THE ROLE OF TYROSINASE IN THE TIME COURSE OF LIGHT-INDUCED CONE MOVEMENTS IN GOLDFISH RETINA

by

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DEDICATION

This thesis is dedicated to a woman who received a call many years ago from a teary eyed teenager ready to give up on her love of science to pursue something a little *'less'* challenging. That teary eyed teenager was me, and the woman my Godmother, Zena Elizabeth Francis. I remember the conversation like it was yesterday. I had just received the final grades from my first semester at university and after looking at them, I was convinced either the letters were wrong or I was in the wrong program.

After she listened to me read each grade out loud and express my desire to give up. She didn't tell me how disappointed she was in me, she didn't tell me to suck it up nor did she advise me to settle for something that was *'less'* challenging. Instead, she gently reminded me that sometimes in life I must fight a litter harder, hold on a little longer and perhaps take the unconventional route in order to achieve my goals. Most importantly, she encouraged me to rediscover the passion that initially led me to Dalhousie University.

Years later, I sit here once again with tears in my eyes typing the dedication page to my Master's thesis project. This time the tears are of joy and gratitude for it has been a wonderful journey thus far.

A wonderful journey I would've completely missed out on had you allowed me to settle for something *'less'* challenging. You have taught me that challenges make life interesting and propel me forward. For your unconditional love and unwavering faith in me I am eternally grateful from the very bottom of my heart.

Thank you Godma!

Love always,
Sophie

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ABSTRACT

Many teleosts regulate the amount of light incident to the retina through retinomotor movements (RMMs), adjustments of the length of photoreceptors and the distribution of melanin within retinal pigment epithelium (RPE). In the dark, rods contract, cones elongate and pigment aggregates within the basal RPE. In the light, rods elongate, cones contract and pigment disperses within the RPE. It is generally thought that RMMs are regulated by the release of dopamine in the light from tyrosine hydroxylase-containing I1 interplexiform cells (IPCs), acting on D2 family dopamine receptors. However, RMMs are not lost when dopaminergic IPCs are destroyed by injection of the neurotoxin 6-hydroxydopamine (6-OHDA). A potential alternate source of dopamine is the RPE, due to the activity of tyrosinase. In this thesis I tested the hypothesis that light-induced cone contraction depends on tyrosinase activity by treating adult goldfish (*Carassius auratus*) with the specific tyrosinase inhibitor phenylthiourea (PTU). PTU did not block light-induced cone contraction, nor did 6-OHDA or the combined treatment of PTU and 6-OHDA. However, PTU slowed the progression of light-induced cone contraction as did 6-OHDA, and the slowest extent of progression was achieved when PTU and 6-OHDA were combined. These results suggest that both neuronal (dopaminergic IPCs) and non-neuronal (RPE) dopamine contribute to light-induced cone contraction. That the combined treatment with PTU and 6-OHDA did not block light-induced cone contraction may indicate that one or both of these treatments do not eliminate completely dopamine signalling in the teleost retina, or that light-induced cone contraction may also involve other mechanisms independent of dopamine.

LIST OF ABBREVIATONS USED

3-MT	3-methoxytyramine
6-OHDA	6-Hydroxydopamine
AADC	Aromatic L-amino Acid Decarboxylase
ANOVA	Analysis of Variance
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanosine Monophosphate
COMT	Catechol-O-Methyl Transferase
Cy3	Cyanine
DAT	Dopamine Active Transporter
DDC	Dopamine Decarboxylase
DOPAC	3,4-dihydroxyphenylacetic acid
FITC	Fluorescein Isothiocyanate
GCL	Ganglion Cell Layer
GPCR	G-Protein Coupled Receptor
HeNe	Helium-Neon Laser
HPLC	High Pressure Liquid Chromatography
HVA	Homovanillic Acid
ILM	Inner Limiting Membrane
INL	Inner Nuclear Layer
IPCs	Interplexiform Cells
IPL	Inner Plexiform Layer
L-DOPA	L-3,4-dihydroxyphenylalanine

MAO	Monoamine Oxidase
MS-222	Buffered Tricaine Methane Sulphonate
N.A.	Numerical Aperture
NGS	Normal Goat Serum
OCT-3	Cation Transporter-3
OLM	Outer Limiting Membrane
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
PBS	Phosphate Buffered Saline
PKA	Protein Kinase A
PRL	Photoreceptor Layer
PTU	Phenylthiourea
RMMs	Retinomotor Movements
RPE	Retinal Pigmented Epithelium
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SD	Standard Deviation
sdy	Sandy Mutants
TOH	Tyrosine Hydroxylase
vVMAT	Vesicular Monoamine Transporter

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I would like to thank my parents **Ram** and **Kopila Thapa** for all their love and support. I am so grateful for all the sacrifices you've made so that I could pursue my dreams. I would not be here if were not for you both.

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I've been very blessed to have encountered so many wonderful, kind, and selfless people on my journey, but to list them all here would mean this acknowledgements section would be longer than my thesis. So if I have not mentioned you please know that your contribution will never be forgotten. To all my loving family, friends, coworkers, colleagues and mentors, I am forever grateful! Thank you!

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CHAPTER 1: INTRODUCTION

This thesis describes experiments that investigated the potential role of tyrosinase in one aspect of retinomotor movements (RMMs): progression of light-induced cone contraction. I first provide some background information concerning the structure and function of the vertebrate retina, with emphasis on the teleost retina, as this was the experimental vertebrate system that was used for this work. I then review the current state of knowledge concerning RMMs with particular emphasis on the regulation of RMMs by dopamine. This will then lead to the formation of a ***hypothesis*** concerning dopaminergic control of RMMs followed by the specific aims that address this hypothesis.

1.1 The Vertebrate Retina: Anatomy

The vertebrate retina is a thin transparent layer of nervous tissue that lines the back of the eye. The retina has been studied in detail for more than a century, notably by Santiago Ramon y Cajal whose book, *La rétine des vértébrés*, published originally in 1893 (for English translation see Rodieck, 1973), continues to guide retinal research to this day. In more recent years, several comprehensive reviews and textbooks have been written concerning the retina (Dowling, 1987; Dowling, 2012; Kolb, 2003; Rodieck, 1973) and the fundamentals of retina structure and function described below were derived from these sources.

Despite its peripheral location relative to the brain, the retina is part of the central nervous system, developing from an invagination of the embryonic forebrain. The retina is ~0.5 mm thick and is composed of six different cell types arranged stereotypically (see Fig. 1).

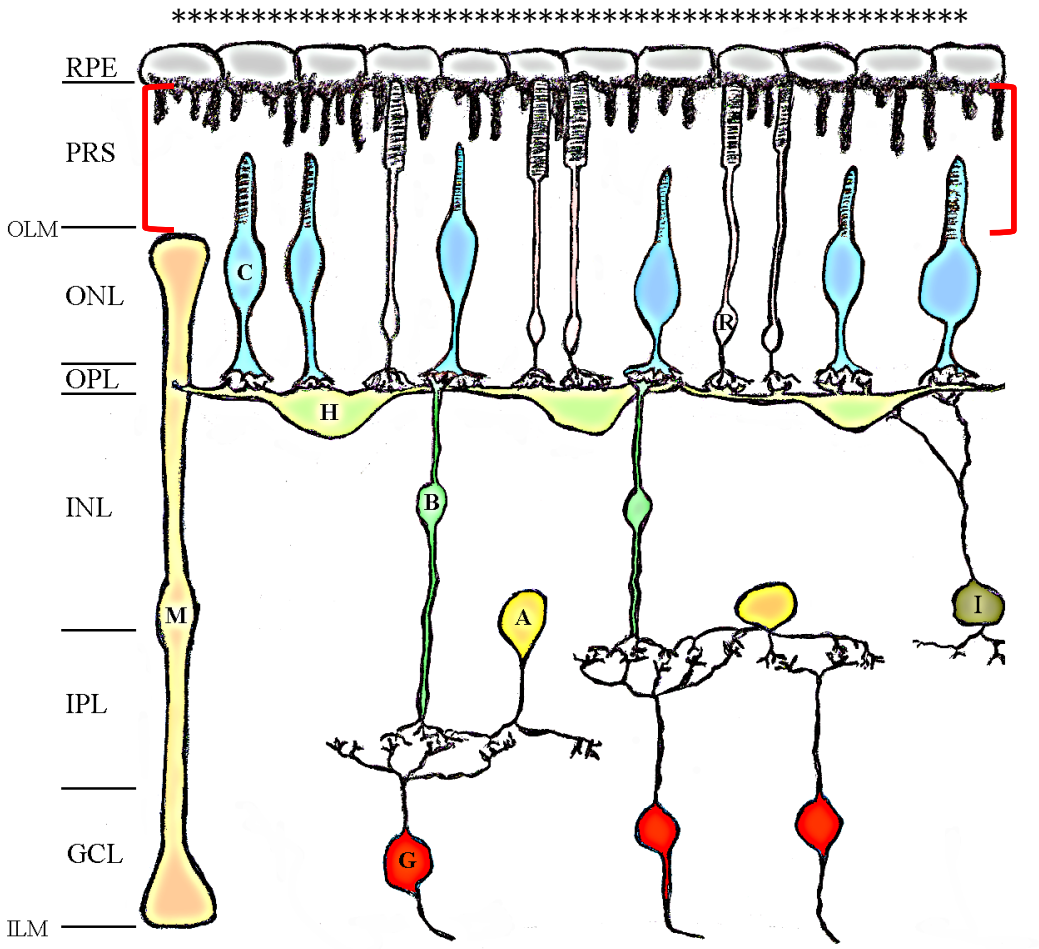


Figure 1: Schematic of the vertebrate retina. RPE, retinal pigmented epithelium; PRS, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; OLM, outer limiting membrane; ILM, inner limiting membrane; C, cone; R, rod; H, horizontal cell; B, bipolar cell; A, amacrine cell; I, interplexiform cell; M, Muller cell; G, ganglion cell. Red brackets indicate the sub-retinal space. Asterisks indicate Bruch's membrane. (Adapted from and used with permission of Dr. Bryan Daniels).

The four cellular layers of the retina, starting at the inner surface (adjacent to the vitreous) are the ganglion cell layer (GCL), the inner nuclear layer (INL), the outer nuclear layer (ONL) and the photoreceptor layer (PRL). Between these cellular layers are two synaptic layers, the inner plexiform (IPL) and outer plexiform (OPL), that house the synaptic connections between the different cell types in the retina.

Müller cells are the primary glial cells of the retina. Although their cell bodies are located in the INL these radial glia span almost the whole thickness of the tissue and form junctions (desmosomes or zonula adherens) with each other and with photoreceptors resulting in the inner and outer limiting membranes (ILM and OLM, respectively). These glia also play a vital role in maintaining homeostatic conditions in the retina with some of their responsibilities including the recycling of neurotransmitter, disposal of neural waste products and forming protective sheaths that surround neuronal cell bodies and processes within the retina.

Photoreceptors are specialized neurons that are responsible for phototransduction, the conversion of light into an electrochemical signal. In most vertebrates there are two principal types of photoreceptors, the rods and the cones. In recent years, other retinal neurons, and even retinal pigmented epithelium (RPE), have been shown to respond directly to light or to contain photopigment (Cheng et al., 2009; Hughes et al., 2016; Peirson et al., 2004). However, rods and cones remain regarded as the principal photosensitive retinal elements associated with vision.

Rods are more sensitive to light than cones and can respond to a single photon of light, thus rods subserve vision in low light conditions and are responsible for vision during night. Cones are responsible for vision when light is abundant, for example during daytime. The cone photoreceptors can be divided further into subtypes and the number of

variations of cone photoreceptors present depends on the species of interest. Teleosts, the group of vertebrates studied in this thesis, possess four kinds of cones, which are distinguished based on their peak sensitivity to different wavelengths of light: long (623 nm), medium (537 nm), short (447 nm) and ultraviolet (356 nm) (Palacios et al., 1998). Each photoreceptor has (from distal to proximal/outer to inner) an outer segment (containing photopigment, see below), an inner segment (containing various cytoplasmic organelles), a region containing the nucleus, and a synaptic terminal. The inner segment is subdivided further into an outer ellipsoid (containing densely-packed mitochondria) and an inner myoid (that contains Golgi apparatus and endoplasmic reticulum).

Photoreceptors are named based on the morphological characteristics of their outer segment; rod photoreceptor outer segments are shaped like rods whereas the cone photoreceptor outer segments are shaped like cones. In most terrestrial vertebrates there is a higher distribution of rods in the periphery of the retina compared to the center. In the center of the retina of some types of terrestrial animals is the fovea (primates) or area centralis (other terrestrial vertebrates), where cones are found at high density enabling high acuity vision. However, in teleosts rods and cones are distributed more or less equally throughout the retina.

As mentioned above, each photoreceptor has an outer segment and respective outer segments (cones or rods) are differentiated based on morphology and the photopigments within. Rod photoreceptor outer segments are long and have membranous discs that contain the visual pigment molecule rhodopsin. Cone photoreceptor outer segments are short and have plasma membrane invaginations that contain a specific cone opsin, which varies based on cone type. These visual pigments result from combining the vitamin A precursor retinaldehyde with proteins that are specific to rods (rhodopsin) and

each type of cone photoreceptor (opsins). Photoreceptors can be further differentiated based on their synaptic terminals. Rod terminals are somewhat spherical and therefore are called spherules whereas cone terminals, called pedicles, tend to be larger and more elongated.

Photoreceptors make synaptic connections with bipolar cells and horizontal cells within the OPL. As suggested by their name, horizontal cells are oriented laterally, relative to most other retinal neurons that are vertically oriented. Bipolar cells receive synaptic input from photoreceptors at dendrites positioned centrally within the photoreceptor terminal whereas horizontal cell dendrites are positioned laterally. Bipolar cells synapse onto amacrine and ganglion cells in the IPL.

1.2 The Vertebrate Retina: Physiology

Early philosophers believed that perception of sight was accomplished by particles emanating from the eyes. However, we now know that formation of a visual percept begins when light particles, called photons, enter the eye. As such, vision is a result of transduction of photons into neural signals. Light enters the eye through the pupil and is focused on the retina via the lens and cornea. At the level of the retina, light is absorbed by the photoreceptors and induces the isomerization of retinal from 11-*cis*-retinal to all-*trans*-retinal. The isomerization of the photopigments results in the activation of an opsin G-protein coupled receptor (GPCR) signal transduction cascade involving the G-protein transducin. This leads ultimately to a reduction in cyclic GMP (cGMP), through the activation of cyclic GMP phosphodiesterase, and a reduction in the activation of cyclic GMP-gated channels on the plasma membrane of the photoreceptor. The result is hyperpolarization of the membrane potential of the photoreceptor. The

activity of rhodopsin or cone opsin is terminated via phosphorylation of the photoreceptor and the binding of arrestin proteins.

Photoreceptors are relatively depolarized in the dark due to steady influx of cations through cGMP-gated channels. Neurotransmitter (glutamate) is released from photoreceptor synaptic terminals by conventional, calcium-dependent release. Therefore, there is relatively greater release of glutamate from photoreceptors in the dark than in the light. The change in glutamate release from photoreceptor synaptic terminals influences horizontal cells and bipolar cells at synapses within the OPL. Like photoreceptors, horizontal cells and bipolar cells respond to light with graded changes in membrane potential. The change in bipolar cell membrane potential governs the release of the neurotransmitter glutamate from these cells, thereby influencing post-synaptic amacrine and ganglion cells at synapses within the IPL. Unlike the preceding retinal neurons, ganglion cells, and some types of amacrine cells, respond by firing action potentials. The ganglion cell signal is carried by axons that ultimately form the optic nerve and project to targets in the brain.

The basic pattern of retinal processing is often described as consisting of a "vertical pathway" from photoreceptors to bipolar cells and then to ganglion cells that is modulated by "horizontal pathways" consisting of horizontal cells, in the OPL, and amacrine cells, in the IPL. The interaction between the vertical and horizontal pathways results in the rich output from different types of retinal ganglion cells. Some features of retinal ganglion cells include center-surround receptive-field organization, directional selectivity and colour opponency.

1.3 Interplexiform Cells

A cell type not yet discussed, but relevant to this thesis, are the interplexiform cells. First described by Ehinger et al., 1969 these cells have neurites in both plexiform layers and hence were named interplexiform cells (IPCs) (Cohen and Dowling, 1983; Dowling and Ehinger, 1978; Ehinger et al., 1969; Hedden and Dowling, 1978). The cell bodies of IPCs are located in the INL and have processes within the IPL where they receive synaptic input from bipolar and amacrine cells and make synaptic connections onto amacrine cell processes and ganglion cell dendrites (Fig. 2). In this way they are similar to amacrine cells. However, they are considered different because, unlike amacrine cells they also send projections to the OPL (Fig. 2).

In teleosts there are three types of IPCs, dopaminergic (I1), glycinergic (12) and a type (I3) that is phenylethanolamine N-methyltransferase (PNMT)-immunoreactive (Baldridge and Ball, 1993; Kalloniatis and Marc, 1990). This thesis is concerned mostly with the subset of IPCs that are thought to release dopamine, the dopaminergic IPCs (Dowling and Ehinger, 1975). These cells do not receive synaptic input in the OPL, leading to the suggestion that these cells receive inputs exclusively within the IPL but send outputs to both IPL and OPL (Hedden and Dowling, 1978; Kalloniatis and Marc, 1990).

1.4 Retinal Pigmented Epithelium Cells

The preceding description of the vertebrate retina is focused entirely on the neural retina. Adjacent to the neural retina, in close proximity to the photoreceptors, is a single layer of hexagonal epithelial cells densely packed with pigment granules, the retinal-pigmented epithelium (RPE). The pigmented cells that make up the RPE are joined

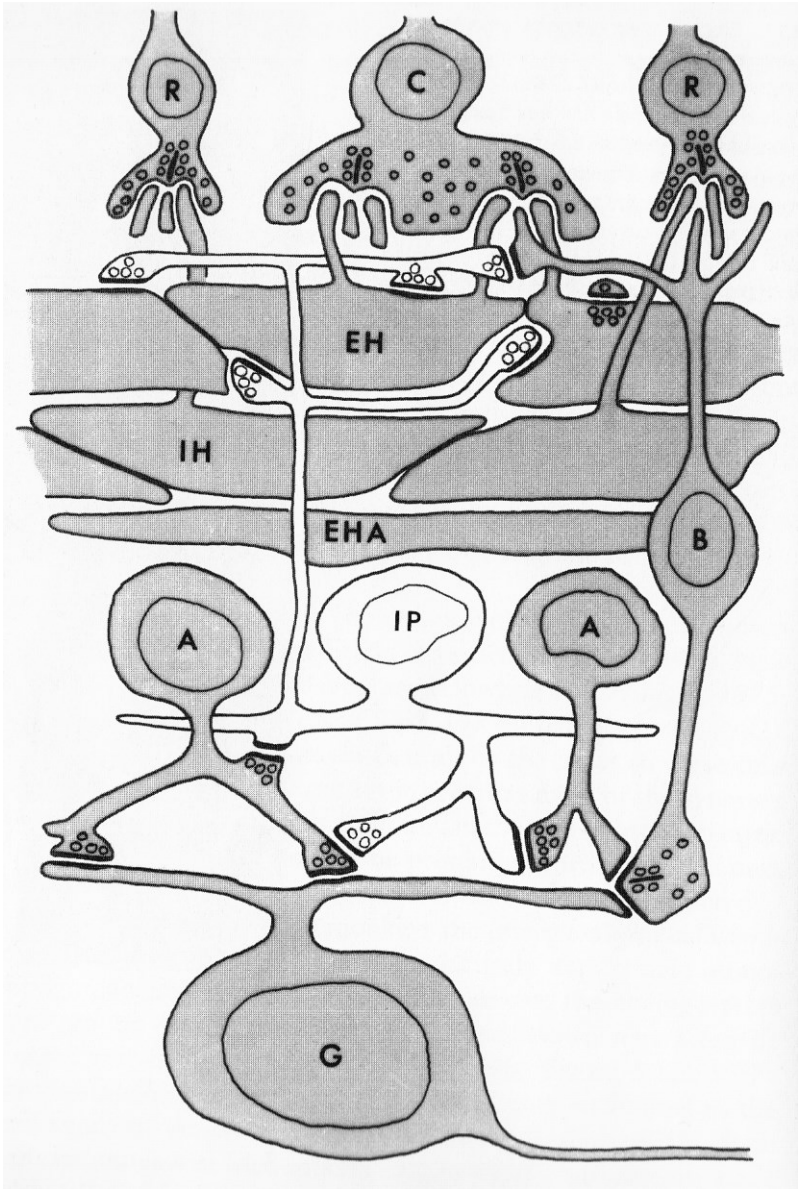


Figure 2: Schematic diagram illustrating the morphology of the I1 dopaminergic interplexiform cell (IP). R, rods; C, cone; EH, external horizontal cell; IH, internal horizontal cell; EHA, external horizontal cell axon terminal; B, bipolar cell; A, amacrine cell; G, ganglion cell. (From Dowling, 2012)

together via tight-junctions and, on the apical side, there are small finger like projections (microvilli) that interdigitate with the photoreceptors in the subretinal space (Steinberg, 1985) (see Figs. 1 and 3). The basal side faces Bruch's membrane, the inner most layer of the choroid and this separates the RPE from the fenestrated capillaries of the choroid (Steinberg, 1985). These pigmented cells are a vital support system for the neural retina and also form part of the blood-retina barrier (Schraermeyer and Heimann, 1999; Strauss, 2005). The pigment contained within RPE cells is melanin, mostly eumelanin (Ito et al., 2013). The RPE is responsible for a variety of functions associated with photoreceptor maintenance. The RPE provides photoreceptors with vitamin A and essential nutrients such as glucose, retinol and fatty acids from the blood (Strauss, 2005). As previously described (Section 1.2) phototransduction in the eye is initiated by photons that induce the isomerization of 11-*cis* retinal to all-*trans*-retinal. Cones have an intrinsic capacity to convert all-*trans*-retinal back to 11-*cis* retinal but rods do not. Therefore, in order to ensure maintained rod activity, all-*trans*-retinal is transported to the RPE where it is converted back to 11-*cis* retinal and then returned to rods (Strauss, 2005). The RPE also facilitates movement of ions, water and metabolic waste from the subretinal space to the blood and phagocytoses outer segment discs that are shed by the photoreceptors (Strauss, 2005). Once the shed outer segment discs are phagocytized any essential compounds, such as retinal, are recycled. The RPE also absorbs photons that have not been captured by the photoreceptors, ensuring two things: first, reducing the possibility of image-degrading light scatter and, second, providing a barrier against light-induced heat that may otherwise damage neural elements (Eckmiller and Burnside, 1983; Steinberg, 1985; Strauss, 2005).

1.5 Retinomotor Movements in the Teleost Retina

The range of light intensities encountered by vertebrates in natural environments is on the order of 10^{12} (Dowling, 2012). However, the dynamic range of retinal photoreceptors, the neurons that transduce the visual scene into an electrochemical signal, is only about 10^{3}. Two types of photoreceptors, rods for dim light and cones for brighter light, collectively account for about 6 log units of visual sensitivity, but clearly additional mechanisms are required if the retina and visual system are to remain sensitive over the full range of natural light intensities.

For many vertebrates the pupillary reflex is a key mechanism that assists the visual system to adapt to the full range of light without saturating the photoreceptors. In most vertebrates, including humans, the size of the pupil, the opening formed by the iris, is adjusted as a function of ambient light level, thereby regulating the amount of light entering the eye. In bright light the pupil is constricted to limit the amount of light entering the eye. The light-induced reduction of the pupillary aperture is regarded as the pupillary reflex proper. In dim light the pupil is dilated increasing the amount of light reaching the retina. The iris contains two separate smooth muscle groups that mediate constriction and dilation. When the sphincter muscle fibers contract, the size of the pupillary opening is reduced (constriction). Conversely, when dilator muscle fibers contract the size of the pupillary opening increases (dilation). The sphincter muscles are thought to be under parasympathetic control and the dilator muscles under sympathetic control.

The pupillary light reflex pathway has been well characterized in mammals. It involves projections from the retina to neurons in the midbrain pre-tectum that in turn, provide input to the Edinger-Westphal Nucleus. The Edinger-Westphal nucleus is located

in the rostral midbrain at the level of the superior colliculus and contains pre-ganglionic parasympathetic neurons that innervate the post-ganglionic parasympathetics in the ciliary ganglion that, in turn, travel with the short ciliary nerves to innervate the iris sphincter muscles. In contrast, post-ganglionic sympathetics from the superior cervical ganglions traveling with long ciliary nerves innervate dilator muscles. Many non-mammalian vertebrates also exhibit a pupillary light reflex with similar, and likely homologous nuclei, involved. Also noteworthy is that the retinal signals that drive the pupillary light reflex are from ganglion cells that receive input not only from rod and cone driven bipolar cells but also ganglion cells that are intrinsically photosensitive, due to the presence of melanopsin (Hughes et al., 2016).

Not all vertebrates posses an iris with a pupil that changes dynamically with changing ambient illumination. Instead several groups of animals, in particular teleost fish, amphibians and birds, exhibit retinomotor movements (RMMs) (see Fig. 3), sometimes referred to as photomechanical movements (Walls, 1963). First described by Khüne in the late 1800's (Khüne, 1878), retinomotor movements involve two major types of cell motility, cell shape change (photoreceptors) and intracellular transport (RPE pigment granules).

Under low light conditions, rod myoids contract, cone myoids elongate and melanin granules aggregate within the basal ends of RPE cells. This arrangement puts rods "first in line" (Burnside and King-Smith, 2010) for photon capture and the aggregation of the melanin granules within the RPE fully exposes the rod outer segments. In the light, rod myoids elongate, cone myoids contract and RPE melanin granules disperse within long apical projections of the RPE.

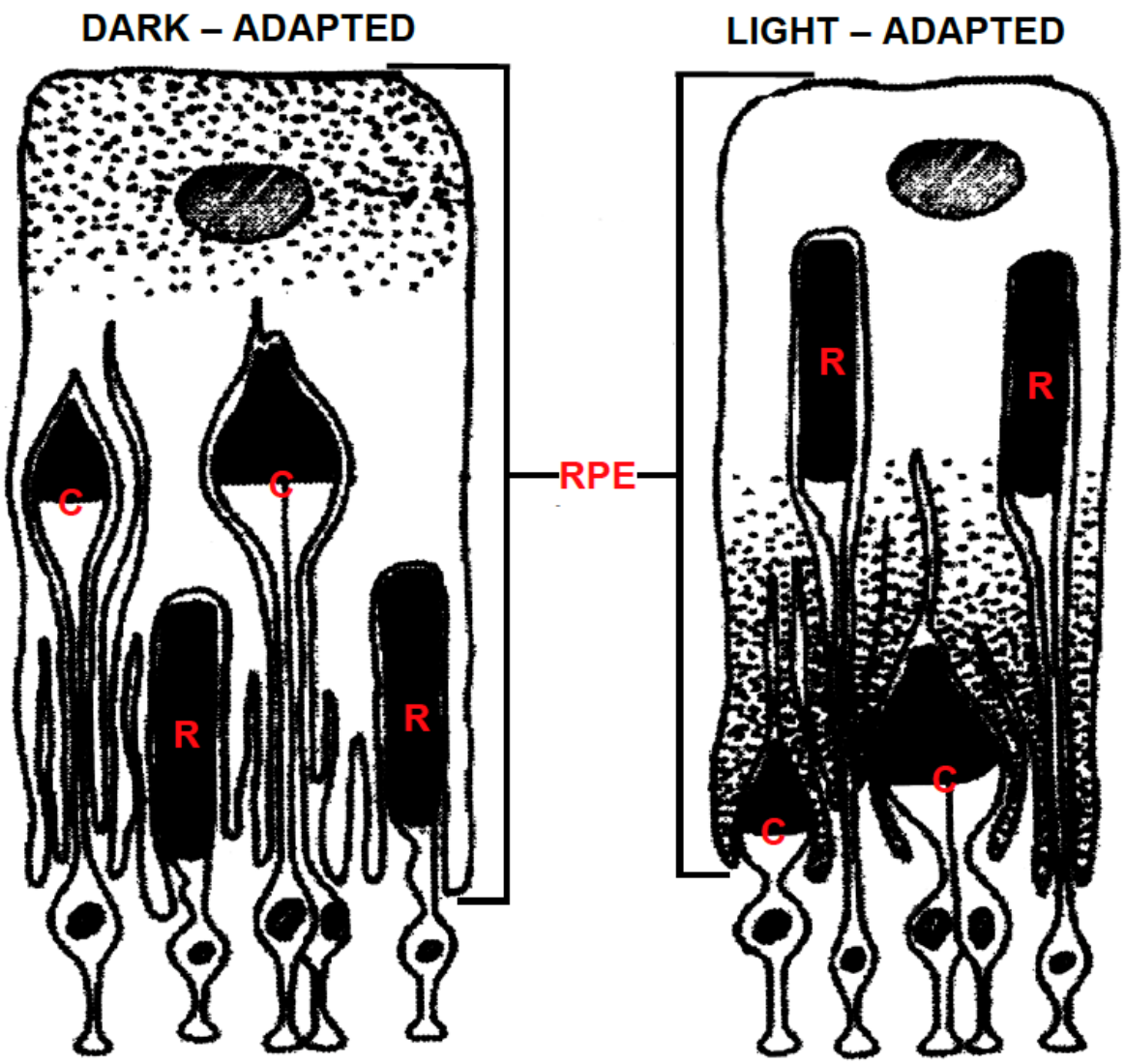


Figure 3: Schematic illustration of retinomotor movements. Under dark-adapted conditions (left) rods (R) contract, cones (C) elongate and pigment granules aggregate within the basal portion of the RPE. Under light-adapted conditions (right) cones contract, rods elongate and pigment granules disperse to the apical end of the RPE. (Modified from Burnside, 2001).

Now cones are “first in line” for photon capture and rod outer segments are shielded by the melanin granules, protecting the sensitive rhodopsin molecules from bleaching. Retinomotor movements are relatively slow processes. For example when a dark-adapted teleost (blue stripe grunt, *Haemulon sciurus)* was placed in light cone photoreceptor contraction was not noted until 3 min and required 20 min to reach full contraction (Burnside et al., 1983). Cone elongation in the dark was even slower. When a light adapted fish was placed in the dark there was a 20 min lag period prior to the start of elongation and ~1.5 hours was required to reach the fully elongated state (Burnside et al., 1983).

Retinomotor movements are affected not only by the level of ambient illumination but also by time of day (circadian signals) (Green et al., 1995; Levinson and Burnside, 1981; McCormack and Burnside, 1991; McCormack and Burnside, 1992; Menger et al., 2005). In constant condition (dark or light), light-adapted-like RMMs occur at subjective dawn and dark-adapted-like movements occur at subjective dusk. However, the magnitude of RMMs at dawn or dusk under constant conditions are greatly reduced. In fact, the extent of circadian RMMs of rods and RPE are so little that it is usually only cone movements that are considered to show a clear circadian rhythm.

The utility of RMMs in teleosts is clear, given the lack of a pupillary light response in most members; in fact, most teleosts lack iris muscles (Walls, 1963). It has been suggested that the lack of a pupillary response reflects adaptation to an aquatic environment. The refractive index of the cornea is almost identical to that of water, therefore the refraction of light occurs solely at the lens. As a result, in teleosts the lens is so large that it precludes or limits the extent of pupillary constriction (Walls, 1963) (Fig. 4). However, this explanation would suggest that pupillary responses should be absent in

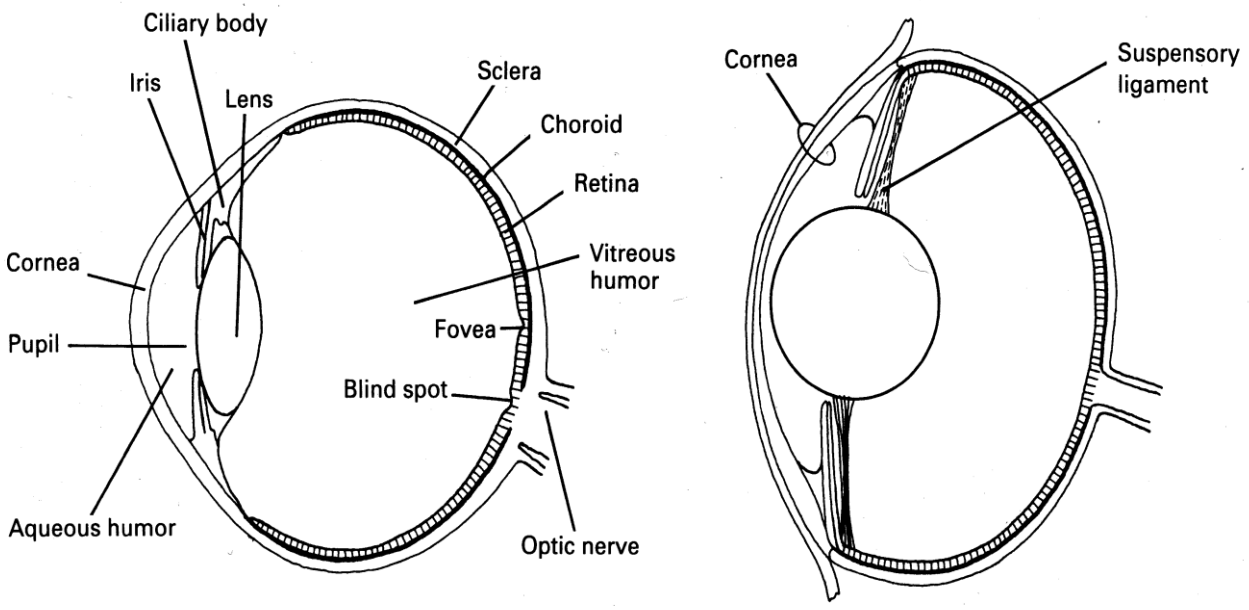


Figure 4: Illustration of a representative mammalian (human) eye (left) and a representative teleost eye (right) (Wolff, 1991).

all aquatic vertebrates. This is not the case, with pupillary responses noted in sharks (elasmobranchs), some teleosts (eels, *Anguilliformes;* stargazers, *Uranoscopus*; monkfish, *Lophius*), and amphibians (Walls, 1963). Such an explanation clearly does not apply to birds where, interestingly, the level of ambient illumination does not strongly influence pupil diameter, being controlled instead by accommodation or acute stress (Walls, 1963).

Initially RMMs, in particular cone contraction and pigment dispersion within RPE, were considered an attractive model system in which to study mechanisms associated with cell shape change (Burnside, 1976; Burnside, 1978; Dearry and Burnside, 1986a; Nagle et al., 1986; Troutt and Burnside, 1989; Warren and Brunside, 1978). Cone myoid elongation (in the dark) was found to be dependent on microtubules and was mimicked by treatments that elevated intracellular 3'-5'-cyclic adenosine monophosphate (cAMP). In contrast, contraction of the cone myoid (during the light) was found to depend on actin and was inhibited by elevated cAMP. Elongation and contraction of rods has been shown to be dependent on actin but not microtubules. Treatments that elevated cAMP mimicked the dark. Similarly, RPE pigment granule dispersion (during the dark) and aggregation (during the light) are both dependent on actin, but not on microtubules, and treatments that elevate cAMP mimicked the dark. Inhibitors of protein kinase A (PKA) blocked dark-adaptive retinomotor movements suggesting that cAMP regulates the photoreceptor cytoskeleton via protein phosphorylation (Liepe and Burnside, 1993a; Liepe and Burnside, 1993b; Rey and Burnside, 1999).

1.6 Dopamine and RMMs

As described above, there is evidence that cAMP (and PKA) are involved in the intracellular signalling that control RMMs. Around the time these studies were conducted, evidence also emerged that RMMs were controlled by an extrinsic mechanism.
Two key results supported this conclusion. The first was that cone movements were triggered by stimuli that matched the spectral sensitivity of rods (Besharse and Witkovsky, 1992; Kirsch et al., 1989). This suggested that cone movements were not due to the intrinsic effect of light on cones but to an indirect mechanism mediated by rods. The second key result was that light-induced RMMs (cone contraction, rod elongation, RPE granule dispersion) were mimicked by dopamine and could be blocked by dopamine D2 family receptor antagonists (Dearry and Burnside, 1988; Douglas et al., 1992). D2 dopamine receptor antagonists also blocked circadian cone contraction (McCormack and Burnside, 1992) suggesting that both light and circadian influences on cone movements are mediated by dopamine. Subsequently, by studying the effectiveness profiles of various dopamine receptor agonists and antagonists, it was concluded that, at least in the case of cone movements, the dopamine receptor responsible was D4-like (Hillman et al., 1995), a member of D2 family of dopamine receptors (see below). This led to a model that, in teleost retina, RMMs are triggered by light acting on rods that then drives the release of dopamine from IPCs that acts on D2 family dopamine receptors on rods, cones and RPE leading to a reduction of cAMP. Before exploring the role of dopamine on RMMs further, I will first review some of the characteristics of dopamine as a neurotransmitter/neuromodulator.

1.6.1 Dopamine

Dopamine (3,4-dihydroxyphenethylamine) was first synthesized in the laboratory in 1910 by George Barger and James Ewens but its biological role was only understood decades later (Barger and Dale, 1910; Carlsson et al., 1957; Carlsson et al., 1958). Arvid Carlsson and colleagues demonstrated that, in addition to being a precursor to other catecholamines, dopamine can also act as a neurotransmitter (Carlsson et al., 1958). Since its discovery dopamine has been extensively studied. The following is a brief summary of current knowledge gathered from various sources (Beaulieu and Gainetdinov, 2011; Dowling et al., 1976; Dowling and Watling, 1981; Gingrich and Caron, 1993; Watling and Dowling, 1981; Zinn and Marmor, 1979). Dopamine is a monoamine neurotransmitter and is grouped with other compounds having similar structure in the catecholamine and phenylamine families.

In neurons dopamine synthesis originates from the amino acid tyrosine, converted to L-3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TOH) (EC1.14.16.2). This is the rate-limiting step of dopamine synthesis (Fig. 5). The final product dopamine is produced when L-DOPA decarboxylase (DDC) removes a carboxyl group from L-DOPA. Dopamine is then packaged into vesicles by the vesicular monoamine transporter (vVMAT) where it is stored prior to release. This transport is driven by exchange of protons across the vesicle membrane. Dopamine release is driven by depolarization of dopaminergic neurons that leads to calcium-dependent vesicle fusion at the pre-synaptic membrane. Once dopamine is released it binds to dopaminergic receptors present on the membranes of post-synaptic neurons. Subsequently, dopamine is either taken back up into the presynaptic neuron by the dopamine active transporter (DAT) or is metabolized.

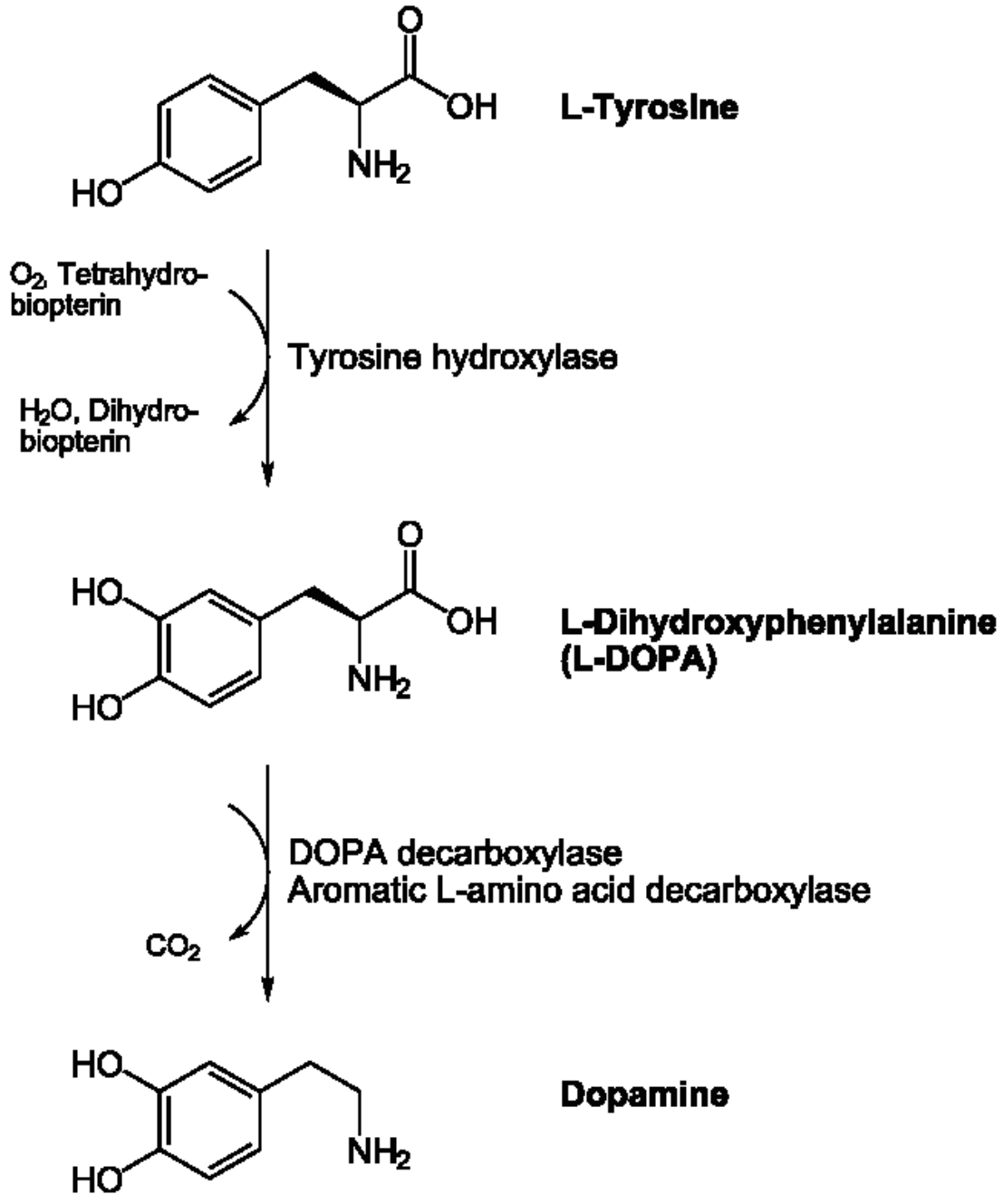


Figure 5: Dopamine synthesis involves the conversion of tyrosine to L-DOPA by tyrosine hydroxylase. L-DOPA is converted into dopamine by DOPA decarboxylase (an aromatic L-amino acid decarboxylase, AADC).

In the case of the former, uptake is a high-affinity, Na^+ dependent and saturable mechanism. If metabolized, dopamine is broken down to 3,4-dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (MAO) or into 3-methoxytyramine (3-MT) by catechol-O-methyl tranferase (COMT). Both metabolites are broken down further to homovanillic acid (HVA) and excreted.

Presently, there are five known dopamine receptors, termed D1-D5, and all are G-protein coupled receptors (Beaulieu and Gainetdinov, 2011; Gingrich and Caron, 1993). Dopamine receptors are grouped into two families D1 and D2. The D1 dopamine receptor family (consisting of D1 and D5 receptors) is coupled to the G-protein G_s and activation results in an increase of cAMP through increased adenylyl cyclase activity. The D2 dopamine receptor family (consisting of D2-D4) is coupled to $G_{i/o}$ that inhibits adenylyl cyclase and results in a decrease of cAMP. With respect to photoreceptor cone RMMs, increase in cAMP levels results in microtubule dependent elongation and decrease in cAMP levels results in actin dependent contraction.

1.6.2 Dopamine in Teleost Retina

In the retina dopamine is a signal of daytime (influenced by circadian rhythms) and light-adaptation (Doyle et al., 2002; Iuvone et al., 1978). In general, terminals of dopaminergic IPCs are not found adjacent to conventional post-synaptic specializations. The release of dopamine in the outer retina (OPL and beyond) is not in close proximity to its target cells. Therefore, dopaminergic signalling in the retina is achieved by volume transmission (Agnati et al., 1995; Dowling et al., 1976; Hedden and Dowling, 1978; Yazulla and Studholme, 1995). The rate-limiting enzyme associated with neuronal dopamine synthesis, tyrosinase hydroxylase, has been identified in dopaminergic IPCs as

well as in the processes and terminals extending from the cell (Dowling and Ehinger, 1975; Witkovsky, 2004).

D1- and D2- like dopamine receptors are found throughout the retina and are expressed by virtually every type of retinal neuron (Popova, 2014). However, in most vertebrates, photoreceptors have been shown (by autoradiography, immunocytochemistry or *in situ* hybridization) to express only D2-like receptors (Wagner et al., 1993). Of the three subtypes in the D2 family, photoreceptors in mice, rats and chicks express the D4 subtype (Cohen et al., 1992; Derouiche and Asan, 1999; Ivanova et al., 2008; Patel et al., 2003). Although the effectiveness profiles of various dopamine receptor agonists and antagonists on teleost cone movements were consistent with D4 receptors (Hillman et al., 1995), direct evidence that teleost photoreceptors express D4 receptors is lacking. It has also not been definitively demonstrated that teleost RPE express D2 family dopamine receptors.

1.6.3 RMMs in the Absence of Dopamine

The blockade of light-induced RMMs by D2 family dopamine receptor antagonists is compelling evidence that dopamine mediates RMMs. The likely source of such dopamine would be dopaminergic I1 IPCs.. To provide direct evidence that this is the case experiments were done where IPCs were selectively abolished by treatment with the neurotoxin 6-hydroxydopamine (6-OHDA). The hypothesis was that if dopaminergic IPCs were no longer present then light-dependent RMMs should be abolished and cones, rods and RPE pigment granules should appear dark-adapted regardless of light condition. It was therefore, puzzling that light-induced RMMs (cone movement and RPE pigment

dispersion) still occurred in teleost retinas where dopaminergic IPCs had been ablated by 6-OHDA lesioning (Ball et al., 1993; Douglas et al., 1992). In these studies the loss of dopaminergic IPCs was verified using various methods such as, loss of high affinity uptake of radioactively labeled dopamine via autoradiography, lack of tyrosine hydroxylase immunoreactivity and the loss of detectable levels of dopamine by HPLC assay.

One possible explanation for continued RMMs in 6-OHDA-lesioned retinas is that there is another source of dopamine within the teleost eye. A possible source of dopamine could be the RPE, where L-DOPA would be produced by the enzyme tyrosinase as a part of the melanin synthesis pathway.

1.6.4 Tyrosinase-derived Dopamine

Tyrosinase (EC 1.14.18.1) is a key enzyme in the biosynthesis of melanin (Cooksey et al., 1997; Mason, 1965; Raper and Wayne, 1928; Schallreuter et al., 2008) (Fig. 6). Tyrosinase catalyzes several reactions that lead to the production of melanin, most notably in the current context, the conversion of tyrosine to L-DOPA. For RPE to be a source of dopamine in the outer retina, would require the conversion of L-DOPA to dopamine (by L-DOPA decarboxylase, DDC) and there must also be a mechanism by which the dopamine could be released. Using RT-PCR and immunoblot methods, Ming et al. (2009) demonstrated that human RPE cells express DDC. They also detected dopamine and the dopamine metabolite homovanillic acid in RPE cells by high-pressure liquid chromatography. However, it has not been demonstrated that RPE cells release dopamine.

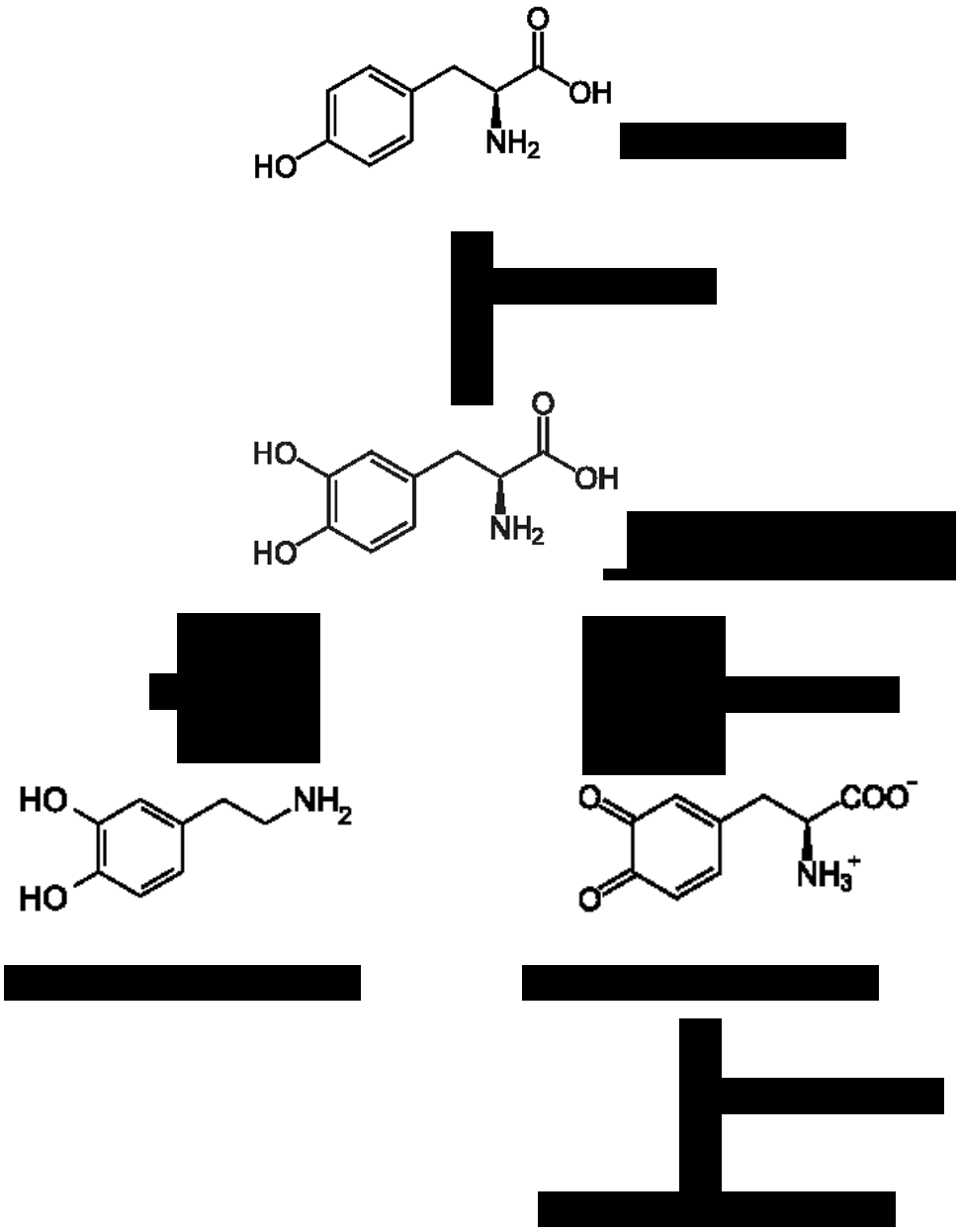


Figure 6: L-DOPA can be synthesized as an intermediate via tyrosinase in the melanin synthesis pathway. L-DOPA is converted into dopamine by DOPA decarboxylase (an aromatic L-amino acid decarboxylase, AADC).

There is evidence that tyrosinase activity is relevant to adaptation processes in the teleost retina. A screen of zebrafish mutants revealed that an albino line, due to the mutation in the tyrosinase *(sdy)* gene, had defective visual behaviour (Neuhauss et al., 1999). Subsequently, light-adaptation (measured by optokinetic response) was slowed in *sdy* fish but with no difference in photoreceptor function (measured by electroretinogram) (Page-McCaw et al., 2004). Interestingly, they also showed delayed light-adaptation in wild-type zebrafish larvae treated with the specific inhibitor of tyrosinase, phenylthiourea (PTU). Their results suggested that tyrosinase activity, normally associated with RPE, plays a role in light-adaptation in the retina, likely as a source of L-DOPA. They also raised the possibility that an alternate source of dopamine, produced by tyrosinase, might explain why 6-OHDA ablation of dopaminergic IPCs does not abolish RMMs.

1.7 Objectives

The guiding ***hypothesis*** for this work was that tyrosinase is required for light-dependent RMMs. To address this hypothesis I determined if the specific tyrosinase inhibitor, PTU, affected light-induced cone contraction. To determine to what extent an effect of PTU might be due to an action on dopaminergic IPCs, experiments were also done in animals lesioned by prior treatment with 6-OHDA. The animal model used was adult goldfish (*Carassius auratus*). These aquatic vertebrates are ideal because of the large magnitude of cone movements and because their retinas are easily accessible**.** If the hypothesis is correct, it is predicted that light-induced cone contraction would be blocked or delayed in PTU-treated goldfish, especially after ablation of dopaminergic IPCs by 6-OHDA.

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental Animals

The maintenance and use of animals for the proposed experiments were conducted according to the guidelines of the Canadian Council on Animal Care and specific protocols approved by the Dalhousie University Animal Care Committee. Common adult goldfish (*Carassius auratus),* were obtained from the Aquatron located in the Life Sciences Center at Dalhousie University and housed in an aerated water at 16ºC under a 10:14 hour light:dark cycle.

2.2 Light Conditions

All experiments were performed during the middle of the 10 hr light portion of the light:dark cycle. In experiments where fish were fully dark-adapted (n=10 eyes), they were placed in complete darkness for 1.5 hrs and then sacrificed and the eyes removed using an infrared imaging system. In experiments where fish were fully light-adapted (n = 10 eyes), they were maintained in a tank subject to 100 lux illumination for 1.5 hrs prior to sacrifice and enucleation under normal lab illumination. Once removed eyes were perforated prior to being placed in fixative to expedite penetration of fixative. For time point studies goldfish were first placed in dark for 1.5 hrs and then exposed to 100 lux illumination for 1.5, 3.5, 5.5, 7.5, 9.5, 13.5, 15 or 30 min (n=10 eyes for each time point) prior to sacrifice and tissue extraction.

2.3 Drug Treatments

A subset of animals were treated with the tyrosinase inhibitor phenylthiourea (PTU; Sigma Chemical Co., Burlington, ON) by placing them in tank water containing

0.2 mM PTU for 48 hrs. The PTU-treatment parameters were based on previous studies in teleosts where PTU was used to prevent melanin formation (Epping, 1970; Karlsson et al., 2001; Whittaker, 1966). In other experiments, dopaminergic IPCs were ablated by intraocular injection of 6-hydroxydopamine (6-OHDA; Sigma) 10 days prior to study. For these experiments fish were anaesthetized by immersion in water containing buffered tricaine methane sulphonate (MS-222; 140 ppm; Sigma). Animals were then given an intraocular injection of 6-OHDA administered via two 10 µl injections of 50 µg 6-OHDA on consecutive days followed by a 10-day time course to allow for the lesioning to take effect. This lesioning protocol is an effective means to remove dopaminergic IPCs as assessed by tyrosine hydroxylase immunohistochemistry and direct measurement of retinal dopamine by HPLC (Baldridge and Ball, 1991; Baldridge et al., 1989). A separate group of fish (n = 4 eyes) were given intraocular injections of 10 µl saline as controls to rule out the possibility that any observable effects were due to the intraocular injection technique.

2.4 Immunohistochemistry

Unless otherwise indicated all reagents were obtained from Sigma Chemical Company (Burlington, ON, Canada). Following the completion of each experimental treatment goldfish were sacrificed by decapitation, eyes enucleated, hemisected and the lens removed. Eye cups were immersed in 6% glutaralaldehyde and 0.5% cetyl-pyridinium chloride (in 0.1 M phosphate buffer saline, PBS) overnight. This fixative enhances the preservation of the photoreceptor cytoskeleton, and the interdigitation between photoreceptor outer segments and RPE, allowing for the visualization necessary for accurate length measurements (Dearry and Burnside, 1986a). Retinas were then

gently removed from the eyecups, edges trimmed and placed in individual wells of a culture dish and rinsed 3 times for 10 min using PBS. Then the wells were filled with 3 ml of PBS + 3% Triton X-100 containing 30 µl of normal goat serum (NGS). The dish was sealed with parafilm, the lid replaced and the tissue incubated overnight in the refrigerator at 4°C. The next morning the tissue was rinsed 3 times for 5 min in PBS. The tissue was then incubated in zpr-1 antibody (Zebrafish International Resource Center, Eugene, OR). Zpr-1, originally called FRet 43, is a monoclonal antibody that was made by immunizing mice with zebrafish retinal cells (Larison and Bremiller, 1990). The antibody was first considered useful because it was found to selectively label double cones in zebrafish and goldfish retina (Larison and Bremiller, 1990; Renninger et al., 2011; Schultz et al., 1997; Zou et al., 2008). The zpr-1 antibody was later found to recognize a single 45-kDa protein in Western blots of zebrafish retinal lysates and was confirmed as arrestin 3-like by mass spectrometry analysis and by loss of zpr-1 immunofluorescence in zebrafish retinas after arrestin-3 knockdown (Ile et al., 2010). The antibody was diluted 1:500 in PBS + 0.1% Triton X-100 and the tissue was incubated at 4°C for 5-7 days. After being rinsed 3 times for 5 min in PBS, the tissue was incubated in secondary (goat anti-mouse IgG) antibody (1:1000 in PBS) conjugated to Cy3 or FITC (Jackson ImmunoResearch, West Grove, PA) for 2.5 days at 4°C and then washed, a final time, 3 times for 5 min in PBS.

Additional retinas (2 naive, 2 saline controls and 2 6-OHDA-treated) were processed for tyrosine hydroxylase immunohistochemistry. The eye cups were immersed in 4% paraformaldehyde (in 0.1 M phosphate buffer, PBS) for 30 mins. Retinas were then carefully removed from the eyecups and placed in individual wells of a culture dish

and rinsed using PBS. The tissue was incubated and treated using the protocol outlined above for zpr-1. The primary antibody was mouse monoclonal anti-tyrosine hydroxylase (MAB 318, Millipore, Temacula, CA). According to manufacturer information the antibody was raised against purified tyrosine hydroxylase from PC12 cells, recognizes an epitope on the outside of the regulatory N-terminus and labels a protein of the expected size (59-61 kDa) by Western blot of mouse brain lysate. Previous studies have demonstrated that the antibody labels IPCs in goldfish and zebrafish retina (Kay et al., 2001; Tyler and Cameron, 2007). The primary antibody was diluted 1:200 in PBS + 0.1% Triton X-100 followed by a secondary antibody (goat anti-mouse IgG, 1:1000 in PBS) conjugated to FITC.

Following zpr-1 immunohistochemistry processing, the retinas were placed in a large petri dish and kept moist with PBS. Using a dissecting microscope and a double edged razor blade, thick (~1-1.5 mm) retinal cross-sections were made within ~5 mm of the optic nerve. Retinal sections were placed on the microscope slide, a drop of mounting media (50% PBS + 50% glycerol) applied followed by a 24 mm x 50 mm glass coverslip (thickness 0) and sealed using nail varnish. Retinas processed for tyrosine hydroxylase immunohistochemistry were removed from the wells and placed into a large petri dish. Using a dissecting microscope and fine spring scissors, four radial cuts were made at equal points on the retina to allow the retina to lie flat. Next the retina was placed vitreal side up on the center of a microscope slide. Once the retina was lying flat, a drop of mounting media was applied followed by a glass coverslip and sealed using nail varnish.

2.5 Confocal microscopy

Zpr-1-immunoreactive cones in thick retinal sections were visualized using either 1) a Nikon Eclipse E800/C1 confocal microscope employing a 40X oil immersion objective (1.40 N.A.) and either a 488 nm (FITC) or 543 nm (Cy3) HeNe laser for excitation and emission filtered at 515 ± 30 nm (FITC) or 586 ± 40 nm (Cy3), respectively; or 2) a Zeiss LSM 510 confocal microscope employing a 40X oil immersion objective (1.30 N.A.) and a 543 nm HeNe laser for excitation and emission band pass filtered at 515-565nm (Cy3). A stack of 1 μm confocal optical sections were collected and used to construct a Z-stack image. The same protocol was followed to obtain images of tyrosine hydroxylase immunoreactive neurons in retinal wholemounts but using a 25X multi immersion objective (N.A. 0.75). Nikon EZ-C1 software or Zeiss LSM 4.2 software was used to capture images and produce the confocal image stacks and was also used to make measurements of cone length.

2.6 Measurements & Data Analysis

In each confocal Z-stack image 5 cone photoreceptors that were fully labelled by zpr-1 immunolabeling were chosen at random. Using the confocal microscope software, measurements were made from the outer edge of the inner segment to the inner edge of the synaptic terminal. Twenty measurements were taken for each eye from which an average value was calculated for each retina. Mean ± standard deviation (SD) cone length were calculated for each group of retinas. Differences between mean data were tested for statistical significance (α=0.05) using Prism Software (GraphPad, LaJolla, CA) employing t-test (for comparison of 2 means) or analysis of variance (ANOVA) when

comparing three or more means, followed, when applicable, by Tukey's multiple comparison test.

CHAPTER 3: RESULTS

The ***aim*** of this study was to examine the role of tyrosinase on light-induced cone movement in the goldfish retina. My stated ***hypothesis*** is that tyrosinase activity is required for dopamine-dependent light-induced cone contraction. Therefore, it was expected that treatment with the specific tyrosinase inhibitor, PTU, would reduce or block light-induced cone contraction in goldfish retinas. To rule out the possible influence of dopaminergic IPCs, additional experiments were done using animals in which these cells were ablated by prior lesioning with 6-OHDA. With respect to the hypothesis, I predicted that any effect of PTU would be maintained or even enhanced in 6-OHDA-lesioned eyes.

I first obtained baseline measurements of cone length in fully dark- or light-adapted control retinas using (in this and all subsequent cone length experiments) zpr-1 immunohistochemistry that labels the cone from the synaptic terminal to the outer segment (see Fig. 7). In fully dark-adapted control retinas (fish kept in the dark for 1.5 hrs and then dissected in complete darkness using an infrared imaging system) cone photoreceptors were elongated. A representative example of dark-adapted goldfish retina, acquired by confocal microscopy and is shown in Fig. 7A where the zpr-1-immunoreactive cones are relatively elongated and a single cone is illustrated with a length of 100 μm. A representative example of light-adapted retina is shown in Fig. 7B and two cones are illustrated having a length of 54 μm. On average, cone length from all control dark-adapted retinas studied was 112 μm ± 3.7 μm (mean ± standard deviation, n=10 retinas, Fig. 8). In fully light-adapted control retinas (exposed to 100 lux illumination for 1.5 hrs) cone photoreceptors were contracted. Mean cone length in light-adapted retinas was 55 μm ± 2.2 μm (n=10 retinas).

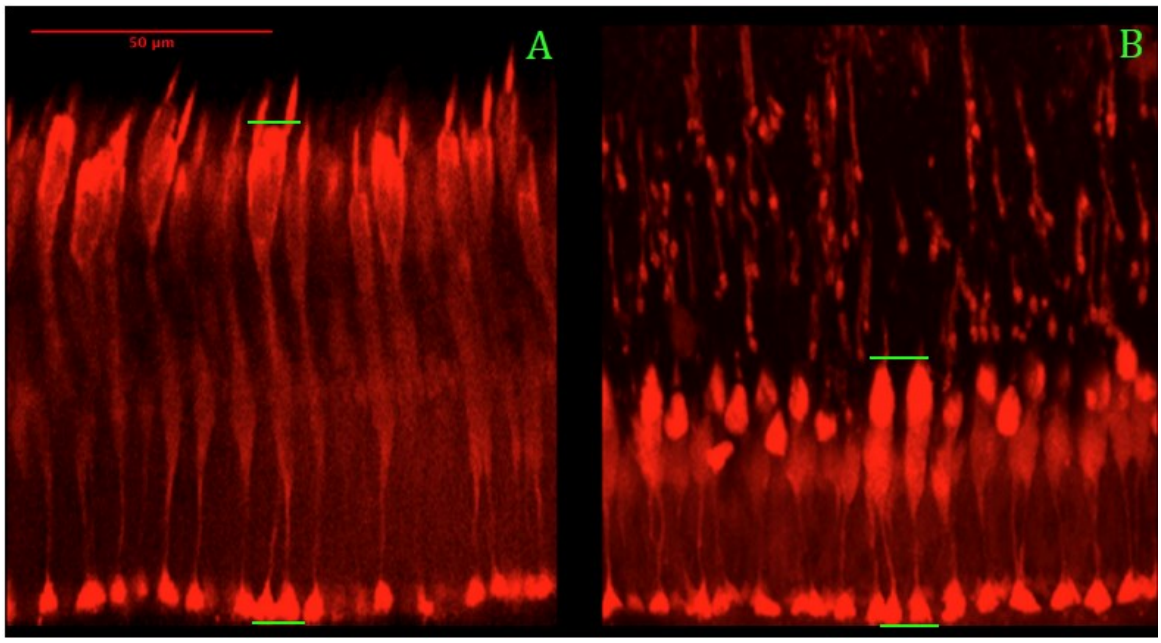


Figure 7. Confocal photomicrograph of retinal thick-sections with cones labeled using the monoclonal antibody zpr- 1 (visualized using a Cy3-conjugated secondary antibody) in (A) dark- and (B) light-adapted retinas. Measurements of cone length were taken from the inner margin of the synaptic terminal (lower green line) to the outer edge of the inner segment (upper green line).

Scale bar = 50 µm.

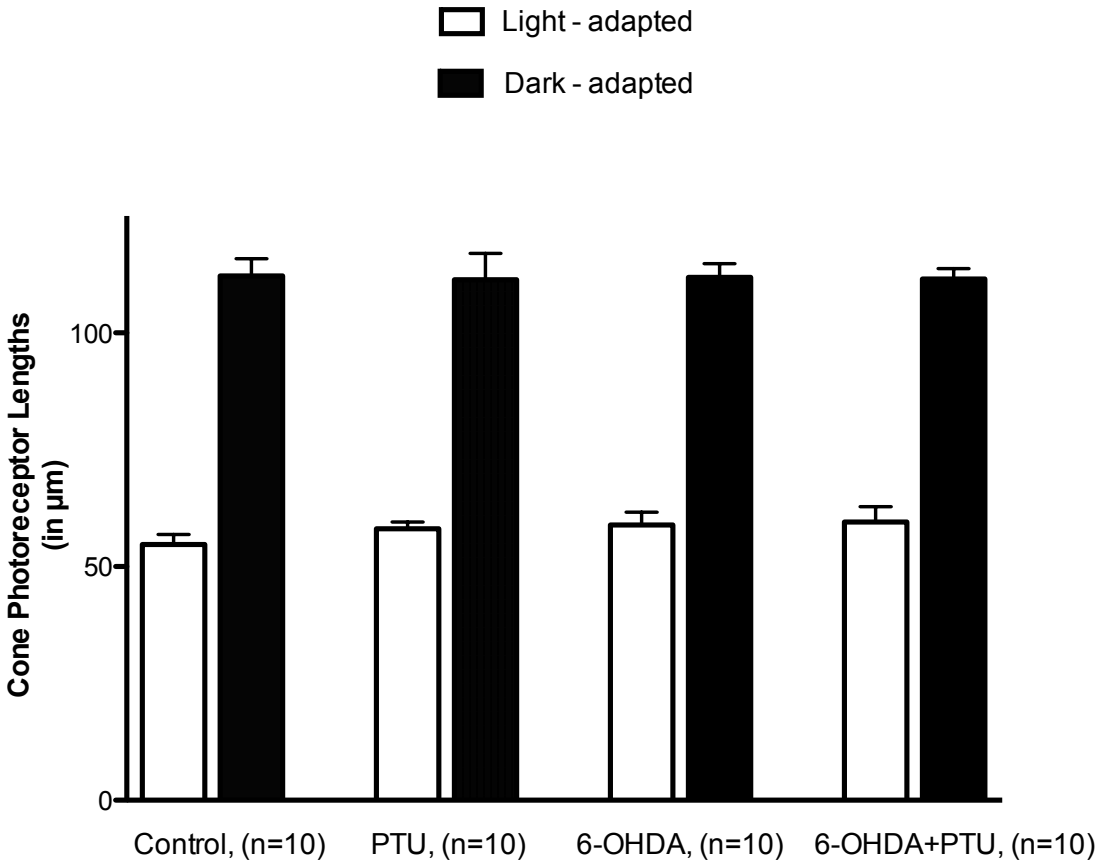


Figure 8. Mean (± SD, n=10) cone length in light- and dark-adapted control, PTU-treated, 6-OHDA lesioned and PTU-treated + 6-OHDA lesioned retinas. In each case mean cone length in light versus dark-adapted retinas were significantly different ($p<0.001$; t-test).

Mean cone length from dark- and light-adapted retinas were significantly different (t-test; $p<0.001$, Fig. 8).

If tyrosinase activity is required for light-induced cone contraction, PTU-treatment should block or reduce the extent of cone contraction in light-adapted retinas. However, I found that cone lengths in retinas from fully light- and dark-adapted fish treated with PTU were essentially identical to that in control light- and dark-adapted retinas (Fig. 8). Retinas from PTU-treated fish that were fully-light adapted exhibited a mean cone length of 58 μm ± 1.5 μm and in fully dark-adapted retinas mean cone length was 111 μm ± 5.6 μm.

In fish treated with PTU, it is possible that there is sufficient dopamine released from dopaminergic IPCs to trigger light-dependent cone contraction. I therefore examined cone position in 6-OHDA lesioned retinas and in retinas lesioned with 6-OHDA from PTU-treated fish. To verify that the 6-OHDA lesioning protocol used in the experiments destroyed the dopaminergic IPCs of the goldfish retina, six retinas (2 control, 2 saline-injection controls and 2 6-OHDA lesioned) were immunolabeled with a monoclonal antibody against tyrosine hydroxylase. Retinas that were not lesioned with 6-OHDA exhibited tyrosine hydroxylase immunoreactive somata in the inner nuclear layer (controls, retinas not injected Fig. 9A and G; saline injected controls Fig. 9 C and E). In the retinas treated with 6-OHDA, tyrosine hydroxylase immunoreactivity could not be detected (Fig. 9 B, D, F, H).

Mean cone length in dark- and light-adapted retinas subject to 6-OHDA lesioning or 6-OHDA lesioning and PTU-treatment were very similar to that of control eyes (see Fig. 8). In 6-OHDA-lesioned eyes, fully light-adapted retinas had a mean cone length of 59 μm ± 2.8 μm and in fully dark-adapted retinas mean cone length was

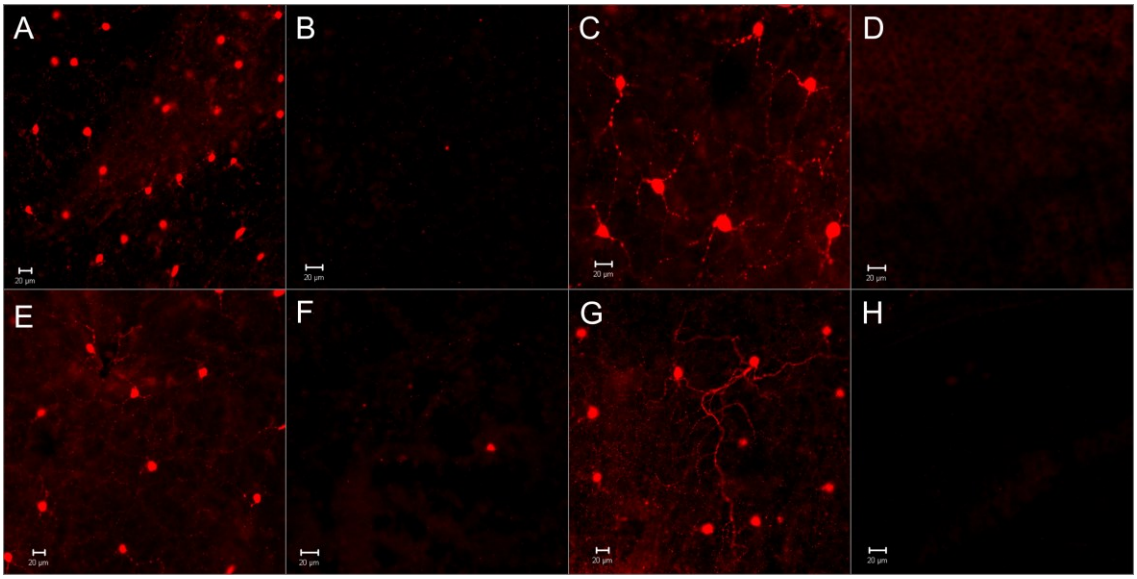


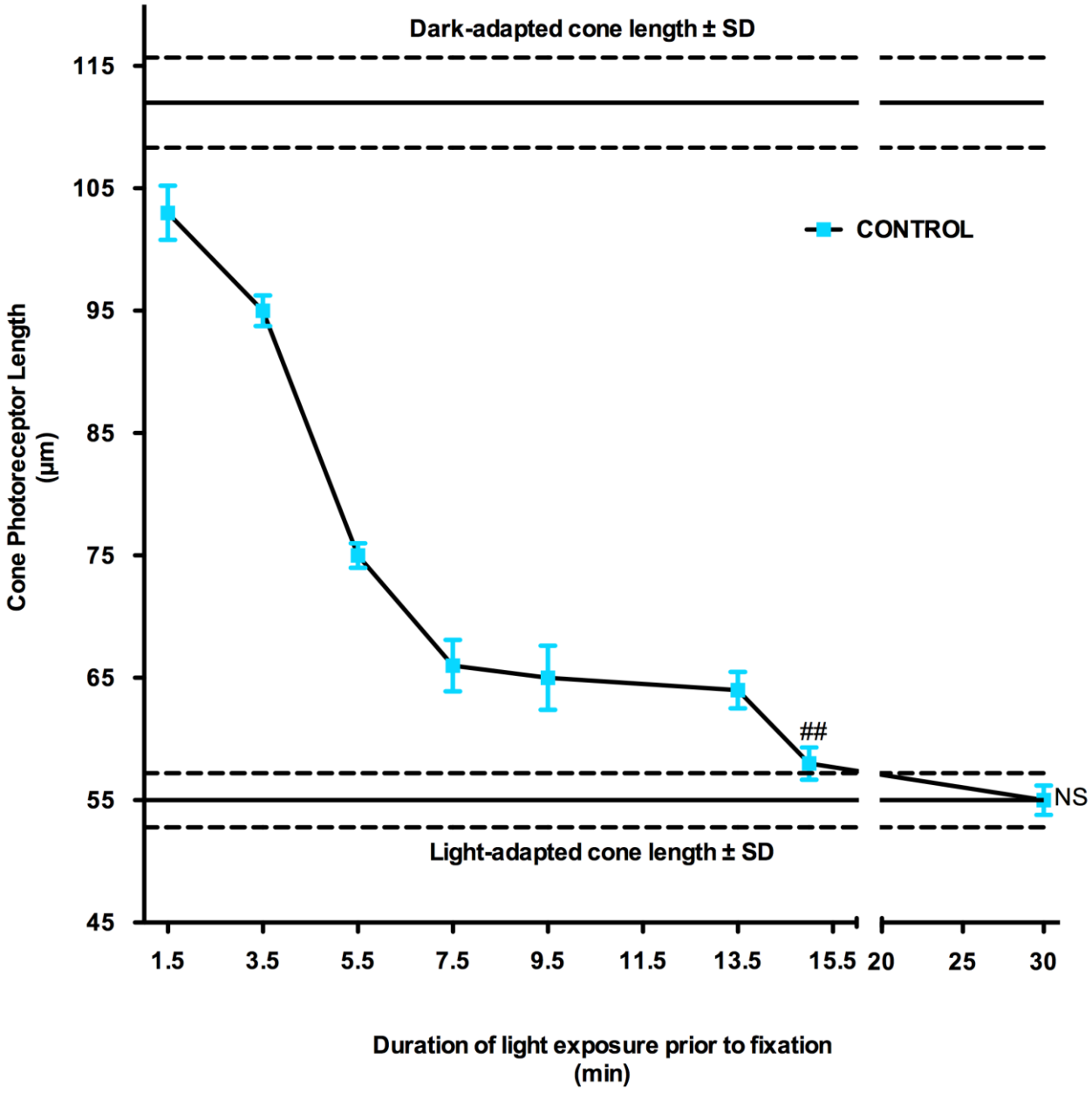
Figure 9. (A, C, E, G) Confocal photomicrograph of the somata and processes of dopaminergic IPCs in the inner nuclear layer of the goldfish retina labeled using a monoclonal antibody against tyrosine hydroxylase (visualized using an FITC-conjugated secondary antibody). A and G control retinas not injected, C and E saline control (same fishes, different eyes. (B, D, F, H) Absence of tyrosine hydroxylase-immunoreactivity in the retina of 6-OHDA-lesioned fish (2 fish, 4 eyes).

All scale bars = 20 μm.

112 μm ± 3.1 μm. In retinas from fish treated with PTU and lesioned with 6-OHDA, cones in fully light-adapted retinas were 60 μm ± 3.3 μm. In fully dark-adapted retinas cones were elongated with a mean cone length of 112 μm ± 2.3 μm. The data presented thus far suggests that PTU has no effect on light-induced cone contraction and that the presence of light-induced cone contraction in 6-OHDA lesioned retinas (as demonstrated here and previously by Douglas et al., 1992) is not explained by dopamine derived from tyrosinase activity. However, given that cone contraction occurs over a period of time, I next examined the effect of PTU and 6-OHDA on the kinetics of light-induced cone contraction.

Control animals were initially dark-adapted for 1.5 hrs followed by light exposure of varying time lengths prior to fixation. As illustrated in Fig. 10 even 1.5 min of illumination produced a significant ($p<0.001$) degree of cone contraction relative to fully dark-adapted retinas. Further contraction was noted with additional periods of light (3.5, 5.5, 7.5, 9.5, 13.5 and 15 min). Although 30 min light exposure was required for cone contraction to reach ($p>0.05$) the fully light-adapted condition, mean cone length at 15 min, while significantly ($p<0.01$) longer than control, was only ~5% longer than the fully light-adapted condition. This suggests that cone contraction, under the experimental condition used here, requires 15-30 min of light exposure to reach the fully light-adapted contracted position. Also noteworthy is the changing rate of contraction over the full extent of time points studied. Following rapid contraction over the first 7.5 min, contraction slowed over the next 6 min, followed by a sudden increase in contraction rate from 13.5 to 15 min and by the final 15 min cones were fully contracted.

Figure 10. Mean cone length (± SD, n=10 eyes at each point) of initially dark-adapted goldfish exposed to different periods of illumination (control conditions) prior to fixation. For comparison, mean cone length from fully dark- and light-adapted retinas (same as Fig. 9) are represented by solid horizontal lines at top and bottom, respectively, with dashed lines indicating ± SD. At each time point, mean cone length was significantly ($p<0.001$) less than mean cone length from fully dark-adapted eyes (significance not indicated). Conversely, mean cone length was significantly ($p<0.001$) greater compared to cone length of fully light-adapted retinas, except where indicated (## $p<0.01$, NS indicates $p>0.05$).


Dark-adapted cone length ± SD
115
105
95
85
75
65
55
45
CONTROL
Cone Photoreceptor Length (µm)
##
NS
Light-adapted cone length ± SD
1.5
3.5
5.5
7.5
9.5
11.5
13.5
15.5
20
25
30
Duration of light exposure prior to fixation (min)

In fish treated with PTU light-induced cone contraction still occurred (first detected at 1.5 min; $p<0.001$ compared to mean cone length in fully dark-adapted retinas) but slower, with longer lengths compared to control at certain time points (Fig. 11). After 1.5 and 3.5 min light exposure, mean cone length was similar ($p>0.05$) in control and PTU-treated fish. After 5.5 min of light, mean cone length was longer ($p<0.001$) in PTU-treated fish compared to control. Further periods of light exposure (7.5 and 9.5 min) produced additional cone contraction but still significantly ($p<0.001$) less than control. After 13.5, 15 and 30 min illumination, cone lengths in control and PTU-treated fish were not significantly ($p>0.05$) different. In fish treated with PTU, 30 min light exposure was required for cone length to reach the fully light-adapted position. At 15 min mean cone length was significantly ($p<0.001$) different and ~10% longer than mean cone length in fully light-adapted retinas. The changing rate of contraction over the full extent of time points studied in PTU-treated animals was similar to that observed in controls. Contraction was relatively rapid over the first 7.5 min but, as noted above, somewhat slower than in control. Contraction rate slowed over the next 6 min but was again followed by the same sudden increase in contraction rate seen in control eyes from 13.5 to 15 min.

Similar results were found in retinas lesioned with 6-OHDA (Fig. 12). Light-induced cone contraction still occurred (cone length reduced even at 1.5 min, significantly ($p<0.001$) less than mean cone length in fully dark-adapted retinas) but slowed compared to control ($p<0.001$) at 5.5, 7.5, 9.5 and 15 min, requiring 30 min to reach the fully light-adapted position. In fact, at 30 min mean cone length in 6-OHDA lesioned retinas was significantly ($p<0.05$) different from mean cone length in fully-light adapted retinas, but was only ~3% longer. The pattern of contraction over time in 6-OHDA lesioned eyes

Figure 11. Mean cone length (± SD, n=10 eyes at each point) of PTU-treated goldfish that were initially dark-adapted and then exposed to different periods of illumination prior to fixation. For comparison, control (same as Fig. 10), fully dark- and light-adapted data are included (same as Fig. 9). At each PTU-treated time point, mean cone length was significantly ($p<0.001$) less compared to fully dark-adapted mean cone length (significance not shown). Conversely, mean cone length was significantly ($p<0.001$; significance not shown) greater compared to mean cone length of fully light-adapted retinas except where indicated (NS indicates $p>0.05$). At certain time points, mean cone length from PTU-treated animals was greater, compared to control, as indicated (*** $p<0.001$).

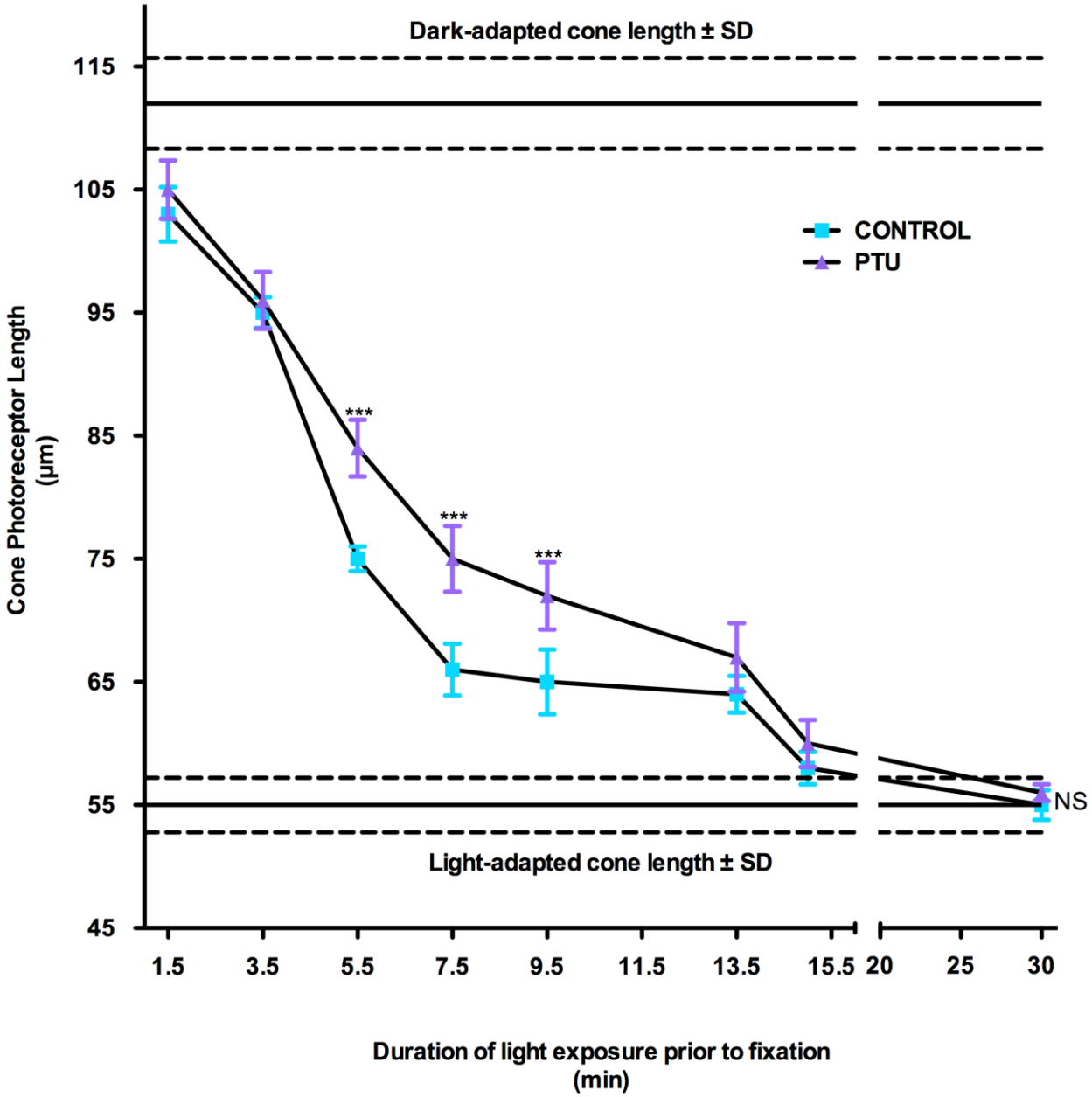
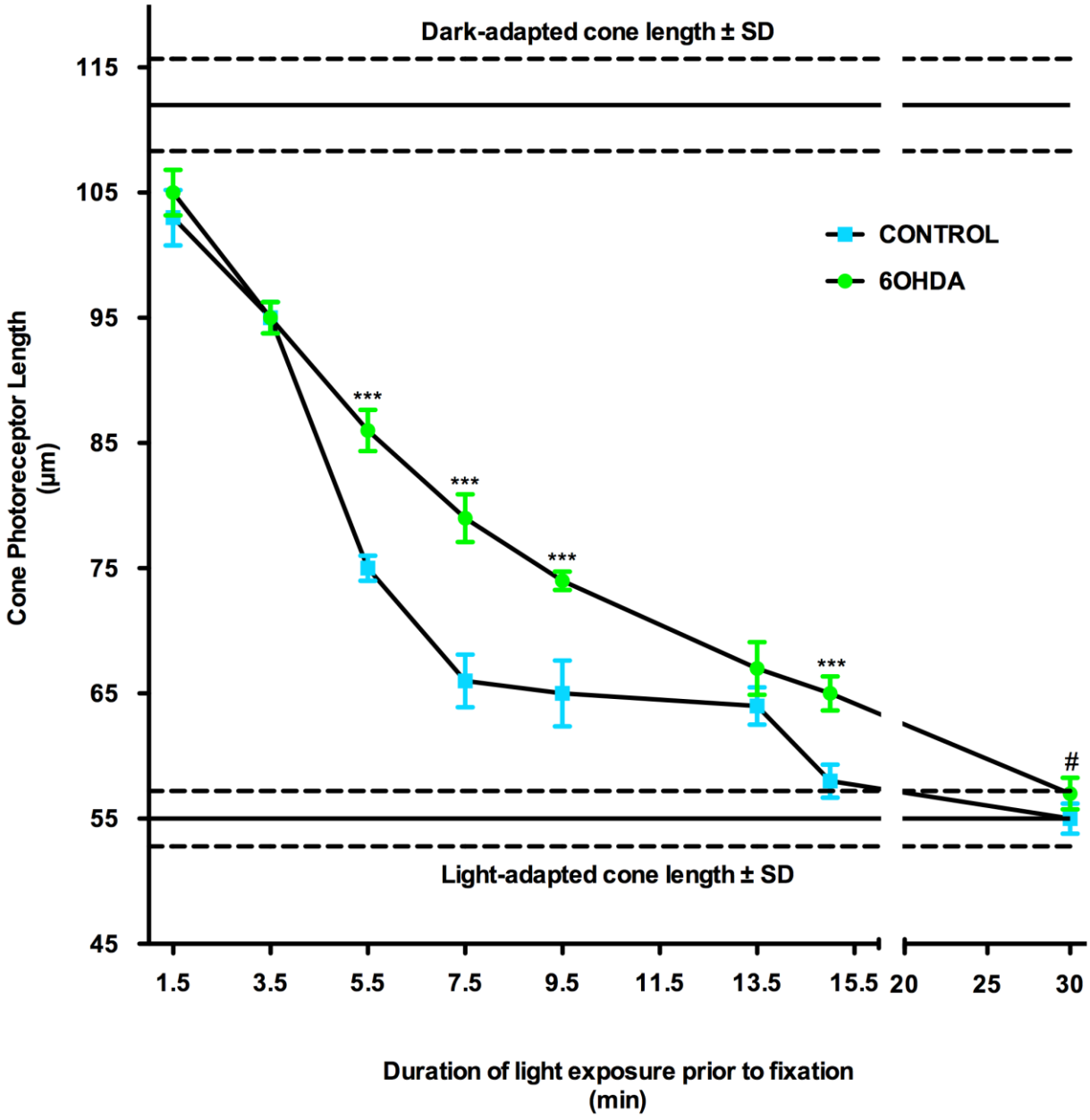

Dark-adapted cone length ± SD
115
105
95
85
75
65
55
45
Cone Photoreceptor Length (µm)
CONTROL
PTU
***
***
***
NS
Light-adapted cone length ± SD
1.5
3.5
5.5
7.5
9.5
11.5
13.5
15.5
20
25
30
Duration of light exposure prior to fixation (min)

Figure 12. Mean cone length (± SD, n=10 eyes at each point) of 6-OHDA-lesioned, initially dark-adapted, goldfish exposed to different periods of illumination prior to fixation. For comparison, control (same as Fig. 10), fully dark- and light-adapted data are included (same as Fig. 9). At each 6-OHDA lesioned time point, mean cone length was significantly ($p<0.001$) less compared to fully dark-adapted mean cone length (significance not indicated). Conversely, mean cone length was significantly ($p<0.001$; significance not shown) greater compared to mean cone length of fully light-adapted retinas except where indicated (# $p<0.05$). At certain time points, mean cone length from PTU-treated animals was greater, compared to control, as indicated (*** $p<0.001$).


Dark-adapted cone length ± SD
115
105
95
85
75
65
55
45
CONTROL
6OHDA
Cone Photoreceptor Length (µm)
***
***
***
***
#
Light-adapted cone length ± SD
1.5
3.5
5.5
7.5
9.5
11.5
13.5
15.5
20
25
30
Duration of light exposure prior to fixation (min)

was different than that in control retinas. Although the fastest change still occurred over the first 7.5 min, the slowing of contraction over the next 6 min was less (compared to control and PTU-treated retinas). There was also no dramatic slowing between 13.5 and 15 min, as seen in control and PTU-treated fish.

To rule out the possibility that the slowing of cone contraction observed in 6-OHDA lesioned retinas was due to injury produced by intraocular injection, I performed the same time point experiments outlined above but instead of 6-OHDA I injected saline. Light-induced cone contraction in retinas of animals that received saline injections was indistinguishable from naïve controls (see Fig. 13). This suggests that the differences seen in retinas lesioned with 6-OHDA was not an artifact of intraocular injection.

These results suggest that inhibition of tyrosinase (PTU) or loss of neuronal dopamine (6-OHDA lesioning) slows the progression of light-induced cone contraction in initially dark-adapted retinas. Another way to consider this is to note that in control retinas mean cone contraction reached ≥ 75% of the fully light-adapted position after 7.5 min illumination. In the case of PTU-treated fish or 6-OHDA lesioned retinas, for mean cone contraction to reach or exceed 75% required 13.5 min of illumination. Both control and PTU-treated fish nearly reached the fully light-adapted condition following 15 min illumination; for 6-OHDA lesioned retinas 30 min of illumination was required. Given the similarity of the effects of PTU and 6-OHDA lesioning, it is possible that both treatments are affecting the same mechanism(s) in the retina that influence light-induced cone contraction. To test this I examined the effect of PTU on light-induced cone contraction in 6-OHDA lesioned retinas. In keeping with the hypothesis that tyrosinase activity is required for light-induced cone contraction, I expected that loss of both proposed sources of dopamine (from RPE, inhibited by PTU; from dopaminergic IPCs,

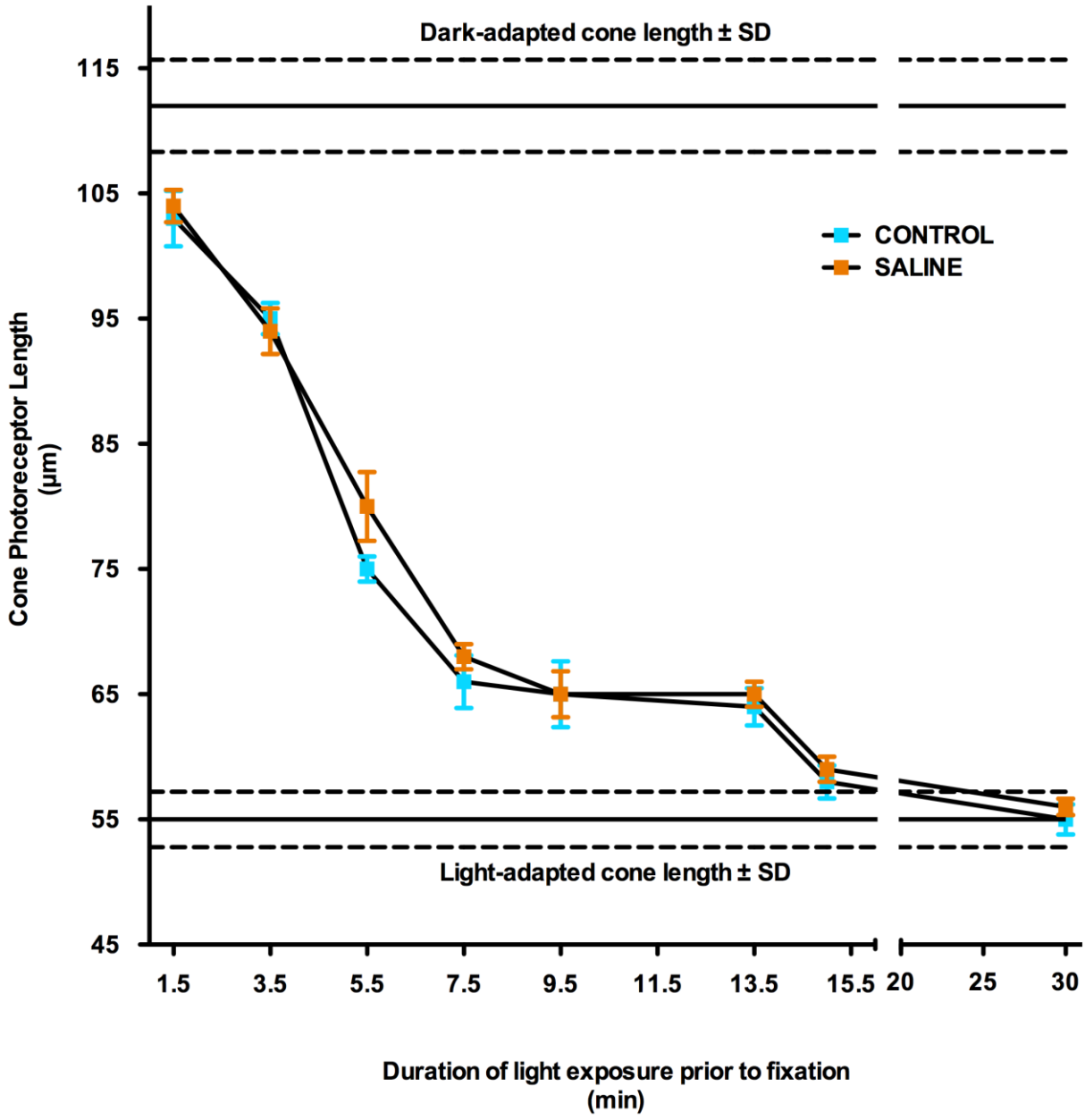


Figure 13: Mean cone length (± SD) from control (same as Fig. 10) and eyes injected with saline (n=6 eyes).

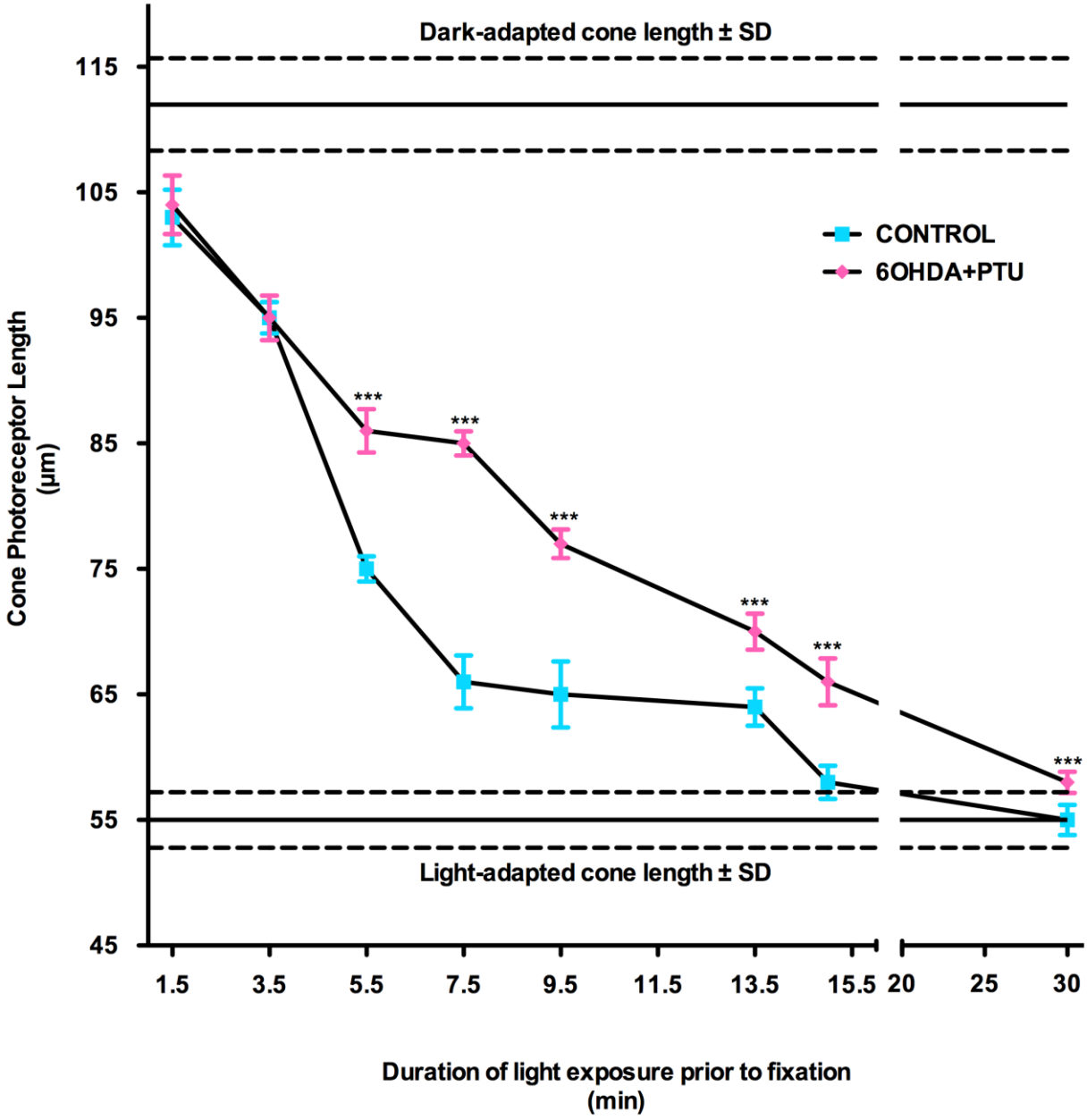
ablated by 6-OHDA) would result in a combined effect and therefore slow light-induced cone contraction even further.

Cone contraction in 6-OHDA lesioned retinas from fish treated with PTU was slowed, compared to control (Fig. 14). Starting at 5.5 min illumination, mean cone length was significantly ($p<0.001$) longer at each time point studied, even after 30 min illumination. As a result, the pattern of contraction over time in 6-OHDA lesioned eyes from fish treated with PTU was different from that in control retinas. Contraction was the fastest over the first 5.5 min but then slowed at 7.5 min followed by fairly consistent contraction over the remaining time.

Cone contraction in 6-OHDA-lesioned retinas from fish treated with PTU was slowed even further than that observed in 6-OHDA lesioned retinas (Fig. 15). Mean cone length was longer in 6-OHDA lesioned retinas from fish treated with PTU (compared to 6-OHDA alone) at 7.5 min, 9.5 min ($p<0.001$) and 13.5 min ($p<0.05$). Although very near (94%) the mean cone length of fully light-adapted retinas, mean cone length in 6-OHDA lesioned retinas from PTU-treated fish was significantly ($p<0.001$) different even after 30 min illumination.

The experiments with PTU and 6-OHDA suggest that the effects of PTU on light-induced cone contraction are not explained by an effect of PTU on dopaminergic IPCs in that cone contraction was slowed even more when PTU-treatment was combined with 6-OHDA lesioning.

Figure 14. Mean cone length (± SD, n=10 eyes at each point) of 6-OHDA-lesioned eyes from PTU-treated fish, initially dark-adapted, goldfish exposed to various periods of illumination prior to fixation. For comparison, control, fully dark- and light-adapted data are included (same as Fig 9). At each 6-OHDA+PTU time point, mean cone length was significantly ($p<0.001$) less compared to fully dark-adapted mean cone length (significance not indicated). Conversely, mean cone length was significantly ($p<0.001$; significance not shown) greater compared to mean cone length of fully light-adapted retinas. At certain time points, mean cone length from 6OHDA+PTU animals was greater, compared to control, as indicated (*** $p<0.001$).


Dark-adapted cone length ± SD
Light-adapted cone length ± SD
CONTROL
6OHDA+PTU
Cone Photoreceptor Length (µm)
115
105
95
85
75
65
55
45
***
1.5
3.5
5.5
7.5
9.5
11.5
13.5
15.5
20
25
30
Duration of light exposure prior to fixation (min)

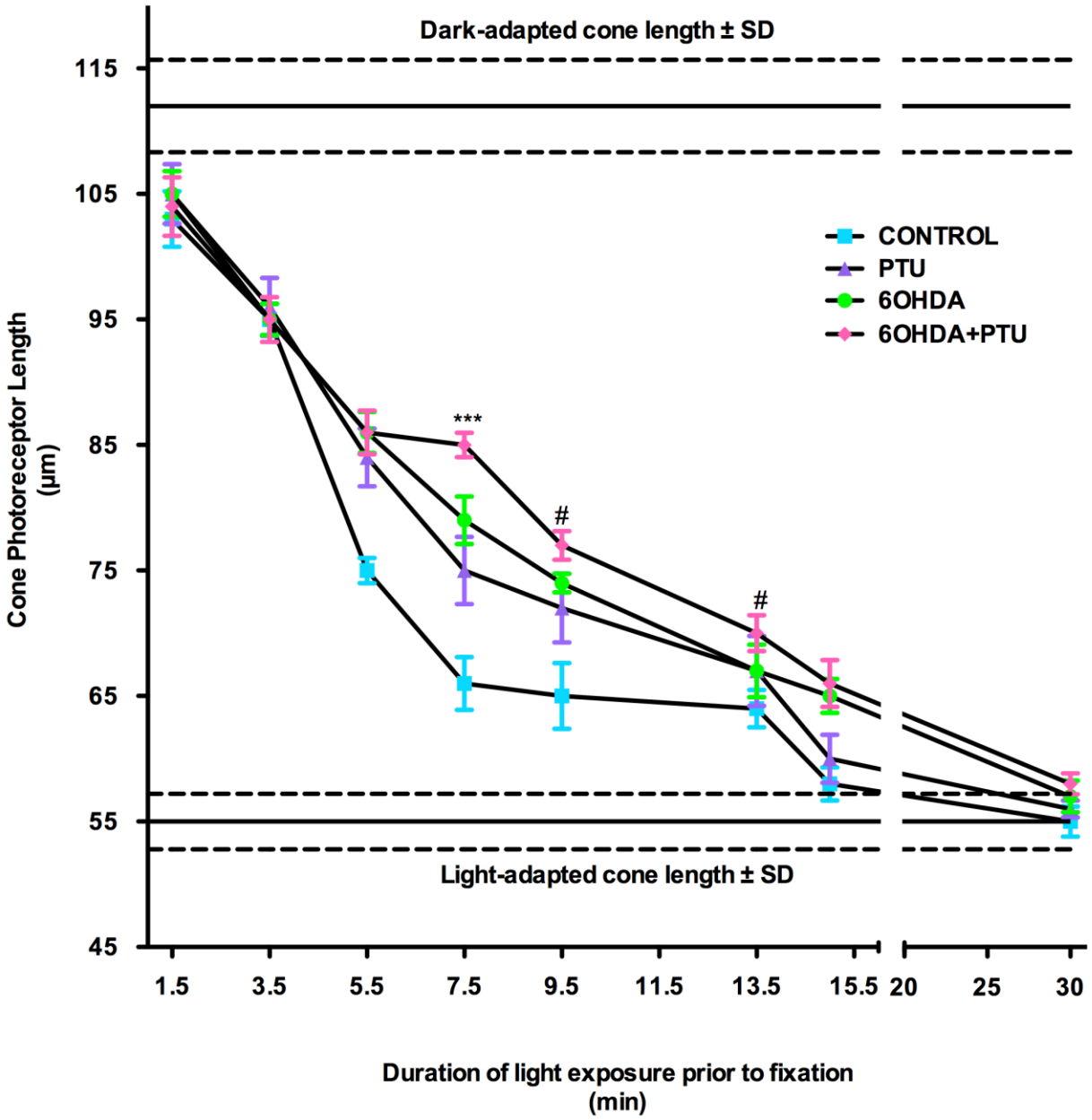


Figure 15. Summary graph illustrating mean cone length (± SD) for all the experiments described in Figs. 10-12, and 14. At certain time points, 6-OHDA lesioned eyes from PTU-treated fish resulted in mean cone lengths greater than 6-OHDA lesioning alone (*** $p<0.001$, # $p<0.05$).

CHAPTER 4: DISCUSSION

The large size of the lens in the eye of teleosts means that many, but not all species, are unable to change the diameter of the pupil in response to ambient light level (Walls, 1963). Instead, teleosts (and some other vertebrates) have evolved a mechanism by which they are able to control the amount of light incident to the photoreceptors of the retina. This mechanism consists of morphological changes that alter the position of rod and cone photoreceptor outer segments, the photopigment-containing light-sensitive region of the photoreceptor, and changes of the distribution of light-absorbing melanin pigment within retinal pigmented epithelium (RPE). Cones elongate in the dark (or at night) and rods contract, repositioning the rod outer segments to allow for optimal exposure to incoming light. Conversely, cones contract when ambient illumination increases (or during the day) so that cone outer segments are positioned to receive optimal exposure to light and rods elongate and become surrounded by melanin-filled processes of RPE, presumably to protect the rod photopigment (rhodopsin) in the rod outer segment from excessive bleaching. These changes are collectively called "retinomotor movements" (RMMs), and have been studied extensively (Ali, 1975; Burnside, 1976; Burnside, 1978; Burnside, 2001; Burnside et al., 1983; Burnside and Basinger, 1983; Burnside et al., 1982; Burnside et al., 1993; Dearry and Burnside, 1986a; Dearry and Burnside, 1986b; Dearry and Burnside, 1989; McCormack and Burnside, 1991; Nagle et al., 1986; Pierce and Besharse, 1985; Troutt and Burnside, 1989).

Retinomotor movements appear to be influenced by dopamine acting via D2 dopamine receptors (Dearry and Burnside, 1986a; Hillman et al., 1995; Rashid et al., 1993). Exogenous application of dopamine mimics the effect of light whereas D2 dopamine receptor antagonists mimic darkness (Dearry and Burnside, 1985; Rashid et al.,

1993). The latter evidence points clearly to endogenous dopamine as a key regulatory neuromodulator governing RMMs. The most likely source of endogenous dopamine in the retina are the dopaminergic IPCs. However, previous studies, where these neurons were ablated by lesioning with 6-OHDA, revealed that light-dependent RMMs persisted (Ball et al., 1993; Douglas et al., 1992). One potential explanation for this result is that another source of dopamine exists within the eye.

In principle, an alternate source of dopamine within the eye could be the RPE (Ming et al., 2009). The synthetic pathway leading to the production of melanin involves the conversion of tyrosine to L-DOPA by the enzyme tyrosinase. L-DOPA is a precursor for melanin but can also be converted to dopamine via the enzyme DOPA decarboxylase. It follows, therefore, that tyrosinase activity could be important for RMMs as an endogenous source of dopamine. While previous studies showed that RMMs persisted in 6-OHDA-lesioned retinas, this does not necessarily mean that dopaminergic IPCs cannot influence RMMs, only that they might not be the sole modulator of RMMs.

The ***aim*** of this thesis was to examine the role of tyrosinase activity on light-induced cone movement in a teleost retina (goldfish). The ***hypothesis*** for this work was that the activity of tyrosinase is required for light-induced cone contraction. Therefore, the specific tyrosinase inhibitor PTU should block light-induced cone contraction. To rule out an effect of PTU on dopaminergic IPCs, I determined if the effect of PTU was maintained or even enhanced in goldfish retinas where the dopaminergic IPCs were lesioned by prior treatment with 6-OHDA.

4.1 Measurement of cone length

Many previous RMM studies, where changes in cone length were measured in sections using bright-field microscopy, determined cone length as the distance from the outer limiting membrane (OLM) to either the cone ellipsoid or the outer segment (Burnside et al., 1982; Dearry and Burnside, 1986a; Dearry and Burnside, 1986b; Douglas et al., 1992). This approach was reasonable given that it is the cone myoid (the inner part of the cone inner segment) that changes in length during RMMs with the portion of the cone from the synaptic terminal to the myoid fixed in length. However, because identifying the position of the OLM, and the borders of the ellipsoid or outer segment, in such preparations can be somewhat arbitrary, for this thesis work I developed a new approach to measuring cone length. Instead of relying on contrast (light microscopy), I labeled a subset of cones in goldfish retina with zpr-1 antibody. Although produced by immunization to retinal cells obtained from wild-type zebrafish, this monoclonal antibody was later found to recognize a type of cone arrestin protein and has been shown to label double-cones in teleosts, specifically in zebrafish and goldfish retinas (Larison and Bremiller, 1990; Renninger et al., 2011; Schultz et al., 1997; Zou et al., 2008). When imaged using confocal microscopy, the labeling obtained with zpr-1 defined clearly the borders of double cones in goldfish retina, from the synaptic terminal to the outer edge of the inner segment, and significant differences of cone length were detected and measured under different adaptation and treatment conditions. Under control conditions, cone lengths typically exceeded 100 μm (112 μm ± 3.7 μm, mean ± SD) under dark-adapted conditions and were reduced by about 50% (55 μm ± 2.2 μm) after full light-adaptation.

4.2 Full dark- and light-adaptation is not affected by PTU or 6-OHDA

Cone length in fully dark- and light-adapted retinas was not affected by PTU. In particular, light-dependent cone contraction was not blocked in fish treated with PTU. However, these first experiments were done in retinas that, in principle, could release dopamine from IPCs. Therefore, to determine if PTU might affect light-dependent cone contraction in the absence of a neuronal dopamine source, I examined the effect of PTU in retinas where the dopaminergic IPCs were destroyed by prior treatment with 6-OHDA. Cone lengths in dark- and light-adapted retinas lesioned with 6-OHDA were indistinguishable from control retinas. This is the same result that was obtained previously by Douglas et al. (1992). Cone position in fish treated with PTU, and eyes injected with 6-OHDA, were also essentially the same as control. These data suggest that neither PTU-treatment nor 6-OHDA lesioning, alone or combined, can block light-dependent cone contraction.

4.2.1 Light-induced cone contraction is slowed by PTU and 6-OHDA

Most studies of RMMs compare dark- and light-adapted retinas, as I did in the first set of experiments described above. I wondered if PTU might alter the transition of cone length from dark- to light-adapted? Therefore, I examined cone length in initially dark-adapted animals exposed to various durations of light (1.5, 3.5, 5.5, 7.5, 9.5, 13.5, 15.0, 30 min) prior to fixation. In control eyes, even 1.5 min of light produced a significant reduction in cone length, reaching the fully light-adapted length by 30 min. In animals treated with PTU, cone contraction was slowed (cone length longer at time points 5.5-15 min) and contraction was not complete until 30 min. These results suggest that PTU slows, but does not block, light-induced cone contraction. To determine the

importance of dopaminergic IPCs, in relation to the effect of PTU, I studied the effect of PTU on fish where the IPCs had been lesioned with 6-OHDA. First I assessed the effect of 6-OHDA-lesioning alone on the transition of cone position from dark- to light-adapted. Interestingly, cone contraction was slowed (cone length longer at time points 5.5-9.5, 15 min) in 6-OHDA-lesioned retinas and even after 30 min of light exposure did not reach the fully light-adapted position. The similarity of cone contraction in PTU-treated fish and 6-OHDA-lesioned retinas might suggest that both manipulations are targeting the same mechanism(s). I believe this is not the case because in 6-OHDA-lesioned retinas from PTU-treated fish, cone contraction was slowed (cone length longer at time points 5.5-15 min) even further compared to 6-OHDA-lesioned alone, and even at 30 min, cone length was longer in 6-OHDA + PTU-treated fish compared to 6-OHDA-lesioned alone.

These results suggest that light-induced cone contraction is sensitive to both 6-OHDA-lesioning and treatment with PTU. Although the effect of PTU-treatment in animals with 6-OHDA-lesioned eyes did not result in a fully additive effect, light-induced cone contraction was slower than in 6-OHDA alone. A reasonable conclusion is that dopamine, derived both from neurons (produced by tyrosine hydroxylase and targeted by 6-OHDA lesioning) and derived from tyrosinase activity (presumably in the RPE and inhibited by PTU) contributes to light-induced cone contraction in the goldfish retina. However, dopamine produced by either source, or even combined, is neither necessary nor sufficient, at least in terms of reaching the fully light-adapted cone position.

The effect of PTU on light-induced cone contraction is similar to the effect of PTU on visual behaviour reported by Page-McCaw et al. (2004). Adaptation of the optokinetic response to light was slowed, but not blocked, in zebrafish larvae (6-7 days post fertilization, dpf) treated and a similar effect was found in a zebrafish with a

mutation in the tyrosinase *(sdy)* gene (Page-McCaw et al., 2004). They suggested that tyrosinase activity, most likely associated with RPE, plays a role in retinal light adaptation. They even speculated that RPE tyrosinase-derived L-DOPA might be a source of dopamine and that this could explain why 6-OHDA ablation of dopaminergic IPCs does not abolish RMMs (Ball et al., 1993; Douglas et al., 1992). While I did not find that PTU abolished light-induced cone contraction, the results presented here are consistent with a potential role for tyrosinase in the time course of light-induced cone contraction.

In principle, the zebrafish *sdy* mutant would be an excellent model system in which to test further the hypothesis that light-induced cone contraction is dependent, at least to some extent, on tyrosinase activity. To this end, Dr. Herwig Baier (University of California San Francisco) very generously supplied our lab with fertilized eggs from *sdy* zebrafish and we were able to generate larvae. Unfortunately, despite repeated efforts, we were unable to maintain viability beyond about 12 dpf and cone movements in zebrafish are not present (or at least detectable) until at least 28 dpf (Hodel et al., 2006). The fact that Page-McCaw et al. (2004) found that the lack *(sdy)* or block (PTU) of tyrosinase affected light-adaptation of optokinetic responses in zebrafish at an age before cone movements begin, suggests that the effects they observed are not due to alterations of light-induced cone contraction. This could indicate that tyrosinase-derived dopamine can affect retinal processes beyond cone contraction.

4.3 RPE as a source of dopamine in the retina

The presence of pigment within teleost RPE cells is evidence that these cells contain tyrosinase and synthesize L-DOPA. But for the L-DOPA to be converted into dopamine requires L-DOPA decarboxylase (DDC) and, if dopamine is produced within

RPE cells, there must be a mechanism by which dopamine can be released or, at the very least, there must be evidence that RPE cells can release dopamine. Very few studies have examined the potential synthesis of dopamine by RPE. However, one study that did address this issue was conducted by Ming et al. (2009) whom were able to detect DDC mRNA (by RT-PCR) and protein (immunoblot) derived from human cultured RPE cells. Even more interesting, they were able to detect (HPLC) dopamine, and the dopamine metabolites DOPAC and HVA, in RPE cell homogenates. They were not able to detect dopamine released by RPE cells placed into a high potassium (56 mM K^+) solution, an approach that had proven effective when applied to ventral mesencephalic neuron cultures (Li et al., 2007). However, RPE cells are not neurons and, even though RPE cells possess voltage-gated calcium channels (Ueda and Steinberg, 1993), elevated potassium may not be an appropriate treatment to produce dopamine release.

What other mechanism(s) might mediate dopamine release from RPE? One possibility is that dopamine within RPE cells could be released by a transporter. There is evidence that RPE cells express the organic cation transporter-3 (OCT-3) (Rajan et al., 2000) that is known to transport various catecholamines. Characterized in astrocytes, from human or animal brain, OCT-3 transport is electrogenic and bidirectional (Cui et al., 2009; Inazu et al., 2003). If these features apply to RPE, elevated intracellular levels of dopamine could be transported out of RPE and this would be greatest when the RPE cell was polarized. This could also explain why dopamine release was not identified when RPE cells were placed in elevated potassium solution (Ming et al., 2009).

Another requirement for RPE to be a source of dopamine associated with light-induced cone contraction is that dopamine would need to be released in response to light. The OCT-3-mediated mechanism described above would require that RPE cells be

relatively polarized in the light and depolarized in the dark. Interestingly, the apical surface of the RPE (facing the photoreceptors) is known to hyperpolarize in response to light (Oakley, 1977; Steinberg et al., 1970), an effect mediated by decreased potassium concentration in the sub-retinal space.

Another possible mechanism is that RPE may themselves be photosensitive. Mouse RPE have been shown to express the photopigment melanopsin (Peirson et al., 2004). Melanopsin is best known because of its expression in specific types of retinal ganglion cells, where it is coupled to G_q G-proteins, leading to activation of phospholipase C and the opening of transient receptor potential C (TRPC) channels (for review see Hughes et al., 2016). RPE also express TRPC channels (for review see Wimmers et al., 2007) and activation of these channels would produce inward currents (leading to depolarization) or increase intracellular calcium ion concentration. How this might lead to dopamine release is uncertain.

Melanin pigment is a defining characteristic of RPE in all non-albino vertebrate retinas. Therefore, there is the potential that RPE could be a source of dopamine in more than just teleost retinas. Although the present work focused on a potential role of RPE (PTU-sensitive) dopamine on light-induced cone contraction in goldfish, there are other targets of dopamine in vertebrate retinas (Popova, 2014). This could mean that RPE-derived dopamine plays a variety of roles in vertebrate retinas. Such a source of dopamine could be particularly important in those vertebrates that do not possess dopaminergic IPCs (see Dowling, 2012). In these vertebrates, dopamine is produced by amacrine cells and released in the inner plexiform layer. To reach targets in the distal (outer) retina, the dopamine needs to diffuse from the inner to outer retina. Although there is evidence that dopamine, released from amacrine cells, can reach targets in the

outer retina (Witkovsky et al., 1993), an additional source of dopamine, from the RPE, could be important in these cases.

4.4 The effectiveness of PTU and 6-OHDA

An underlying assumption of this work is that both endogenous sources of dopamine have been removed, either abolished (neuronal) by 6-OHDA, or inhibited (tyrosinase-dependent) by PTU. However, the nM affinity of D2 (D4) dopamine receptors for dopamine (Gingrich and Caron, 1993) means that if some dopamine, from either source, remains, it might not lead to a complete block of cone contraction.

In the current work, I assessed the effectiveness of the 6-OHDA-lesioning protocol using tyrosine hydroxylase immunohistochemistry in 2 eyes from 2 different fish and compared them to naïve (2 eyes) or saline-injected (2 eyes) controls. Tyrosine hydroxylase-immunoreactive neurons were easy to detect in the control eyes but there was no evidence of tyrosine hydroxylase-immunoreactive neurons in the 6-OHDA-lesioned retinas. This result is consistent with previous work that showed that the 6-OHDA-lesioning protocol employed was effective and specific (Baldridge and Ball, 1991; Ball et al., 1993). In these studies, the 6-OHDA-lesioning protocol not only resulted in a loss of tyrosine hydroxylase-immunoreactive neurons, but also a loss of cells showing high affinity uptake of ^{3}H-dopamine and, most convincingly of all, levels of dopamine measured by HPLC that were at or below the level of detection.

Whether the PTU-treatment produced an effective and specific inhibition of tyrosinase in the goldfish eye was not independently verified. PTU has been used extensively as a means to prevent melanin synthesis during the development of various aquatic vertebrates, increasing the transparency of these organisms (Epping, 1970;

Whittaker, 1966), and in recent years, in particular in zebrafish (Karlsson et al., 2001). A major advantage of using PTU in aquatic animals is the ease of application: it can simply be added to the tank water. I used the same concentration (0.2 mM) and time protocol (48 hrs) that has been deemed effective for the prevention of melanin formation in zebrafish with limited toxicity (Karlsson et al., 2001).

It is not possible to rule out unequivocally that light-dependent dopamine release is still present in the retinas of 6-OHDA-lesioned eyes and PTU-treated fish and that this is sufficient to lead ultimately, though somewhat slower, to full cone contraction. A way to examine this question further would be to examine the effect of a D2 dopamine receptor antagonist in eyes that have been lesioned with 6-OHDA and fish treated with PTU. If the full light-dependent cone contraction observed in this study, in PTU-treated fish, 6-OHDA-lesioned eyes, or in 6-OHDA lesioned eye from PTU-treated fish is blocked by the D2 antagonist this would suggest that sufficient dopamine remains to trigger cone contraction. If the D2 antagonist does not block the effect of light this would suggest that there must be an additional mechanism (or mechanisms) involved with light-dependent cone contraction.

4.5 Other mechanisms mediating light-dependent cone contraction

Neither PTU-treatment nor 6-OHDA lesioning, alone or in combination, blocked light-induced cone contraction. Assuming that these treatments, especially when combined, eliminated dopamine as a light signal within the retina, this suggests that other mechanisms exist that lead to light-induced cone contraction. In addition, the initial phase of light-induced cone contraction (over the time period 0-3.5 min) was very similar in controls and fish treated with PTU, 6-OHDA or both. This may indicate that the earlier

phases of light–induced cone contraction, in particular, occur independent of dopamine and may be influenced by an alternate mechanism.

The contraction of cones by an indirect effect of light on rods, leading to dopamine release, suggested that there was no direct, intrinsic effect of light on cone length (Besharse and Witkovsky, 1992; Kirsch et al., 1989). However, isolated cones (*in vitro*) were shown to be elongated when maintained in the dark relative to cone length when treated with exogenous dopamine or exposed to light (Burnside et al., 1993). The authors of this study suggested that this indicated an intrinsic mechanism in cones that could contribute to light-induced cone contraction. However, what their data actually showed was that isolated cones gradually elongate when maintained in the dark and this elongation is slowed by light and blocked by dopamine. Although an intrinsic effect of light on cone length could explain why PTU-treatment and 6-OHDA lesioning, especially when combined, failed to block light-induced cone contraction, it is not clear that the data supporting an intrinsic mechanism is that convincing. Even more curious is the fact that treatment with D2/D4 dopamine receptor antagonists block completely light-induced cone contraction (Dearry and Burnside, 1988; Douglas et al., 1992; Hillman et al., 1995). In relation to the results I obtained, this suggests that either sufficient dopamine remains to produce cone contraction, even after PTU-treatment and 6-OHDA lesioning, or that some other messenger, capable of acting at D2 dopamine receptors, contributes to the light signal to cones in the retina.

4.6 Summary of limitations and future directions

The results obtained from this thesis work indicate that treatment with the tyrosinase inhibitor PTU or the neurotoxin 6-OHDA slows the progression of light-

induced cone contraction in the goldfish retina. The greatest effect was obtained when both treatments were combined. I interpret these effects as indication that dopamine, produced both by RPE (PTU-sensitive) or dopaminergic IPCs (6-OHDA-sensitive), contribute to light-induced cone contraction. That light-induced cone contraction was not blocked by either treatment, or even when the treatments were combined, may indicate that there are additional mechanisms, not affected by PTU or 6-OHDA, that contribute to light-induced cone contraction.

A key limitation, as described above, is that I did not verify that the treatments resulted in the loss of dopamine in the retina. Although the 6-OHDA protocol used has been well characterized, and I did provide evidence that the protocol abolished tyrosine hydroxylase-immunoreactive neurons, there was no assessment of the effectiveness of PTU. There are a number of ways that this limitation could be addressed. Some of these approaches would focus on fundamental issues associated with the hypothesis that RPE is a source of dopamine. For example, RPE from goldfish could be isolated and maintained in culture and their dopamine content measured (as was done by Ming et al., 2009 using human RPE). The effect of PTU on RPE dopamine levels could then be investigated. But another approach that could be used to determine to what extent retinal dopamine signalling remains after PTU and 6-OHDA treatment would be to perform an additional treatment with a D2/D4 dopamine receptor antagonist. If the observed light-induced cone contraction that remains following PTU and 6-OHDA treatment is blocked by D2/D4 dopamine receptor antagonist this could suggest that sufficient dopamine is present to allow for full light-induced cone contraction. If D2/D4 dopamine receptor antagonist treatment was not effective, this could suggest that there is an additional mechanism or

mechanisms that can lead to light-induced cone contraction independent of D4 dopamine receptors.

Another limitation of the study was the use of a single inhibitor of tyrosinase, PTU. Although PTU has been used the most in teleost systems, in particular to render zebrafish transparent (Epping, 1970; Karlsson et al., 2001; Whittaker, 1966), it would be useful to test the effect of other tyrosinase inhibitors such as deoxyarbutin (Boissy et al., 2005). Another consideration related to PTU is whether or not it might have an effect on retinal function independent of an effect on cone length. For example, if PTU were to reduce the responsiveness of photoreceptors to light, this might slow light-induced cone contraction. I think this is unlikely for two reasons. First, Page-McCaw et al. (2004) did not find that photoreceptor function (measured by ERG) was affected in *sdy* zebrafish or in wildtype fish treated with PTU. Second, Mr. Sunghan Jung, working in our (Baldridge) lab, recorded the light responses of horizontal cells in the isolated retinas of control and PTU-treated goldfish. The responses of horizontal cells from PTU-treated fish were no different from control.

There are many experiments that could be done to extend the study of tyrosinase in the teleost retina. An obvious experiment would be to examine the other component of retinomotor movements, pigment migration within RPE. Pigment movement is also thought to depend on the action of dopamine on the family of D2 type dopamine receptors but is not abolished by lesioning of dopaminergic IPCs by 6-OHDA (Ball et al., 1993). Additional experiments could also be conducted to explore the potential mechanism of dopamine synthesis in, and release from, the RPE. I already mentioned above experiments to examine whether dopamine can be detected in teleost RPE. It would also be useful to determine if OCT-3, the transporter that could mediate dopamine

release from RPE, is present on teleost RPE using in situ hybridization or RT-PCR (as done in mice by Rajan et al., 2000). To determine if OCT-3 is involved in the release of dopamine from RPE it would be interesting to test the effect of an inhibitor of OCT-3, decynium-22 (Inazu et al., 2003), on light-induced cone contraction.

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