

mesopic-housed control mouse which were run on every blot. α-tubulin was detected as a loading control.

Table 3.2 Antibodies used to measure DA related proteins in retinal tissue.

Protein Target	Source	Concentration Used	Secondary Antibody Used
TH	EMD Millipore (AB152)	1:1,000	Pierce Goat AntiRabbit IgG (31460, 1:5,000)
pTH^{Ser40}	Sigma Aldrich (T9573)	1:1,000	Pierce Goat AntiRabbit IgG (31460, 1:5,000)
VMAT2	Custom-made*	1:1,000	Pierce Goat AntiRabbit IgG (31460, 1:5,000)
α-Tubulin	Abcam (ab4074)	1:5,000	EMD Millipore Goat AntiRabbit IgG (AP132, 1:10,000)

*Custom-made VMAT2 antibody (Cliburn et al., 2016).

3.3.5 Eye cup perfusion system to measure dopamine release

LIM and control mice housed in each light level (n=4/group) were dark adapted at P35 for 30 minutes – 2 hours. Mice were sacrificed, and eyes were enucleated and immediately placed in the Braincubator™ [Kingswood, New South Wales, Australia (Buskila et al., 2014)], a self-contained incubation system with circulating, oxygenated, and temperature controlled artificial cerebrospinal fluid (aCSF). Eyes were enucleated individually and dissected into eye cups, with the cornea and lens removed (**Figure 3.1A, B**) then returned to the Braincubator™. Dissections were done in oxygenated aCSF and took no longer than 3 minutes each. Eye cups were then moved into 1.7mL epi tubes with 30µL of oxygenated aCSF, placed up-right such that the anterior side of the eye faced up, and exposed to bright light stimulus (16,000 lux) or darkness for 8 minutes. All right eyes including lens defocus treated eyes were exposed to light and left eyes were exposed to darkness, differences between eyes were determined as measures of the effect of both light and lens defocus on the response to light (**Figure 3.1**). Eye cups were then removed from the aCSF and discarded, aCSF was immediately frozen on dry ice and stored in -80° C.

Once all samples had been collected, aCSF was thawed and samples were processed for HPLC detection of DA. Briefly, 0.1M perchloric acid was added and samples were centrifuged to remove debris from eye cups. Supernatant was used to detect DA content with HPLC. For HPLC, an ESA 5600A CoulArray detection system was used. Separations were performed at 30ºC using an MD-150 × 3.2 mm C18 column. The mobile phase consisted of 1.7 mM 1-octanesulfonic acid sodium, 75 mM NaH2PO4, 0.025% triethylamine, and 8% acetonitrile at pH 2.93. 20 µl of sample was injected. The analytes were identified by the matching criteria of

retention time to known standards (Sigma Chemical Co., St. Louis, MO). Compounds were quantified by comparing peak areas to those of standards on the dominant sensor.

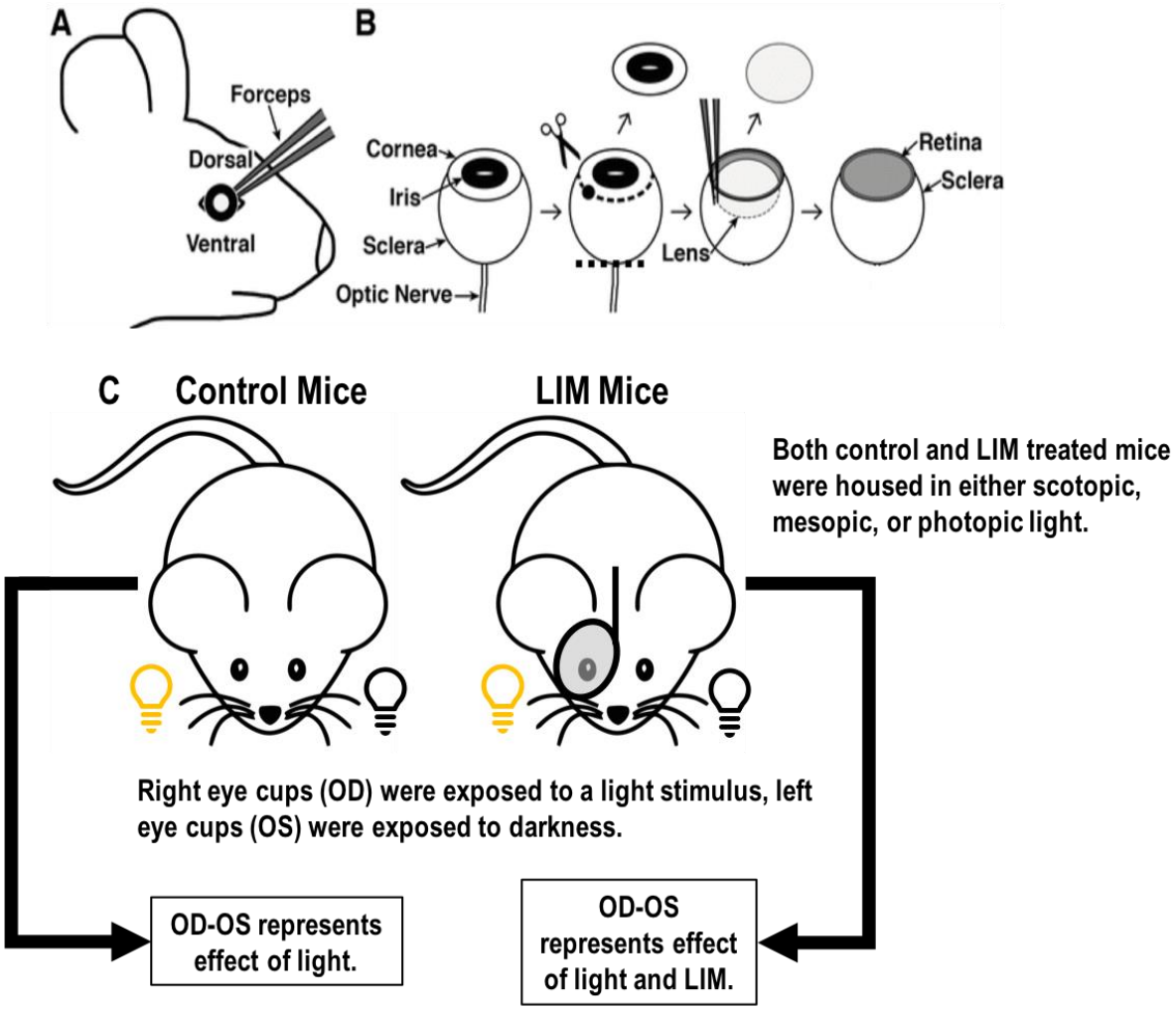

Figure 3.1. Experimental design to measure extracellular DA after light stimulus. (A) Immediately after cervical dislocation, eyes are enucleated with forceps and placed in oxygenated aCSF. (B) In aCSF, eyes are dissected into eye cups by removing the optic nerve, cornea, iris, and lens. Eye cups are returned to oxygenated aCSF. (C) In this experiment, eye cups from control and LIM treated animals from each light level were collected. Right eye cups (OD) from both groups were exposed to light stimulus for 8 minutes while in aCSF, then discarded. Left eye cups (OS) were exposed to darkness for 8 minutes in aCSF, then eye cups were discarded. aCSF was immediately frozen on dry ice for HPLC analysis of DA content. Images (A) and (B) are from (Simmons and Fuerst, 2018), (C) was created by the author.

3.3.6 Statistical analysis

The effects of light and LIM were compared by determining means for control eyes (average of both eyes of control mice), lens treated eyes, and naïve contralateral eyes. Results of experiments done to measure DA and DOPAC, gene expression levels, and DA related protein levels were analyzed using Two-way ANOVAs (GraphPad Prism, La Jolla, CA) and Holm Sidak post hoc comparisons. The extracellular DA content measured by the eye cup perfusion assay were analyzed by comparing the DA content from light exposed right eyes to dark exposed left eyes; however, no statistical analysis was done as the current dataset is underpowered and more data needs to be collected. For all analyses, significance was set at alpha of 0.05. Data shown here are means ± standard error of the mean (Vugler et al., 2007).

3.4 Results

3.4.1 Retinal dopamine levels and metabolism increases with bright light

To evaluate the potential role of DA in the refractive states of the mice housed under the three light levels, DA, DOPAC, and the DOPAC/DA ratio (indicative of DA turnover) were measured in the retina using HPLC. Across luminance levels, scotopic exposed mice had the lowest levels of DOPAC (controls: 42.09 ± 1.70 pg/mg retinal protein, lens defocus: 42.68 ± 1.71 pg/mg, naïve: 38.74 ± 0.98 pg/mg), followed by mesopic (controls: 68.52 ± 2.24 pg/mg, lens defocus: 73.27 ± 2.43 pg/mg, naïve: 77.78 ±2.61 pg/mg), and photopic exposed mice (controls: 86.82 ± 4.51 pg/mg, lens defocus: 90.04 ± 2.87 pg/mg) [Two-way ANOVA, main effect of light; $F(2,132) = 294.5$, $p<0.001$; **Figure 3.2A**]. Within each light level, the three treatment groups (control mice, lens defocus treated eyes, contralateral naïve eyes) were not significantly different for DOPAC levels. For all three treatment conditions, DA levels in

scotopic and photopic exposed retinas were within 10% of the mesopic values. Despite very small changes to DA levels across light and LIM, a significant effect of both the light and lens treatment was found [Two-way ANOVA, interaction effect, $F(4,132)=3.688$, $p= 0.007$, **Figure 3.2B**]. Differences were found between control eyes in mesopic and photopic light ($p=0.002$), between contralateral, naïve eyes of mice in scotopic and mesopic light compared to photopic (scotopic: $p<0.001$, mesopic: $p<0.001$) and between lens defocus treated eyes in scotopic and photopic light ($p=0.001$). The DOPAC/DA ratio was not affected by the changes in DA levels and showed the same increase across light levels as the DOPAC levels [Two-way ANOVA, main effect of light, $F(4,132)=306.3$, $p<0.001$, **Figure 3.2C**]. DOPAC/DA ratios were significantly higher in photopic exposed mice [controls: 0.08 ± 0.001, lens defocus: 0.08 ± 0.002, naïve: 0.008 ± 0.002, Two-way ANOVA, main effect of light, $F(2,132)=306.3$, $p<0.001$] than mesopic exposed mice (control: 0.07 ± 0.002, lens defocus: 0.07 ± 0.003, naïve: 0.07 ± 0.002) and scotopic mice (control: 0.04 ± 0.003, lens defocus: 0.04 ± 0.001, naïve: 0.04 ± 0.001) regardless of LIM treatment. The three treatment groups (control mice, lens defocus treated eyes, contralateral naïve eyes) were not significantly different within each light level.

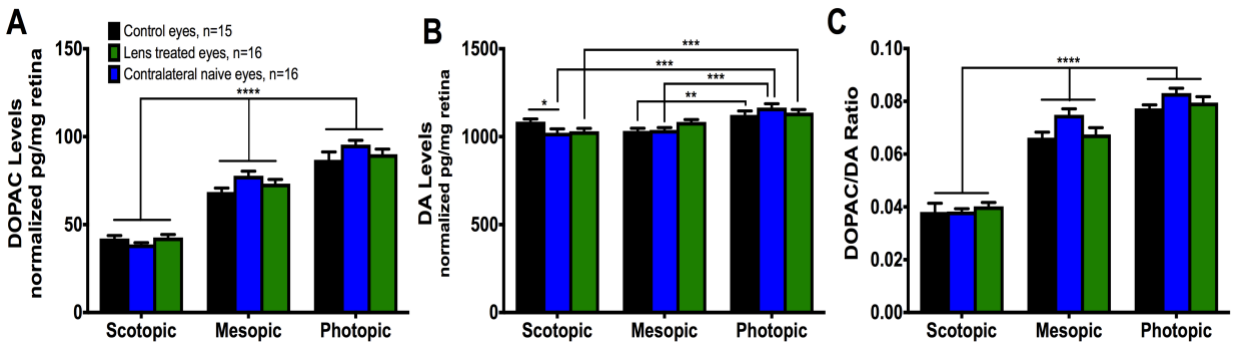

Figure 3.2. DA levels are maintained across light and lens treatment groups while DOPAC increases with light intensity. Retinas were collected 4-6 hours after light onset under the lumination each animal was housed in. (A) After 2 weeks of ambient light exposure, DOPAC levels were increased with higher light intensities [Two-way ANOVA, main effect of light, $F(2,132)= 294.5$, $p<0.001$]. (B) Some experimental groups showed changes in DA levels with light and lens defocus [Two-way ANOVA, interaction effect, $F(4,132)=3.69$, $p=0.007$] In control eyes (*black*), photopic housed mice had higher DA content than mesopic housed mice ($p=0.002$). In lens defocus treated eyes (*green*), scotopic mice had less DA than photopic mice ($p<0.001$), and in naïve eyes (*blue*), photopic housed eyes had more DA than both scotopic ($p<0.001$) and mesopic mice ($p<0.001$). (C) DOPAC/DA ratio increased with light indicating higher dopamine metabolism [Two-way ANOVA, main effect of light, $F(2,132)=306.3$, $p<0.001$]. Bars represent mean ± SEM. For all plots, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.4.2 Light and lens treatment interact to alter gene expression and presence of dopamine related proteins

Th expression was significantly altered by both light and LIM [Two-way ANOVA, interaction effect, $F(4,38)=6.32$, $p<0.001$, **Figure 3.3A**]. In control mice, there was an increase in *Th* expression between scotopic and photopic housed mice (scotopic: 0.014 ± 0.001 arbitrary units (a.u.), photopic: 0.020 ± 0.002 a.u., $p=0.012$). For both lens defocus treated and contralateral, naïve eyes, *Th* expression was significantly higher in mesopic exposed mice than either scotopic or photopic exposed mice. In lens defocus, mesopic housed mice had higher levels of *Th* expression (0.021 ± 0.001 a.u.) than scotopic housed mice (0.015 ± 0.001 a.u., $p=0.043$) and photopic light housed mice (0.013 ± 0.000 a.u., $p=0.007$). Contralateral naïve eyes also showed higher levels of *Th* expression in mesopic light (0.023 ± 0.002 a.u.) than scotopic housed mice (0.017 ± 0.001 a.u., $p=0.025$) and photopic light housed mice (0.015 ± 0.000 a.u., $p=0.004$). No significant differences in expression of *Slc6a3* or *Slc18a2* were found between treatment groups (**Figure 3.3B, C**). *Maoa* expression was significantly different across treatment groups such that mice given lens defocus showed higher expression levels in both lens defocused (0.037±0.002 a.u.) and contralateral, naïve eyes (0.038±0.002 a.u.) relative to control mice (0.030±0.001 a.u.) across all three light levels [Two-way ANOVA, main effect of treatment, $F(2,38)=9.21$, $p<0.001$, **Figure 3.3D**]. *Maob* expression also showed an effect of treatment, suggesting an increase in expression in lens defocus (0.004±0.0003 a.u.) and naïve, contralateral eyes (0.005±0.0003 a.u.) relative to control mice [0.004±0.0002 a.u., Two-way ANOVA, main effect of treatment, $F(2,38)=3.91$, $p=0.029$, **Figure 3.3E**].

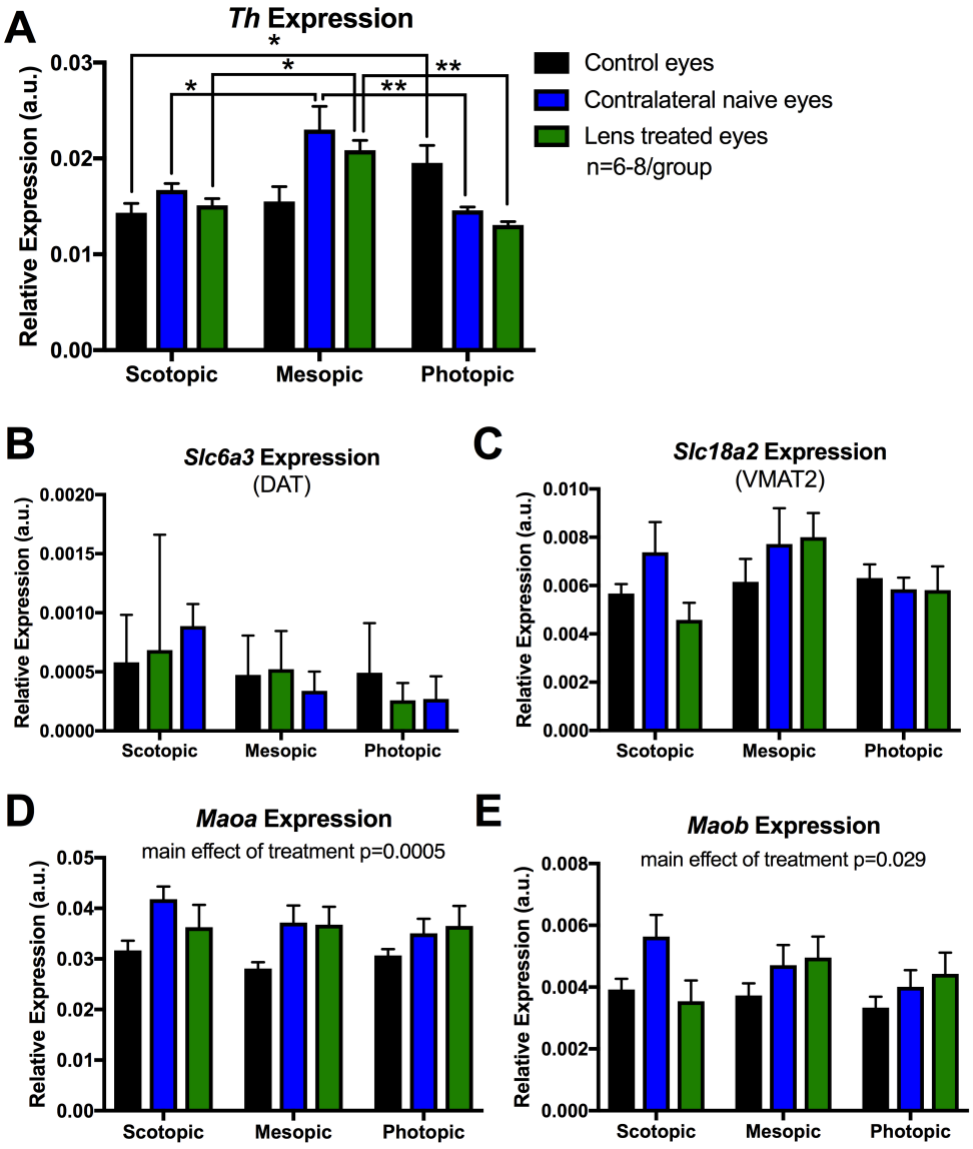

Figure 3.3. *Th* mRNA expression is highest in mesopic light housed mice, but only with lens defocus. The expression levels of DA signaling genes were measured with ddPCR after light and LIM treatment. (A) In control mice (*black*), *Th* expression was significantly higher in mice housed in photopic light compared to scotopic [Two-way ANOVA, $F(4,38)=6.32$, $p<0.001$, post-hoc comparison: $p=0.012$]. Both lens defocus retinas (*green*) and contralateral, naïve retinas (*blue*) showed higher levels of Th expression under mesopic light housing (scotopic: $p<0.05$, photopic: $p<0.01$ for both treatment groups). (B,C) No significant differences were found in expression of *Slc6a3* (DAT) or *Slc18a2* (VMAT2). (D) LIM and naïve contralateral eyes were significantly different than control eyes for expression levels of both *Maoa* [Two-way ANOVA, $F(2,38)=9.21$, $p<0.001$, post-hoc analysis done with One-way ANOVA] and (E) *Maob* expression was highest in contralateral, naïve eyes [Two-way ANOVA, $F(2,38)=3.91$, $p=0.029$, post-hoc analysis with One-way ANOVA]. Data are mean ± SEM measured in arbitrary units normalized to levels of HPRT. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

An analysis of the gene expression for genes related to DA activity in DACs was followed by an analysis of the levels of these proteins in the retinas of light and lens defocus treated animals. Here, western blots of the DA related proteins TH, pTH^{Ser40}, and VMAT2 were used to compared differences between lens defocus treated eyes and the right eyes of control animals.

No significant differences in TH levels were observed between control and LIM treated eyes across light levels. However, a nonsignificant trend was observed such that lens defocus treated eyes had higher levels of TH in mesopic housed mice, but lower levels in scotopic or photopic exposed mice relative to the control group in each light level (**Figure 3.4A**). Contrary to expectations, levels of pTH^{Ser40} decreased with increasing light intensity [Two-way ANOVA, main effect of light, $F(1,33)=8.34$, $p=0.007$, **Figure 3.4B**]. The ratio of phosphorylated to total levels of TH was calculated to determine what portion of TH was actively synthesizing DA under each condition. No significant differences between groups were found (**Figure 3.4C**). Finally, levels of VMAT2 were also not changed with light or lens defocus treatment (**Figure 3.4D**).

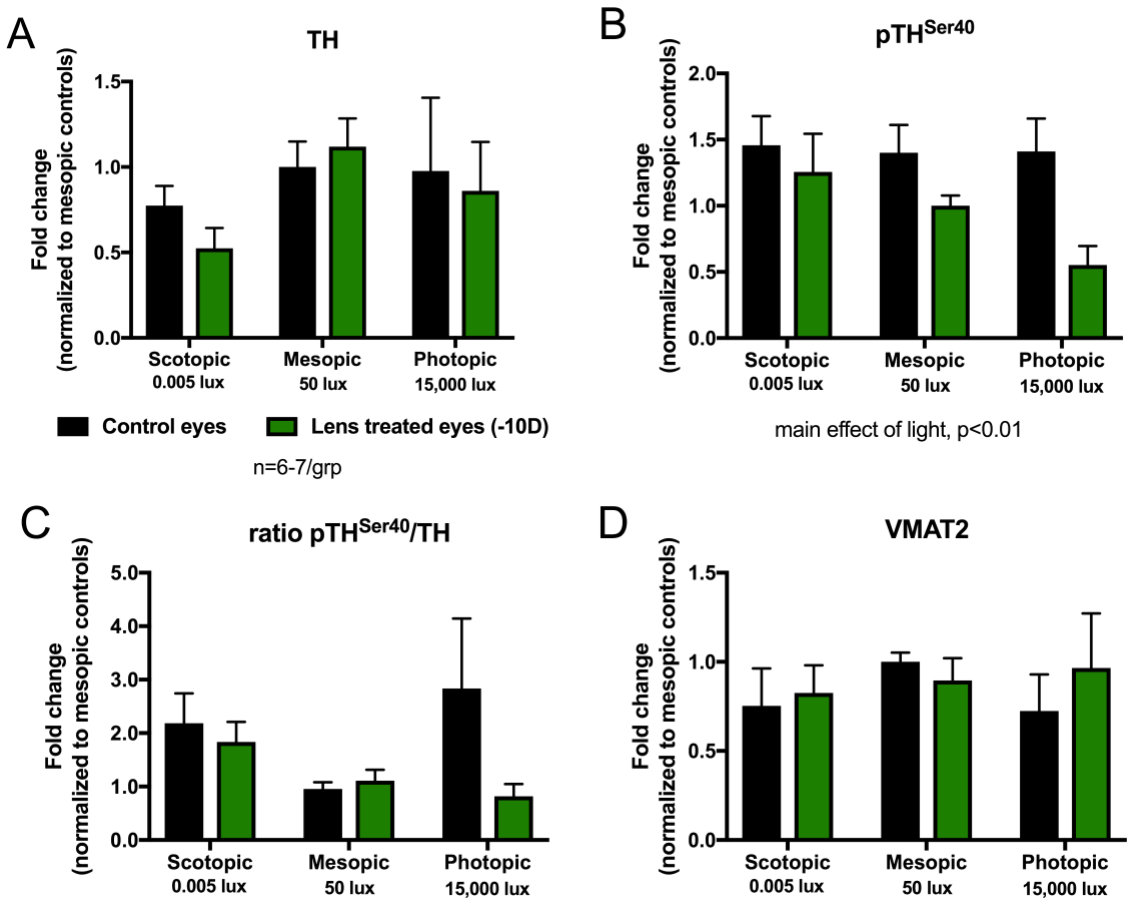

Figure 3.4. DA related proteins are affected by an interaction between ambient light and lens defocus. (A) Levels of TH did not change significantly with ambient light or LIM treatment. A trend was found indicating lens defocus (*green*) decreased TH in scotopic or photopic light but increased TH under mesopic light relative to control retinas (*black*). (B) Phosphorylated TH, pTH^{Ser40}, was inversely related to light intensity [Two-way ANOVA, main effect of light, $F(1,33)=8.34$, $p<0.01$]. This effect was likely driven by decreases in pTH^{Ser40} in lens defocus treated retinas. (C) The ratio of pTH^{Ser40} to total TH showed a non-significant relationship between control and lens defocused retinas under each light level. (D) VMAT2 protein levels were not significantly affected by light or lens. Data are mean ± SEM.

3.4.3 Extracellular dopamine in the retina increases with bright light exposure in normal retinas

In control animals, aCSF used with light-probed eye cups had higher levels of DA than aCSF used with dark exposed eye cups (**Figure 3.5A**). This response did not change with different ambient light levels with the exception of one mesopic housed animal. The light probed eyes of mice treated with lens defocus did not show consistently higher DA content in aCSF after light exposure compare to naïve eyes exposed to darkness. In both scotopic and photopic housed LIM treated mice, two animals showed more aCSF DA content after light exposure and the others showed less (**Figure 3.5B**). Eye cups from mice housed in mesopic light, the extracellular DA content in lens defocused eyes with light stimulus was very similar to that in contralateral, naïve eyes exposed to darkness (**Figure 3.5B**).

Finally, the difference between eye cups (light exposed OD – dark exposed OS) from each animal was determined to compare the effect of ambient lighting and LIM on the retinal response to the light stimulus (**Figure 3.5C**). Control mice had similar levels of DA content across all three ambient light housing conditions (scotopic: 0.065±0.012 ng/mL, mesopic: 0.039±0.026 ng/mL, photopic: 0.045±0.009 ng/mL). LIM treated mice however did not show similar increases in DA content from the light stimulus and instead showed variable changes when housed in scotopic or photopic light (scotopic: 0.060±0.0.073 ng/mL, photopic: 0.002±0.055 ng/mL) and a negative difference when housed in mesopic light (-0.014±0.0.014 ng/mL).

Due to the small sample size in this experiment (n=4/group, statistical power 0.34), no statistical analysis was performed.

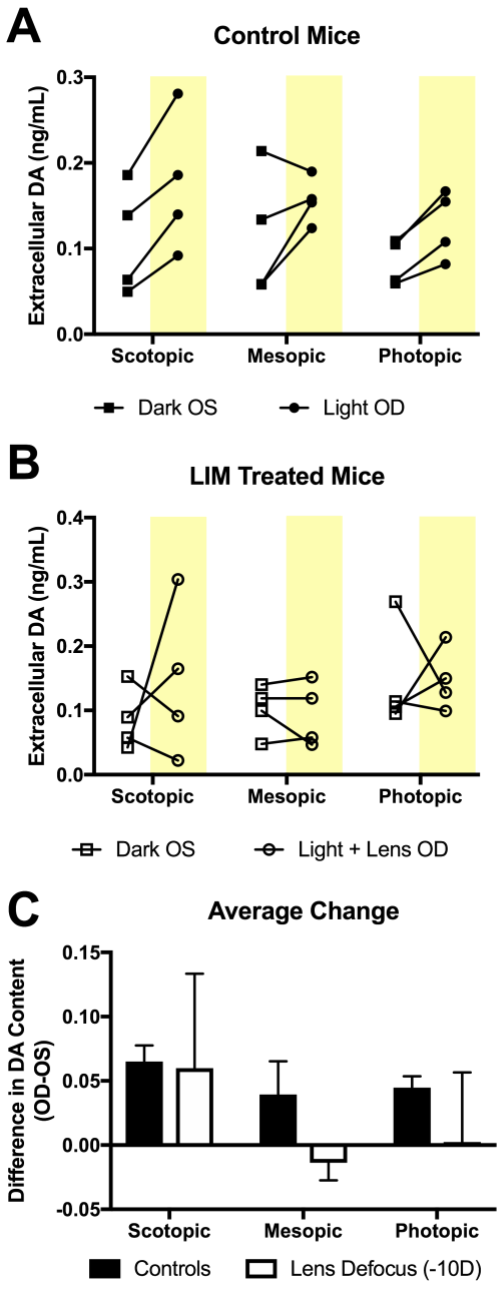

Figure 3.5. Extracellular DA analysis reveals a change in DA activity after light stimulus exposure with lens defocus, potentially dependent on ambient light intensity. (A) In control mice, right eyes (OD) exposed to a light probe (*yellow shading*) showed higher levels of extracellular DA than dark exposed left eyes (OS) across all ambient light housing levels. (B) In LIM, the lens treated eye cups (OD, *circles*) were exposed to light and the contralateral, naïve eye cups were exposed to darkness (OS, *squares*) for the same time. In mesopic light, extracellular DA levels were very similar between the two eye cups. (C) Differences between eye cups (OD-OS) for both control and LIM treated mice indicate an effect of the lens on the retinal response to light stimulus. Data are mean ± SEM. As a pilot study (n=4/grp), no statistical analysis was done.

3.5 Discussion

3.5.1 Dopamine activity increases with increasing light intensity

As previously described, housing mice in either scotopic or photopic light prevented the effects of LIM relative to the exaggerated response seen in animals housed in mesopic light (CHAPTER 2). The role of DA in this light mediated protection from myopia was investigated here. DA has previously been implicated as an anti-myopigenic signal in refractive development (Iuvone et al., 1989; Stone et al., 1989; Feldkaemper and Schaeffel, 2013) and animal experiments suggest that DA levels are decreased in some animal models of myopia (Stone et al., 1989). In these experiments, we found an interaction between ambient light and lens defocus on DA dynamics in the retina. This was seen in the gene expression and levels of DA related proteins which were differentially affected by lens defocus treatment across various ambient light levels. The extracellular DA measured from eye cups after light stimulus which showed little response to light in mesopic housed mice, but a varied response from mice in scotopic or photopic mice with lens defocus. This interaction is supported by novel findings in DA signaling, which have likely been masked in previous reports of DA in myopia, which only use DA and DOPAC levels as measurements of DA signaling.

DA plays a role in the protective effects of bright light on form deprivation myopia [for review (Norton and Siegwart, 2013)]. It is well established that the synthesis of retinal DA increases with light onset (Iuvone et al., 1978) and DA activity is stimulated by increasing light intensity (Cohen et al., 2012). This was repeated here with small but significant increases in DA as the intensity of ambient light housing increased. DOPAC levels and DOPAC/DA ratios were highest in mice housed under photopic light and lowest in mice under scotopic light, as expected,

suggesting that DA release and consequent degradation increased in response to bright light levels.

Here, DOPAC levels were not dependent on lens defocus treatment. This agrees with past studies in which DA activity does not seem to change in response to form deprivation in mice (Wu et al., 2015a). However, examination of DA turnover at the time of goggling in several different mutant mouse strains shows that a lower rate of DA metabolism (DOPAC levels) is associated with increased susceptibility to form deprivation (Chakraborty and Pardue, 2015). Furthermore, elimination of the DA synthesis protein, TH, in the mouse retina results in relative myopia compared to strain-matched controls (Bergen et al., 2015). Thus, endogenous levels of retinal DA may influence the refractive development and the response to form deprivation or lens defocus in mice.

In this experiment, we housed mice in the three different light levels for five days prior to lens defocus to allow for the endogenous retinal DA system to adapt to each light level. This short-term exposure did not alter the refractive error (CHAPTER 2), but likely altered the levels of DA signaling that was occurring at the time of goggling. This hypothesis is supported by data not shown here demonstrating that DA levels begin to change with light level as soon as 3 hours after treatment began. After 2 weeks of exposure to each light level, the retina appears to have adjusted to the anticipated need for DA by altering DA levels slightly (**Figure 3.2B**). Further studies are needed to examine how DA activity changes with duration of various ambient illuminances and whether the “pre-conditioning” of retinal DA levels is needed for the protective effects of scotopic and photopic lighting on lens defocus in mice.

3.5.2 Lens treatment and light intensity alter dopamine dynamics by changing dopamine related proteins

An increased DOPAC/DA ratio with increasing light intensity indicates changes to DA dynamics in the DAC, likely related to the synthesis, release, or degradation of DA. In order to study these potential changes to DA synthesis (TH and pTH^{Ser40}), storage (VMAT2), uptake (DAT), and degradation (MAO A/B), DA related genes and proteins were analyzed after both light and lens defocus treatment. The proteins studied were chosen based on their importance to DA dynamics and the feasibility of measuring them in the retina.

Expression levels of the gene *Th*, which encodes the protein that converts tyrosine to L-DOPA (TH), were measured with ddPCR while levels of the protein were measured with western blots. Generally, *Th* expression increased under mesopic light, relative to scotopic and photopic light housing (**Figure 3.3A**). This change was significant in both lens defocus treated and contralateral, naïve eyes. As expected, expression levels of *Th* in control animals increased with light intensity and were highest in photopic housed mice. Levels of TH protein also indicated unexpected changes with light and lens defocus with a trend for higher values for lens treated eyes in mesopic lighting (**Figure 3.4A**). A separate study, the only other to examine levels of TH protein in the myopic retina, found no significant differences between FDM and control retinas in guinea pigs raised in mesopic level light (100 lux) (Wu et al., 2015a). The results here are likely different because of the range of ambient light conditions used and the differences in FDM and LIM which have been shown to affect the role of DA in refractive error (Feldkaemper and Schaeffel, 2013).

Surprisingly, levels of pTH^{Ser40} were significantly decreased in lens treated eyes with increasing light intensity. Again, these data show an interaction between the lens defocus and the

response to ambient light intensity to modify DA activity. While *Th* expression and levels of TH protein might be expected to increase with luminance between scotopic and mesopic lighting, less *Th* expression and TH under photopic light with lens defocus is surprising. Contrasted with the increases in retinal DA levels with increasing light intensity, these findings indicate a more complex response to light with lens defocus than had previously been hypothesized.

The protein responsible for storing DA, VMAT2, complexes with TH in dopaminergic cells to prevent extra DA from diffusing through the cytosol (Daubner et al., 2011). However, in this study, the *Slc18a2* expression and VMAT2 levels did not significantly change with either LIM or ambient illumination. However, like *Th*, expression of *Slc18a2* in lens defocus treated retinas was highest in mesopic light. Genes related to DAergic reuptake and degradation were also investigated. The gene expression of *Slc6a3,* which encodes DAT, was very low in all treatment groups, and was undetectable in some samples making it unlikely that changes in response to either ambient light or lens defocus would be observed. *Maoa* and *Maob* were significantly higher in LIM retinas compared to controls regardless of light. Unfortunately, due to a lack of reliable primary antibodies for retinal tissue, the levels of these proteins (DAT, MAO-A, and MAO-B) could not be included at this time.

3.5.3 Dopamine activity is dysregulated under lens defocus

By measuring extracellular DA in response to a light stimulus, it is possible to hypothesize about the activity of TH and VMAT2. In control mice, retinas exposed to light consistently showed higher extracellular DA than contralateral retinas exposed to the darkness for the same time period. As expected, this confirms previous reports that DA is released under light stimulation (Iuvone et al., 1978; Cohen et al., 1983; Iuvone, 1984). In animals with LIM, the response to light stimulation is less regulated. In mesopic housed mice, there was no change

in released DA between lens defocused retinas and naïve retinas indicating a dysfunction in the lens defocused eye which prevents DA release in response to light. This finding is contradictory to the increased levels of TH and VMAT2 protein under mesopic light and highlights the importance of investigating protein activity in addition to content. Interestingly, mice housed in either scotopic or photopic light with LIM showed either more or less DA after light stimulation than in the naïve eye. These extracellular DA levels seem to be the clearest evidence of an interaction effect between light and LIM, which several of the experiments here have shown. These findings need to be replicated and it will be important to establish the noise associated with this novel technique as well as how the retina responds to varying light stimulus intensities.

The potential for an interaction effect between ambient light levels and LIM observed here for retinal DA signaling would suggest that ambient light determines DA activity when the eye responds to lens defocus. The end result of this process in scotopic and photopic light is protection from LIM, despite different DA dynamics across the two light levels. More work will be necessary to determine how DA signaling, as measured by DOPAC, is increased under bright light without significant increases in DA content or the proteins related to DA synthesis or release. Other studies of DA activity after FDM in mice have shown little change in retinal DA regulation (Wu et al., 2015a). However, in investigations of photopic and mesopic (referred to as normal animal housing in most studies) light, others have also found changes to DA activity with myopia in mesopic light, but not photopic, which would suggest also an interaction between light and lens defocus (Chen et al., 2017).

Another important consideration in DA mediated protection from myopia is the effect of DA on other retinal cell types and pathways. The pattern of protection for myopia across light levels used here closely resembles the pattern of DA-mediated cell-to-cell coupling across

similar light levels. Coupling between AII amacrine cells is known to be highest in mesopic light and lowest in both scotopic and photopic light (Bloomfield et al., 1997); a mechanism to enhance visual acuity by altering signal to noise ratios in very dim and bright light. The inhibition of this coupling is mediated by the release of DA from dopaminergic amacrine cells acting on D1-like receptors found on AII amacrine cells (Hampson et al., 1992; Urschel et al., 2006; Kothmann et al., 2009). DA receptor activity is dependent on receptor affinity and DA levels (Witkovsky, 2004), thus, different DA receptor activities are modulated by retinal pathways to optimize vision under different lighting conditions (Qiao et al., 2016).

One possible explanation for the action of DA under different light levels on myopia susceptibility may be the binding to different classes of DA receptors. Blocking DA activity with the DA receptor 2 (D2R) antagonist spiperone can ameliorate the protective effect of photopic lighting (Ashby and Schaeffel, 2010b). It is not known if the D2R is involved in lens induced myopia under scotopic lighting, but D2Rs have been involved in LIM in several studies in chicks housed under mesopic lighting (McCarthy et al., 2007; Nickla et al., 2010; Huang et al., 2014). Transgenic D2R knock-out mice are not susceptible to FDM, indicating the receptors importance in both forms of experimental myopia (Huang et al., 2014). Further investigations are needed to determine if DA has greater affinity for different DA receptors under scotopic, mesopic, and photopic lighting.

3.5.4 Potential alternative signaling mechanisms mediating scotopic light protection

Scotopic lighting protected the mouse eye from lens induced myopia. While DA signaling did seem to be altered by lens defocus under scotopic light, it is possible that a different signaling mechanisms is also playing a role in scotopic drivel protection from myopia. Many other retinal mechanisms have been proposed to signal visually-driven eye growth, including

nitric oxide, acetylcholine, Egr-1, GABA, and others (Kennedy, 1995; Nickla et al., 2009; Ashby et al., 2010; Wu et al., 2015a). These neuromodulators/neurotransmitters have all been shown to be related to DA signaling, suggesting that the potential influence of DA on refractive eye growth could be more complex than is generally thought. Another possible explanation for the protective effect of scotopic lighting is that dim light does not provide enough visual information during the lens defocus to trigger a response for refractive eye growth, however earlier circadian rhythm data (CHAPTER 2) would indicate this is unlikely.

Despite lower levels of DOPAC in scotopic housed mice, the gene expression and extracellular DA findings presented here suggest there are similarities between DA signaling in scotopic light and in photopic light. Additional work will be needed to determine whether the DA signaling and/or the retinal pathways in each light level is directly influencing the response to LIM. In addition to the potential role of gap junctions and cell-to-cell coupling on myopia development, recent evidence has suggested that rod photoreceptors play an important role in retinal signaling under both dim and bright light (Cameron et al., 2009; Cameron et al., 2018). Further, mice with knocked-out melanopsin, the photopigment in ipRGCs which responds to a wide range of light conditions, have high susceptibility to LIM (Chakraborty et al., 2015b). This may be because ipRGCs interact reciprocally with DACs (Vugler et al., 2007; Zhang et al., 2008; Vuong et al., 2015). Together, these findings indicate that the retinal signaling pathways which respond to light and defocus are far more complex than simple increases or decreases in DA content which were previously only studied with HPLC. Further study will be needed to determine how visual signals are integrated to influence ocular development and myopia.

CHAPTER 4: Preventing myopia by increasing endogenous dopamine

4.1 Abstract

Dopamine (DA) may modulate refractive eye growth. We determined if increasing DA activity using pharmacological or genetic approaches decreased myopia susceptibility in mice. First, systemic injections of L-3,4-dihydroxyphenylalanine (L-DOPA) were tested on form deprivation myopia (FDM) in wild-type (WT) and retinal degeneration (*rd10*) mice with low DA activity. Second, we tested genetically engineered mice with increased expression of vesicular monoamine transporter 2 (VMAT2 HI) under normal and FD conditions. At post-natal day 28 (P28), monocular FD was induced in all mice, and WT and *rd10* mice received daily systemic injections of L-DOPA Only (n=29), L-DOPA + ascorbic acid (AA, n=38), AA Only (n=36), or Saline (n=28). Weekly measurements of refractive error, corneal curvature, and ocular biometry were performed until P42 or P49. WT mice exposed to FD developed a significant myopic shift (treated-contralateral eye) with AA Only treatment (-3.27±0.73D) or Saline treatment (-3.71±0.80D) that was significantly decreased by L-DOPA only (-0.73±0.90D, p=0.0002) or L-DOPA + AA (-0.11±0.46D, p=0.0103). *rd10* mice showed the opposite response such that L-DOPA + AA (-3.21±0.73D, p<0.05) or L-DOPA Only treatments (-5.75±0.90D, p<0.001) did not provide protection (Saline:-5.81±0.98D) while AA Only treatment significantly reduced FDM (-0.77±0.80D). Both VMAT2 HI and WT mice developed significant myopic shifts to FD (VMAT2 HI: -3.10 ± 0.39D; WT: -4.24 ± 0.55D, p=n.s.) These results indicate that L-DOPA treatment protects WT mice from FD myopia. However, L-DOPA treatment in *rd10* mice or genetically altered DA release does not provide protection from FD, suggesting complexity in DA signaling for myopia.

4.2 Introduction

Proper development of the eye includes a process of emmetropization, in which the eye grows to match the optical power of the lens and cornea to focus incoming light directly on the retina. This process results in high acuity vision and clear images of objects in the environment. However, for a growing number of individuals around the world, emmetropization is disrupted by either genetic or environmental factors which cause the eye to develop a refractive error (Fricke et al., 2018; Morgan et al., 2018). The most common refractive error, myopia, is defined by an elongated eye such that light is focused in front of the retina causing a blurred perception of objects at a distance. Though relatively easy to treat with corrective lenses, myopia increases the risk for blinding vision loss later in life such as cataracts, retina detachments, and glaucoma (Verkicharla et al., 2015; Morgan et al., 2017). Myopia dramatically increases the burden on health care systems and negatively impacts educational and economic productivity when left untreated (Resnikoff et al., 2008).

Despite growing attention to myopia, the factors influencing its development and the ocular signaling mechanisms controlling it are still largely unknown. The retinal mechanisms by which the eye detects and responds to defocus during development and emmetropization are currently being investigated in animal models with experimentally induced myopia. Through these studies, several chemical messengers have been proposed as regulators in refractive eye growth (Stone et al., 1991; Stone et al., 2003; Ashby et al., 2007; Nickla et al., 2009). One chemical signal in the retina shown to be protective against myopia is the neuromodulator dopamine (DA). DA is the most ubiquitous neuromodulator in the retina, produced by dopaminergic amacrine cells (DACs) with dendrites in the interplexiform layer spanning the entire retina, and is detected by DA receptors found throughout the retina. DA is responsible for

many important functions of the retina such as gap junction regulation and control of retinal circadian rhythms [for review (Witkovsky, 2004)].

DA has been implicated in myopia and refractive development in many animal studies (Feldkaemper and Schaeffel, 2013). In animals with form deprivation (FD) or lens induced myopia (LIM), retinas show decreased levels of DA relative to control eyes (Stone et al., 1989). DA receptor agonists generally decrease the response to both FD or LIM (Iuvone et al., 1991; Schmid and Wildsoet, 2004; Nickla et al., 2010; Huang et al., 2018), while decreasing DA signaling through receptor antagonists generally exaggerates the response (Rohrer et al., 1993; Schaeffel et al., 1995; McCarthy et al., 2007). While DA itself is not believed to directly impact ocular growth, it is likely that DA activity is an early signaling mechanism triggering downstream effects on the choroid, sclera, or even cornea. Therefore, increasing DA levels and DA activity in the retina may prevent myopia development.

While most people with myopia have no other major visual disorders, many neurological or visual disorders are accompanied by refractive errors. Several types of retinal degenerations include symptoms of either hyperopia or myopia (Miyake et al., 1986; Laties and Stone, 1991; Smith et al., 2007b; Lavanya et al., 2010; Chui et al., 2012). However, the biological connection between retinal degeneration and refractive error is unknown. In order to determine how retinal degeneration changes the refractive development of the eye, the *rd10* mouse, which has an inherited photoreceptor degeneration was exposed to form deprivation myopia (Park et al., 2013). It was found that *rd10* mice were significantly more hyperopic than wild-type (WT) mice under normal visual conditions but showed greater susceptibility to form deprivation myopia (FDM) than WT mice, resulting in a response more than twice as severe after only two weeks of treatment (**Figure 4.1**). It was hypothesized that the high susceptibility to FDM in *rd10* mice was

mediated by low DA activity in the retina. The same study found that *rd10* mice, at all ages, had slightly higher levels of retinal DA but dramatically lower levels of retinal DOPAC relative to WT control mice (Park et al., 2013). This would indicate that less DA is released from DACs and is instead kept in storage vesicles in those cells. Other studies of retinal DA activity in *rd10* mice have shown structural and physiological changes to the DACs including limited ability to release DA in response to light (Nir and Iuvone, 1994), decreased signaling to AII amacrine cells (Ivanova et al., 2016), and a decreased effect on horizontal cell coupling (Hankins and Ikeda, 1994). Together, these studies indicate that decreased DAC activity caused by decreased stimulation from photoreceptor pathways disrupts the entire DAergic system in the retina.

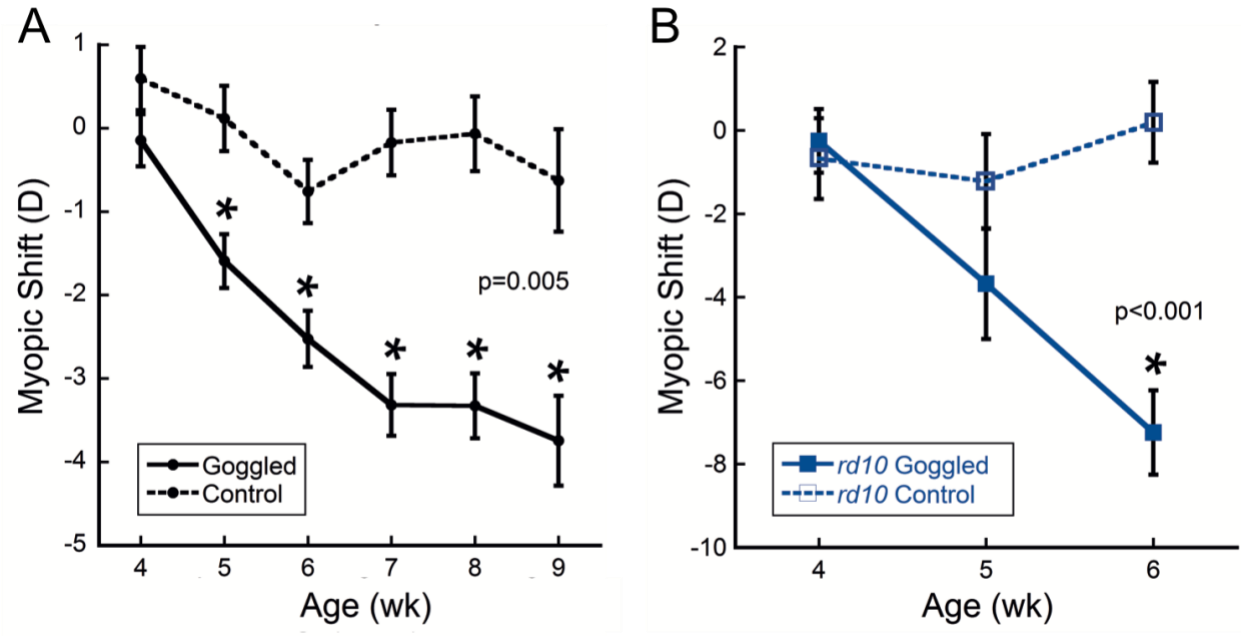

Figure 4.1. Myopic shift of WT and *rd10* mice after form deprivation. At P28 WT and *rd10* mice were treated with FDM and refractive errors were measured for 2 – 5 weeks. (A) WT mice showed significant myopic shift with FDM after only one week of treatment, and at 2 weeks after FDM showed ~2 diopters (D) myopic shift. (B) In *rd10* mice, 2 weeks of FDM resulted in a significant myopic shift relative to control mice, ~7D. Symbols and bars represent mean ± SEM. +p<0.005; **p<0.001. Figure was originally published in (Park et al., 2013) and is used here with permission.

In these experiments we determined the effect of increasing DA by treating form deprived mice with L-DOPA, the DA precursor. DA in the retina is synthesized when tyrosine hydroxylase (TH) is stimulated by light and converts tyrosine to L-DOPA which is then converted to DA by amino acid decarboxylase (AADC). Treating animals with L-DOPA could increase the levels of DA synthesized by TH and therefore increase the amounts of DA released into extracellular space, increase downstream signaling, and prevent myopia. Previous work with L-DOPA to prevent myopia in guinea pigs showed that the drug was effective at increasing DA levels and preventing the response to form deprivation (Mao et al., 2010). However, the effect of L-DOPA has not been tested in mice and we hypothesized that providing L-DOPA to WT or rd10 mice would endogenously increase DA levels and have a protective effect on FDM.

Once generated, DA in the cytosol is packaged into vesicles by the vesicular monoamine transporter 2 (VMAT2), a H^+/ATPase antiporter which utilizes an electrochemical gradient to exchange two protons from within the vesicle for a monoamine molecule (Rudnick et al., 1990; Parsons et al., 1993; Reimer et al., 1998). VMAT2 is a critical piece of DA signaling in neural tissue and just as TH is the rate limiting step in DA synthesis, VMAT2 is vital for maintaining cellular DA capacity.

In the brain, VMAT2 dysfunction and loss has been associated with several disorders including Parkinsonism (Mooslehner et al., 2001; Lohr et al., 2014; Segura-Aguilar et al., 2014), affective disorders including bipolar disorder and schizophrenia (Zubieta et al., 2001; Zucker et al., 2002b; Zucker et al., 2002a), some features of ADHD (Toren et al., 2005), and others. Investigations into the actions of VMAT2 in these disordered states as well as in neurotypical, healthy individuals have used pharmaceutical interventions and a range of transgenic mouse models to better understand the mechanisms of action behind each issue. The VMAT2 HI mouse,

with BAC-mediated, increased expression of VMAT2 is one model used to study how altering DA activity could impact DA-related disease states (Lohr et al., 2014).

Early characterization of the VMAT2 HI mouse has shown increased expression of VMAT2 in the striatum, leading to increased vesicle capacity and volume, increased DA levels, and increased dopamine release upon cellular stimulation (Lohr et al., 2014). This increased DA signaling in VMAT2 HI mice resulted in a resistance to neurotoxicity from methamphetamines and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Lohr et al., 2015; Lohr et al., 2016). Together, these studies show that VMAT2 has dramatic regulatory effects on DA dynamics and related health outcomes.

In the retina, VMAT2 has not been closely studied, despite its potential role in DA signaling. Studies characterizing VMAT2 in the retina have localized it to DACs and shown how its expression is increased as DACs mature. However, none of this work has been done in mice (Witkovsky et al., 2005; Burger et al., 2011; Hirasawa et al., 2012). One study used mice with a knock-down mutation of the VMAT2 gene and found no effect on retinal function in response to light despite lower levels of retinal DA (Taylor et al., 2009). Some of the initial studies on DA dynamics in myopia development and the effect of changes to DA on susceptibility to myopia used the non-specific VMAT inhibitor reserpine to dampen DA activity by depleting DA stores (Peter et al., 1994). Surprisingly, most of these studies done in chicks found an inhibitory effect on form deprivation (FD) and lens induced (LI) myopia (Schaeffel et al., 1995; Diether and Schaeffel, 1997; Ohngemach et al., 1997). A more recent investigation of the role of VMAT2 in myopia used PET/CT imaging of FD treated guinea pigs and showed that VMAT2 levels were decreased with myopia. They also observed a decrease in DA levels corresponding with the decrease in VMAT2 under FD (Sun et al., 2018).

Thus, this study aims to test the hypothesis that myopia can be rescued through endogenous increases in DA levels. WT and *rd10* mice with FD were given DA precursor L-DOPA to increase DA activity in the retina and we examined whether increased levels of DA in the VMAT2 HI mouse can prevent FDM. By studying the effect of endogenous DA, the nature of refractive development and potential preventative mechanisms will be better understood.

4.3 Methods

4.3.1 Transgenic and wild-type animals

Male and female C57BL/6J wild-type mice (WT, n= 66) and *rd10* (n= 65) mice on a C57BL/6J background were used. The *rd10* mouse has a missense mutation in exon 13 of the *Pde6b* gene, which encodes the β-subunit of the phototransduction protein cyclic nucleotide phosphodiesterase-6 in rod photoreceptors. These animals experience an inherited retinal degeneration and progressive photoreceptor death beginning at post-natal day 16 (P16) until P60 when degeneration is complete (Chang et al., 2007).

Both male and female VMAT2 WT (n=36) and VMAT2 HI (n=40) mice were used. In all cases, VMAT2 WT mice were littermates of the VMAT2 HI mice. VMAT2 HI mice were generated with a bacterial artificial chromosome (BAC)-mediated chromosomal insert using pronuclear injections into C57BL6 embryos (Lohr et al., 2014). The BAC insert contained the entire VMAT2 locus (*Slc18a2*). Each pup was genotyped for the BAC insert before experimental testing began - non-carriers were categorized as VMAT2 WT mice. All genotyping was done by Transnetyx, Inc. (Cordova, TN). Copy numbers of inserted VMAT2 were kept consistent by mating VMAT2 HI mice with C57BL/6 mice obtained from Charles River Labs (Wilmington,

MA). In this way, both VMAT2 WT and VMAT2 HI mice were derived from each litter, and VMAT2 overexpression in the VMAT2 HI mice was kept consistent.

All animals were bred and raised in the animal facility of the Atlanta VA Medical Center in Atlanta, GA. Mice were given unrestricted access to food and water, kept on a 12:12 hr light:dark cycle at approximately 20-200 lux during the light phase, and their health was checked daily. All mice were used according to the approved Institutional Animal Care and Use Committee protocol and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

4.3.2 Refractive and ocular Measurements

At P28, baseline measurements of refractive error and ocular biometrics were taken for all animals. As previously described, refractive error was measured with a custom-made automated photorefractor (Schaeffel, 2008). Mouse eyes were dilated using 1% tropicamide, then measurements were taken while the animal was awake and free moving. This "awake" recording establishes a baseline refractive error with the mouse in a natural position - none of these measurements were presented as results. The mice were then anesthetized [ketamine (80 mg/kg) and xylazine (16 mg/kg)], and the refractive error was measured again while the animal's head rested in a natural position; these recordings were used in our analysis. Animals that showed a baseline difference between right (OD) and left (OS) eyes greater than 2.0 diopters (D) in either direction were eliminated from the study.

Following refractive error measurements, the corneal curvature of each eye was recorded using a custom-made keratometer (Schaeffel, 2008). Finally, the axial length and retinal thicknesses were measured using a Bioptogen 4300 nm spectral domain optical coherence tomography (SD-OCT) system. Corneal thickness, lens thickness, and anterior and posterior

chamber depths were also measured. Following measurements, a subset of animals were treated with form deprivation, described below, then given yohimbine (2.1mg/kg) to aid in recovery and saline eye drops. All measurements were repeated weekly for FD experiments until P42 (L-DOPA with WT and *rd10* mice) or P56 (VMAT2 WT and VMAT2 HI mice). A subset of VMAT2 WT (n=8) and VMAT2 HI (n=15) mice were measured biweekly beginning at P28 until P112 to determine the effect of VMAT2 overexpression on refractive development under normal visual conditions i.e. no myopiagenic treatment.

4.3.3 L-DOPA and ascorbic acid administration

WT and *rd10* mice were divided into four treatment groups; L-DOPA Only (1.0 mg/kg body weight, MiliporeSigma, St. Louis, MO), Ascorbic acid (Simmons and Fuerst, 2018) Only (1.0 mg/kg body weight), L-DOPA + AA (1.0 mg/kg body weight each), and saline (0.9% NaCl). AA was added to solutions of L-DOPA to prevent oxidation. All drug treatments were prepared in saline immediately before administration, kept in light tight containers, and administered daily via intraperitoneal injection between 9-11 hours after light onset. Treatments for all groups began at P28, when a subset of animals from each strain and treatment group were given FD to trigger myopia.

4.3.4 Form deprivation treatment

Immediately following baseline measurements and before yohimbine administration, FD mice were given a surgically attached, head mounted diffuser goggle over the right eye (Faulkner et al., 2007). These custom-made goggles were painted white to deprive the eye of a clear image without obstructing incoming light. Diffuser goggles were “threaded” through a surgically implanted head pedestal and held in place with an aluminum cube around the frame. The cube also held a balance bar which rested on the left side of the face. Following all measurements,

mice were given the xylazine reversal agent yohimbine (2.1 mg/kg) and kept on an electric heating pad until they fully recovered and could right themselves. Goggles were removed weekly for cleaning and to measure changes in refractive error. For FD experiments, C57 WT (n=31), rd10 (n=31), VMAT2 WT (n=5) and VMAT2 HI (n=4) mice were used. VMAT2 WT and VMAT2 HI mice without FDM were used as controls (VMAT2 WT: n=7, VMAT2 HI: n=5).

4.3.5 HPLC detection of dopamine and DOPAC in the retina

Two days after the final ocular measurements, all FD and control mice were sacrificed via cervical dislocation 4-6 hours after light onset. Retinas were immediately frozen on dry ice and stored at -80° C until all tissue samples had been collected. To measure DA and DOPAC content in the retinas, high performance liquid chromatography (HPLC) with electrochemical detection and a 0.1 M sodium phosphate, 0.1 mM EDTA, 0.35 mM sodium octyl-sulfate, and 6% acetonitrile (pH 2.7) mobile phase was used as described previously (Nir et al., 2000; Pozdeyev et al., 2008). The retinas were homogenized in 0.1 N perchloric acid (PCA; C57 and *rd10* treated mice) or 0.1 N HClO4 solution (0.01% sodium metabisulfite and 50 ng/mL internal standard 3,4-dihydroxybenzylamine hydro- bromide; VMAT2 mice) and centrifuged to separate debris, the supernatant was then filtered and collected for testing. All samples were kept cold during preparation. Resulting HPLC peaks were analyzed based on a DA standard curve with 0.1 to 1 ng DA and DOPAC. Resulting values were normalized to total protein concentration (ng/mg) as determined by Lowry Assay. Retinas from left and right eyes of each mouse were tested individually, then averaged in later analyses.

4.3.6 Contrast sensitivity and visual acuity with VMAT2 overexpression

Visual function of each animal was tested using the virtual optomotor system (OptoMotry system; Cerebral-Mechanics), as described previously (Douglas et al., 2005; Aung et al., 2013).

Briefly, the animal was placed on a platform surrounded by four computer monitors that displayed vertical sine wave gratings rotating at 12 degrees/s. A video camera positioned above the animal allowed an observer, blinded to genotype, to determine the presence or absence of visual tracking. Tracking was defined as slow head movements, known as the optomotor response (OMR), in the same direction and speed as the rotating gratings. To measure the spatial frequency threshold, the grating started at a spatial frequency of 0.042 cycles/degree with 100% contrast and increased in a staircase paradigm until the response threshold was crossed three times. Contrast sensitivity was determined by reducing the contrast between the black and white gradients from 100% at a controlled spatial frequency in a staircase paradigm until the OMR was no longer observed. All OMR tests were done on P28 mice (WT: n=3, VMAT2 HI: n=3).

4.3.7 Statistical analysis

For each experiment, results are plotted as means with standard error. All analyses were done using Graphpad Prism 7 (La Jolla, CA). The response to FD was quantified as a myopic shift; the difference between the refractive error of the FD treated right eye and the naïve contralateral eye (OD-OS). For comparison, the interocular difference is also calculated for control animals which did not receive lens defocus. The myopic shift and other ocular biometric differences across treatment groups were tested using Repeated Measures Two-way ANOVAs with Holm-Sidak post-hoc comparisons. Final myopic shifts across all mouse genotypes and treatment groups were compared using Two-way ANOVAs. Refractive development values under normal conditions in VMAT2 mice were compared across genotypes using Two-way repeated measures ANOVAs with Holm-Sidak post-hoc tests to determine significant effects of genotype or treatment, and age. DA and DOPAC levels are also analyzed with Students t-tests.

The ungoggled left eye was used as an internal control, here referred to as the naïve, contralateral eye.

4.4 Results

4.4.1 L-DOPA prevents the effects of form deprivation in WT mice

Systemic L-DOPA treatment beginning at P28, the day FD treatment began, was sufficient to prevent changes in refractive error in WT mice. With 2 weeks of treatment, AA Only and Saline injection mice showed significant myopic refractions compared to both contralateral and control eyes. At P42, mice treated with Saline had significant myopia in response to FD (0.68±0.56D), relative to the contralateral and control eyes [contralateral: 4.99±0.40D; control: 4.08±0.52D; RM Two-way ANOVA, interaction effect, $F(4,40)=6.78$, $p<0.001$; **Figure 4.2A**]. In AA Only treated mice, the FDM eyes (0.07±0.75D) became significantly more myopic by P42 than either the contralateral eyes (3.35±1.05D) and the eyes of control mice [3.97±0.71D; RM Two-way ANOVA, interaction effect, $F(4,44)=3.66$, $p=0.012$, **Figure 4.2B**]. WT mice given L-DOPA + AA or L-DOPA Only showed a low susceptibility to FDM after one week of treatment. After two weeks, mice treated with L-DOPA + AA and FDM (3.04±0.75D) showed no significant differences in refractive error compared to their contralateral, untreated eyes (2.83±0.68D) and eyes from control mice with L-DOPA + AA (4.40±0.46D, **Figure 4.2C**). With L-DOPA Only treatment, both the FDM (1.81±1.63D) and contralateral eyes (2.54±0.90D) trended to be less hyperopic than control mice, but the differences were not statistically significant [5.64±0.62D; RM Two-way ANOVA, main effect of age, $F(2,30)=4.69$, $p=0.017$, **Figure 4.2D**].

To measure the effect of the L-DOPA treatment on refractive error we also compared the myopic shifts of these treatment groups to the ungoggled control animals with the same drug treatment after 2 weeks of FD. Significant myopic shifts were found for both AA Only (FD: -3.27±0.73D, controls:-0.75±0.59D, $p<0.05$; Two-way ANOVA, interaction effect, $F(3,58)=3.65$, $p=0.018$, **Figure 4.2E**) and Saline treated animals (FD:-3.71±0.80D, controls:0.31±0.31D, $p<0.001$, **Figure 4.2E**). In contrast, L-DOPA treatments prevented myopic shifts such that there were no statistical differences with the control groups (L-DOPA + AA FD:-0.11±0.46D, controls: 0.39±0.36D, $p=0.99$; L-DOPA Only FD: -0.73±0.90D, controls: 0.60±0.037D, $p=0.92$; **Figure 4.2E**). Among the myopic shifts of FDM animals, Saline treated groups had significantly higher shifts than L-DOPA + AA ($p<0.001$) and L-DOPA Only mice ($p=0.035$). Mice given FDM and AA Only were significantly more myopic than mice with FDM and L-DOPA + AA ($p=0.004$). No significant differences between control groups were found.

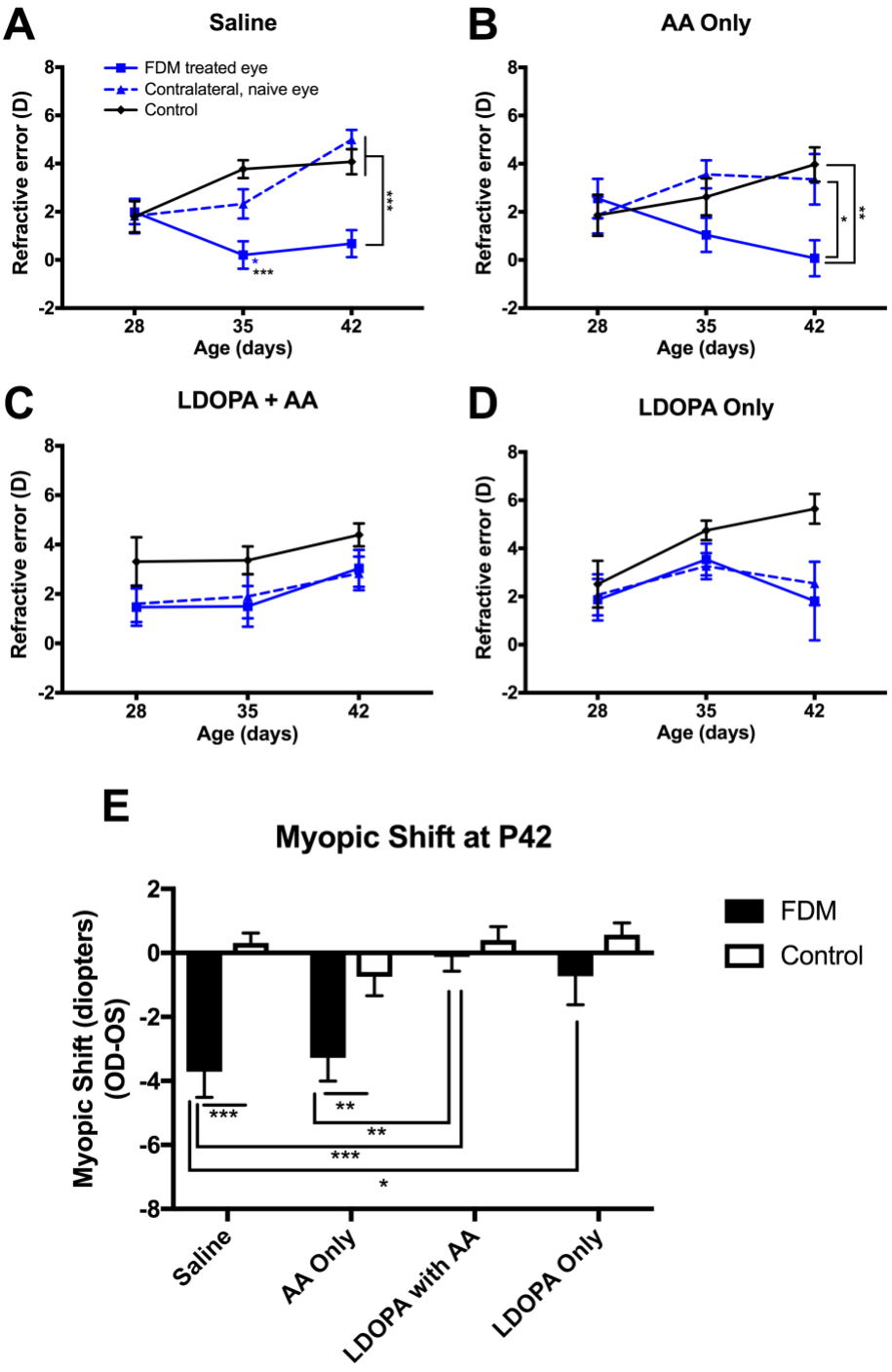

Figure 4.2. L-DOPA and L-DOPA + AA prevented FDM in WT mice. WT mice were treated with FD and either L-DOPA + AA, L-DOPA Only, AA Only, or Saline in daily systemic injections. The refractive error of all FD treated eyes (*blue lines*) were compared to the contralateral, treated eyes (*blue dashed lines*), and control eyes (*black lines*). (A) Saline injections did not prevent a significant myopic growth in the FDM eye compared to both the contralateral and control eyes at P35 and 42 [RM Two-way ANOVA, interaction effect,

$F(4,40)=6.78$, $p<0.001$] (B) After two weeks of AA Only treatment, the FDM treatment resulted in significant myopia relative to both contralateral and control eyes [RM Two-way ANOVA, interaction effect, $F(4,44)=3.66$, $p=0.012$] (C) With L-DOPA+AA treatment, no significant differences were found. (D) With L-DOPA Only treatment, FDM and contralateral eyes showed a trend for relative myopia compared to eyes of control mice, but did not reach significance [RM Two-way ANOVA, main effect of age, $F(2,30)=4.69$, $p=0.017$] (E) Comparing the myopic shifts of control and FDM animals in each treatment groups shows significant myopia in animals treated with AA Only or Saline after FDM [Two-way ANOVA, interaction effect, $F(3,58)=3.65$, $p=0.018$]. Data are mean ± SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

The corneal curvatures of each animal were measured immediately following photorefraction. No differences were found between the corneal curvature of the control eyes, FD treated eyes, and contralateral naïve eyes compared across time (data not shown). To isolate the effects of drug treatment on the response to FD, the difference between FD and contralateral eyes were compared across drug treatment groups, termed "shift" (**Figure 4.3A-B**). No differences in corneal curvature shifts were found.

Other ocular parameters measured included retinal thickness and axial length, both measured with SD-OCT. WT mice showed no differences in retinal thickness regardless of time, deprivation treatment, or drug administration (data not shown). The retinal thickness shift showed a main effect of drug treatment with FDM [RM Two-way ANOVA, main effect of treatment, $F(3,96)=2.81$, $p=0.044$, **Figure 4.3C**]. However, this likely does not explain the differences in refractive error under FD and with L-DOPA treatments. No differences between retinal thickness shifts in control mice were found (**Figure 4.3D**). Axial length shifts were also analyzed and no differences were found across FDM and control groups (**Figure 4.3E and F**). Other ocular parameters that were measured include corneal thickness, anterior chamber depth, lens thickness, and posterior chamber depth. No differences were found in these parameters across treatment group (data not shown).

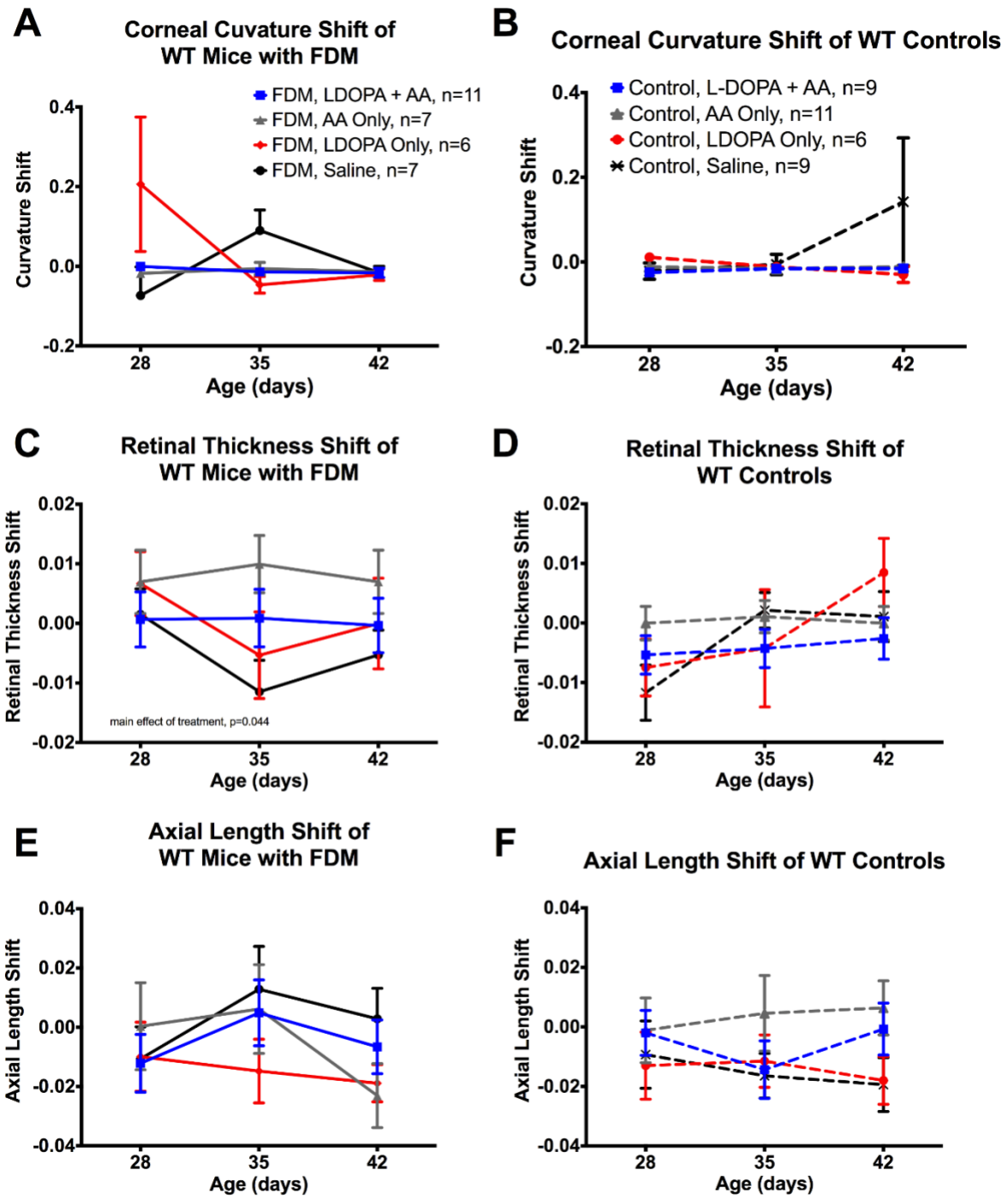

Figure 4.3. Ocular parameters of WT FD and Control mice are not altered by L-DOPA or AA treatment. The effects of FD with drug treatment (A, C, E) and drug treatment alone (B, D, F) were measured by studying the shifts (OD-OS) of corneal curvature, retinal thickness, and axial length of each group from P28 to P42. No differences with treatment, or between FD and control mice, were found except in the retinal thicknesses of FD treated mice in which a significant difference between drug groups was found [RM Two-way ANOVA, main effect of treatment, $F(3,96)=2.81$, $p=0.044$]. Data are means ± SEM. L-DOPA + AA (*red*), L-DOPA Only (*blue*), AA Only (*grey*), Saline (*black*). Control animals are represented by dashed lines.

4.4.2 Ascorbic acid, not L-DOPA, prevents form deprivation in *rd10* mice

L-DOPA treatment groups differentially affected the FDM response of *rd10* mice [RM Two-way ANOVA interaction effect $F(6,54)=4.82$, $p<0.001$, not shown]. With Saline only treatment, the FD eyes of treated mice (-0.46±0.86D; post-hoc $p<0.001$) showed significantly less hyperopia relative to the contralateral (5.39±0.96D; post-hoc $p=0.003$) and control eyes (4.23±0.52D; RM Two-way ANOVA, interaction effect, $F(4,36)=3.65$, $p=0.014$; **Figure 4.4A**). Contrary to WT mice, *rd10* mice given L-DOPA treatments showed a similar myopic shift in response to FDM as the Saline treated mice. Treatment with L-DOPA + AA resulted in a myopic refractive error at P42 in FDM treated eyes (-0.12±1.12D) compared to contralateral (3.09±0.86; post-hoc $p=0.028$) and control eyes (4.80±0.50D; post-hoc $p<0.001$; RM Two-way ANOVA main effect of treatment, $F(2,24)=9.70$, $p<0.001$; **Figure 4.4B**). L-DOPA Only treatment has a similar effect (FDM eyes: 0.67±1.11D, Contralateral: 6.42±0.55, $p<0.001$ compared to FDM; control eyes: 5.42±0.68D, $p=0.002$ compared to FDM; RM Two-way ANOVA, interaction effect, $F(4,46)=7.03$, $p<0.001$; **Figure 4.4C**). Surprisingly, *rd10* mice treated with AA Only showed no response to FD across the experimental period. After two weeks of FD, FD eyes (1.45±1.54D) were indistinguishable in post-hoc comparisons from contralateral (2.22±1.20D) control mice (3.83±0.49D; RM Two-way ANOVA, interaction effect, $F(4,46)=2.80$, $p=0.037$; **Figure 4.4D**).

Three of the treatment groups showed significant differences in myopic shifts after the FD [Two-way ANOVA, interaction effect, $F(3,57)=5.97$, $p=0.002$; **Figure 4.4E**]. *rd10* mice given Saline and FDM had higher myopic shifts (-5.81±0.98D) than the control and Saline treated mice (0.34±0.69D; $p<0.001$). The same effect was found in L-DOPA + AA treated mice (FDM: -3.21±0.73D; controls: 0.31±0.46; $p=0.02$) and in L-DOPA Only treated mice (FDM: -

5.75±0.90D; controls: -0.48±0.90D; $p<0.001$). However, no difference was found between the myopic shift of AA Only and FDM treated mice (-0.77±0.80D) and control mice (-0.62±0.62D; $p=0.99$). The three treatment groups that responded to FD had statistically similar responses (FDM + Saline compared to FDM + L-DOPA + AA: $p=0.35$; compared to FDM + L-DOPA Only; $p=0.99$; FDM + L-DOPA + AA compared to FDM + L-DOPA Only; $p=0.20$).

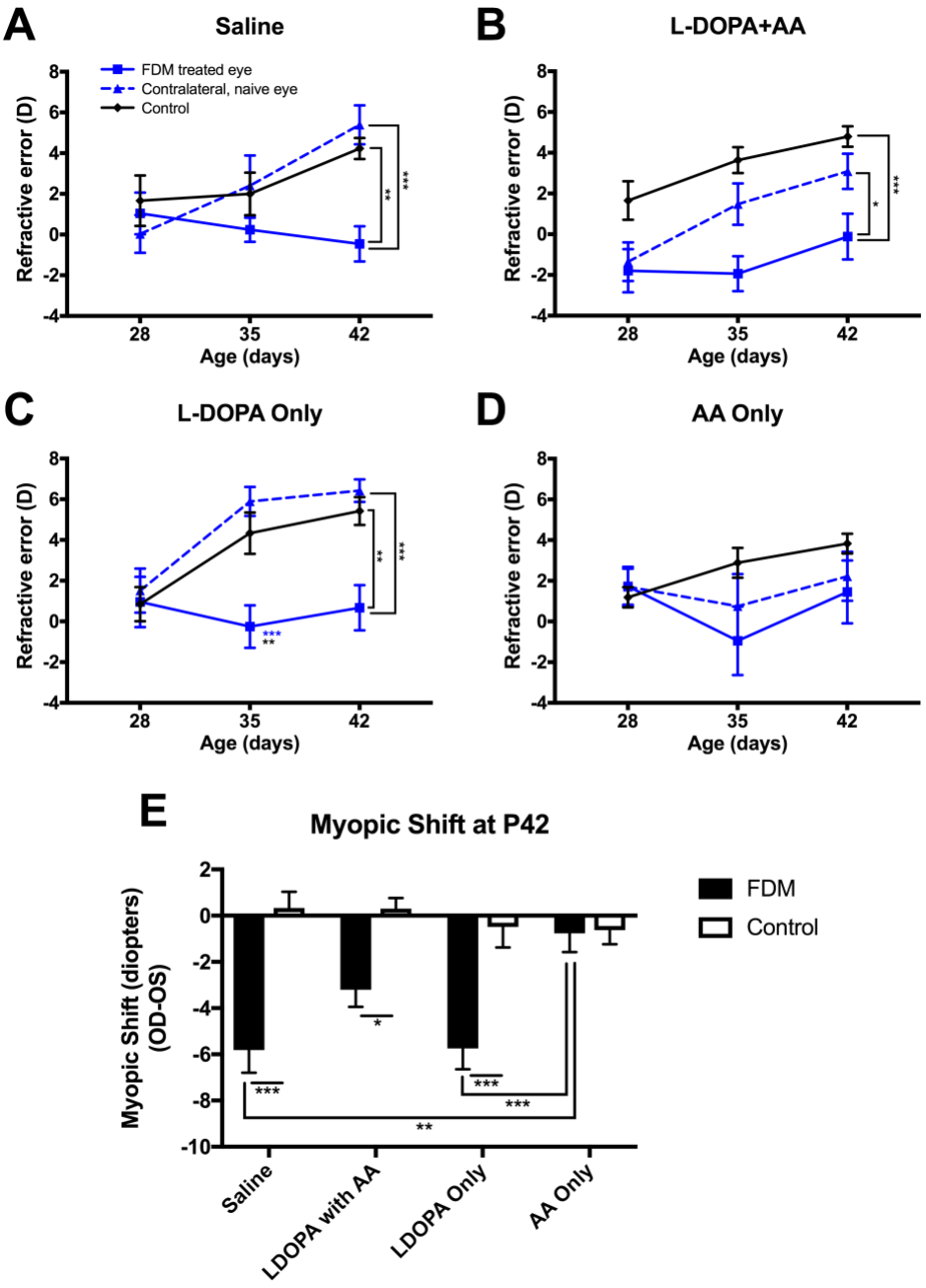

Figure 4.4. *Rd10* mice are protected from FDM with AA, but not L-DOPA. (A) As expected, FDM eyes (*blue lines*) of Saline treated mice had significant myopic refractive errors compared to the contralateral (*blue dashed lines*) and control eyes (*black lines*) [RM Two-way ANOVA, interaction effect, $F(4,36)=3.65$, $p=0.014$]. (B) *rd10* mice were treated with FD and L-DOPA + AA in daily systemic injections showed a significant effect of FDM compared to contralateral and control mice [RM Two-way ANOVA main effect of treatment, $F(2,24)=9.70$, $p<0.001$]. (C) L-DOPA Only treatment also resulted in significant myopic response to FDM [RM Two-way ANOVA, interaction effect, $F(4,46)=7.03$, $p<0.001$]. (D) After AA Only treatment, the FDM had no effect on refractive error relative to contralateral eyes or control mice [RM Two-way

ANOVA, interaction effect, $F(4,46)=2.80$, $p=0.037$] (E) Myopic shifts of the mice from each treatment groups indicated significant effects of FDM in L-DOPA + AA, L-DOPA Only, and Saline treatment groups [Two-way ANOVA, interaction effect, $F(3,57)=5.97$, $p=0.002$]. Data are mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Corneal curvature, retinal thickness, and axial length were measured weekly in *rd10* mice in this experiment using keratometry and SD-OCT (**Figure 4.5**). In *rd10* mice, we observed no changes in corneal curvature or retinal thickness shifts across treatment groups (Figure 4.4A-D). A main effect of drug treatment was found to affect the axial length shift in FDM treated mice. The AA only group had significantly smaller axial length shifts than the other treatment groups [RM Two-way ANOVA, main effect of treatment, $F(3,87)=3.845$, $p=0.012$]. However, these axial length changes are unlikely to explain the differences in myopic shift in these mice. In order to test if L-DOPA and/or AA could prevent retinal degeneration in *rd10* mice, we also analyzed the average retinal thickness of ungoggled control mice across all drug treatment groups. In this analysis, we found no significant differences, indicating retinal health was not affected by drug treatment (**Figure 4.6**).

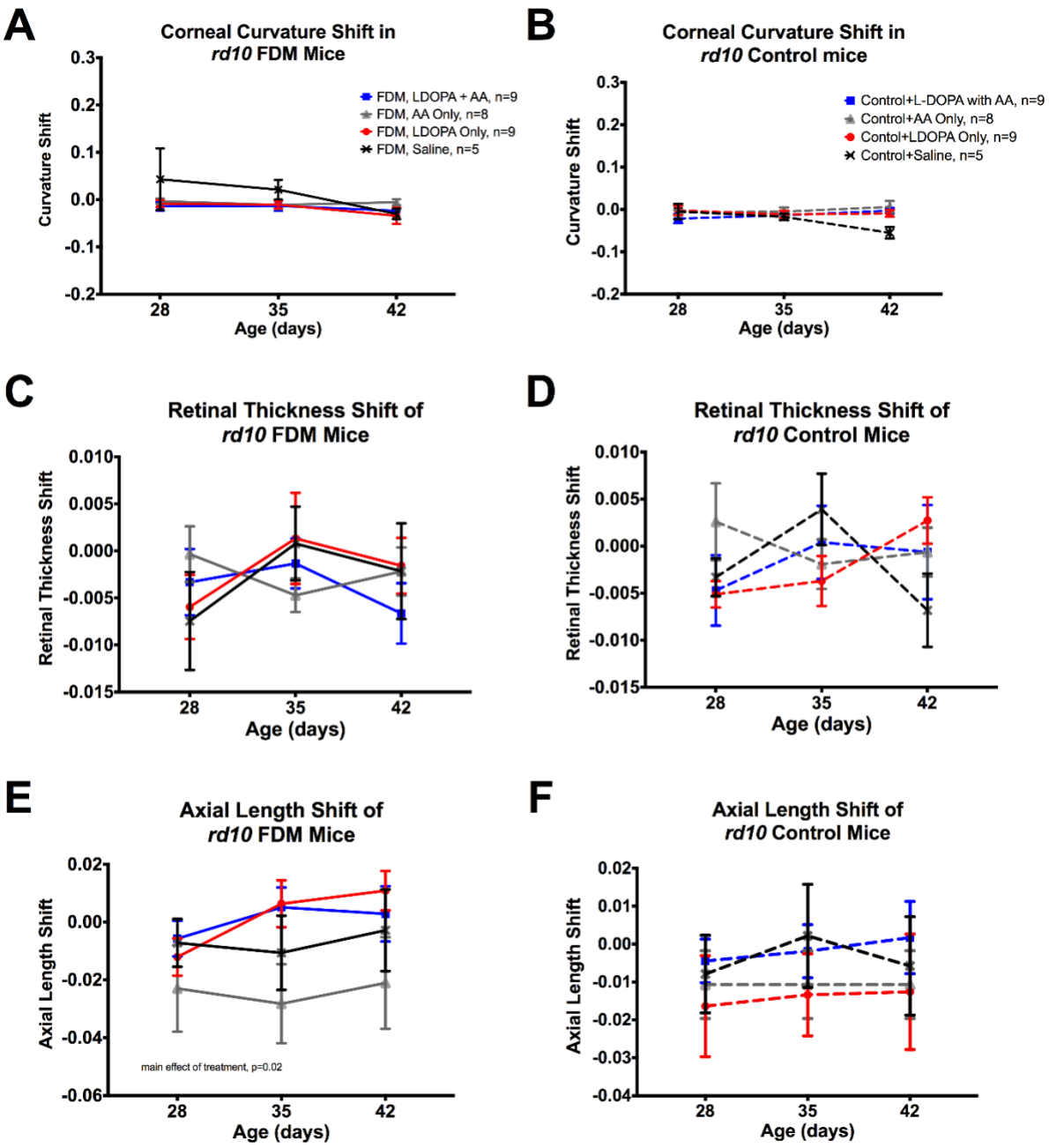

Figure 4.5. FD and drug treatment had no effect on ocular parameters in *rd10* mice. (A-B) The corneal curvature shift was measured in FD (*solid lines*) and control (*dashed lines*) mice treated with L-DOPA + AA (red), L-DOPA Only (*blue*), AA Only (*grey*), or Saline (*black*). No significant differences between treatment groups were found. (C-D) Retinal thickness shift did not vary between FD or control mice with drug treatment. (E) In *rd10* mice, a main effect of drug treatment was found across axial length shifts [RM Two-way ANOVA, main effect of treatment, $F(3,87)=3.845$, $p=0.012$]. (F) Axial length shifts in control mice did not vary with treatment. Data are means ± SEM.

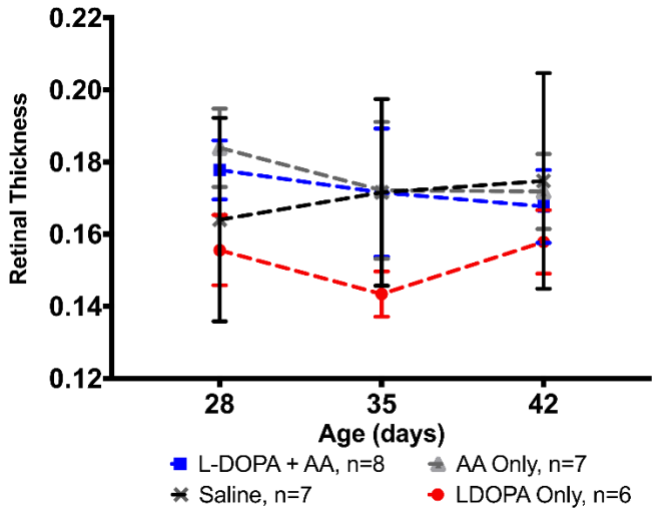

Figure 4.6. Retinal health in *rd10* mice is not affected by L-DOPA or AA administration. The mean retinal thickness values of control mice from each treatment groups did not significantly differ throughout the experiment. Also, retinal thicknesses do not decrease with time. Data represent mean ± SEM. L-DOPA + AA (*red*), L-DOPA Only (*blue*), AA Only (*grey*), Saline (*black*).

4.4.3 Dopamine activity is not altered by VMAT2 overexpression

To study DA levels in the retinas of VMAT2 HI mice, DA and DOPAC were measured from adult retinas with HPLC. VMAT2 WT (1638 ± 73.5 mg/pg) and VMAT2 HI (1749 ± 82.05 mg/pg) mice had statistically similar levels of DA in the retina (Student's t-test, t=1.01, df=50, p=0.3174, **Figure 4.7A**). Levels of DOPAC, the DA metabolite, also did not change between the two genotypes (VMAT2 WT: 151.2 ± 10.4 mg/pg; VMAT2 HI: 157.6 ± 11.22 mg/pg, Student's t-test, t=0.4175, df=50, p=0.678, **Figure 4.7B**).

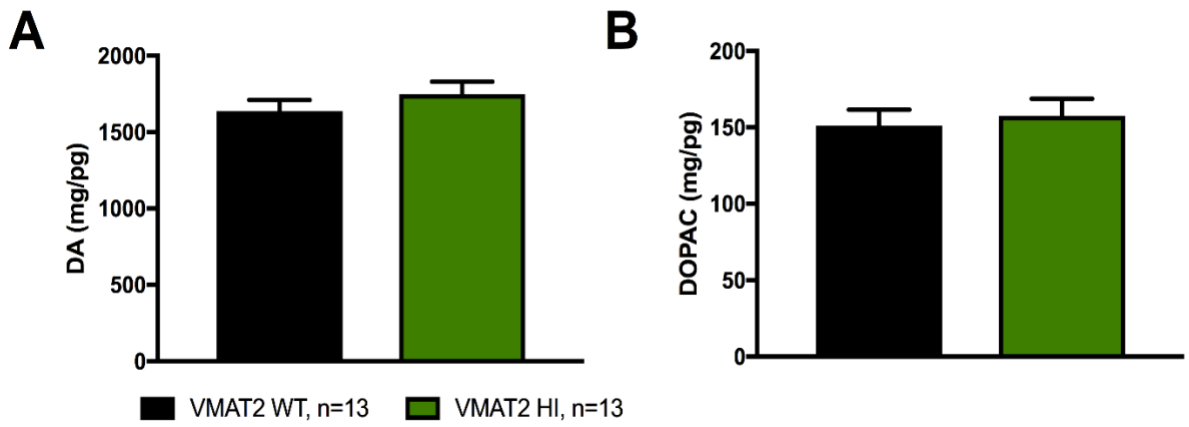

Figure 4.7. DA and DOPAC levels are not altered by increased VMAT2 expression. (A) Retinal DOPAC content was measured in the retinas of adult VMAT2 WT (*black*) and VMAT2 HI (*green*) mice. No significant difference was found between the two groups. (B) Additionally, no change was found in DA levels with overexpression of VMAT2. Data shown are mean ± SEM**.**

4.4.4 Visual function is not altered by VMAT2 overexpression

Spatial frequency and contrast sensitivity thresholds were measured in VMAT2 WT and VMAT2 HI mice using OMR. We found that VMAT2 WT (0.296 ± 0.01 cyc/deg) and VMAT2 HI (0.271 ± 0.02 cyc/deg), mice had nearly identical spatial frequency thresholds (Student's t-test, t=0.961, df=4, p=0.390, **Figure 4.8A**). Similarly, contrast sensitivity did not vary between groups (VMAT2 WT:2.38 ± 0.201 HI:2.24 ± 0.154, Student's t-test, t=0.609, df=4, p=0.554, **Figure 4.8B**).

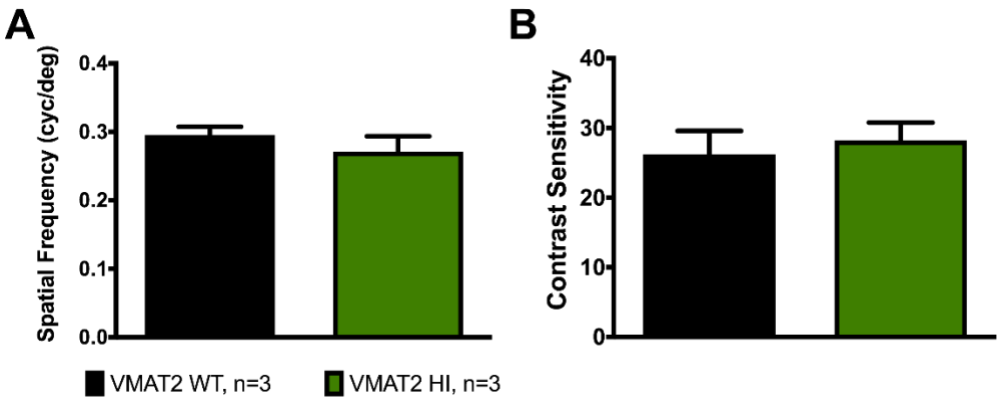

Figure 4.8. VMAT2 WT and VMAT2 HI mice show similar visual function. (A) Spatial frequency thresholds did not change across genotypes. (B) Contrast sensitivity thresholds also did not vary between VMAT2 WT (*black*) and VMAT2 HI (*green*) mice. Data shown are mean ± SEM**.**

4.4.5 Altered VMAT2 expression does not alter normal refractive development in mice

Refractive error in VMAT2 WT and VMAT2 HI mice was measured every two weeks from P28 to P112. At P28, both groups had refractive errors of approximately 2-3 D. As the animals aged, both groups became more hyperopic. By P112 at the end of the experiment, VMAT2 WT (4.88 ± 0.42D) and VMAT2 HI (4.84 ± 0.27D) mice had similar refractive errors. While no statistical differences were found between genotypes, a significant effect of age was found [RM Two-way ANOVA, main effect of age, $F(6,126)=6.24$, $p<0.001$, **Figure 4.9**].

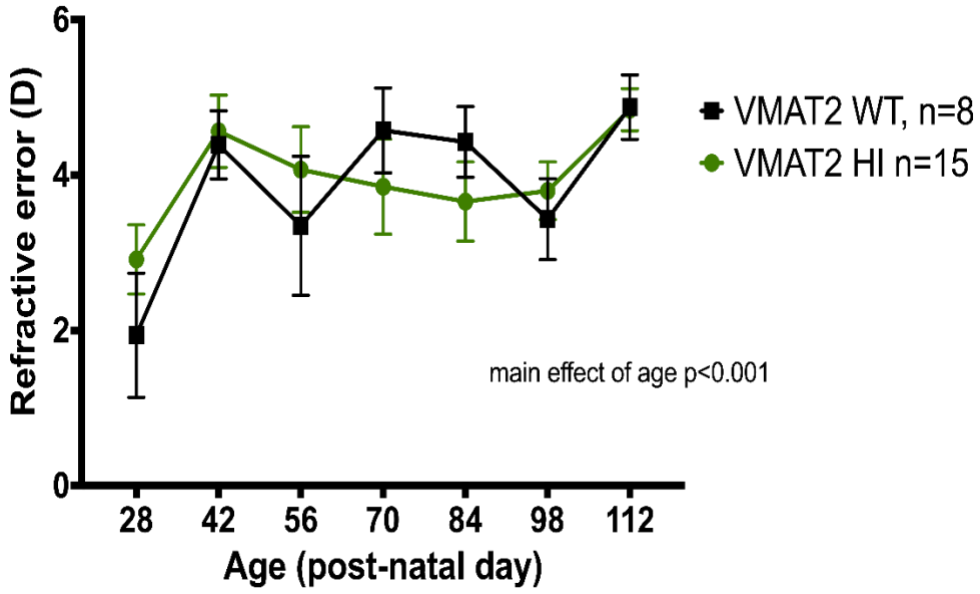

Figure 4.9. Refractive development of VMAT2 WT and VMAT2 HI mice. Both VMAT2 HI (*green*) and VMAT2 WT (*black*) mice became more hyperopic at P42 and remained at approximately 4D through the experimental period [RM Two-way ANOVA, main effect of age, $F(6,126)=6.24$, $p<0.001$). Data show mean ± SEM].

4.4.6 FDM unaltered with increased VMAT2 expression

Both VMAT2 WT and VMAT2 HI mice showed a response to FD [RM Two-way ANOVA, interaction effect, $F(9,51)=4.527$, $p<0.001$, **Figure 4.10**]. VMAT2 WT mice developed a myopic shift after two weeks (-3.30 ± 0.73D) relative to untreated, control VMAT2 WT mice (-0.54 ± 0.64D, $p=0.002$). This significant response grew more robust after three weeks of FD (VMAT2 WT FD: -4.24 ± 0.55D, VMAT2 WT Controls: 0.74 ± 0.25D, $p<0.001$). VMAT2 HI mice also showed a significant myopic shift in response to FD after three weeks of treatment (VMAT2 HI FD: -3.10 ± 0.39D, VMAT2 HI Controls: 0.20 ± 0.48D, $p<0.001$). There were no significant differences found between the VMAT2 WT and VMAT2 HI control mice. Additionally, the myopic shifts between VMAT2 HI and VMAT2 WT mice were not statistically different at any time point and a linear regression analysis showed no differences between the rates of the myopia development between the two groups (linear regression, $F=0.57$, $DFn=1$, $DFd=4$, $p=0.49$) indicating no differences in progression.

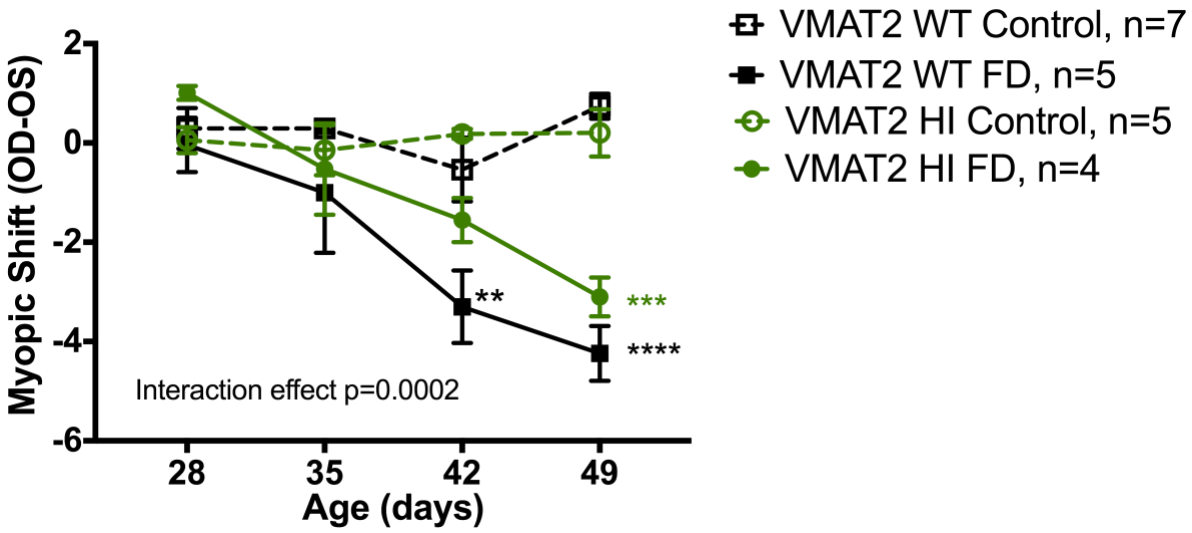

Figure 4.10. Both VMAT2 WT and VMAT2 HI mice are susceptible to FDM. In response to FD treatment, both VMAT2 WT (*black*) and VMAT2 HI (*green*) mice develop a myopic shift [RM Two-way ANOVA, interaction effect, $F(9,51)=4.527$, $p<0.001$]. VMAT2 WT mice develop a significant myopic shift relative to the control WT mice after 2 weeks of treatment, at P42 ($p<0.001$, indicated by black asterisks). This difference between VMAT2 WT FDM treated mice and controls continued after 3 weeks of treatment ($p<0.001$, black asterisks).VMAT2 HI mice develop a myopic shift after 3 weeks of treatment, at P49 ($p<0.001$, green asterisks). There were no significant differences found between the VMAT2 WT and VMAT2 HI FD treated mice. All data shown are mean ± SEM. **$p<0.01$, ***$p<0.001$.

4.5 Discussion

4.5.1 Increasing endogenous DA pharmacologically prevents myopia in a mouse model

The administration of L-DOPA, the DA precursor, prevented the myopiagenic effects of FD in WT mice. This finding supports the previously held hypothesis that DA activity plays an important role in myopia development, specifically as a stop signal for myopic eye growth (Feldkaemper and Schaeffel, 2013). This finding also suggests myopia can be prevented by increasing DA in the retina. Previous studies have shown increasing DA receptor activity can prevent the effects of FDM or LIM (Schaeffel et al., 1995; McCarthy et al., 2007; Nickla et al., 2010). Additionally, a previous study of guinea pigs with FDM showed a protective effect of increased DA through L-DOPA administration (Mao et al., 2010). However, this is the first study showing prevention of myopia with increased DA in mice.

We have shown that L-DOPA administration alone is sufficient to prevent the effects of FDM. Thus, while other eye growth signals may occur in the retina, the effects of DA may dominate or preclude the signaling mechanisms implicated in other studies (Arumugam and McBrien, 2010). It seems unlikely that DA is directly impacting the development of the choroid, sclera, or cornea; however, it is possible that by increasing DA signaling, the downstream effects of FDM on those tissues are prevented. Here, systemic injections, rather than previously used intravitreal injections, prevented FDM (Ward et al., 2016).

4.5.2 With degenerating retinas, dopamine dynamics are altered such that increasing DA has no effect on the susceptibility to FD

Contrary to WT mice, *rd10* mice, with retinal degeneration and low DA activity, are not protected from FDM by L-DOPA treatments. The *rd10* mouse has a higher susceptibility to FDM than WT mice, likely due to lower DA activity (Park et al., 2013). In a surprising finding,

the antioxidant AA completely ameliorated the myopic shift from FD in *rd10* mice, and the protective effect of AA treatment was decreased when L-DOPA was added. The mechanisms by which AA is acting to prevent FDM are unknown, however, it seems likely that the drug might be preventing the oxidation of DA in the retina and thereby increasing DA receptor activation (Neal et al., 1999; Kang et al., 2016). L-DOPA Only treatment had no benefit to *rd10* mice undergoing FD. L-DOPA Only may be ineffective because the rd10 mice have already undergone significant photoreceptor degeneration at the start of the treatment period (Chang et al., 2007) and the DACs may also be significantly damaged at this timepoint which would prevent normal DA release (Ivanova et al., 2016). Data not included here showed that treatment with L-DOPA + AA in drinking water from birth did prevent the myopic effects of FDM given at P28, suggesting that early treatment of *rd10* mice, before degeneration and DAC loss does provide some protection.

We have considered several possible explanations as to how AA is providing a protective effect for FDM in the *rd10* mice. One explanation may be due to consequences of retinal degeneration in *rd10* mice. During the development of retinal degeneration in *rd10* mice, many retinal cell types display hyperactivity, an increase in spontaneous firing which likely occurs because of the absence of photoreceptor stimulation (Ivanova et al., 2016). DACs, however, decrease spontaneous firing (Atkinson et al., 2013). We hypothesize that with decreased firing, any DA released is being protected from extracellular oxidation by AA and therefore preserving the downstream signaling effects of DA to prevent form deprivation. Without AA, it is possible that DA is depleted as it is released and without functional TH, DA levels are not replenished, leading to an increased response to FDM.

A second possibility is that AA may be preventing retinal degeneration; as AA is an antioxidant it could be delaying the effects of degeneration (Zhao et al., 2014; Martínez-Fernández de la Cámara et al., 2015; Kang et al., 2016). This may also slow the loss of DACs which may allow for more normal DA activity that provides the protective effect. While retinal degeneration is occurring in these mice during this experiment, there were no observed changes in retinal thickness across treatment groups at the timepoints measured here. A previous study reported that most of the outer nuclear layer loss has occurred by P30 in these mice indicating thickness changes would occur before these measurements began and therefore retinal thickness may not be the best measure of damage here (Chang et al., 2007). An alternative technique would look histologically at cellular structure to determine how treatments were affecting damage. Also, the protective effect of AA on FDM in rd10 mice was nearly complete, which would suggest other mechanisms at play other than normal DA signaling.

4.5.3 VMAT2 does not alter retinal dopamine or visual function in mice

By testing retinal DA and DOPAC levels of VMAT2 WT and VMAT2 HI mice, we determined that increasing expression of VMAT2 protein in the retina did not alter total DA content. In this study, DA levels were measured from whole retina tissue using HPLC. This method does not differentiate where DA resides in the tissue: intracellular vs extracellular. Therefore, while overall levels of DA in VMAT2 HI retinas are similar to that in VMAT2 WT retinas, the localization of DA is unknown. It is possible that DA was localized to storage vesicles controlled by VMAT2 in the VMAT2 HI mice versus more DA outside the vesicles in VMAT2 WT mice. DOPAC levels were also similar across genotypes, indicating that DA activity and metabolism does not change with increased expression of VMAT2.

Unfortunately, due to lack of established techniques for measuring retinal DA, it is not possible to determine if these results are masking changes to DA localization caused by VMAT2. Measurement of extracellular DA after light stimulation through the eye cup perfusion assay described previously (CHAPTER 3) may be useful for determining how DA release might be altered with VMAT2 overexpression. DOPAC levels have generally been used in retinal research as a proxy for DA metabolism, under the assumption that released DA is taken back into the cell and degraded into DOPAC. The absence of changes to both DA and DOPAC levels across genotypes indicate that either DA related proteins are altering their activity to compensate for changes in VMAT2 expression and keep DA signaling the same, or that the VMAT2 mutant is not complete in the retina. In tests of VMAT2 HI striatal tissue, small but significant increases in DA were found (Lohr et al., 2014).

No effects of increased VMAT2 expression on visual function, contrast sensitivity and visual acuity, were observed. This was unexpected given the effects of DA signaling on visual function in other studies. Mice with a conditional knock-out of TH show significant deficits in both contrast sensitivity and spatial frequency (Jackson et al., 2012). Studies have also linked the effects of DA on circadian rhythms to daily changes in contrast sensitivity (Hwang et al., 2013; Jackson et al., 2014). This finding again supports the hypothesis that the VMAT2 overexpression is either not complete in the retina, or some unknown compensatory mechanism is activated in VMAT2 HI mice to prevent these psychophysical changes.

4.5.4 Increasing VMAT2 does not affect refractive development or myopia susceptibility

Both VMAT2 WT and VMAT2 HI mice had similar refractive development with normal laboratory conditions. Other studies of mouse refractive error development have shown similar developmental curves in WT mice with normal visual experience (Pardue et al., 2008; Park et al.,

2013; Park et al., 2014). These results indicate that overexpression of VMAT2, despite potentially increasing DA release in the retina, does not affect normal refractive development. It is possible that without a myopiagenic factor, such as FD, DA activity does not impact refractive error. However, other transgenic mice with low levels of DA show myopic refractive errors throughout development (Bergen et al., 2016).

VMAT2 HI mice also did not show a protection from FD, as would be expected in animals with higher DA activity. Both VMAT2 WT and VMAT2 HI mice developed a myopic shift after three weeks of FDM. Therefore, overexpression of VMAT2 did not offer protection from experimental myopia.

A potential explanation for these findings could be an incomplete mutation in the VMAT2 HI mouse which results in normal levels of VMAT2 in the retina. Previously, this BAC-mediated mutation was assumed to be complete, however western blots of sample retinas from adult VMAT2 WT and VMAT2 HI mice showed no clear differences in band size or intensity (data not shown). An important step to verify the presence of VMAT2 overexpression in the retina that is similar to other tissues will be to measure gene copy number with genomic quantitative PCR. Previously, VMAT2 HI brain regions have shown 2.5 fold higher gene copies than VMAT2 WT mice (Lohr et al., 2014). Another potential explanation for the lack of changes with increasing VMAT2 expression would be a simultaneous change in other DA related proteins to compensate and bring DA activity back to WT levels. Due to the cytotoxicity of DA and the importance of small changes in DA signaling, the proteins which control the DAergic system are highly adaptable, and therefore, a closer study of the mechanisms of action in DA signaling are necessary to fully determine the effects of VMAT2 in the retina.

Finally, an important addition to this work would be a study of a complementary VMAT2 LO (VMAT2 deficient) transgenic mouse (Caudle et al., 2007; Mooslehner et al., 2001; Taylor et al., 2009). These mice express significantly less VMAT2 protein than WT mice and have less DA content and signaling. Previous investigation of these mice have shown no effects of decreased VMAT2 gene expression on retinal signaling after light exposure. If VMAT2 has no effect on refractive development or myopia susceptibility, then VMAT2 LO mice should have similar refractive error development curves and myopic shifts after FD as the VMAT2 WT and VMAT2 HI mice presented here. A more thorough study of the role of VMAT2 in the retina, which is likely to be different than its role in the brain, will expand on these findings to determine the role of VMAT2 in myopia directly.

4.5.5 Potential clinical applications for increasing endogenous retinal dopamine

The protection from FDM seen in WT mice with L-DOPA treatment highlights the importance of DA activity in myopia and the potential for increasing DA activity as a preventative mechanism clinically. While several studies have associated DA activity with myopia, L-DOPA administration clearly shows how increasing DA directly might benefit those at risk of myopia. Treatment with L-DOPA for children at risk of developing myopia is unlikely as it can have dramatic impacts on movement and other DA related neural functioning (Pardue and Allen, 2018).

Fortunately, increasing endogenous levels of DA in the retina could be achieved by increasing exposure to bright light (Iuvone, 1984). Higher intensity light increases the activity of tyrosine hydroxylase and therefore the synthesis of DA (Iuvone et al., 1978). Bright light also reduces experimental myopia in animal models - likely by increasing DA synthesis and signaling (Ashby et al., 2009; Siegwart Jr et al., 2012; Smith et al., 2012). Early studies in human

populations have shown that spending more time outdoors in bright sunlight reduces the incidence and progression of myopia (Wu et al., 2018). The mechanism behind this bright light protection are not completely known, although DA has been implicated [for review (Norton and Siegwart, 2013)]. Future investigations of how increased DA activity alters refractive error development are necessary to uncover safe and effective prevention strategies.

4.6 Summary

Increasing endogenous DA with systemic L-DOPA administration in mice with FD showed that the effects on refractive error could be prevented with increased DA. These results support the hypothesis that DA activity is involved in myopic eye growth and that myopia could be prevented by increasing DA in the retina. Our second approach to increasing DA activity by potentially increasing DA release through increased expression of the monoamine transporter VMAT2 did not affect susceptibility to FDM. It is possible that the potential complications with the VMAT2 HI mouse discussed above have prevented any effect in this experiment. A more careful characterization of this mouse model will be necessary to confirm the genotype and its effect on retinal DA release. Because levels of DA and DOPAC are not altered by VMAT2 overexpression, a different transgenic model with increased DA or DA activity in the retina could be used to investigate the role of genetic manipulation of DA activity and further confirm the role of DA in myopia prevention.

CHAPTER 5: Dim light exposure and myopia in children

*This chapter was originally published in Landis EG, Yang V, Brown DM, Pardue MT, Read SA. Dim Light Exposure and Myopia in Children. Investigative Ophthalmology & Visual Science 2018;59:4804-4811.

5.1 Abstract

Experimental myopia in animal models suggests that bright light can influence refractive error and prevent myopia. Additionally, animal research indicates activation of rod pathways and circadian rhythms may influence eye growth. In children, objective measures of personal light exposure, recorded by wearable light sensors, have been used to examine the effects of bright light exposure on myopia. The effect of time spent in a broad range of light intensities on childhood refractive development is not known. This study aims to evaluate dim light exposure in myopia. We reanalyzed previously published data to investigate differences in dim light exposure across myopic and non-myopic children from the Role of Outdoor Activity in Myopia (ROAM) study in Queensland, Australia. The amount of time children spent in scotopic (<1-1 lux), mesopic (1-30 lux), indoor photopic (>30-1000 lux), and outdoor photopic (>1000 lux) light over both weekdays and weekends was measured with wearable light sensors. We found significant differences in average daily light exposure between myopes and non-myopes. On weekends myopic children received significantly less scotopic light ($p=0.024$) and less outdoor photopic light than non-myopic children ($p<0.001$). In myopes, lower refractive errors were correlated with increased time in mesopic light ($R=-0.46$, $p=0.002$). These findings suggest that in addition to bright light exposure, rod pathways stimulated by dim light exposure could be important to human myopia development. Optimal strategies for preventing myopia with environmental light may include both dim and bright light exposure.

5.2 Introduction

Myopia, or nearsightedness, is a significant public health concern in many developed and developing countries. It is estimated that by 2050, approximately 50% of the world population will be affected by myopia (Holden et al., 2016). This raises concerns about a growing population with increased risk of high myopia and associated visually debilitating disorders later in life including glaucoma, retinal detachment, and cataracts. One strategy to combat this rise in myopia is to prevent its onset in childhood. Behaviors that protect against myopia or slow the progression of myopia are of particular interest as they can be implemented at the population level and would not involve pharmaceutical interventions in children.

Behavioral comparisons of children with and without myopia have identified time spent outdoors as a risk factor for both the presence of myopia and the progression of axial elongation [for review (French et al., 2013a)]. The first study to identify this correlation analyzed myopia progression of school children in Finland and found increased time outdoors correlated with decreased progression (Parssinen and Lyyra, 1993; Parssinen et al., 2014). Subsequently, multiple studies confirmed that myopic children spent less time outdoors than non-myopic children or that time outdoors negatively correlated with myopia development (Mutti et al., 2002; Saw et al., 2002; Khader et al., 2006; Jones et al., 2007; Rose et al., 2008b; Rose et al., 2008a; Wu et al., 2010; Jones-Jordan et al., 2011). Multifactorial analyses have suggested that bright light exposure during time outdoors, over other factors like physical activity, was the most likely candidate for driving the protection against myopia (Rose et al., 2008b; Sherwin et al., 2012; French et al., 2013a; Ma et al., 2014b; McKnight et al., 2014).

These findings changed our understanding of how environmental conditions during development could alter the prevalence and severity of myopia. However, most of these studies

relied on questionnaire data, usually answered by parents who could be influenced by recall bias. Objective studies of the association between light exposure, not just time outdoors, and myopia are therefore needed to more comprehensively understand these environmental impacts, and to evaluate what amount of bright light is necessary for myopia prevention. In 2015, Read et al. utilized wearable light sensors (the Actiwatch, a wrist watch style device capable of measuring personal ambient light exposure every 30 seconds) to assess the daily light exposure patterns of myopic and non-myopic children, and to examine the relationship between light exposure and longitudinal changes in axial eye growth (Read et al., 2015). When children aged 10-15 had their light exposure assessed over two, 14-day periods in one year, it was observed that myopic children spent significantly less time in light >1000 lux than non-myopic children, and a significant association between slower axial eye growth and greater time spent in light >3000 lux was found. By using objective personal light exposure measures, in addition to questionnaires, the authors could make more specific conclusions about the timing and brightness of light which might be necessary to slow or prevent myopia progression.

The focus on bright light exposure as a preventative measure against myopia has been well supported by human and animal studies (Ashby et al., 2009; Cohen et al., 2012; Smith et al., 2012; Wu et al., 2013; Karouta and Ashby, 2014; He et al., 2015). In animal models of experimental myopia where single factors are more easily controlled, bright light exposure was also shown to be protective during myopia development (Ashby et al., 2009; Ashby and Schaeffel, 2010b; Siegwart et al., 2012; Smith et al., 2012; Karouta and Ashby, 2014). Importantly, randomized and controlled clinical intervention studies in children have replicated this finding. Children who were assigned to spend daily recess in a gymnasium, getting physical activity without outdoor light, were more likely to develop myopia over a one-year period than

children who spent the same amount of time outside (Wu et al., 2013). Additionally, two clinical trials that administered additional outdoor time to children during the school day showed decreased onset and development of myopia compared to the control groups (He et al., 2015; Jin et al., 2015). However, most of these studies did not use objective methods to assess personal daily light exposure, so we cannot know the full range of light that children were exposed to during the day.

Studies have shown that the portion of the day children spend in bright light (greater than 1000 lux) is relatively small, roughly 1-2 hours on weekdays, even for non-myopic children (Rose et al., 2008a; Dharani et al., 2012; Read et al., 2014, 2015; Verkicharla et al., 2017; Wu et al., 2018). Thus, many hours of each day fit into a general category of "less than bright" exposure including indoor light and dim light. Evidence that dim light exposure, specifically, could be important for refractive eye growth has been found in several animal models. First, a previous study by our group has shown that illuminance levels of 0.005 lux, similar to starlight, can prevent lens-induced myopia in a mouse model (Landis E, IOVS, 2015, 56, ARVO EAbstract, 2152-2152). Additionally, mice with dysfunctional rod photoreceptors have no response to form deprivation myopia compared to wild-type animals, indicating rod driven vision may be essential for detecting the visual input needed for correct refractive eye growth (Park et al., 2014). In animals with foveas, the peripheral retina is dominated by rod photoreceptors compared to the cone-rich fovea and has been implicated in the development of myopia. When myopiagenic inputs are projected to the peripheral retina of rhesus monkeys or chickens, with the central retina either ablated by laser or given normal vision, myopic refractions and elongated axial lengths are observed (Smith et al., 2005; Smith et al., 2007a; Smith et al., 2009; Huang et al., 2011; Liu and Wildsoet, 2011). In humans, myopia progression

is slowed in children treated with contact lenses which reduce peripheral hyperopia (Sankaridurg et al., 2011). However, the relative contributions of the peripheral and central retina are still debated, and it is unknown if these protective inputs are due to rod versus cone stimulation to the retina or optical consequences (Walline et al., 2013; Wang et al., 2016; Zhang et al., 2016b; Zhang et al., 2016a; Ghosh et al., 2017; Bowrey et al., 2017).

The implications that dim light exposure may modulate myopia development through rod activity leads to the question of how much dim light exposure children typically receive and whether the patterns of dim light exposure differ between myopic and non-myopic children. In order to address this gap in knowledge, we used the ROAM study light exposure data set first published by Read et al. to analyze the amount of dim light to which myopic and non-myopic children are typically exposed (Read et al., 2015).

5.3 Methods

5.3.1 Participants and data collection

For this experiment, data was collected as described previously (Read et al., 2014, 2015). Briefly, Actiwatch-2 Activity Monitors (Philips, Nevada, U.S.A) were worn by 102 children between 10 and 15 years of age from the Brisbane area in Queensland, Australia who were enrolled in the Role of Outdoor Activity in Myopia (ROAM) study. The Queensland University of Technology human research ethics committee approved all study procedures before data collection began and all parents and children gave written, informed consent. All participants were treated according to the guidelines set by the Declaration of Helsinki. At the beginning of the study, the refractive error of all children was determined by non-cycloplegic subjective refraction aiming for maximum plus/least minus for best visual acuity. No participant had a

history of ocular disease. All the children exhibited best corrected visual acuity of logMAR 0.00 or better in each eye. Each child was classified as either myopic (average non-cycloplegic spherical equivalent subjective refractive error (SER) from right and left eyes of -0.50 diopters (D) of myopia or more, with at least one eye exhibiting -0.75 D or more myopia) or non-myopic (average SER from right and left eyes between < +1.25 and > -0.50, with neither eye exhibiting -0.75 D or more myopia). Forty children were classified as myopes with a mean SER of -2.39 ± 1.50 D and each myopic child was paired with a non-myopic child (mean SER of 0.34 ± 0.30 D) of the same sex and similar age, who wore the Actiwatch device over the exact same period as the matched myopic child. Classification as either myopes or non-myopes did not change for any of the children throughout the first 12-months of the study. One pair of participants were excluded during follow-up visits due to the development of ocular pathology in one non-myope. Therefore, data from 80 children were included in the final analysis presented here. A small group of older non-myopes (n=20) also participated in the study but wore the Actiwatch device at a different time compared to the matched myopes and non-myopes. This group was excluded from our analyses to ensure that daily and seasonal variations in dim light exposure did not influence the findings.

Baseline ocular measurements were taken between May and November of 2012 with objective measures of light exposure taken by an Actiwatch over the following 14-day period. A second 14-day period of light exposure measurements was conducted six months later. Therefore, light exposure measurements were spread across seasons, and all were collected during school term. Ocular measurements were taken at the initial visit, and six-months later and again one year after the initial measurements were taken.

The Actiwatch is a wristwatch style device that contains a silicon photodiode light sensor capable of measuring visible light within 400 to 900 nm (peak sensitivity 570 nm). The light sensors were programmed to record illuminance data every 30 seconds, resulting in approximately 80,640 measurements per child. The sensitivity of the Actiwatch at dim illuminance levels was measured by comparison to a calibrated luxmeter (Extech HD450 Datalogging light meter; Extech Instruments, USA) across a range of 16 dim (<50 lux) light levels. The Actiwatch sensor showed high agreement with the luxmeter (mean difference 2.1 ± 1.1 lux) indicating a high level of sensitivity for assessing dim light levels (**Figure 5.1**). Each participant completed an activity diary which was used to estimate illuminance during times when the Actiwatch was removed, e.g. for sports practice or bathing. If the watch was removed for over 90% of any day, that day was eliminated from analysis. This resulted in an average of 23.5±0.34 days per participant over both collection periods. Data from both collection periods were included and combined in this analysis as there were no significant differences in light exposure or time spent awake between the two periods. Only data taken during waking hours was used; this was determined by the Actiwatch sleep and wake detection algorithms and the activity diaries (Weiss et al., 2010; O'Hare et al., 2015; Toon et al., 2016). Myopes and non-myopes spent equal amounts of time awake per day (myopes: 14.73±1.75 hrs., non-myopes: 14.62 ±1.63 hrs. t-test; t=0.046, df=78, p=0.9632, **Table 5.1**).

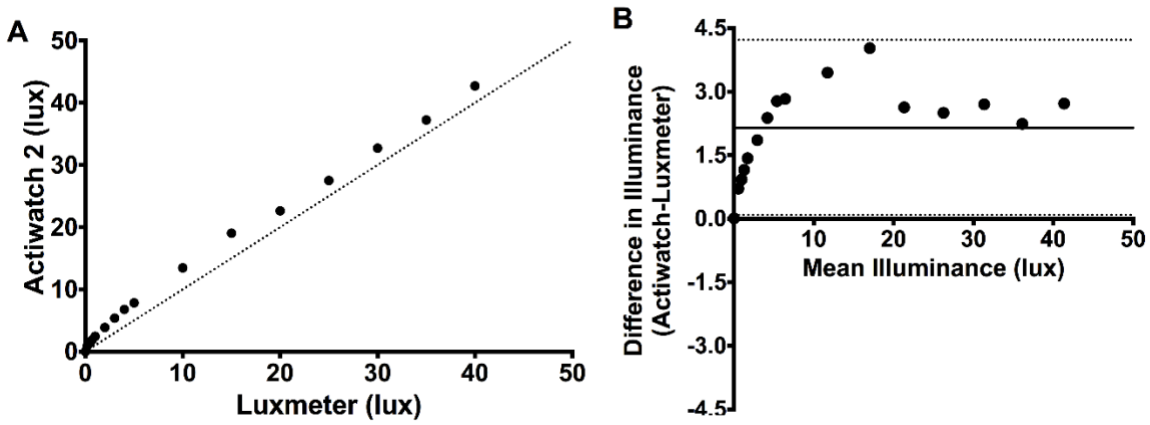

Figure 5.1. Philips Actiwatch 2 accurately detects dim light levels. To determine the accuracy of the Actiwatch 2 at scotopic light levels the lux values of the Actiwatch were compared to a luxmeter (Extech HD450 Datalogging light meter; Extech Instruments, USA). The Actiwatch and luxmeter were placed in the same position in a room (both facing vertically upwards) and the ambient lights were changed through 16 different levels between 0 lux and 40 lux. The Actiwatch took 3 readings at each level which were averaged here. (A) There is a close correlation between the lux readings of the two light sensors (R^2=0.9958). Black dots represent recordings by Actiwatch and luxmeter at each brightness step, line represents an exact match of the values. (B) Differences between the Actiwatch and the luxmeter were found and compared to the mean readings between the two devices. This comparison shows that the Actiwatch overestimated lux by no more than 2.5 lux and that differences were greater at higher illuminance levels. Solid line represents the mean difference between Actiwatch and luxmeter readings, dotted lines represent upper and lower 95% limits of agreement. Figure used with permission from (Landis et al., 2018).

Table 5.1. Demographics and Sleep Patterns of Myopic and Non-myopic Children in the ROAM Study Cohort. All data is shown as mean ± SD.

	Age	Sex, m	Waking time	Sleeping time	Time spent awake (hrs)
All (n=80)	12.97 (±1.39)	40	6:40AM (±1:05)	9:21PM (±1:24)	14.66 (±1.66)
Myopes (n=40)	12.98 (±1.53)	20	6:51AM (±1:10)	9:35PM (±0:57)	14.73 (±1.75)
Non-myopes	12.95 (±1.26)	20	6:32AM (±0:59)	9:09PM (±1:39)	14.62 (±1.63)

5.3.2 Data Analysis

All data cleaning and analysis was done using R and R Studio (https://cran.r-project.org) or Python for SPSS (New York, U.S.A). Once recordings had been cleaned for missing data as described above and measurements taken during sleeping hours were eliminated, the light exposure data for each subject was binned into four different light intensity levels; scotopic light (<1-1 lux), mesopic (>1-30 lux), indoor photopic (>30-1000 lux), and outdoor photopic (>1000 lux). These light bins were chosen to provide an overview of the children's habitual light exposure patterns across a variety of conditions, with a particular focus on the dimmer end of the light exposure spectrum. This also allowed us to isolate the light that would be activating rod pathways in the retina. To analyze the typical light exposure patterns of myopic and non-myopic children across the day, the time in each light level per half hour after waking each day was averaged for both groups of participants across each of the four light levels. Total time spent in each light level each day was averaged for myopes and non-myopes and compared. For these analyses, data was also divided into weekdays (Monday-Friday) and weekends (Saturday-Sunday) to reflect the differences in behavior across the week. Significant differences in these patterns were assessed using ANCOVAs with Tukey post-hoc comparisons, treating time as a 3rd order covariate due to its nonlinear relationship with light over the day. Significance for these comparisons was set at $p<0.03$ (Benjamini and Hochberg, 1995). All reported interactions with time (refraction x time or time x day) are on the quadratic term. Correlations between refractive error in myopes and non-myopes and the time spent in each light bin per day were determined through Pearson correlation analysis. The refractive error of myopic participants in this study were not normally distributed. Therefore, in myopes these correlations were performed on refractive error transformed by taking the cube root.

5.4 Results

5.4.1 Non-myopic children spend more time in photopic and scotopic light on weekends

We examined the periods of day when exposure to each light level occurs for myopic and non-myopic children and found significant differences in all light levels across the week and with refractive status (**Figure 5.2**). The pattern of light exposure throughout the day was different for each of the four light levels. Scotopic light exposure primarily occurred in the hours before bed and immediately after waking, discrediting the possibility that this time is a false recording from the Actiwatch being covered by clothes or other items throughout the day. Myopic children received significantly less scotopic light during weekends than non-myopic children [ANCOVA, $F(1,53) = 5.38$, $p = 0.024$, **Figure 5.2A**], with exposure during evening hours showing the largest differences (approximately three hours before falling asleep). In addition, scotopic light exposure for non-myopes was increased on weekends compared to weekdays [$F(1,53) = 16.58$, $p<0.001$]. Non-myopes spent less time in mesopic light on weekdays than on weekends [$F(1,53) = 16.91$, $p<0.001$, **Figure 5.2B**]. However, on weekends myopes generally spent more time in mesopic light than non-myopes [$F(1,53) = 6.09$, $p=0.017$]. Indoor photopic light was increased in both groups on weekdays compared to weekends [myopes: $F(1,53) = 10.90$, $p=0.002$, non-myopes: $F(1,53) = 47.10$, $p<0.001$, **Figure 5.2C**]. Outdoor photopic light was most prevalent in the middle of the day, corresponding with breaks in the school day or after school. Consistent with previously published findings, myopic children were exposed to less outdoor photopic light on weekends than non-myopic children [ANCOVA, $F(1, 53) = 60.76$, $p<0.001$, **Figure 5.2D**].

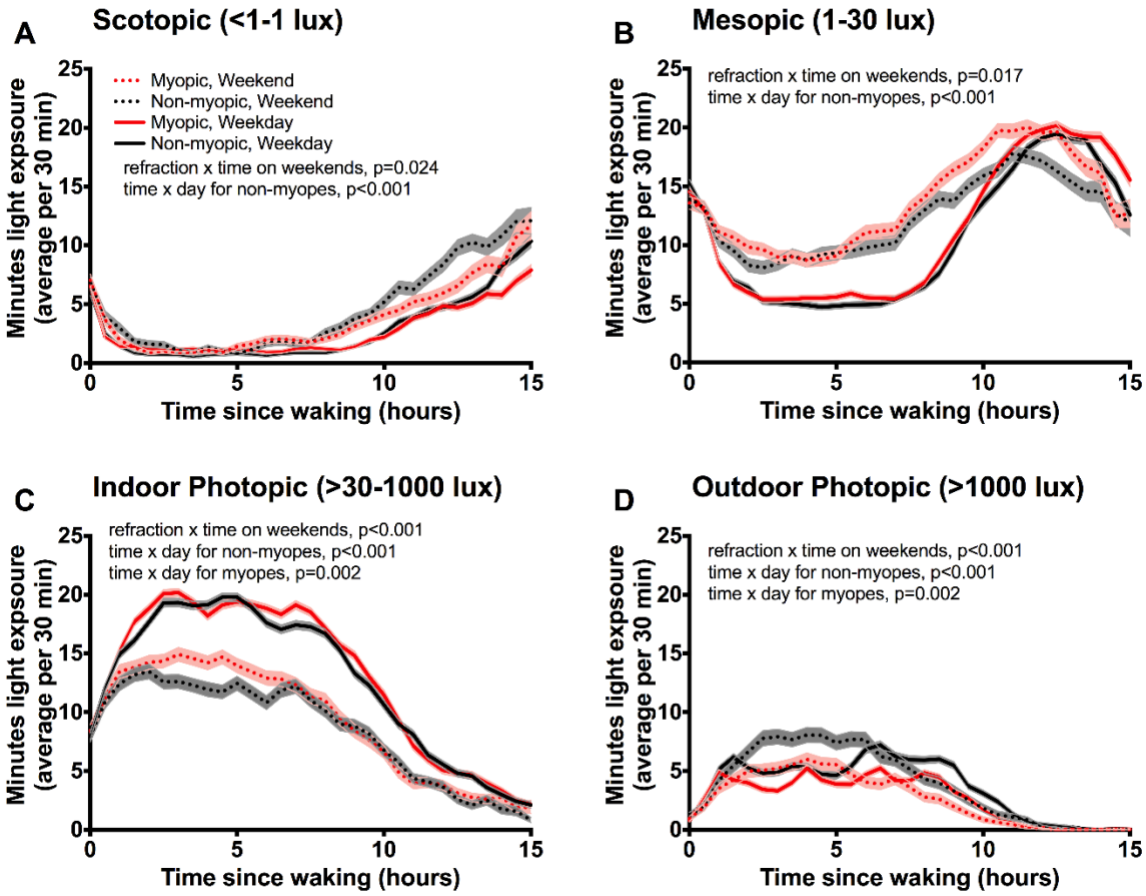

Figure 5.2. Patterns of daily light exposure across four intensity levels show differences in both dim and bright light as well as differences in behavior across the week. (A-D) Light exposure patterns of myopic (*red*) and non-myopic (*black*) participants were mapped across the average 15 hours of awake time per day for each level of illuminance then divided by weekday (Cavallotti et al.) or weekend (*dotted*). (A) Recordings of scotopic light were observed immediately after waking and in the hours before bedtime. Myopic children received significantly less scotopic light during weekends than non-myopic children [ANCOVA, $F(1,53) = 5.38$, $p = 0.024$]. Scotopic light exposure patterns in non-myopes were higher on weekends compared to weekdays [$F(1,53) = 16.58$, $p<0.001$]. (B) Mesopic light peaks in the evening then drops off approaching the average bedtime. Non-myopes spend more time in mesopic light on weekends compared to weekdays [$F(1,53) = 16.91$, $p<0.001$]. On weekends, myopes generally spend more time in mesopic light than non-myopes [$F(1,53) = 6.09$, $p=0.017$]. (C) For each refractive group, exposure to indoor photopic light was significantly higher on weekdays compared to weekends [myopes: $F(1,53) = 10.90$, $p=0.002$, non-myopes: $F(1,53) = 47.10$, $p<0.001$]. On weekends, myopes have significantly more indoor photopic light exposure [$F(1,53) = 14.32$, $p<0.001$]. (D) Outdoor photopic light was highest in midday. Both myopes and non-myopes received more outdoor photopic light on weekends than on weekdays [myopes: $F(1,53) = 8.41$, $p=0.005$, non-myopes: $F(1,53) = 20.39$, $p<0.001$]. On weekends, non-myopic participants have significantly more outdoor photopic exposure than myopic ones [$F(1,53) = 60.76$, $p<0.001$]. P-values shown in graphs represent significant interaction effects. Black lines represent non-myopes, red lines represent myopes, and dashed lines represent weekends, data shown as mean ±

SEM minutes of all subjects (n=40/group) in bins of 30 minutes. Figure used with permission from (Landis et al., 2018).

We also found a significant difference between myopes and non-myopes in the average daily light exposure in each light level summed across waking hours [multivariate ANOVA $F(4, 77) = 3.87$, $p = 0.006$; Wilk's $\Lambda = 0.83$, partial $\eta 2 = 0.17$, **Figure 5.3**]. During weekdays myopes spend significantly more time in mesopic light than non-myopes (myopic: 5.56±0.22 hrs, non-myopes: 5.16±0.16 hrs, p=0.001; **Figure 5.3B**). The differences between myopes and non-myopes did not reach significance for the other light levels during weekdays. However, there was a trend for myopes spending less time than non-myopes in outdoor photopic light (1.35±0.09 hrs of waking time vs. 1.85±0.10 hrs, p=0.08; **Figure 5.3D**). On weekends, myopes spend significantly more time in mesopic light (myopes: 6.40±0.25 hrs, non-myopes: 5.75±0.21 hrs, p<0.001, **Figure 5.3B**) and less time in outdoor photopic light than non-myopes (myopes: 1.27±0.15 hrs, non-myopes: 1.93±0.21 hrs, p =0.008, **Figure 5.3D**). The exposure to each light level was also different between weekdays and weekends. Both myopes and non-myopes spend more time in mesopic light on weekends compared to weekdays (myopes, p<0.0005, non-myopes, p<0.0005; **Figure 5.3B**) and less time in indoor photopic (myopes, p<0.0005, non-myopes, p<0.0005, **Figure 5.3C**). However, only non-myopes spend more time in scotopic light on weekends compared to weekdays (p=0.026, **Figure 5.3A**). Furthermore, the additional amount of time non-myopes spent in scotopic light on the weekends was less than the additional amount of time spent in outdoor photopic light. Thus, non-myopes are spending more time in dim light outside of school hours.

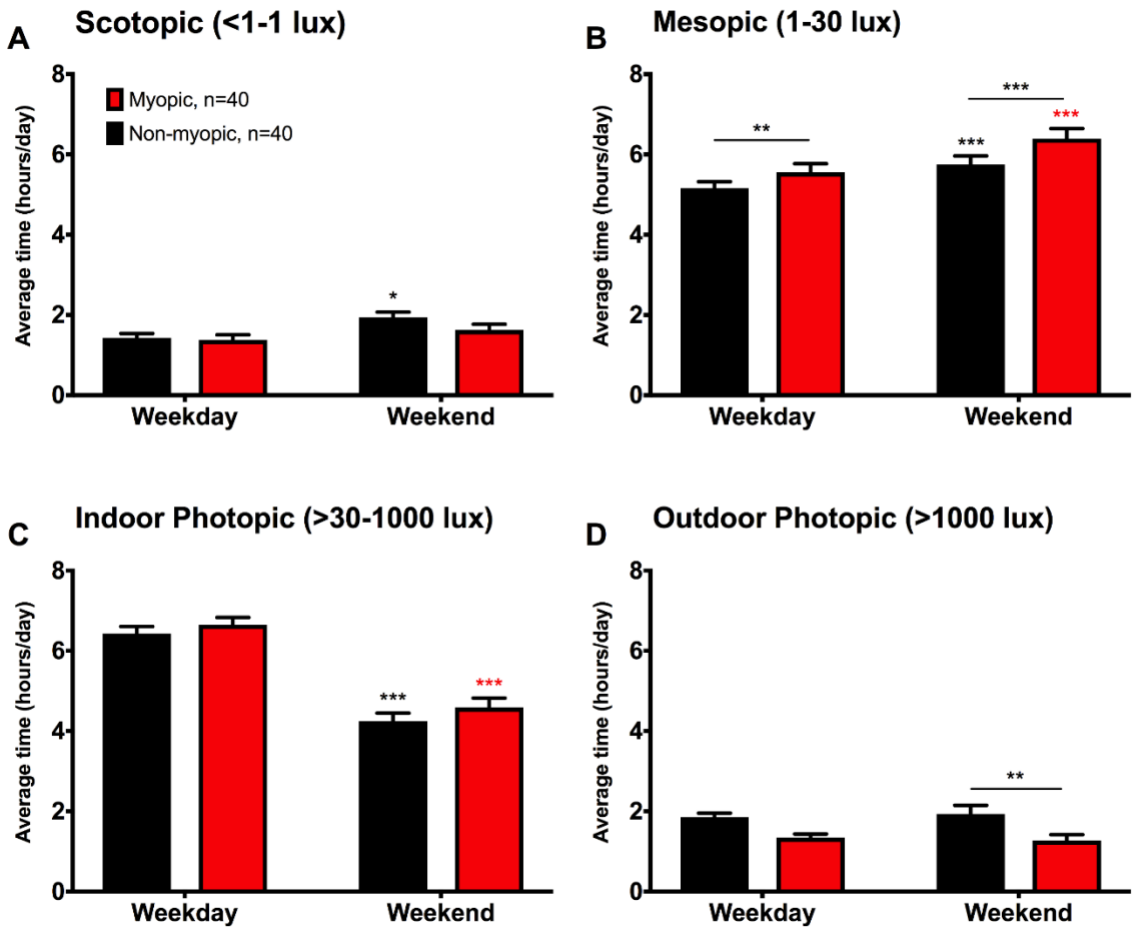

Figure 5.3. Myopes spend less time in outdoor photopic and more time in mesopic light. Recordings of light intensity exposure during all waking hours were binned into four light levels and compared across myopic (*red*) and non-myopic (*black*) children for weekdays and weekends. Data shown are the mean ± SEM of time spent in each light level per 15-hour awake period. (A) Non-myopes spend more time in scotopic light during the weekend compared to the weekdays (p=0.026). (B) On weekdays and weekends, myopes spend more time in mesopic light than non-myopes (p=0.001, p<0.001). Both myopes and non-myopes spend more time in mesopic light on weekends compared to weekdays (p<0.001). (C) Both groups spend less time in indoor photopic light on weekends (myopes: p<0.001, non-myopes: p<0.001). (D) On weekends, myopic children spent significantly less time in outdoor photopic light (p=0.008). Asterisks above lines represent differences in refractive status groups, asterisks directly above bars represent differences across days, *p<0.05, **p<0.01, ***p<0.001. Figure used with permission from (Landis et al., 2018).

5.4.2 Increased time in outdoor photopic light is correlated with less severe refractive errors in myopes.

An important consideration when developing recommendations for light exposure to prevent myopia is the relationship between the amount of time spent in different light levels and refractive error. The average of the initial refractive error measurements at the beginning of the study and the refractive measurement collected one year later was compared to the amount of time the children spent in each light intensity bin during the day (**Figure 5.4**). No significant association between light and refractive error was found for the non-myopes across all four light bins, including non-myopes that spend little time in outdoor photopic light. In this data set, non-myopes had a very small range of refractive error but the range of time in each light level matched that of the myopes. Lower daily outdoor photopic light exposure was significantly correlated with more myopic refractive errors in myopes (R=0.33, p=0.005, **Figure 5.4D**). The correlations between time in both scotopic and outdoor photopic light and refractive error showed similar patterns (scotopic: slope: 0.007, R=0.10, p=0.43, photopic: slope: 0.009, R=0.33, p=0.005). As expected, myopes showed a significant negative correlation for time in mesopic light (R=-0.46, p=0.002, **Figure 5.4B**) such that myopes who spent more time in mesopic light had more severe myopia. Indoor photopic light had no relationship to refractive error in myopes or non-myopes.

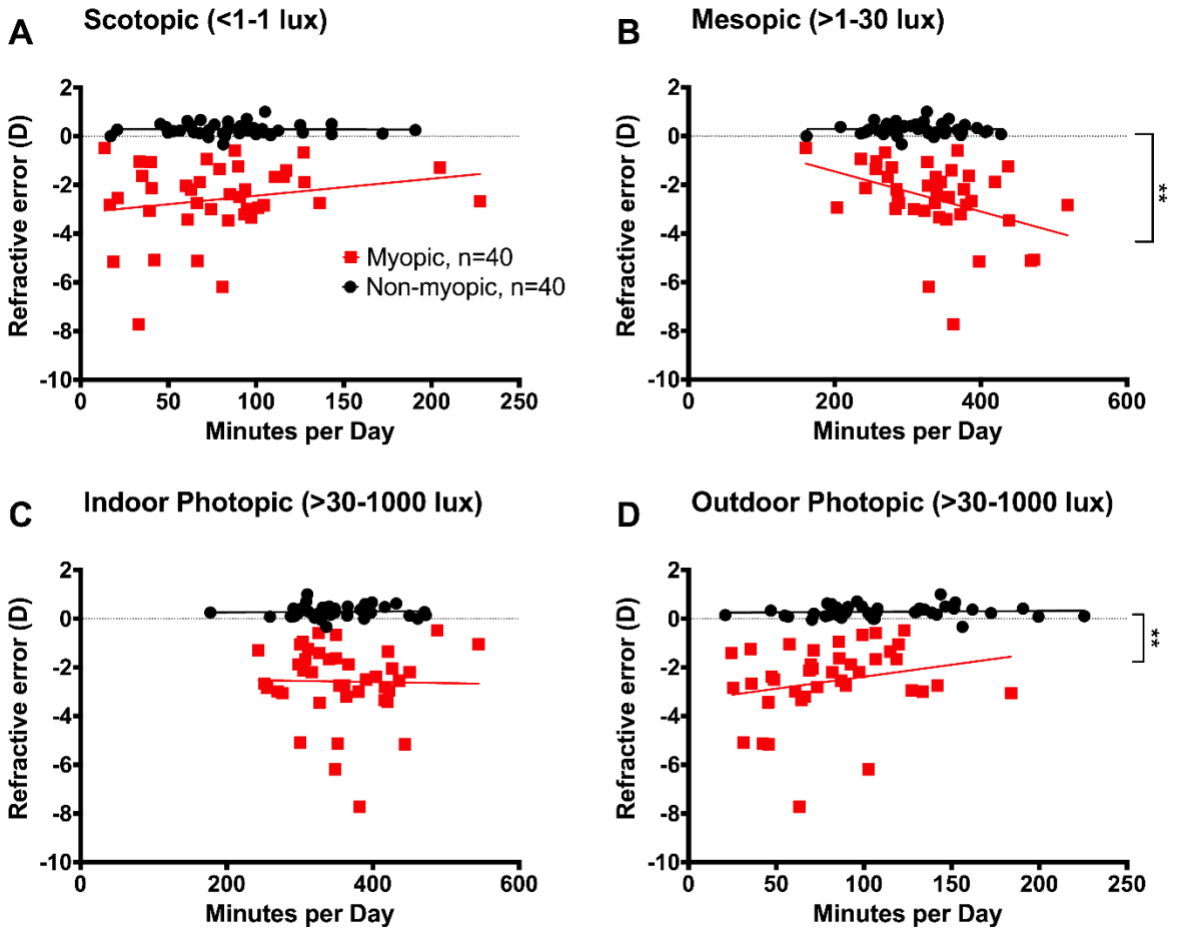

Figure 5.4. Significant correlation of mesopic and outdoor photopic light exposure and refractive errors of myopes. (A) Time spent in scotopic light was not significantly associated with refractive error in myopes (*red*) or non-myopes (*black*). (B) Increased time in mesopic light was significantly correlated with more severe myopia (R=-0.46, p=0.002). (C) Indoor photopic light exposure times were similar in myopes and non-myopes and were not significantly associated with refractive error. (D) The time participants spent in outdoor photopic light was significantly associated with the refractive error of myopes (R=0.33, p=0.005). Each point represents an individual participant. Figure used here with permission from (Landis et al., 2018).

5.5 Discussion

The data presented here demonstrate that myopic children spent less time in both scotopic and outdoor photopic light conditions compared to non-myopes. While the association between less bright light exposure and myopia was expected based on previously reported studies in both humans and animal models, a novel finding here was the significantly greater scotopic light exposure in the non-myopic children. This suggests a potentially protective effect of both dim and bright light exposure in myopia development and potential myopiagenic effects of mesopic and indoor photopic light. Here, children spent more time in dim light levels than we had hypothesized, roughly equal to the amount of time spent in outdoor photopic light. Therefore, the potential of dim light exposure as a prevention approach in myopia should be considered in further studies.

The retinal signaling mechanisms that would underlie the protection of myopia by bright and dim light are likely different given the differences in photoreceptor activation under such a broad range of illumination. Previously, no studies of human light exposure and myopia have directed attention to rod dominated light levels. Here, we find that dim light, and potentially rod signaling mechanisms in the retina, could be playing a role in human myopia development, as shown in animal studies of myopia (Landis E., IOVS, 2015, 56, ARVO EAbstract, 2152-2152; (Park et al., 2014). Cones, and potentially melanopsin photoreceptors, would be stimulated under bright light (Lall et al., 2010; Tikidji-Hamburyan et al., 2017) and may initiate signaling cascades that play a role in myopia prevention in bright light (Zhang et al., 2008; Qiao et al., 2016). Most investigations of time outdoors and the impact of light on refractive error in animal models have shown that increased dopamine activity triggered by bright light protects from induced myopia (Feldkaemper and Schaeffel, 2013). This mechanism might explain how

children who spend more time in bright light are protected from myopia. Potential protection from myopia under dim, scotopic light is likely through a different retinal signaling mechanism. Whether this mechanism also utilizes dopamine signaling is unclear but possible given the demonstrated connection between rod photoreceptors and dopamine release (Herrmann et al., 2011; Zhao et al., 2017).

The daily patterns of light exposure on both weekdays and weekends were similar across each of the light levels between myopes and non-myopes, even though the amount of time in each light level differed. One possible explanation for increased scotopic light at night could be the use of electronic devices before bed. Studies indicate that the use of tablets and cell phones before bed is very common in teenage children and that scotopic light from these devices could disrupt the circadian rhythms of participants (Van den Bulck, 2007; Gradisar et al., 2013). However, the lack of differences in the general daily patterns of light exposure between refractive groups along with the lack of significant differences in awake/sleep behavior would suggest that it is unlikely that there were differences in circadian rhythms associated with refractive error in this study. There is increasing evidence from animal models that circadian rhythms may play a role in myopia (Chakraborty et al., 2018b). Circadian rhythm genes have been implicated in myopia and the chick model has shown that the timing of both light exposure and lens defocus are important factors in myopia development (Stone et al., 2013; Nickla and Totonelly, 2016; Stone et al., 2016; Nickla et al., 2017a; Nickla et al., 2017b). It is possible therefore that any changes in circadian rhythms that may lead to refractive error in humans may be subtler than what could be detected by the measurement methods and sample of subjects examined in the current study. It is also possible that myopes experienced altered circadian rhythms at an age outside of the study dates.

The analysis used here differed in two ways from the analysis originally published; the number of children included in the final analysis differs from the Read et al. (2015) analysis and the waking hours were determined by activity recordings from the Actiwatch whereas the original ROAM study analyzed readings from 6:00AM to 6:00PM. While these changes made slight differences in the mean time of light exposure, the major finding that myopes spend significantly less time in light >1000 lux than non-myopes was replicated. A limitation of the study is the position of the Actiwatch on the wrist instead of near the eye; however, previous reports of wrist-worn light sensor recordings have correlated strongly to eye-level light sensors except in late evening and at night, when the authors suspected bedding covered the wrist but not the eye-level sensor (Okudaira et al., 1983). This potential underestimation due to the participant's position in bed is a possible confounding factor in the findings presented here as well. However, the elimination of data collected while participants were sleeping, as measured through analysis of the physical activity data with validated algorithms (Weiss et al., 2010; O'Hare et al., 2015; Toon et al., 2016), likely mitigates this complication. This limitation also raises the question of whether increased time spent in scotopic light was simply a reflection of decreased time spent in mesopic light, which would be protective against myopia. To investigate this possibility, total time spent in each light level was univariately correlated to time spent in scotopic light. This analysis showed that time spent in scotopic light was associated with less time in indoor photopic light only, indicating no significant connection between scotopic and mesopic light (**Figure 5.5**). The thresholds between the light levels used here were chosen based on similar studies in the case of higher intensity light exposure and on the ability of the Actiwatch to detect dim light in the case of the scotopic light threshold, set at 1 lux. The slight overestimation of light by the Actiwatch compared to a calibrated Luxmeter for dim light levels

(**Figure 5.1**) would indicate the scotopic threshold might be more stringent than is implied. The use of more accurate light sensors that provide better estimates of illuminance and spectral content of light at the plane of the eye in future analyses of dim light exposure is likely to provide a more comprehensive understanding of exposure patterns. Future analyses should also include cycloplegic refraction of study participants. The ROAM study included non-cycloplegic refractions which are known to be less reliable than cycloplegic, creating a risk of misclassification of refractive error groups (e.g. non-myopic participants with undetected hyperopia). However, all myopic participants had previously been diagnosed with myopia and wore corrective lenses at the time of the study. Additionally, none of the participants changed between refractive groups during the first 12-months of the study, indicating consistency in their refractive classification over the course of the study.

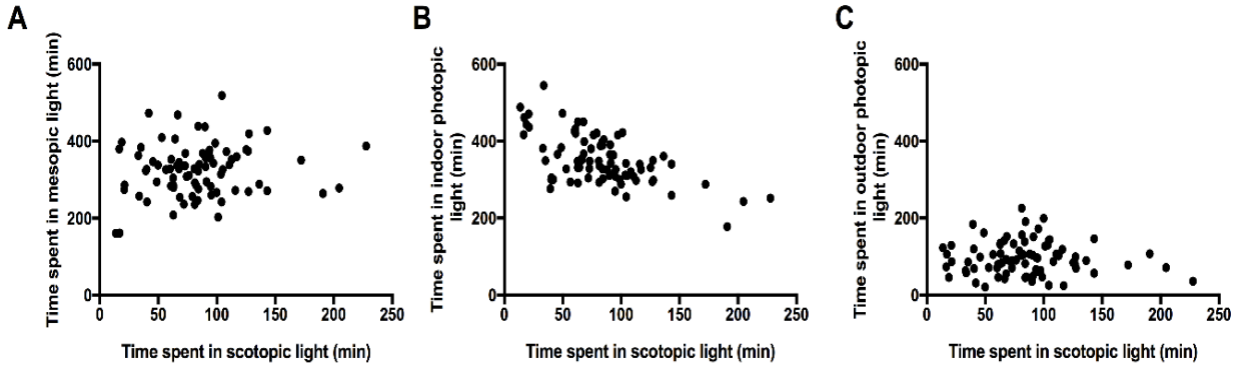

Figure 5.5. Time spent in scotopic light is associated with time spent in indoor photopic light. To determine if effects of light exposure at one level might be driving any effects seen at other light levels, correlations between time spent in scotopic light and the other light levels were examined. While a significant correlation exists between scotopic light and indoor photopic (B; $R=-0.646$, $p<0.001$), we found no relationship between scotopic exposure times and time in either mesopic (A) or outdoor photopic (C) light. For this analysis, Actiwatch data from all days were averaged for each subject and pooled. Figure used here with permission (Landis et al., 2018).

Another limitation of this study is the relatively small sample size. With a larger population of children, it is possible that the differences in time spent under different levels of light would increase. Larger studies could aim to explore the behaviors of younger children; here, participants were between ages 10-15, and many were already myopic. By investigating light exposure in younger children, we might be able to determine what type of light exposure and signaling in the retina precedes the development of myopia.

We recommend that future studies on light exposure during refractive development in childhood include an analysis of dim light, especially in studies of younger children with larger populations. Since the findings reported here do not directly assess the effectiveness of light to prevent myopia through intervention, future studies may also be designed to establish causation. Finally, only data from waking hours was analyzed here; however, it is possible that light exposure while sleeping could also play a role in myopia development and progression (Quinn et al., 1999).

These findings, shown here for the first time in the human eye, are consistent with reports in animal models of myopia that have also demonstrated scotopic light exposure can be protective against myopia development. The results support a potentially key role for rod-signaling in myopia development. Although the exact mechanisms underlying these findings remain unknown, early work in animal models could suggest different mechanisms across the light intensity range presented here depend on the specific pattern of photoreceptor activation. Therefore, these results provide a catalyst for future research to investigate the role of rod photoreceptors in myopia development.

CHAPTER 6: Conclusions

6.1 Summary of results

Over the last several decades, rates of myopia in developed nations have increased dramatically (Holden et al., 2016). In the United States myopia affects almost half of the population and rates in Asian countries reach over 90% in some studies [(Vitale et al., 2009; Pan et al., 2012b), for review (Morgan et al., 2012)]. The effect of increased myopia has been an added burden to health care systems world-wide and lowers educational and economic achievements both personally and nationally (Fricke et al., 2012; Holden et al., 2014; Fricke et al., 2018). These issues are not likely to diminish soon as the rates of myopia in many developed countries are expected to increase. By 2050 an estimated half of the world's population will be myopic (Holden et al., 2016). Despite growing concern about the rates and effects of myopia, the mechanisms behind myopic eye growth and refractive development broadly are still unknown.

Investigations to determine why rates of myopia have increased in recent decades have identified time spent outdoors and particularly bright light exposure, like sunlight, as protective against myopia. Children who spend more time in bright, outdoor light are less likely to be myopic and show slower progression [for review (French et al., 2013a)]. These findings have been replicated in many study populations crossing age, ethnicity, and geographic location. Recently, studies have begun to test bright light exposure as an intervention technique to prevent myopia development (Wu et al., 2013; He et al., 2015; Jin et al., 2015). As an easily accessible and low-cost treatment, bright light has the potential to stem the rising tide of myopia prevalence. However, these recent studies would indicate that bright light interventions do not prevent myopia in all participants and the timing, intensity, and color of light may be important. To determine the most effective use of light to prevent myopia, a better understanding of how

visual experience guides ocular and refractive development is needed. The work described in this dissertation aims to determine how a wide range of ambient light intensities encountered in everyday life could impact myopia development and how DA, a stop-signal in myopia, could be involved in the retinal signaling behind light-based myopia protection.

Based on evidence supporting bright light as an anti-myopia signal, and evidence that rod photoreceptors are necessary for normal refractive development, I tested the effect of housing mice in bright (photopic), intermediate (mesopic), and dim (scotopic) light on myopia susceptibility (CHAPTER 2). Each ambient light level was chosen based on the receptive ranges of rod and cone photoreceptors. Scotopic light activated rods, mesopic light triggered mixed combination of rods and cones, and cone photoreceptors stimulated cones while saturating rods. By stimulating each photoreceptor type, the downstream retinal pathways could be isolated as well. Across several cohorts, scotopic and photopic light prevented the effect of lens defocus and protected the treated eye from becoming myopic (**Figure 6.1**). While the protection from myopia under photopic light was expected based on other animal studies [for review (Norton and Siegwart, 2013)] protection from myopia with scotopic light was a novel finding. Protection from both sides of the light intensity spectrum indicate the importance of a broad range of ambient light in ocular development.

Studies of myopia in animal models have shown that increased DA and DAergic activity can prevent myopic eye growth [(Stone et al., 1989), for review (Feldkaemper and Schaeffel, 2013)] and that using bright light to increase DA activity in the retina has been protective in other models of myopia (McCarthy et al., 2007; Ashby et al., 2009; Ashby and Schaeffel, 2010b). For the second part of this work, I investigated DA signaling in the retina after ambient light housing and lens defocus to determine if increased DAergic activity was driving myopia

prevention in photopic or scotopic light (CHAPTER 3). Testing DA and proteins related to synthesis, packaging, uptake, and degradation revealed an interaction between ambient light housing and lens defocus such that while DA levels subtly increased across treatment groups, the protein and gene expression levels and the response to light stimuli were altered by both lens defocus and light (**Figure 6.1**). While these findings need to be replicated, they indicate an effect not only of light on DA signaling and myopia susceptibility but a more complicated response to lens defocus than has previously been shown.

To gain a better understanding of DA in myopia susceptibility, two approaches were pursued to test how endogenously increased retinal DA and signaling could affect the response to form deprivation (CHAPTER 4). With systemic injections of L-DOPA, mice with FDM did not show myopic eye growth, indicating protection from FD. Interestingly, mice with low DA levels due to retinal degeneration (*rd10*) showed no effect of L-DOPA but protection from AA. For the second approach, I used a transgenic VMAT2 HI mouse, which overexpresses VMAT2 to determine if genetic changes to DA signaling could prevent FDM. No differences were found between the VMAT2 HI and VMAT2 WT mice across any of the parameters tested. I hypothesize that either the VMAT2 overexpression is not complete in the retina in these mice, or that with higher levels of VMAT2 from birth the animals adapt and are not protected from FD later in life.

With evidence that dim light was protective against myopia development in the mouse model, I was interested in whether or not this finding was clinically relevant. In the final study included in this dissertation, I collaborated with the lead author of the Role of Outdoor Activity in Myopia Study to study the light exposure patterns of a group of myopic and non-myopic children. By analyzing the light each participant was exposed to while awake, we found that

myopes spent less time in both outdoor photopic (>1,000 lux) and scotopic (<1 lux) light on weekends than non-myopes. All children spent more time in scotopic light than was previously expected. Future studies of light exposure in myopic children should include an investigation of dim, scotopic light in order to fully understand the range of light which may be protecting non-myopes from refractive error.

To conclude this dissertation, I will discuss the relevance of these studies to the field of myopia research, potential next steps for continuing this work, and possible clinical implications for my findings.

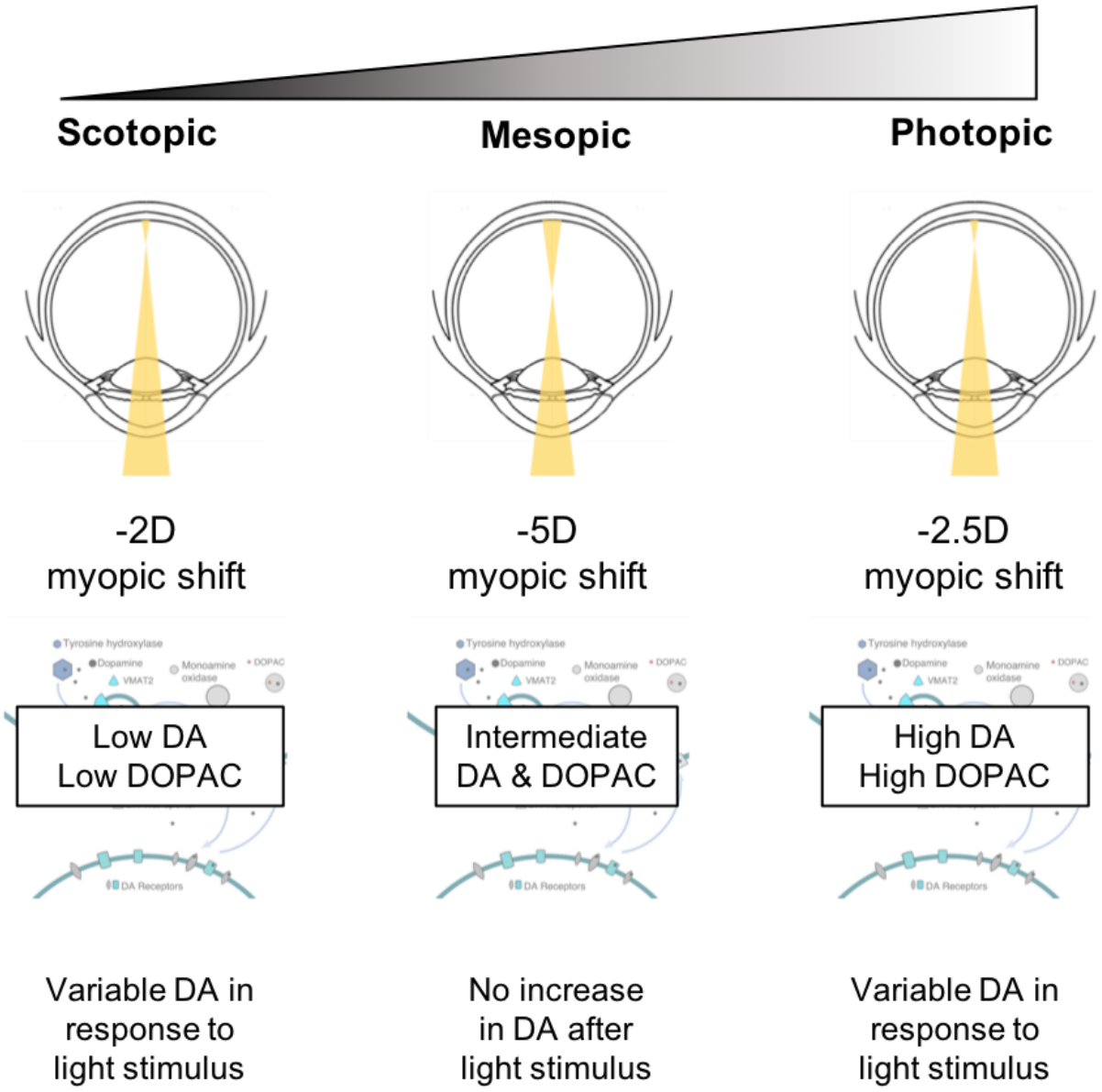

Figure 6.1. Effect of ambient light on myopic response to lens defocus and DA signaling. The experiments described in this dissertation have shown that when housed in either scotopic or photopic light, mice treated with lens induced myopia (LIM) show only a small response to the defocus, resulting in a small myopic shift. Mice housed in mesopic light during LIM develop a significant myopic shift relative to the control eye in the same time period. Further studies examining the role of DA on protection from myopia in scotopic and photopic light revealed very different DA signaling conditions across treatment groups. With HPLC, each light level showed different levels of DA and DOPAC in the retina. Later, experiments with light stimuli to measure extracellular DA indicated that with LIM a dysfunction occurred in mice housed in mesopic light such that no additional DA was found after light exposure. Scotopic and photopic housed mice showed more varied responses. Together these findings indicate an effect of both light and lens defocus on DA signaling in the retina. Original image by author.

6.2 Innovating techniques in myopia research

A major goal of this dissertation was to use and establish techniques not commonly used in myopia research. The use of the mouse model for myopia research is the first innovative technique induced here. Though mouse research is very common in other fields including retinal neurobiology, it has only recently been added to the many varied animals used in myopia research [for review (Pardue et al., 2013)]. By using a mouse model to study myopia, I have been able to take advantage of transgenic models like the VMAT2 HI mouse, available assays for mouse tissue like antibodies for western blots, as well as the research done on the mouse retina from other fields. A common criticism of using a mouse model in myopia is that the mouse is behaviorally nocturnal. Despite this trait, there is no evidence that nocturnal behavior would alter refractive development or myopia susceptibility.

A challenge to using mice in myopia research has been the resolution of devices used to measure eye size. The optical coherence tomography (SD-OCT) used in these experiments has a resolution below that of our expected changes to axial length; the resolution of the SD-OCT is 4.1 ± 2.3 μm and the average size of the mouse eye is 3mm with expected changes of approximately 5μm per 1D of refractive change. Therefore, it is unlikely that the characteristic myopia trait of elongated eyes will be consistently detectable in these experiments until higher resolution systems are developed.

Overall, mice are a good model for human myopia as they respond to light similarly and their use may elucidate important aspects of retinal neurobiology in myopia. Many of the molecular assays used in this dissertation are not commonly found in other studies of myopia. Previously, HPLC for measuring DA and DOPAC was the most common measurement of DA activity in the myopic retina [for review (Feldkaemper and Schaeffel, 2013)]. While agonists and

antagonists of DA receptors have been used in many studies, a thorough investigation of activity in DACs has not been done in myopia. Additionally, changes to DA and DOPAC seen by HPLC can be caused by a number of changes to DA related proteins making it possible that experimental groups with similar DA levels would have different signaling mechanisms driving that homeostasis. In this dissertation, I have hypothesized that these DA related proteins in DACs play an important role in the DAergic mechanisms behind refractive error development; therefore, using novel techniques to study these proteins was critical.

Each protein related to DA signaling in DACs can be modified in expression, localization or activity to impact DA dynamics in the retina. In order to investigate DA signaling more closely, I have utilized ddPCR and western blots. These techniques, though relatively common in other fields, have not been widely utilized in myopia research. This is likely due to the broad range of animal models used to study myopia. Many of the necessary molecular tools for PCR and western blots including antibodies are manufactured for research in mice, making their application for other animals, like the chick, difficult. However, similar techniques to measure DA related proteins which could be used across species, are still not employed. This indicates an assumption that HPLC measurements of DOPAC adequately represent DA activity; I hope that the work I present here will help to dispel that assumption and prompt a more careful examination of under-utilized techniques to measure DA activity.

A novel assay developed here to measure the activity of DA related proteins through extracellular DA after a light stimulus demonstrates the importance of measuring protein activity, in addition to expression. With this eye cup perfusion technique, I was able to find differences in how LIM and control retinas responded to light which would not have been discovered with the other techniques used here or with HPLC alone. While this technique needs

to be validated further, it has great potential to measure the effect of environmental conditions, myopia, and pharmacological intervention on DA signaling in the retina. Other studies of the role of DA in myopia, should look more closely at including measures of protein activity, such as this assay, to fully understand DA dynamics in myopia.

A final aspect of protein activity which was not studied here was localization. The careful localization of DA related proteins in DACs is important for their proper function. For example, TH and VMAT2 are often localized together outside vesicles to ensure rapid loading of DA and prevent cytosolic toxicity (Cartier et al., 2010). Immunocytochemistry of the retina would indicate if in addition to the gene expression, levels, and activity of DA related proteins, ambient light affected their localization within the cell. This information might illuminate the effect of each protein on myopiagenic signaling.

Finally, previous reports on the effect of bright light in myopia have focused on differences between bright and what many refer to as dim light, but which is actually mesopic, intermediate light. By establishing the light intensity spectrum as a dichotomy, the myopia field has found support for the protection of photopic light and myopiagenic effects of mesopic light. Unfortunately, this does not capture the full spectrum of visual experience and ignores the importance of rod photoreceptor activation in myopia development. For the first time, this investigation has studied the role of dim, scotopic light in myopia. Here, evidence shows scotopic light does play a role in myopic eye growth by interacting with lens defocus to disrupt DA signaling.

Future studies of myopia and ambient light should include a full light intensity spectrum to mirror the true visual experience and study the effect on DA dynamics. For many experiments, this would mean including a scotopic light exposed group as was done here. Other experiments

could examine the role of scotopic light as an anti-myopiagenic factor by exposing FDM animals to brief periods of scotopic light as was done in early investigations of photopic light. It will also be important to identify the range of light at which each of these results are occurring. Preliminary data not included here has shown that housing in dim mesopic light (3 lux) was not as myopiagenic as mesopic light, but also not as protective as scotopic light resulting in an intermediate level of protection from LIM. It is possible that there are not defined cut-offs between protective and non-protective lighting but a spectrum; investigating more ambient light conditions will answer this question. Pharmaceutical intervention with DA receptor agonists and antagonists in animals housed in scotopic light could also identify the downstream effects of DA under scotopic light. The use of scotopic light in clinical studies of light exposure is discussed later in this chapter. Ultimately, it will be important that the work here is replicated and continued in follow-up experiments.

6.3 Potential retinal mechanisms for light driven protection from myopia

The experiments described here outline the potential for a broad range of environmental light to impact the development of the eye and myopia (**Figure 6.2**). While there are several examples of environmental factors which influence neurological development, few have the potential to impact so many individuals, or be as easily implemented. If specific levels and periods of light exposure can prevent or slow the progression of myopic refractive error, it will greatly decrease the number of individuals affected by myopia and prevent the later-in-life risks associated with high myopia. However, though both animal and human studies of myopia have shown the potential for prevention by light, the mechanisms driving this protection are largely

unknown. Here, I discuss two cell types with potential roles in light-driven protection from myopia.

6.3.1 Potential role of rod photoreceptors across light levels

Rod photoreceptors, expressing rhodopsin, were traditionally thought of as dim light detectors. They can detect single photons but become saturated at higher light intensities; their activity while saturated has been largely ignored. In these experiments, rods were stimulated by housing mice in scotopic light while cones were isolated by housing mice in photopic light which saturated rods. The role of DA activity after activation of each retinal pathway could then be investigated.

However, some studies of rod photoreceptors under bright light have shown that they are more responsive than previously thought. First, there is a growing body of evidence that rods do not completely saturate in bright light conditions (Yin et al., 2006) and can mediate physiological function at a wide range of light intensities (Demontis et al., 1993; Altimus et al., 2010). Mice with retinal cone deficiencies have shown rod responses to bright light (Naarendorp et al., 2010) including a study of contrast sensitivity with bright background illumination which showed that rod driven contrast sensitivity recovered after several minutes of bright light exposure (Tikidji-Hamburyan et al., 2017). The mice in this experiment were exposed to bright light for 12 hours daily, however, these experiments indicate there may be an adaption to bright light occurring which would allow rods to play a role in LIM even under bright light. Rod photoreceptors may be responsive to both the dim and bright ambient lighting in these experiments and would perhaps drive physiological functions previously ascribed to cone pathways.

DA is released through ON pathway stimulation, meaning rod photoreceptors play an important role in DA signaling (Boatright et al., 1994; Boelen et al., 1998; Dumitrescu et al.,

2009). Therefore, it is possible that rod photoreceptors, active under dim light and after adaption to bright light, could mediate changes to DA activity in both of the protective light housing conditions used here. Future experiments could examine the role of rods more closely by exposing transgenic rod knock-out and rod only mice to the same ambient light levels used here. Based on the results described here, we predict that that the protective effects of both dim and bright on LIM would be eliminated without functional rod photoreceptors, as suggested by a previous study (Park et al., 2014). In this study, mice with dysfunctional rod cells showed no susceptibility to FDM under normal animal housing lighting, therefore, if the protection from scotopic and photopic light is rod driven I would predict that there would be no response to LIM in those light conditions.

6.3.2 Gap junctions in light with lens defocus

The three ambient light levels studied in these experiments were chosen to target specific photoreceptor signaling pathways. While scotopic light activates rod photoreceptors, the mesopic light activates mixed rod and cone signaling, and photopic light activates cone photoreceptors (**Figure 6.2**). An interesting aspect of these different signaling cascades is the use of open gap junctions by the retina in mesopic light [for review (Bloomfield and Volgyi, 2009)]. In the retina, gap junctions are opened during mesopic light to pool signaling from rods through rod-rod and rod-cone coupling. This pooling through open gap junctions does not occur under scotopic or photopic light. In photopic light, gap junctions are closed by an excess of DA which is released by activated DACs (Hampson et al., 1992; Kothmann et al., 2009; Li et al., 2013). However, DA content and metabolism, as seen here with HPLC, is low under scotopic light indicating that gap junctions under dim light are closed by some other mechanism. I hypothesize that the varied release of DA after light stimulus as detected here in the eye cup perfusion assay may provide a

hint for what mechanisms may be acting on gap junctions in scotopic light (**Figure 6.2**). Here, several similarities in DA signaling between scotopic and photopic light have been shown including gene expression of *Th* and response to light stimulus in the eye cups, however there are still dramatic differences in DOPAC, indicating that despite similarities DA signaling is still highest in photopic light. It is possible that one of the similarities shown here acts on gap junctions in scotopic light despite low levels of DA in the whole retina.

Open gap junctions during mesopic light could potentially increase the effect of lens defocus. When signals from neighboring photoreceptors are pooled the visual acuity is decreased which could effect the response to lens defocus. Future experiments will need to determine the role of gap junctions on myopia susceptibility, including connexin knock-out animals housed under scotopic, mesopic, and photopic light. Future work should also include a more complete analysis of gap junction activity under each of the light conditions used here. As stated previously, the terms used to define light levels in current literature are not always consistent across studies which may lead to misinterpretation. In part, this is because the thresholds between scotopic, mesopic, and photopic light are not well defined in the mouse. Using transgenic animals in this research, with dysfunctional photoreceptors in addition to connexin knock-outs, will help determine the exact range of each pathway described here and its role on myopia susceptibility.

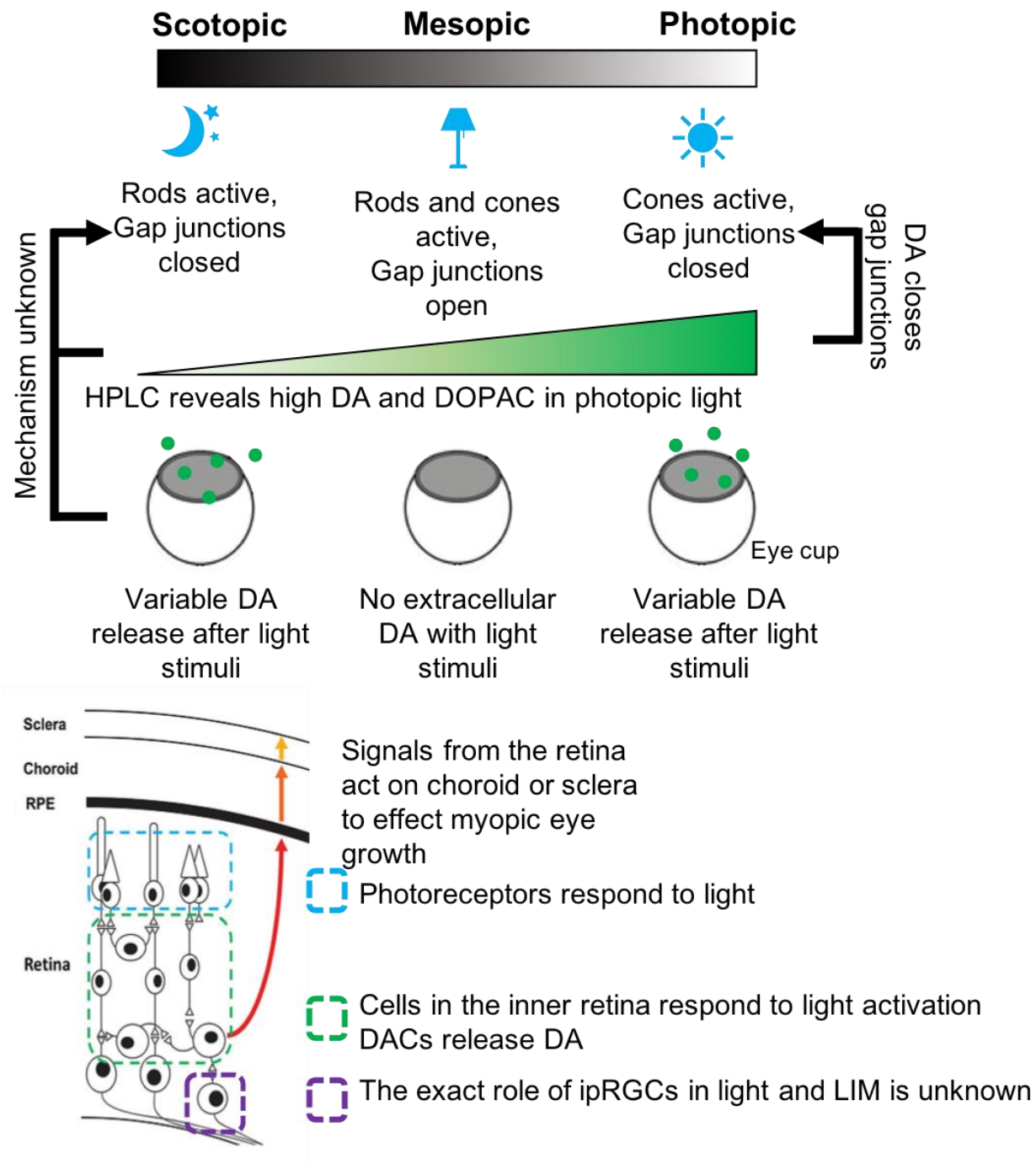

Figure 6.2. Potential signaling mechanisms behind the effect of light on refractive eye growth. Scotopic, mesopic, and photopic light each activate different photoreceptor pathways, *top*. Gap junctions connecting photoreceptors are likely open under mesopic light, but not scotopic or photopic. These pathways effect DACs to alter the synthesis, packaging, release, uptake, and degradation of DA in unique ways. High DA release closes gap junctions under photopic light, but how junctions close under scotopic light is unknown; the preliminary results here may indicate DA also plays a role in that process. Once DA has been released, it either acts through volume transmission on the posterior structures of the eye or mediates a second

downstream messenger which ultimately has the same effect, *bottom*. In order to alter myopic eye growth, these signals must reach either the choroid or sclera, however how this happens is still unclear. This work has shown that tight control of DA in DACs after ambient light and lens defocus can affect the downstream signaling to the posterior eye. Top is original image by author, bottom is modified with permission from http://webvision.med.utah.edu.

6.3.3 Light sensitive retinal ganglion cells and myopia susceptibility

Despite using ambient light to target specific photoreceptor pathways, the exact signaling mechanisms behind the role of DA in these responses remains unknown. One potential regulatory cell is the intrinsically photosensitive retinal ganglion cells (ipRGCs). ipRGCs expressing the photopigment melanopsin have been implicated in both image and non-image forming vision [for review (Munch and Kawasaki, 2013; Tu et al., 2005; Schmidt and Kofuji, 2009)]. Of the five different sub-types of ipRGCs, all have the potential to influence the response to LIM or FDM. These cells can respond to a very broad range of light intensities and signal to DACs and therefore should be investigated more closely for their role in light driven myopia protection.

ipRGCs respond to light differently than rod and cone photoreceptors. First, it has recently been discovered that at least one ipRGC subtype (the M4 cells) respond to a wide range of light intensities including the range of scotopic and photopic light included in the experiments reported here (Sonoda et al., 2018). Second, ipRGCs show a sustained response to light stimulus, up to 10 seconds long (Schmidt et al., 2011), much longer than other photoreceptors indicating that they may be more responsive to lens defocus treatment than rods or cones.

Additionally, evidence of reciprocal signaling mechanisms between ipRGCs and DACs would indicate they may be contributing to the changes in DA signaling with light housing. The two cell types synapse in the inner plexiform layer (Vugler et al., 2007; Dumitrescu et al., 2009). DA signaling impacts signaling in ipRGCs which express D1 receptors (Van Hook et al., 2012) and regulate expression of melanopsin mRNA (Sakamoto et al., 2005). Whether melanopsin signaling regulates DA activity in DACs is still unclear (Zhang et al., 2008; Cameron et al., 2009; Vuong et al., 2015). If ipRGCs regulate or respond to DA signaling after light exposure,

and play a role in the detection of defocus, it is likely that they could be impacting the results found here.

Preliminary work on the role of ipRGCs and melanopsin in myopia susceptibility have shown that ipRGCs respond to defocus such that chicks with LIM had decreased levels of melanopsin (Stone et al., 2011). Preliminary work in mice with transgenic knock-out of melanopsin have shown increased susceptibility to FDM relative to WT controls (Chakraborty et al., 2015b). Additionally, ipRGCs regulate circadian rhythms (Sexton et al., 2012) which have also been implicated in myopia development (Chakraborty et al., 2018b).

6.4 Clinical potential

My work has shown that the response to ambient light and lens defocus may interact to influence refractive development. A main conclusion from the work presented here is that myopiagenic and anti-myopiagenic factors such as bright light and lens defocus, interact in specific ways to determine physiological changes to the eye growth. Without investigating a wide range of light levels and corresponding retinal signaling pathways, the previous reports on ambient light as a factor in myopia have not identified these interactions, though they may have a large impact on how we can prevent myopia for individuals in the future. The findings I have presented here support the argument that other studies of objective light exposure in children should be reanalyzed to determine whether or not scotopic light had an impact on other myopic populations.

Several early clinical trials which aim to prevent or slow myopia in human populations by increasing bright light exposure, have seen that some participants ultimately still become myopic (Wu et al., 2013; He et al., 2015). It is possible that based on genetic risk, lifestyle, or

other factors, some light exposure paradigms will work well for only certain individuals. The interactions between ambient light exposure and these factors may mirror the interactions shown here and prevent some individuals from responding to bright light protection. To identify which prevention strategy might work for individual patients, more should be known about the retinal biology behind the response to each light level and its interaction with other myopiagenic factors.

The findings reported here are novel and will need to be replicated in other models and populations. Evidence for the effect of dim, scotopic light protection in both the mouse model and an analysis with human participants are encouraging. However, like bright light protection, it is possible that dim light protection will interact with other factors and therefore should be studied more closely. A major, clinically relevant finding of this work is that both myopic and non-myopic children spend more time in dim light than was expected, and that the amount of time non-myopic children spent in dim light over myopic children was roughly equal to the increased time spent in outdoor photopic light. This means that not only is dim light protective, but its potential as an intervention technique might be equal to that of bright light, which is already being used as an intervention in some places.

An aspect of light exposure, not fully investigated here, which may be important in clinical prevention of myopia is the timing of specific levels of light exposure. In this study, myopic and non-myopic children were exposed to light at roughly the same time of day, especially on weekdays when there were no differences between groups. It is likely that these similarities are driven by cultural practices. However, given the recent evidence of a role for circadian rhythms in animal models of myopia (Chakraborty et al., 2018b), it is possible that the timing of scotopic and photopic light exposure affects its efficiency in altering refractive error.

Indeed, recent unpublished data has demonstrated an effect of time of day on the ocular response to myopic and hyperopic defocus in human participants (Chakraborty et al., 2018a). This is more likely if light driven protection in human myopia is driven by DA signaling which changes on a daily rhythm. By finding which light intensities are most protective for each patient, and when that light could be most effective, we may be able to take advantage of circadian rhythms in retinal neurobiology to prevent myopia.

6.5 Future directions for this work

With the work presented here, the effect of ambient light and DA activity on myopia susceptibility is revealed to be more complex than previously thought. While DA does prevent myopia in mice, similar to the protection seen in other species, DA activity in mice that have been protected from myopia by bright or dim light do not show the changes that would be expected. The hypothesis that bright light would prevent myopia by increasing DA synthesis and activity was supported by the levels of DA and DOPAC found in the retina, but levels and activity of DA related proteins do not support such a clear relationship. Additionally, the mechanism behind the myopia prevention caused by scotopic light is still unclear. While DA signaling in scotopic and photopic light showed some similarities, such as expression of *Th*, there are few studies which would support a role of increased DA activity under scotopic light (Weiler et al., 1997). Therefore, the similarities detected here should be more fully studied, in addition to other myopiagenic signaling molecules which might be responsive to light. Future studies should also include an investigation of these proteins in other animal models of myopia. The role of DA in myopia has been shown in several species including chick, guinea pig, tree shrew, and marmosets [for review (Feldkaemper and Schaeffel, 2013)]. It will be important to know how

changes to DA signaling with myopia are conserved across species in order to best understand how this signaling may occur in humans where tests of retinal DA are not currently possible.

Before more conclusions are made, a few of the tests described here need further validation or increased statistical power. The eye cup perfusion assay used here as a measure of the DAergic response to light stimuli has the potential to probe the activity of DA in myopic retinas more closely than has been done previously. However, more work will be needed to validate this test. A project is currently underway to determine whether extracellular DA is increased after exposure to ambient light stimuli of increasing intensity or duration, this will more rigorously indicate that the DA measured in aCSF is a result of light stimulated DA release. Other studies should use this and similar systems to measure the activity levels of different DA related proteins after FDM or LIM. By combining this or similar assays with different light stimuli, pharmaceutical intervention, or transgenic animal models, the activity of a number of DA related proteins could be discovered. With this information, we could begin to understand more about how DA is being trafficked in a myopic retina.

Overall, the major finding of this dissertation which I feel should be continued in other studies is the careful study of DA signaling more broadly and the interactions which may occur between visual factors affecting refractive error. With the new techniques described here, including the use of dim light, these relationships can be more fully investigated. By studying them, we may be able to develop more personalized techniques to prevent myopia; is it possible that certain combinations or timings of ambient light and defocus have more severe effects on myopia than others or that certain interactions would have larger effects on some patients than others. Only by investigating interactions between factors effecting refractive error development can a full picture of how to prevent myopia be uncovered.

6.6 Final thoughts

In conclusion, my thesis work establishes several key and novel findings which will contribute to the field of myopia.

1. The mouse model of myopia shows several key similarities with other models of myopia, namely a protective response under bright light and protection from myopia by L-DOPA administration.
2. Dim light exposure, in addition to bright light, could prevent the development of refractive error and could be used as an intervention technique in human myopia.
3. DA signaling and activity in myopia and protection from myopia by ambient lighting may be more complex than previously thought such that the ability of the retinal DAergic system to adapt and maintain homeostasis may dictate which retinal pathways are important in myopia development and prevention.
4. Environmental lighting conditions and myopiagenic stimuli like defocus influencing ocular development may interact in unique ways which will need to be investigated further in the future.

Overall, this thesis uncovers important insights into the retinal DA mechanisms behind light as a factor in myopia development and provides a framework for future investigations of dim light as a myopia prevention.

REFERENCES

Altimus CM, Guler AD, Alam NM, Arman AC, Prusky GT, Sampath AP, Hattar S (2010) Rod photoreceptors drive circadian photoentrainment across a wide range of light intensities. Nat Neurosci 13:1107-1112.

Anera RG, Soler M, de la Cruz Cardona J, Salas C, Ortiz C (2009) Prevalence of refractive errors in school-age children in Morocco. Clin Exp Ophthalmol 37:191-196.

Arumugam B, McBrien N (2010) The D2 Antagonist Spiperone Prevents Muscarinic Antagonist Control of Experimentally-Induced Myopia in Chick. Invest Ophthalmol Vis Sci 51:1195-1195.

Ashby R, Schaeffel F (2010a) The Effect of Bright Light on Lens Compensation in Chicks. Invest Ophthalmol Vis Sci 51:5247-5253.

Ashby R, Ohlendorf A, Schaeffel F (2009) The Effect of Ambient Illuminance on the Development of Deprivation Myopia in Chicks. Invest Ophthalmol Vis Sci 50:5348-5354.

Ashby R, Kozulin P, Megaw PL, Morgan IG (2010) Alterations in ZENK and glucagon RNA transcript expression during increased ocular growth in chickens. Mol Vis 16:639-649.

Ashby R, McCarthy CS, Maleszka R, Megaw P, Morgan IG (2007) A muscarinic cholinergic antagonist and a dopamine agonist rapidly increase ZENK mRNA expression in the form-deprived chicken retina. Exp Eye Res 85:15-22.

Ashby RS, Schaeffel F (2010b) The Effect of Bright Light on Lens Compensation in Chicks. Invest Ophthalmol Vis Sci 51:5247-5253.

Atkinson CL, Feng J, Zhang DQ (2013) Functional integrity and modification of retinal dopaminergic neurons in the rd1 mutant mouse: roles of melanopsin and GABA. J Neurophysiol 109:1589-1599.

Aung MH, Kim MK, Olson DE, Thule PM, Pardue MT (2013) Early visual deficits in streptozotocin-induced diabetic long evans rats. Invest Ophthalmol Vis Sci 54:1370-1377.

Backhouse S, Collins AV, Phillips JR (2013) Influence of periodic vs continuous daily bright light exposure on development of experimental myopia in the chick. Ophthal Physl Opt 33:563-572.

Barathi VA, Boopathi VG, Yap EP, Beuerman RW (2008) Two models of experimental myopia in the mouse. Vision Res 48:904-916.

Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J Roy Stat Soc B Met 57:289-300.

Bergen MA, Park Hn, Chakraborty R, Landis EG, Sidhu C, Iuvone PM, Pardue MT (2015) Retinal-specific Dopamine Knock-out Mice are Myopic. Invest Ophthalmol Vis Sci 56:2153-2153.

Bergen MA, Park HN, Chakraborty R, Landis EG, Sidhu C, He L, Iuvone PM, Pardue MT (2016) Altered Refractive Development in Mice With Reduced Levels of Retinal Dopamine. Invest Ophthalmol Vis Sci 57:4412-4419.

Berson DM, Dunn FA, Takao M (2002) Phototransduction by retinal ganglion cells that set the circadian clock. Science 295:1070-1073.

Bloomfield SA, Volgyi B (2009) The diverse functional roles and regulation of neuronal gap junctions in the retina. Nat Rev Neurosci 10:495-506.

Bloomfield SA, Xin D, Osbourne T (1997) Light-induced modulation of coupling between AII amacrine cells in the rabbit retina. Visual Neurosci 14:565-576.

Boatright JH, Gordon JR, Iuvone PM (1994) Inhibition of endogenous dopamine release in amphibian retina by L-2-amino-4-phosphonobutyric acid (L-AP4) and trans-2-aminocyclopentane-1,3-dicarboxylate (ACPD). Brain Res 649:339-342.

Boelen MK, Boelen MG, Marshak DW (1998) Light-stimulated release of dopamine from the primate retina is blocked by l-2-amino-4-phosphonobutyric acid (APB). Visual Neurosci 15:97-103.

Borchert MS, Varma R, Cotter SA, Tarczy-Hornoch K, McKean-Cowdin R, Lin JH, Wen G, Azen SP, Torres M, Tielsch JM, Friedman DS, Repka MX, Katz J, Ibironke J, Giordano L (2011) Risk factors for hyperopia and myopia in preschool children the multi-ethnic pediatric eye disease and Baltimore pediatric eye disease studies. Ophthalmology 118:1966-1973.

Brainard GC, Morgan WW (1987) Light-induced stimulation of retinal dopamine: a dose-response relationship. Brain Res 424:199-203.

Burger S, Doring B, Hardt M, Beuerlein K, Gerstberger R, Geyer J (2011) Co-expression studies of the orphan carrier protein Slc10a4 and the vesicular carriers VAChT and VMAT2 in the rat central and peripheral nervous system. Neuroscience 193:109-121.

Buskila Y, Breen PP, Tapson J, van Schaik A, Barton M, Morley JW (2014) Extending the viability of acute brain slices. Sci Rep 4:5309.

Cameron MA, Perez Fernandez V, Milosavljevic N, Morley JW (2018) Rod input drives, and supresses, dopamine release in the mouse retina. Invest Ophthalmol Vis Sci 59:3099-3099.

Cameron MA, Pozdeyev N, Vugler AA, Cooper H, Iuvone PM, Lucas RJ (2009) Light regulation of retinal dopamine that is independent of melanopsin phototransduction. Eur J Neurosci 29:761-767.

Cartier EA, Parra LA, Baust TB, Quiroz M, Salazar G, Faundez V, Egana L, Torres GE (2010) A biochemical and functional protein complex involving dopamine synthesis and transport into synaptic vesicles. J Biol Chem 285:1957-1966.

Casson RJ, Kahawita S, Kong A, Muecke J, Sisaleumsak S, Visonnavong V (2012) Exceptionally low prevalence of refractive error and visual impairment in schoolchildren from Lao People's Democratic Republic. Ophthalmology 119:2021-2027.

Caudle WM, Richardson JR, Wang MZ, Taylor TN, Guillot TS, McCormack AL, Colebrooke RE, Di Monte DA, Emson PC, Miller GW (2007) Reduced vesicular storage of dopamine causes progressive nigrostriatal neurodegeneration. J Neurosci 27:8138-8148.

Cavallotti C, Pescosolido N, Artico M, Feher J (1999) Localization of dopamine receptors in the rabbit cornea. Cornea 18:721-728.

Chakraborty R, Pardue MT (2015) Molecular and Biochemical Aspects of the Retina on Refraction. Prog Mol Biol Transl 134:249-267.

Chakraborty R, Park HN, Hanif AM, Sidhu CS, Iuvone PM, Pardue MT (2015a) ON pathway mutations increase susceptibility to form-deprivation myopia. Exp Eye Res 137:79-83.

Chakraborty R, Do M, Hobbs S, Lam V, Moderiano D, Sarin S (2018a) Time of the day influences the response to optical defocus in human eyes. Invest Ophthalmol Vis Sci 59:1176-1176.

Chakraborty R, Ostrin LA, Nickla DL, Iuvone PM, Pardue MT, Stone RA (2018b) Circadian rhythms, refractive development, and myopia. Ophthal Physl Opt 38:217-245.

Chakraborty R, Lee DC, Landis EG, Bergen MA, Park Hn, Sidhu C, Hattar S, Iuvone PM, Stone RA, Pardue MT (2015b) Melanopsin knock-out mice have abnormal refractive

development and increased susceptibility to form-deprivation myopia. Invest Ophthalmol Vis Sci 56:5843-5843.

Chang B, Hawes NL, Pardue MT, German AM, Hurd RE, Davisson MT, Nusinowitz S, Rengarajan K, Boyd AP, Sidney SS, Phillips MJ, Stewart RE, Chaudhury R, Nickerson JM, Heckenlively JR, Boatright JH (2007) Two mouse retinal degenerations caused by missense mutations in the beta-subunit of rod cGMP phosphodiesterase gene. Vision Res 47:624-633.

Chen N, Reith ME (2000) Structure and function of the dopamine transporter. Eur J Pharmacol 405:329-339.

Chen S, Zhi Z, Ruan Q, Liu Q, Li F, Wan F, Reinach PS, Chen J, Qu J, Zhou X (2017) Bright Light Suppresses Form-Deprivation Myopia Development With Activation of Dopamine D1 Receptor Signaling in the ON Pathway in Retina. Invest Ophthalmol Vis Sci 58:2306-2316.

Chen Y, Wang W, Han X, Yan W, He M (2016) What Twin Studies Have Taught Us About Myopia. Asia Pac J Ophthal 5:411-414.

Cheung N, Nagra P, Hammersmith K (2016) Emerging trends in contact lens-related infections. Curr Opin Ophthalmol 27:327-332.

Chui TY, Bissig D, Berkowitz BA, Akula JD (2012) Refractive Development in the "ROP Rat". J Ophthalmol 2012:956705.

Cliburn RA, Dunn AR, Stout KA, Hoffman CA, Lohr KM, Bernstein AI, Winokur EJ, Burkett J, Shmitz Y, Caudle WM, Miller GW (2016) Immunochemical localization of vesicular monoamine transporter 2 (VMAT2) in mouse brain. J Chem Neuroanat 83-84:82-90.

Cohen J, Hadjiconstantinou M, Neff NH (1983) Activation of dopamine-containing amacrine cells of retina: light-induced increase of acidic dopamine metabolites. Brain Res 260:125-127.

Cohen Y, Belkin M, Yehezkel O, Avni I, Polat U (2008) Light intensity modulates corneal power and refraction in the chick eye exposed to continuous light. Vision Res 48:2329-2335.

Cohen Y, Belkin M, Yehezkel O, Solomon AS, Polat U (2011) Dependency between light intensity and refractive development under light–dark cycles. Exp Eye Res 92:40-46.

Cohen Y, Peleg E, Belkin M, Polat U, Solomon AS (2012) Ambient illuminance, retinal dopamine release and refractive development in chicks. Exp Eye Res 103:33-40.

Cooper JR, Bloom FE, Roth RH (2003) The biochemical basis of neuropharmacology. New York: Oxford University Press.

Cottriall CL, Brew J, Vessey KA, McBrien NA (2001) Diisopropylfluorophosphate alters retinal neurotransmitter levels and reduces experimentally-induced myopia. N-S Arch Pharmacol 364:372-382.

Crewther SG, Barutchu A, Murphy MJ, Crewther DP (2006) Low frequency temporal modulation of light promotes a myopic shift in refractive compensation to all spectacle lenses. Exp Eye Res 83:322-328.

Cumberland PM, Bao Y, Hysi PG, Foster PJ, Hammond CJ, Rahi JS (2015) Frequency and Distribution of Refractive Error in Adult Life: Methodology and Findings of the UK Biobank Study. PloS one 10:e0139780.

Dacey DM, Liao HW, Peterson BB, Robinson FR, Smith VC, Pokorny J, Yau KW, Gamlin PD (2005) Melanopsin-expressing ganglion cells in primate retina signal colour and irradiance and project to the LGN. Nature 433:749-754.

Dandona R, Dandona L, Srinivas M, Sahare P, Narsaiah S, Munoz SR, Pokharel GP, Ellwein LB (2002) Refractive error in children in a rural population in India. Invest Ophthalmol Vis Sci 43:615-622.

Daubner SC, Le T, Wang S (2011) Tyrosine Hydroxylase and Regulation of Dopamine Synthesis. Arch Biochem Biophys 508:1-12.

Demontis GC, Bisti S, Cervetto L (1993) Light sensitivity, adaptation and saturation in mammalian rods. Prog Brain Res 95:15-24.

Dharani R, Lee CF, Theng ZX, Drury VB, Ngo C, Sandar M, Wong TY, Finkelstein EA, Saw SM (2012a) Comparison of measurements of time outdoors and light levels as risk factors for myopia in young Singapore children. Eye 26:911.

Diether S, Schaeffel F (1997) Local changes in eye growth induced by imposed local refractive error despite active accommodation. Vision Res 37:659-668.

Ding BY, Shih YF, Lin LLK, Hsiao CK, Wang IJ (2017) Myopia among schoolchildren in East Asia and Singapore. Surv Ophthalmol 62:677-697.

Dong F, Zhi Z, Pan M, Xie R, Qin X, Lu R, Mao X, Chen JF, Willcox MD, Qu J, Zhou X (2011) Inhibition of experimental myopia by a dopamine agonist: different effectiveness between form deprivation and hyperopic defocus in guinea pigs. Mol Vis 17:2824-2834.

Douglas RM, Alam NM, Silver BD, McGill TJ, Tschetter WW, Prusky GT (2005) Independent visual threshold measurements in the two eyes of freely moving rats and mice using a virtual-reality optokinetic system. Visual Neurosci 22:677-684.

Doyle SE, McIvor WE, Menaker M (2002a) Circadian rhythmicity in dopamine content of mammalian retina: role of the photoreceptors. J Neurochem 83:211-219.

Doyle SE, Grace MS, McIvor W, Menaker M (2002b) Circadian rhythms of dopamine in mouse retina: the role of melatonin. Visual Neurosci 19:593-601.

Dumitrescu ON, Pucci FG, Wong KY, Berson DM (2009) Ectopic retinal ON bipolar cell synapses in the OFF inner plexiform layer: contacts with dopaminergic amacrine cells and melanopsin ganglion cells. J Comp Neurol 517:226-244.

Ecker JL, Dumitrescu ON, Wong KY, Alam NM, Chen SK, LeGates T, Renna JM, Prusky GT, Berson DM, Hattar S (2010) Melanopsin-expressing retinal ganglion-cell photoreceptors: cellular diversity and role in pattern vision. Neuron 67:49-60.

Eggers ED, Lukasiewicz PD (2011) Multiple pathways of inhibition shape bipolar cell responses in the retina. Visual Neurosci 28:95-108.

Faulkner AE, Kim MK, Iuvone PM, Pardue MT (2007) Head-mounted goggles for murine form deprivation myopia. J Neurosci Meth 161:96-100.

Feldkaemper M, Schaeffel F (2013) An updated view on the role of dopamine in myopia. Exp Eye Res 114:106-119.

French AN, Ashby RS, Morgan IG, Rose KA (2013a) Time outdoors and the prevention of myopia. Exp Eye Res 114:58-68.

French AN, Morgan IG, Mitchell P, Rose KA (2013b) Patterns of myopigenic activities with age, gender and ethnicity in Sydney schoolchildren. Ophthalmic Physl Opt 33:318-328.

Fricke TR, Holden BA, Wilson DA, Schlenther G, Naidoo KS, Resnikoff S, Frick KD (2012) Global cost of correcting vision impairment from uncorrected refractive error. Bulletin of the World Health Organization 90:728-738.

Fricke TR, Jong M, Naidoo KS, Sankaridurg P, Naduvilath TJ, Ho SM, Wong TY, Resnikoff S (2018) Global prevalence of visual impairment associated with myopic macular

degeneration and temporal trends from 2000 through 2050: systematic review, meta-analysis and modelling. Br J Ophthalmol 102:855-862.
Gao Z, Meng N, Muecke J, Chan WO, Piseth H, Kong A, Jnguyenphamhh T, Dehghan Y, Selva D, Casson R, Ang K (2012) Refractive error in school children in an urban and rural setting in Cambodia. Ophthalmic Epidemiol 19:16-22.
Ghosh A, Zheleznyak L, Barbot A, Jung H, Yoon G (2017) Neural adaptation to peripheral blur in myopes and emmetropes. Vision Res 132:69-77.
Gingrich JA, Caron MG (1993) Recent advances in the molecular biology of dopamine receptors. Annu Rev Neurosci 16:299-321.
Goldschmidt E, Jacobsen N (2014) Genetic and environmental effects on myopia development and progression. Eye 28:126-133.
Goss DA, Criswell MH (1981) Myopia development in experimental animals-a literature review. Amer J Optom Phys Opt 58:859-869.
Gradisar M, Wolfson AR, Harvey AG, Hale L, Rosenberg R, Czeisler CA (2013) The sleep and technology use of Americans: findings from the National Sleep Foundation's 2011 Sleep in America poll. J Clin Sleep Med 9:1291-1299.
Group M-EPEDS (2010) Prevalence of myopia and hyperopia in 6- to 72-month-old african american and Hispanic children: the multi-ethnic pediatric eye disease study. Ophthalmology 117:140-147.e143.
Gu Y, Xu B, Feng C, Ni Y, Wu Q, Du C, Hong N, Li P, Ding Z, Jiang B (2016) A Head-Mounted Spectacle Frame for the Study of Mouse Lens-Induced Myopia. J Ophthalmol 8497278.
Guggenheim JA, Northstone K, McMahon G, Ness AR, Deere K, Mattocks C, Pourcain BS, Williams C (2012) Time Outdoors and Physical Activity as Predictors of Incident Myopia in Childhood: A Prospective Cohort Study. Invest Ophthalmol Vis Sci 53:2856-2865.
Guler AD, Ecker JL, Lall GS, Haq S, Altimus CM, Liao HW, Barnard AR, Cahill H, Badea TC, Zhao H, Hankins MW, Berson DM, Lucas RJ, Yau KW, Hattar S (2008) Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision. Nature 453:102-105.
Guo SS, Sivak JG, Callender MG, Diehl-Jones B (1995) Retinal dopamine and lens-induced refractive errors in chicks. Curr Eye Res 14:385-389.
Gwiazda J, Thorn F, Bauer J, Held R (1993) Myopic children show insufficient accommodative response to blur. Invest Ophthalmol Vis Sci 34:690-694.
Bowery HE, Zeng G, Tse DY, Leotta AJ, Wu Y, To CH, Wildsoet CF, McFadden SA (2017) The Effect of Spectacle Lenses Containing Peripheral Defocus on Refractive Error and Horizontal Eye Shape in the Guinea Pig. Invest Ophthalmol Vis Sci 58:2705-2714.
Hampson EC, Vaney DI, Weiler R (1992) Dopaminergic modulation of gap junction permeability between amacrine cells in mammalian retina. J Neurosci 12:4911-4922.
Hankins M, Ikeda H (1994) Early abnormalities of retinal dopamine pathways in rats with hereditary retinal dystrophy. Doc Ophthalmol 86:325-334.
Hattar S, Liao HW, Takao M, Berson DM, Yau KW (2002) Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science 295:1065-1070.

He M, Xiang F, Zeng Y, Mai J, Chen Q, Zhang J, Smith W, Rose K, Morgan IG (2015) Effect of Time Spent Outdoors at School on the Development of Myopia Among Children in China: A Randomized Clinical Trial. JAMA 314:1142-1148.

Herrmann R, Heflin SJ, Hammond T, Lee B, Wang J, Gainetdinov RR, Caron MG, Eggers ED, Frishman LJ, McCall MA, Arshavsky VY (2011) Rod vision is controlled by dopamine-dependent sensitization of rod bipolar cells by GABA. Neuron 72:101-110.

Hirasawa H, Betensky RA, Raviola E (2012) Corelease of dopamine and GABA by a retinal dopaminergic neuron. J Neurosci 32:13281-13291.

Hodos W, Kuenzel WJ (1984) Retinal-image degradation produces ocular enlargement in chicks. Invest Ophthalmol Vis Sci 25:652-659.

Hokoc JN, Mariani AP (1987) Tyrosine hydroxylase immunoreactivity in the rhesus monkey retina reveals synapses from bipolar cells to dopaminergic amacrine cells. J Neurosci 7:2785-2793.

Holden B, Sankaridurg P, Smith E, Aller T, Jong M, He M (2014) Myopia, an underrated global challenge to vision: where the current data takes us on myopia control. Eye 28:142-146.

Holden BA, Fricke TR, Wilson DA, Jong M, Naidoo KS, Sankaridurg P, Wong TY, Naduvilath TJ, Resnikoff S (2016) Global Prevalence of Myopia and High Myopia and Temporal Trends from 2000 through 2050. Ophthalmology 123:1036-1042.

Honda S, Fujii S, Sekiya Y, Yamamoto M (1996) Retinal control on the axial length mediated by transforming growth factor-beta in chick eye. Invest Ophthalmol Vis Sci 37:2519-2526.

Howlett MH, McFadden SA (2006) Form-deprivation myopia in the guinea pig (Cavia porcellus). Vision Res 46:267-283.

Howlett MH, McFadden SA (2007) Emmetropization and schematic eye models in developing pigmented guinea pigs. Vision Res 47:1178-1190.

Howlett MH, McFadden SA (2009) Spectacle lens compensation in the pigmented guinea pig. Vision Res 49:219-227.

Hsueh HA, Molnar A, Werblin FS (2008) Amacrine-to-amacrine cell inhibition in the rabbit retina. J Neurophysiol 100:2077-2088.

Hua WJ, Jin JX, Wu XY, Yang JW, Jiang X, Gao GP, Tao FB (2015) Elevated light levels in schools have a protective effect on myopia. Ophthalmic Physl Opt 35:252-262.

Huang F, Zhang L, Wang Q, Yang Y, Li Q, Wu Y, Chen J, Qu J, Zhou X (2018) Dopamine D1 Receptors Contribute Critically to the Apomorphine-Induced Inhibition of Form-Deprivation Myopia in Mice. Invest Ophthalmol Vis Sci 59:2623-2634.

Huang F, Yan T, Shi F, An J, Xie R, Zheng F, Li Y, Chen J, Qu J, Zhou X (2014) Activation of Dopamine D2 Receptor Is Critical for the Development of Form-Deprivation Myopia in the C57BL/6 MouseActivation of Dopamine D2 Receptor Is Critical. Invest Ophthalmol Vis Sci 55:5537-5544.

Huang HM, Chang DS, Wu PC (2015) The Association between Near Work Activities and Myopia in Children-A Systematic Review and Meta-Analysis. PloS one 10:e0140419.

Huang J, Hung LF, Smith EL, 3rd (2011) Effects of foveal ablation on the pattern of peripheral refractive errors in normal and form-deprived infant rhesus monkeys (Macaca mulatta). Invest Ophthalmol Vis Sci 52:6428-6434.

Hunter JJ, Morgan JI, Merigan WH, Sliney DH, Sparrow JR, Williams DR (2012) The susceptibility of the retina to photochemical damage from visible light. Prog Retin Eye Res 31:28-42.

Hwang CK, Chaurasia SS, Jackson CR, Chan GC, Storm DR, Iuvone PM (2013) Circadian rhythm of contrast sensitivity is regulated by a dopamine-neuronal PAS-domain protein 2-adenylyl cyclase 1 signaling pathway in retinal ganglion cells. J Neurosci 33:14989-14997.

Ip JM, Saw SM, Rose KA, Morgan IG, Kifley A, Wang JJ, Mitchell P (2008) Role of near work in myopia: findings in a sample of Australian school children. Invest Ophthalmol Vis Sci 49:2903-2910.

Iuvone PM (1984) Regulation of retinal dopamine biosynthesis and tyrosine hydroxylase activity by light. Fed Proc 43:2709-2713.

Iuvone PM, Galli CL, Garrison-Gund CK, Neff NH (1978) Light stimulates tyrosine hydroxylase activity and dopamine synthesis in retinal amacrine neurons. Science 202:901-902.

Iuvone PM, Tigges M, Fernandes A, Tigges J (1989) Dopamine synthesis and metabolism in rhesus monkey retina: development, aging, and the effects of monocular visual deprivation. Visual Neurosci 2:465-471.

Iuvone PM, Tigges M, Stone RA, Lambert S, Laties AM (1991) Effects of apomorphine, a dopamine receptor agonist, on ocular refraction and axial elongation in a primate model of myopia. Invest Ophthalmol Vis Sci 32:1674-1677.

Ivanova E, Yee CW, Sagdullaev BT (2016) Disruption in dopaminergic innervation during photoreceptor degeneration. J Comp Neurol 524:1208-1221.

Jackson CR, Capozzi M, Dai H, McMahon DG (2014) Circadian perinatal photoperiod has enduring effects on retinal dopamine and visual function. J Neurosci 34:4627-4633.

Jackson CR, Ruan GX, Aseem F, Abey J, Gamble K, Stanwood G, Palmiter RD, Iuvone PM, McMahon DG (2012) Retinal dopamine mediates multiple dimensions of light-adapted vision. J Neurosci 32:9359-9368.

Jiang L, Long K, Schaeffel F, Zhou X, Zheng Y, Ying H, Lu F, Stell WK, Qu J (2014) Effects of dopaminergic agents on progression of naturally occurring myopia in albino guinea pigs (Cavia porcellus). Invest Ophthalmol Vis Sci 55:7508-7519.

Jiang X, Kurihara T, Kunimi H, Miyauchi M, Ikeda SI, Mori K, Tsubota K, Torii H, Tsubota K (2018) A highly efficient murine model of experimental myopia. Sci Rep 8:2026.

Jimenez JR, Bermudez J, Rubino M, Gomez L, Anera RG (2004) Prevalence of myopia in an adult population of two different ethnic groups in the Ecuadorian Amazon. Jpn J Ophthalmol 48:163-165.

Jimenez R, Soler M, Anera RG, Castro JJ, Perez MA, Salas C (2012) Ametropias in school-age children in Fada N'Gourma (Burkina Faso, Africa). Optometry Vision Sci 89:33-37.

Jin JX, Hua WJ, Jiang X, Wu XY, Yang JW, Gao GP, Fang Y, Pei CL, Wang S, Zhang JZ, Tao LM, Tao FB (2015) Effect of outdoor activity on myopia onset and progression in school-aged children in northeast China: the Sujiatun Eye Care Study. BMC Ophthalmol 15:73.

Jones LA, Sinnott LT, Mutti DO, Mitchell GL, Moeschberger ML, Zadnik K (2007) Parental history of myopia, sports and outdoor activities, and future myopia. Invest Ophthalmol Vis Sci 48:3524-3532.

Jones-Jordan LA, Sinnott LT, Cotter SA, Kleinstein RN, Manny RE, Mutti DO, Twelker JD, Zadnik K (2012) Time outdoors, visual activity, and myopia progression in juvenile-onset myopes. Invest Ophthalmol Vis Sci 53:7169-7175.

Jones-Jordan LA, Mitchell GL, Cotter SA, Kleinstein RN, Manny RE, Mutti DO, Twelker JD, Sims JR, Zadnik K (2011) Visual activity before and after the onset of juvenile myopia. Invest Ophthalmol Vis Sci 52:1841-1850.

Jung S-K, Lee JH, Kakizaki H, Jee D (2012) Prevalence of Myopia and its Association with Body Stature and Educational Level in 19-Year-Old Male Conscripts in Seoul, South Korea Prevalence on Myopia in Young Males in Korea. Invest Ophthalmol Vis Sci 53:5579-5583.

Kang K, Tarchick MJ, Yu X, Beight C, Bu P, Yu M (2016) Carnosic acid slows photoreceptor degeneration in the Pde6b(rd10) mouse model of retinitis pigmentosa. Sci Rep 6:22632.

Karouta C, Ashby RS (2014) Correlation between light levels and the development of deprivation myopia. Invest Ophthalmol Vis Sci 56:299-309.

Kennedy RH (1995) Progression of myopia. T Am Ophthalmol Soc 93:755-800.

Khader YS, Batayha WQ, Abdul-Aziz SM, Al-Shiekh-Khalil MI (2006) Prevalence and risk indicators of myopia among schoolchildren in Amman, Jordan. E Mediterr Health J 12:434-439.

Khandekar RB, Abdu-Helmi S (2004) Magnitude and determinants of refractive error in Omani school children. Saudi Med J 25:1388-1393.

Kim EC, Morgan IG, Kakizaki H, Kang S, Jee D (2013) Prevalence and risk factors for refractive errors: Korean National Health and Nutrition Examination Survey 2008-2011. PloS one 8:e80361.

Koh V, Yang A, Saw SM, Chan YH, Lin ST, Tan MM, Tey F, Nah G, Ikram MK (2014) Differences in prevalence of refractive errors in young Asian males in Singapore between 1996-1997 and 2009-2010. Ophthalmic Epidemiol 21:247-255.

Kothmann WW, Massey SC, O'Brien J (2009) Dopamine-stimulated dephosphorylation of connexin 36 mediates AII amacrine cell uncoupling. J Neurosci 29:14903-14911.

Ksendzovsky A, Pomeraniec IJ, Zaghloul KA, Provencio JJ, Provencio I (2017) Clinical implications of the melanopsin-based non-image-forming visual system. Neurology 88:1282-1290.

Lall GS, Revell VL, Momiji H, Al Enezi J, Altimus CM, Güler AD, Aguilar C, Cameron MA, Allender S, Hankins MW, Lucas RJ (2010) Distinct Contributions of Rod, Cone, and Melanopsin Photoreceptors to Encoding Irradiance. Neuron 66:417-428.

Lan W, Feldkaemper M, Schaeffel F (2014) Intermittent episodes of bright light suppress myopia in the chicken more than continuous bright light. PloS one 9:e110906.

Landis EG, Yang V, Brown DM, Pardue MT, Read SA (2018) Dim Light Exposure and Myopia in Children. Invest Ophthalmol Vis Sci 59:4804-4811.

Laties A, Stone R (1991) Ametropia in retinal disorders. Retinal Degenerations CRC Press, Boca Raton, pp 383e390.

Lavanya R, Kawasaki R, Tay WT, Cheung GC, Mitchell P, Saw SM, Aung T, Wong TY (2010) Hyperopic refractive error and shorter axial length are associated with age-related macular degeneration: the Singapore Malay Eye Study. Invest Ophthalmol Vis Sci 51:6247-6252.

Lazzerini Ospri L, Prusky G, Hattar S (2017) Mood, the Circadian System, and Melanopsin Retinal Ganglion Cells. Annu Rev Neurosci 40:539-556.

Lee DC, Chakraborty R, Iuvone PM, Stone RA, Pardue MT (2016) Eliminating the Master Clock Gene in the Murine Retina Produces Myopia. Invest Ophthalmol Vis Sci 57:3616-3616.

Li H, Zhang Z, Blackburn MR, Wang SW, Ribelayga CP, O'Brien J (2013) Adenosine and dopamine receptors coregulate photoreceptor coupling via gap junction phosphorylation in mouse retina. J Neurosci 33:3135-3150.

Li XX, Schaeffel F, Kohler K, Zrenner E (1992) Dose-dependent effects of 6-hydroxy dopamine on deprivation myopia, electroretinograms, and dopaminergic amacrine cells in chickens. Visual Neurosci 9:483-492.

Lin B, Masland RH (2006) Populations of wide-field amacrine cells in the mouse retina. J Comp Neurol 499:797-809.

Lindquist AC, Cama A, Keeffe JE (2011) Screening for uncorrected refractive error in secondary school-age students in Fiji. Clin Exp Ophthalmol 39:330-335.

Liu J, Pendrak K, Capehart C, Sugimoto R, Schmid GF, Stone RA (2004) Emmetropisation under continuous but non-constant light in chicks. Exp Eye Res 79:719-728.

Liu Y, Wildsoet C (2011) The effect of two-zone concentric bifocal spectacle lenses on refractive error development and eye growth in young chicks. Invest Ophthalmol Vis Sci 52:1078-1086.

Llorente L, Barbero S, Cano D, Dorronsoro C, Marcos S (2004) Myopic versus hyperopic eyes: axial length, corneal shape and optical aberrations. J Vision 4:288-298.

Lohr KM, Stout KA, Dunn AR, Wang M, Salahpour A, Guillot TS, Miller GW (2015) Increased Vesicular Monoamine Transporter 2 (VMAT2; Slc18a2) Protects against Methamphetamine Toxicity. ACS Chem Neurosci 6:790-799.

Lohr KM, Chen M, Hoffman CA, McDaniel MJ, Stout KA, Dunn AR, Wang M, Bernstein AI, Miller GW (2016) Vesicular Monoamine Transporter 2 (VMAT2) Level Regulates MPTP Vulnerability and Clearance of Excess Dopamine in Mouse Striatal Terminals. Toxicol Sci 153:79-88.

Lohr KM, Bernstein AI, Stout KA, Dunn AR, Lazo CR, Alter SP, Wang M, Li Y, Fan X, Hess EJ, Yi H, Vecchio LM, Goldstein DS, Guillot TS, Salahpour A, Miller GW (2014) Increased vesicular monoamine transporter enhances dopamine release and opposes Parkinsonian disease-related neurodegeneration in vivo. P Natl Acad Sci USA 111:9977-9982.

Low W, Dirani M, Gazzard G, Chan YH, Zhou HJ, Selvaraj P, Au Eong KG, Young TL, Mitchell P, Wong TY, Saw SM (2010) Family history, near work, outdoor activity, and myopia in Singapore Chinese preschool children. Br J Ophthalmol 94:1012-1016.

Ma M, Zhang Z, Du E, Zheng W, Gu Q, Xu X, Ke B (2014a) Wnt Signaling in Form Deprivation Myopia of the Mice Retina. PloS one 9:e91086.

Ma Q, Xu W, Zhou X, Cui C, Pan CW (2014b) The relationship of season of birth with refractive error in very young children in eastern China. PloS one 9:e100472.

Mao J, Liu S (2017) Different roles of retinal dopamine in albino Guinea pig myopia. Neurosci Lett 639:94-97.

Mao J, Liu S, Qin W, Xiang Q, Wu X (2011) Exogenous levodopa increases the neuro retinal dopamine of guinea pig myopic eyes in vitro. Eye Sci 26:211-216.

Mao J, Liu S, Qin W, Li F, Wu X, Tan Q (2010) Levodopa inhibits the development of form-deprivation myopia in guinea pigs. Optometry Vis Sci 87:53-60.

Martínez-Fernández de la Cámara C, Hernández-Pinto AM, Olivares-González L, Cuevas-Martín C, Sánchez-Aragó M, Hervás D, Salom D, Cuezva JM, de la Rosa EJ, Millán JM, Rodrigo R (2015) Adalimumab Reduces Photoreceptor Cell Death in A Mouse Model of Retinal Degeneration. Sci Rep 5:11764.

McBrien NA, Cottriall CL, Annies R (2001) Retinal acetylcholine content in normal and myopic eyes: a role in ocular growth control? Visual Neurosci 18:571-580.
McBrien NA, Moghaddam HO, Cottriall CL, Leech EM, Cornell LM (1995) The effects of blockade of retinal cell action potentials on ocular growth, emmetropization and form deprivation myopia in young chicks. Vision Res 35:1141-1152.
McCarthy CS, Megaw P, Devadas M, Morgan IG (2007) Dopaminergic agents affect the ability of brief periods of normal vision to prevent form-deprivation myopia. Exp Eye Res 84:100-107.
McKnight CM, Sherwin JC, Yazar S, Forward H, Tan AX, Hewitt AW, Pennell CE, McAllister IL, Young TL, Coroneo MT, Mackey DA (2014) Myopia in young adults is inversely related to an objective marker of ocular sun exposure: the Western Australian Raine cohort study. Am J Ophthalmol 158:1079-1085.
Miyake Y, Yagasaki K, Horiguchi M, Kawase Y, Kanda T (1986) Congenital stationary night blindness with negative electroretinogram. A new classification. Arch Ophthalmol 104:1013-1020.
Mooslehner KA, Chan PM, Xu W, Liu L, Smadja C, Humby T, Allen ND, Wilkinson LS, Emson PC (2001) Mice with very low expression of the vesicular monoamine transporter 2 gene survive into adulthood: potential mouse model for parkinsonism. Mol Cell Biol 21:5321-5331.
Morgan I, Rose K (2005) How genetic is school myopia? Prog Retin Eye Res 24:1-38.
Morgan IG, Ohno-Matsui K, Saw S-M (2012) Myopia. Lancet 379:1739-1748.
Morgan IG, He M, Rose KA (2017) EPIDEMIC OF PATHOLOGIC MYOPIA: What Can Laboratory Studies and Epidemiology Tell Us? Retina 37:989-997.
Morgan IG, French AN, Ashby RS, Guo X, Ding X, He M, Rose KA (2018) The epidemics of myopia: Aetiology and prevention. Prog Retin Eye Res 62:134-149.
Munch M, Kawasaki A (2013) Intrinsically photosensitive retinal ganglion cells: classification, function and clinical implications. Curr Opin Neurol 26:45-51.
Mutti DO, Zadnik K (2009) Has near work's star fallen? Optometry Vis Sci 86:76-78.
Mutti DO, Mitchell GL, Moeschberger ML, Jones LA, Zadnik K (2002) Parental myopia, near work, school achievement, and children's refractive error. Invest Ophthalmol Vis Sci 43:3633-3640.
Naarendorp F, Esdaille TM, Banden SM, Andrews-Labenski J, Gross OP, Pugh EN, Jr. (2010) Dark light, rod saturation, and the absolute and incremental sensitivity of mouse cone vision. J Neurosci 30:12495-12507.
Neal MJ, Cunningham JR, Matthews KL (1999) Release of Endogenous Ascorbic Acid Preserves Extracellular Dopamine in the Mammalian Retina. Invest Ophthalmol Vis Sci 40:2983-2987.
Nguyen-Legros J, Versaux-Botteri C, Savy C (1997) Dopaminergic and GABAergic retinal cell populations in mammals. Microsc Res Techniq 36:26-42.
Nickla DL, Totonelly K (2011) Dopamine antagonists and brief vision distinguish lens-induced- and form-deprivation-induced myopia. Exp Eye Res 93:782-785.
Nickla DL, Totonelly K (2016) Brief light exposure at night disrupts the circadian rhythms in eye growth and choroidal thickness in chicks. Exp Eye Res 146:189-195.
Nickla DL, Damyanova P, Lytle G (2009) Inhibiting the neuronal isoform of nitric oxide synthase has similar effects on the compensatory choroidal and axial responses to myopic

defocus in chicks as does the non-specific inhibitor L-NAME. Exp Eye Res 88:1092-1099.
Nickla DL, Totonelly K, Dhillon B (2010) Dopaminergic agonists that result in ocular growth inhibition also elicit transient increases in choroidal thickness in chicks. Exp Eye Res 91:715-720.
Nickla DL, Jordan K, Yang J, Totonelly K (2017a) Brief hyperopic defocus or form deprivation have varying effects on eye growth and ocular rhythms depending on the time-of-day of exposure. Exp Eye Res 161:132-142.
Nickla DL, Thai P, Zanzerkia Trahan R, Totonelly K (2017b) Myopic defocus in the evening is more effective at inhibiting eye growth than defocus in the morning: Effects on rhythms in axial length and choroid thickness in chicks. Exp Eye Res 154:104-115.
Nir I, Michael Iuvone P (1994) Alterations in light-evoked dopamine metabolism in dystrophic retinas of mutant rds mice. Brain Res 649:85-94.
Nir I, Haque, R., Iuvone, P.M. (2000) Diurnal metabolism of dopamine in the mouse retina. Brain Res 870:118-125.
Norton TT, Siegwart JT, Jr. (1995) Animal models of emmetropization: matching axial length to the focal plane. J Optom Assoc 66:405-414.
Norton TT, Siegwart JT (2013) Light levels, refractive development, and myopia - A speculative review. Exp Eye Res 114:48-53.
Norton TT, Essinger JA, McBrien NA (1994) Lid-suture myopia in tree shrews with retinal ganglion cell blockade. Visual Neurosci 11:143-153.
Norton TT, Amedo AO, Siegwart JT, Jr. (2010) The effect of age on compensation for a negative lens and recovery from lens-induced myopia in tree shrews (Tupaia glis belangeri). Vision Res 50:564-576.
O'Hare E, Flanagan D, Penzel T, Garcia C, Frohberg D, Heneghan C (2015) A comparison of radio-frequency biomotion sensors and actigraphy versus polysomnography for the assessment of sleep in normal subjects. Sleep Breath 19:91-98.
Ohngemach S, Hagel G, Schaeffel F (1997) Concentrations of biogenic amines in fundal layers in chickens with normal visual experience, deprivation, and after reserpine application. Visual Neurosci 14:493-505.
Okudaira N, Kripke DF, Webster JB (1983) Naturalistic studies of human light exposure. The Am J Physiol 245:R613-615.
Onal S, Toker E, Akingol Z, Arslan G, Ertan S, Turan C, Kaplan O (2007) Refractive errors of medical students in Turkey: one year follow-up of refraction and biometry. Optometry Vis Sci 84:175-180.
Ostrin LA, Sajjadi A, Benoit JS (2018) Objectively Measured Light Exposure During School and Summer in Children. Optom Vis Sci 95:332-342.
Pan CW, Ramamurthy D, Saw SM (2012a) Worldwide prevalence and risk factors for myopia. Ophthal Physl Opt 32:3-16.
Pan CW, Zheng YF, Wong TY, Lavanya R, Wu RY, Gazzard G, Saw SM (2012b) Variation in prevalence of myopia between generations of migrant indians living in Singapore. Am J Ophthalmol 154:376-381.e371.
Papastergiou GI, Schmid GF, Laties AM, Pendrak K, Lin T, Stone RA (1998) Induction of axial eye elongation and myopic refractive shift in one-year-old chickens. Vision Res 38:1883-1888.

Pardue MT, Allen RS (2018) Neuroprotective strategies for retinal disease. Prog Retin Eye Res 65:50-76.
Pardue MT, Stone RA, Iuvone PM (2013) Investigating mechanisms of myopia in mice. Exp Eye Res 114:96-105.
Pardue MT, Faulkner AE, Fernandes A, Yin H, Schaeffel F, Williams RW, Pozdeyev N, Iuvone PM (2008) High susceptibility to experimental myopia in a mouse model with a retinal on pathway defect. Invest Ophthalmol Vis Sci 49:706-712.
Park H, Tan CC, Faulkner A, Jabbar SB, Schmid G, Abey J, Iuvone PM, Pardue MT (2013) Retinal degeneration increases susceptibility to myopia in mice. Mol Vis 19:2068-2079.
Park H, Jabbar SB, Tan CC, Sidhu CS, Abey J, Aseem F, Schmid G, Iuvone PM, Pardue MT (2014) Visually driven ocular growth in mice requires functional rod photoreceptors. Invest Ophthalmol Vis Sci 55:6272-6279.
Parsons SM, Bahr BA, Rogers GA, Clarkson ED, Noremberg K, Hicks BW (1993) Acetylcholine transporter--vesamicol receptor pharmacology and structure. Prog Brain Res 98:175-181.
Parssinen O, Lyyra A (1993) Myopia and myopic progression among schoolchildren: a three-year follow-up study. Invest Ophthalmol Vis Sci 34:2794-2802.
Parssinen O, Kauppinen M, Viljanen A (2014) The progression of myopia from its onset at age 8-12 to adulthood and the influence of heredity and external factors on myopic progression. A 23-year follow-up study. Acta Ophthalmol 92:730-739.
Peter D, Jimenez J, Liu Y, Kim J, Edwards RH (1994) The chromaffin granule and synaptic vesicle amine transporters differ in substrate recognition and sensitivity to inhibitors. J Biol Chem 269:7231-7237.
Pokharel GP, Negrel AD, Munoz SR, Ellwein LB (2000) Refractive Error Study in Children: results from Mechi Zone, Nepal. Am J Ophthalmol 129:436-444.
Popova E (2014) Ionotropic GABA Receptors and Distal Retinal ON and OFF Responses. Scientifica 2014:23.
Pozdeyev N, Tosini G, Li L, Ali F, Rozov S, Lee RH, Iuvone PM (2008) Dopamine modulates diurnal and circadian rhythms of protein phosphorylation in photoreceptor cells of mouse retina. Eur J Neurosci 27:2691-2700.
Puopolo M, Hochstetler SE, Gustincich S, Wightman RM, Raviola E (2001) Extrasynaptic release of dopamine in a retinal neuron: activity dependence and transmitter modulation. Neuron 30:211-225.
Qiao S-N, Zhang Z, Ribelayga CP, Zhong Y-M, Zhang D-Q (2016) Multiple cone pathways are involved in photic regulation of retinal dopamine. Sci Rep 6:28916.
Quek TP, Chua CG, Chong CS, Chong JH, Hey HW, Lee J, Lim YF, Saw SM (2004) Prevalence of refractive errors in teenage high school students in Singapore. Ophthalmic Physl Opt 24:47-55.
Quinn GE, Shin CH, Maguire MG, Stone RA (1999) Myopia and ambient lighting at night. Nature 399:113-114.
Raviola E, Wiesel TN (1990) Neural control of eye growth and experimental myopia in primates. Ciba F Symp 155:22-44.
Read SA, Collins MJ, Vincent SJ (2014) Light exposure and physical activity in myopic and emmetropic children. Optom Vis Sci 91:330-341.
Read SA, Collins MJ, Vincent SJ (2015) Light Exposure and Eye Growth in Childhood. Invest Ophthalmol Vis Sci 56:6779-6787.

Read SA, Vincent SJ, Tan CS, Ngo C, Collins MJ, Saw SM (2018) Patterns of Daily Outdoor Light Exposure in Australian and Singaporean Children. Trans Vis Sci Technol 7:8.
Reimer RJ, Fon EA, Edwards RH (1998) Vesicular neurotransmitter transport and the presynaptic regulation of quantal size. Curr Opin Neurobiol 8:405-412.
Resnikoff S, Pascolini D, Mariotti SP, Pokharel GP (2008) Global magnitude of visual impairment caused by uncorrected refractive errors in 2004. Bulletin of the World Health Organization 86:63-70.
Rohrer B, Spira AW, Stell WK (1993) Apomorphine blocks form-deprivation myopia in chickens by a dopamine D2-receptor mechanism acting in retina or pigmented epithelium. Visual Neurosci 10:447-453.
Rose KA, French AN, Morgan IG (2016) Environmental Factors and Myopia: Paradoxes and Prospects for Prevention. Asia Pac J Ophthalmol 5:403-410.
Rose KA, Morgan IG, Smith W, Burlutsky G, Mitchell P, Saw SM (2008a) Myopia, lifestyle, and schooling in students of Chinese ethnicity in Singapore and Sydney. Arch Ophthalmol 126:527-530.
Rose KA, Morgan IG, Ip J, Kifley A, Huynh S, Smith W, Mitchell P (2008b) Outdoor activity reduces the prevalence of myopia in children. Ophthalmology 115:1279-1285.
Rosner M, Belkin M (1987) Intelligence, education, and myopia in males. Arch Ophthalmol 105:1508-1511.
Rucker FJ (2013) The role of luminance and chromatic cues in emmetropisation. Ophthal Physl Opt 33:196–214.
Rudnick G, Steiner-Mordoch SS, Fishkes H, Stern-Bach Y, Schuldiner S (1990) Energetics of reserpine binding and occlusion by the chromaffin granule biogenic amine transporter. Biochemistry 29:603-608.
Sakamoto K, Liu C, Kasamatsu M, Pozdeyev NV, Iuvone PM, Tosini G (2005) Dopamine regulates melanopsin mRNA expression in intrinsically photosensitive retinal ganglion cells. Eur J Neurosci 22:3129-3136.
Sankaridurg P, Holden B, Smith E, 3rd, Naduvilath T, Chen X, de la Jara PL, Martinez A, Kwan J, Ho A, Frick K, Ge J (2011) Decrease in rate of myopia progression with a contact lens designed to reduce relative peripheral hyperopia: one-year results. Invest Ophthalmol Vis Sci 52:9362-9367.
Saw S, Zhang M, Hong R, Fu Fu Z, Pang M, Tan DH (2002) Near-work activity, night-lights, and myopia in the singapore-china study. Arch Ophthalmol 120:620-627.
Saw S-M, Shankar A, Tan S-B, Taylor H, Tan DTH, Stone RA, Wong T-Y (2006) A Cohort Study of Incident Myopia in Singaporean Children. Invest Ophthalmol Vis Sci 47:1839-1844.
Saw SM, Chua WH, Wu HM, Yap E, Chia KS, Stone RA (2000) Myopia: gene-environment interaction. Ann Acad Med Singap 29:290-297.
Saw SM, Cheng A, Fong A, Gazzard G, Tan DT, Morgan I (2007) School grades and myopia. Ophthalmic Physl Opt 27:126-129.
Schaeffel F (2008) Test systems for measuring ocular parameters and visual function in mice. Front Biosci 13:4904-4911.
Schaeffel F, Feldkaemper M (2015) Animal models in myopia research. Clin Exp Optom 98:507-517.
Schaeffel F, Glasser A, Howland HC (1988) Accommodation, refractive error and eye growth in chickens. Vision Res 28:639-657.

Schaeffel F, Bartmann M, Hagel G, Zrenner E (1995) Studies on the role of the retinal dopamine/melatonin system in experimental refractive errors in chickens. Vision Res 35:1247-1264.
Schaeffel F, Burkhardt E, Howland HC, Williams RW (2004) Measurement of refractive state and deprivation myopia in two strains of mice. Optom Vis Sci 81:99-110.
Schippert R, Burkhardt E, Feldkaemper M, Schaeffel F (2007) Relative axial myopia in Egr-1 (ZENK) knockout mice. Invest Ophthalmol Vis Sci 48:11-17.
Schmid KL, Wildsoet CF (2004) Inhibitory effects of apomorphine and atropine and their combination on myopia in chicks. Optometry Vis Sci 81:137-147.
Schmidt TM, Kofuji P (2009) Functional and morphological differences among intrinsically photosensitive retinal ganglion cells. J Neurosci 29:476-482.
Schmidt TM, Chen SK, Hattar S (2011) Intrinsically photosensitive retinal ganglion cells: many subtypes, diverse functions. Trends Neurosci 34:572-580.
Schmucker C, Seeliger M, Humphries P, Biel M, Schaeffel F (2005) Grating Acuity at Different Luminances in Wild-Type Mice and in Mice Lacking Rod or Cone Function. Invest Ophthalmol Vis Sci 46:398-407.
Schwahn HN, Schaeffel F (1997) Flicker parameters are different for suppression of myopia and hyperopia. Vision Res 37:2661-2673.
Seeman P, Van Tol HH (1994) Dopamine receptor pharmacology. Trends Pharmacol Sci 15:264-270.
Segura-Aguilar J, Paris I, Munoz P, Ferrari E, Zecca L, Zucca FA (2014) Protective and toxic roles of dopamine in Parkinson's disease. J Neurochem 129:898-915.
Sexton T, Buhr E, Van Gelder RN (2012) Melanopsin and mechanisms of non-visual ocular photoreception. J Biol Chem 287:1649-1656.
Shah RL, Huang Y, Guggenheim JA, Williams C (2017) Time Outdoors at Specific Ages During Early Childhood and the Risk of Incident Myopia. Invest Ophthalmol Vis Sci 58:1158-1166.
Sherman SM, Norton TT, Casagrande VA (1977) Myopia in the lid-sutured tree shrew (Tupaia glis). Brain Res 124:154-157.
Sherwin JC, Hewitt AW, Coroneo MT, Kearns LS, Griffiths LR, Mackey DA (2012) The association between time spent outdoors and myopia using a novel biomarker of outdoor light exposure. Invest Ophthalmol Vis Sci 53:4363-4370.
Siegwart Jr JT, Ward AH, Norton TT (2012) Moderately elevated fluorescent light levels slow form deprivation and minus lens-induced myopia development in tree shrews. Invest Ophthalmol Vis Sci 53:3457-3457.
Siegwart JT, Ward AH, Norton TT (2012) Moderately Elevated Fluorescent Light Levels Slow Form Deprivation and Minus Lens-Induced Myopia Development in Tree Shrews. Invest Ophthalmol Vis Sci 53:3457-3457.
Simmons AB, Fuerst PG (2018) Analysis of Retinal Vascular Plexuses and Interplexus Connections. In: Mouse Retinal Phenotyping: Methods and Protocols (Tanimoto N, ed), pp 317-330. New York, NY: Springer New York.
Sivak JG, Barrie DL, Callender MG, Doughty MJ, Seltner RL, West JA (1990) Optical causes of experimental myopia. Ciba F Symp 155:160-172.
Smith EL, 3rd, Hung LF, Huang J (2009) Relative peripheral hyperopic defocus alters central refractive development in infant monkeys. Vision Res 49:2386-2392.

Smith EL, 3rd, Hung LF, Huang J (2012) Protective effects of high ambient lighting on the development of form-deprivation myopia in rhesus monkeys. Invest Ophthalmol Vis Sci 53:421-428.
Smith EL, 3rd, Bradley DV, Fernandes A, Boothe RG (1999) Form deprivation myopia in adolescent monkeys. Optom Vis Sci 76:428-432.
Smith EL, 3rd, Kee CS, Ramamirtham R, Qiao-Grider Y, Hung LF (2005) Peripheral vision can influence eye growth and refractive development in infant monkeys. Invest Ophthalmol Vis Sci 46:3965-3972.
Smith EL, 3rd, Hung LF, Huang J, Blasdel TL, Humbird TL, Bockhorst KH (2010) Effects of optical defocus on refractive development in monkeys: evidence for local, regionally selective mechanisms. Invest Ophthalmol Vis Sci 51:3864-3873.
Smith EL, 3rd, Ramamirtham R, Qiao-Grider Y, Hung LF, Huang J, Kee CS, Coats D, Paysse E (2007a) Effects of foveal ablation on emmetropization and form-deprivation myopia. Invest Ophthalmol Vis Sci 48:3914-3922.
Smith IIIEL, Hung L-F, Arumugam B, Huang J (2013) Negative Lens–Induced Myopia in Infant Monkeys: Effects of High Ambient LightingLens-Induced Myopia and Lighting Levels. Invest Ophthalmol Vis Sci 54:2959-2969.
Smith M, Whittock N, Searle A, Croft M, Brewer C, Cole M (2007b) Phenotype of autosomal dominant cone-rod dystrophy due to the R838C mutation of the GUCY2D gene encoding retinal guanylate cyclase-1. Eye 21:1220-1225.
Soler M, Anera RG, Castro JJ, Jimenez R, Jimenez JR (2015) Prevalence of refractive errors in children in Equatorial Guinea. Optom Vis Sci 92:53-58.
Sonoda T, Lee SK, Birnbaumer L, Schmidt TM (2018) Melanopsin Phototransduction Is Repurposed by ipRGC Subtypes to Shape the Function of Distinct Visual Circuits. Neuron 99:754-767.e4.
Stone RA, Lin T, Iuvone PM (1989) Retinal dopamine and form-deprivation myopia. Proc Natl Acad Sci U S A 86:704-706.
Stone RA, Lin T, Laties AM (1991) Muscarinic antagonist effects on experimental chick myopia. Exp Eye Res 52:755-758.
Stone RA, Pardue MT, Iuvone PM, Khurana TS (2013) Pharmacology of myopia and potential role for intrinsic retinal circadian rhythms. Exp Eye Res 114:35-47.
Stone RA, Liu J, Sugimoto R, Capehart C, Zhu X, Pendrak K (2003) GABA, experimental myopia, and ocular growth in chick. Invest Ophthalmol Vis Sci 44:3933-3946.
Stone RA, McGlinn AM, Baldwin DA, Tobias JW, Iuvone PM, Khurana TS (2011) Image Defocus and Altered Retinal Gene Expression in Chick: Clues to the Pathogenesis of Ametropia. Invest Ophthalmol Vis Sci 52:5765-5777.
Stone RA, Pendrak K, Sugimoto R, Lin T, Gill AS, Capehart C, Liu J (2006) Local patterns of image degradation differentially affect refraction and eye shape in chick. Curr Eye Res 31:91-105.
Stone RA, Cohen Y, McGlinn AM, Davison S, Casavant S, Shaffer J, Khurana TS, Pardue MT, Iuvone PM (2016a) Development of Experimental Myopia in Chicks in a Natural Environment. Invest Ophthalmol Vis Sci 57:4779-4789.
Sun Y, Zhao N, Liu W, Liu M, Ju Z, Li J, Cheng Z, Liu X (2018) Study of Vesicular Monoamine Transporter 2 in Myopic Retina Using [18F]FP-(+)-DTBZ. Mol Imaging Biol 20:771-779.
Sung C-H, Chuang J-Z (2010) The cell biology of vision. J Cell Biol 190:953.

Taylor TN, Caudle WM, Shepherd KR, Noorian A, Jackson CR, Iuvone PM, Weinshenker D, Greene JG, Miller GW (2009) Nonmotor symptoms of Parkinson's disease revealed in an animal model with reduced monoamine storage capacity. J Neurosci 29:8103-8113.

Tikidji-Hamburyan A, Reinhard K, Storchi R, Dietter J, Seitter H, Davis KE, Idrees S, Mutter M, Walmsley L, Bedford RA, Ueffing M, Ala-Laurila P, Brown TM, Lucas RJ, Munch TA (2017) Rods progressively escape saturation to drive visual responses in daylight conditions. Nat Commun 8:1813.

Tkatchenko TV, Shen Y, Tkatchenko AV (2010) Mouse experimental myopia has features of primate myopia. Invest Ophthalmol Vis Sci 51:1297-1303.

Toon E, Davey MJ, Hollis SL, Nixon GM, Horne RS, Biggs SN (2016) Comparison of Commercial Wrist-Based and Smartphone Accelerometers, Actigraphy, and PSG in a Clinical Cohort of Children and Adolescents. J Clin Sleep Med 12:343-350.

Toren P, Rehavi M, Luski A, Roz N, Laor N, Lask M, Weizman A (2005) Decreased platelet vesicular monoamine transporter density in children and adolescents with attention deficit/hyperactivity disorder. Eur Neuropsychopharm 15:159-162.

Troilo D, Judge SJ (1993) Ocular development and visual deprivation myopia in the common marmoset (Callithrix jacchus). Vision Res 33:1311-1324.

Troilo D, Gottlieb MD, Wallman J (1987) Visual deprivation causes myopia in chicks with optic nerve section. Curr Eye Res 6:993-999.

Tu DC, Zhang D, Demas J, Slutsky EB, Provencio I, Holy TE, Van Gelder RN (2005) Physiologic diversity and development of intrinsically photosensitive retinal ganglion cells. Neuron 48:987-999.

Turner PV, Albassam MA (2005) Susceptibility of rats to corneal lesions after injectable anesthesia. Comparative Med 55:175-182.

Urschel S, Hoher T, Schubert T, Alev C, Sohl G, Worsdorfer P, Asahara T, Dermietzel R, Weiler R, Willecke K (2006) Protein kinase A-mediated phosphorylation of connexin36 in mouse retina results in decreased gap junctional communication between AII amacrine cells. J Biol Chem 281:33163-33171.

Van den Bulck J (2007) Adolescent use of mobile phones for calling and for sending text messages after lights out: results from a prospective cohort study with a one-year follow-up. Sleep 30:1220-1223.

Van Hook MJ, Wong KY, Berson DM (2012) Dopaminergic modulation of ganglion-cell photoreceptors in rat. Eur J Neurosci 35:507-518.

Verkicharla PK, Ohno-Matsui K, Saw SM (2015) Current and predicted demographics of high myopia and an update of its associated pathological changes. Ophthalmic Physiol Opt 35:465-475.

Verkicharla PK, Ramamurthy D, Nguyen QD, Zhang X, Pu SH, Malhotra R, Ostbye T, Lamoureux EL, Saw SM (2017) Development of the FitSight Fitness Tracker to Increase Time Outdoors to Prevent Myopia. Translational vision science & technology 6:20.

Vitale S, Sperduto RD, Ferris FL, 3rd (2009) Increased prevalence of myopia in the United States between 1971-1972 and 1999-2004. Arch Ophthalmol 127:1632-1639.

Vugler AA, Redgrave P, Semo M, Lawrence J, Greenwood J, Coffey PJ (2007) Dopamine neurones form a discrete plexus with melanopsin cells in normal and degenerating retina. Exp Neurol 205:26-35.

Vuong HE, Hardi CN, Barnes S, Brecha NC (2015) Parallel Inhibition of Dopamine Amacrine Cells and Intrinsically Photosensitive Retinal Ganglion Cells in a Non-Image-Forming Visual Circuit of the Mouse Retina. J Neurosci 35:15955-15970.

Walline JJ, Greiner KL, McVey ME, Jones-Jordan LA (2013) Multifocal contact lens myopia control. Optom Vis Sci 90:1207-1214.

Wallman J, Adams JI (1987) Developmental aspects of experimental myopia in chicks: susceptibility, recovery and relation to emmetropization. Vision Res 27:1139-1163.

Wallman J, Winawer J (2004) Homeostasis of eye growth and the question of myopia. Neuron 43:447-468.

Wallman J, Turkel J, Trachtman J (1978) Extreme myopia produced by modest change in early visual experience. Science 201:1249-1251.

Wang D, Chun RK, Liu M, Lee RP, Sun Y, Zhang T, Lam C, Liu Q, To CH (2016) Optical Defocus Rapidly Changes Choroidal Thickness in Schoolchildren. PloS one 11:e0161535.

Ward AH, Siegwart JT, Jr., Frost MR, Norton TT (2016) The effect of intravitreal injection of vehicle solutions on form deprivation myopia in tree shrews. Exp Eye Res 145:289-296.

Weiler R, Baldridge WH, Mangel SC, Dowling JE (1997) Modulation of endogenous dopamine release in the fish retina by light and prolonged darkness. Visual Neurosci 14:351-356.

Weiss AR, Johnson NL, Berger NA, Redline S (2010) Validity of activity-based devices to estimate sleep. J Clin Sleep Med 6:336-342.

Wiesel TN, Raviola E (1977) Myopia and eye enlargement after neonatal lid fusion in monkeys. Nature 266:66-68.

Wildsoet C (2003) Neural pathways subserving negative lens-induced emmetropization in chicks--insights from selective lesions of the optic nerve and ciliary nerve. Curr Eye Res 27:371-385.

Wildsoet C, Clark I (1993) Interacting effects of kainic acid and 6-hydroxydopamine on eye growth and refraction in the chick. Invest Ophthalmol Vis Sci, pp 1210-1210.

Wildsoet C, Wallman J (1995) Choroidal and scleral mechanisms of compensation for spectacle lenses in chicks. Vision Res 35:1175-1194.

Williams KM et al. (2015) Prevalence of refractive error in Europe: the European Eye Epidemiology (E(3)) Consortium. Eur J Epidemiol 30:305-315.

Witkovsky P (2004) Dopamine and retinal function. Doc Ophthalmol 108:17-39.

Witkovsky P, Gabriel R, Krizaj D (2008) Anatomical and Neurochemical Characterization of Dopaminergic Interplexiform Processes in Mouse and Rat Retinas. J Comp Neurol 510:158-174.

Witkovsky P, Arango-Gonzalez B, Haycock JW, Kohler K (2005) Rat retinal dopaminergic neurons: differential maturation of somatodendritic and axonal compartments. J Comp Neurol 481:352-362.

Witkovsky P, Veisenberger E, Haycock JW, Akopian A, Garcia-Espana A, Meller E (2004) Activity-dependent phosphorylation of tyrosine hydroxylase in dopaminergic neurons of the rat retina. J Neurosci 24:4242-4249.

Wojciechowski R (2011) Nature and nurture: the complex genetics of myopia and refractive error. Clin Genet 79:301-320.

Wu PC, Tsai CL, Hu CH, Yang YH (2010) Effects of outdoor activities on myopia among rural school children in Taiwan. Ophthalmic Epidemiol 17:338-342.

Wu PC, Tsai CL, Wu HL, Yang YH, Kuo HK (2013) Outdoor activity during class recess reduces myopia onset and progression in school children. Ophthalmology 120:1080-1085.

Wu PC, Chen CT, Lin KK, Sun CC, Kuo CN, Huang HM, Poon YC, Yang ML, Chen CY, Huang JC, Wu PC, Yang IH, Yu HJ, Fang PC, Tsai CL, Chiou ST, Yang YH (2018) Myopia Prevention and Outdoor Light Intensity in a School-Based Cluster Randomized Trial. Ophthalmology 125:1239-1250.

Wu XH, Li YY, Zhang PP, Qian KW, Ding JH, Hu G, Weng SJ, Yang XL, Zhong YM (2015a) Unaltered retinal dopamine levels in a C57BL/6 mouse model of form-deprivation myopia. Invest Ophthalmol Vis Sci 56:967-977.

Wu YT, Willcox M, Zhu H, Stapleton F (2015b) Contact lens hygiene compliance and lens case contamination: A review. Contact Lens Anterio 38:307-316.

Yaffe D, Forrest LR, Schuldiner S (2018) The ins and outs of vesicular monoamine transporters. J Gen Physiol 150:671-682.

Yazulla S, Zucker CL (1988) Synaptic organization of dopaminergic interplexiform cells in the goldfish retina. Visual Neurosci 1:13-29.

Yin L, Smith RG, Sterling P, Brainard DH (2006) Chromatic properties of horizontal and ganglion cell responses follow a dual gradient in cone opsin expression. J Neurosci 26:12351-12361.

Yingyong P (2010) Risk factors for refractive errors in primary school children (6-12 years old) in Nakhon Pathom Province. J Med Assoc Thailand 93:1288-1293.

Zhang DQ, Wong KY, Sollars PJ, Berson DM, Pickard GE, McMahon DG (2008) Intraretinal signaling by ganglion cell photoreceptors to dopaminergic amacrine neurons. Proc Natl Acad Sci U S A 105:14181-14186.

Zhang Y, Raychaudhuri S, Wildsoet CF (2016a) Imposed Optical Defocus Induces Isoform-Specific Up-Regulation of TGFbeta Gene Expression in Chick Retinal Pigment Epithelium and Choroid but Not Neural Retina. PloS one 11:e0155356.

Zhang Y, Liu Y, Hang A, Phan E, Wildsoet CF (2016b) Differential gene expression of BMP2 and BMP receptors in chick retina & choroid induced by imposed optical defocus. Visual Neurosci 33:E015.

Zhao J, Qu X, Qi Y, Zhou W, Liu X (2010) Study on retinal dopamine transporter in form deprivation myopia using the radiopharmaceutical tracer 99mTc-TRODAT-1. Nucl Med Commun 31:910-915.

Zhao L, Wang C, Song D, Li Y, Song Y, Su G, Dunaief JL (2014) Systemic administration of the antioxidant/iron chelator alpha-lipoic acid protects against light-induced photoreceptor degeneration in the mouse retina. Invest Ophthalmol Vis Sci 55:5979-5988.

Zhao X, Wong KY, Zhang DQ (2017) Mapping physiological inputs from multiple photoreceptor systems to dopaminergic amacrine cells in the mouse retina. Sci Rep 7:7920.

Zhou X, Pardue MT, Iuvone PM, Qu J (2017) Dopamine signaling and myopia development: What are the key challenges. Prog Retin Eye Res 61:60-71.

Zubieta JK, Taylor SF, Huguelet P, Koeppe RA, Kilbourn MR, Frey KA (2001) Vesicular monoamine transporter concentrations in bipolar disorder type I, schizophrenia, and healthy subjects. Biol Psychiat 49:110-116.

Zucker M, Valevski A, Weizman A, Rehavi M (2002a) Increased platelet vesicular monoamine transporter density in adult schizophrenia patients. Eur Neuropsychopharm 12:343-347.
Zucker M, Aviv A, Shelef A, Weizman A, Rehavi M (2002b) Elevated platelet vesicular monoamine transporter density in untreated patients diagnosed with major depression. Psychiat Res 112:251-256.