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# ADENOSINE PROTECTS ZEBRAFISH PHOTORECEPTORS FROM DAMAGE AND APOPTOSIS IN A LIGHT-INDUCED RETINAL DEGENERATION MODEL OF AGE-RELATED MACULAR DEGENERATION

A Dissertation in

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by

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## ABSTRACT

Age-related macular degeneration (AMD) is the leading cause of blindness among our aging population. AMD is characterized by the loss or impairment of central vision due to macular dropout of the retinal pigment epithelium and photoreceptor death. While there are treatments that can prevent vision loss in the "wet" form of AMD, there are currently no viable treatments for the more common "dry" form of AMD. Adenosine has been shown to be neuroprotective in the CNS and the inner retina under a number of pathological conditions. Adenosine receptor activation has been shown to inhibit voltage-dependent $Ca^{2+}$ influx in photoreceptors, which could potentially protect these cells from neurodegeneration. This suggests that adenosine could provide a protective role in degenerative photoreceptor diseases like AMD. The central goal of this thesis was to explore whether adenosine is neuroprotective in a light-induced-retinal degeneration (LIRD) model of AMD in pigmented zebrafish.

One powerful approach to model AMD utilizes a prolonged period of dark-adaptation (7-14 days) followed by high-intensity light exposure to generate retinal degeneration. This LIRD model results in the thinning of the outer retina and the loss of photoreceptors, which recapitulates the pathology observed in dry AMD. As a critical change for my thesis, we modified the original LIRD model by reducing the dark-adaptation period to just 24 hours. Our findings showed that a short period of dark-adaptation was sufficient to produce retinal damage that was comparable to the damage seen in other zebrafish studies using the original LIRD paradigm. The timing, progression, and severity of photoreceptor damage, apoptosis, and loss was similar to the timeline of events reported in the original model.

Our laboratory previously reported that adult zebrafish photoreceptors express $A_{2a}$ and $A_3$ adenosine receptors. One major aim of this thesis was to test whether the activation of adenosine receptors present on photoreceptors reduces the amount of damage observed in our LIRD model in pigmented zebrafish. Our findings revealed that adenosine prevented LIRD of photoreceptors in a dose-dependent manner. This included a decrease in apoptosis and overall cell loss by preserving the outer segments, inner segments, and synaptic terminal structure of both rods and cones. Moreover, the normal regenerative response in zebrafish was not observed in adenosine-treated retinas following light damage, meaning that if there was damage present, it was not sufficient to produce a regeneration response in the retina, suggesting a strong neuroprotective mechanism. Surprisingly, we discovered that stimulation of both $A_1$Rs and $A_3$Rs conveyed the

protective effects of adenosine on photoreceptors in zebrafish. Based on what we know about adenosine receptor expression in the zebrafish retina, the actions of $A_3R$ are likely conferred through receptors present on photoreceptors, whereas the effects of the $A_1R$ agonist could be mediated by stimulation of receptors on microglia, which likely suppresses microglial activation and subsequent pro-inflammatory signaling in the outer retina.

Our second major aim was to test whether adenosine can preserve visual function. In our studies, the optomotor response (OMR) was tested and used as a measure of visual acuity and contrast sensitivity. Impairment of visual acuity and contrast sensitivity threshold (CST) were significantly reduced in fish treated with adenosine in a concentration-dependent manner. Contrast sensitivity, although not significant, showed a small improvement in adenosine-treated fish after LIRD.

Altogether, this thesis demonstrates that stimulation of $A_1Rs$ and $A_3Rs$ by adenosine conveys neuroprotection for photoreceptors and provides strong evidence of a potential novel therapeutic target for the treatment of dry AMD.

# TABLE OF CONTENTS

# LIST OF FIGURES

# ABBREVIATIONS

ADP - adenosine diphosphate
AMD - age-related macular degeneration
AMP - adenosine monophosphate
AREDS - Age-Related Eye Disease Study
$A_1R$ - adenosine 1 receptor
$A_{2A}R$ - adenosine 2A receptor
$A_{2B}R$ - adenosine 2B receptor
$A_3R$ - adenosine 3 receptor
ATP - adenosine triphosphate
BAY- 60 - BAY 60-6583
cAMP - cyclic adenosine monophosphate
cGMP - cyclic guanosine monophosphate
CGS - CGS-21680 hydrochloride
CNS - central nervous system
CPA - $N^6$-Cyclopentyladenosine
CS - contrast sensitivity
CST - contrast sensitivity threshold
DHA - docosahexanoic acid
DNA - deoxyribose nucleic acid
dpf - days post fertilization
$EC_{50}$ - half maximal effective concentration
ER - endoplasmic reticulum
ERG - electroretinogram
FDA - Food and Drug Administration
GDP - guanosine diphosphate
GTP - guanosine triphosphate
GC - guanylyl cyclase
GCL - ganglion cell layer
h - hour
HEMADO - 2-(1-Hexynyl)-N-methyladenosine
hpf - hours post fertilization
$IC_{50}$ - half maximal inhibitory concentration
INL - inner nuclear layer
iNOS - inducible nitric oxide synthetase
IPL - inner plexiform layer
LED - light-emitting diode
LIRD - light-induced retinal degeneration
LWS1 - long wavelength sensitive cone
MDA - malondialdehyde
min - minute
mRNA - messenger ribonucleic acid
M-system - Mauthner cell system
MWS - middle wavelength sensitive cone
NOS - nitric oxide synthetase
Nrf2 - erythroid 2-related factor
OKR - optokinetic response
OMR - optomotor response
ONL - outer nuclear layer
OPL - outer plexiform layer
PCNA - proliferating cell nuclear antigen
PDE - phosphodiesterase
PKM2 - pyruvate kinase isozyme type M2
RGC - retinal ganglion cell
ROS - reactive oxygen species
RPE - retinal pigment epithelium
SFT - spatial frequency threshold
SOD1 - superoxide dismutase
SWS1 - short single cone

SWS2 - long single cone

TNFα - tumor necrosis factor alpha

TON - traumatic optic nerve neuropathy

TUNEL - terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

VEGF - vascular endothelial growth factor

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# Chapter 1

# Introduction

## 1.1 The Retina

The vertebrate eye is composed of three concentric layers: the fibrous layer, the vascular layer, and the retina (Fig. 1.1)[1,2]. The fibrous layer is the outermost layer of the eye and consists of the sclera and cornea. The middle layer is the vascular layer, which consists of the choroid, the ciliary body, and the iris. The innermost layer is composed of the retina and is closely associated with a pigmented layer posteriorly (Fig. 1.2)[3]. Internally, the retina is associated with the vitreous body (vitreous humor). External to the pigmented layer is the choroid, which contains a dense capillary network that provides the retina with oxygen and nutrients[1].

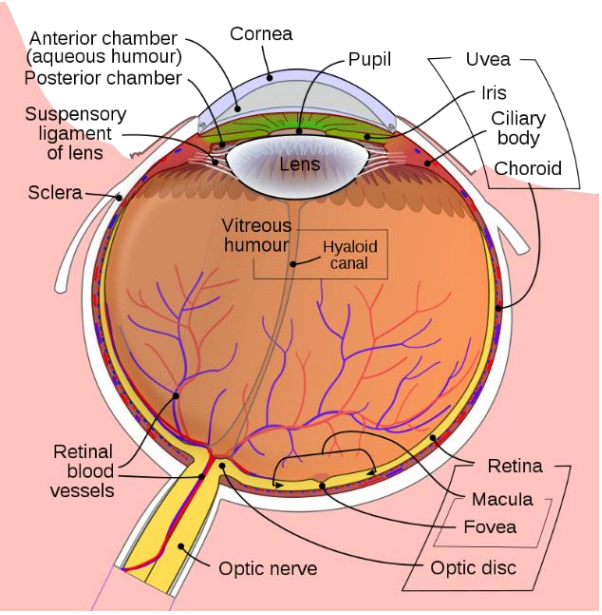


**Figure 1.1:** Illustration of the layers and components of the vertebrate eye. See text for description. Image from Rhcastilhos and Jmarchn, 2020. (Wikimedia Commons).

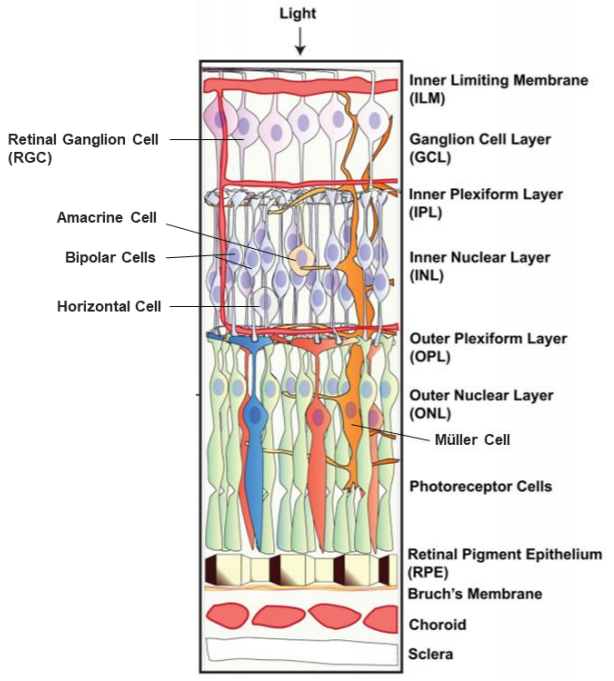


**Figure 1.2:** Section of the layers of the human eye illustrating the multi-layered retina and the outer pigmented layer with RPE cells. See text for description. Adapted from Jarrett and Boulton, 2012.

The retina is the light sensitive layer where photoreceptors and other retinal neurons reside. The retina is

considered to be a part of the central nervous system (CNS) and is essentially an outgrowth of the CNS[4]. In early human development, starting at 22 days post-fertilization (dpf), the retina begins as a diverticulum of the forebrain primordium and stays connected to the brain through the axons forming the optic nerve[5]. The pigmented layer, which has a close relationship with the photoreceptors of the retina, is a single layer of simple cuboidal, melanin-containing cells known as the retinal pigment epithelium (RPE). The RPE functions to 1) absorb extraneous light to reduce the scattering of light in the eye, 2) phagocytoses the shedding membranous discs of photoreceptor outer segments, 3) participates in regeneration of photopigments, 4) fulfills the metabolic needs of the photoreceptors, and 5) forms the outer blood-retinal barrier[6].

The retina is the first structure in the primary visual pathway. It converts light into electrical and chemical signals, which are then sent to the visual centers of the brain via the optic nerve[4]. When light passes through the eye, it is focused on the retina. Clinically, the internal aspect of the posterior part of the eye is known as the fundus (Fig. 1.3)[7,8]. The retina of the fundus includes several important structural landmarks. The first is a distinct circular feature known as the optic disc, which is an area where the axons of retinal ganglion cells pass through to enter the optic nerve. Many mammalian retinas have a specialized region were the cones are concentrated[9]. In humans, this area is known as the

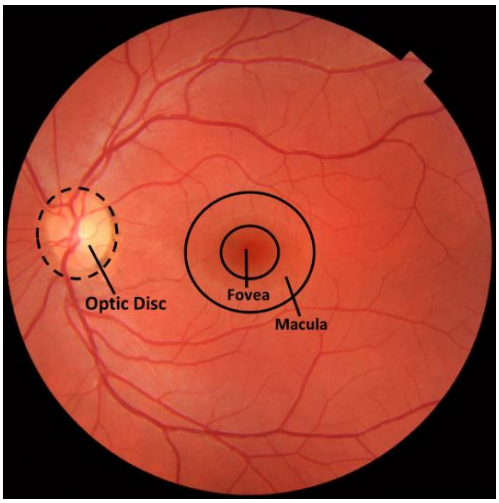


**Figure 1.3:** Ophthalmoscopic view of the fundus of a normal retina showing the macula, fovea, and optic disc. Adapted from Häggström, M., 2014.

macula lutea and is located lateral to the optic disc, appearing as a small oval region that is yellowish in color (Fig. 1.3). The macula is responsible for our central, high-resolution color vision and is cone-dominated[1]. Within the macula, there is a central depression or pit devoid of rods known as the fovea centralis. In primates, the fovea contains only red and green cones or a pure cone region and exhibits the highest visual acuity[9,10].

The macula gets its yellowish color from three carotenoid pigments: lutein, zeaxanthin, and meso-zeaxanthin. The macular pigments function to optimize visual performance and image quality by attenuating chromatic aberration, the adverse impacts of light scatter (veiling luminance), and glare[11]. Lutein and zeaxanthin are derived from diet and can be found in brightly colored fruits and vegetables as well as eggs, while meso-zeaxanthin has non-dietary origins and is produced in the macula following isomerization of retinal[11]. In terms of distribution, lutein is dominant in the peripheral macula, zeaxanthin is concentrated in the mid-peripheral macula, and meso-zeaxanthin is dominant at the center in the fovea[12]. All carotenoids have a characteristic linear conjugated polyene chain and are classified into either hydrocarbon carotenoids or oxycarotenoids. Macular pigments belong in the oxycarotenoid category and consist of a typical C40 carotenoid structure, substituted with hydroxy groups at 3 and 3’ carbon positions[12]. The light-absorbing properties of the macular pigments are provided by their inherent conjugated double-bond system and their absorption spectra peaks at 460 nm[12].

The macular pigments have generated interest among researchers because they are believed to play a protective role in the eye in several different ways. The macular pigments filter blue light and protect cone photoreceptors against the deleterious effects of short wavelengths[10,11]. By pre-absorbing blue light, the macular pigments may protect

the central retina from oxidative injury by limiting light-induced generation of reactive oxygen species (ROS)[11,13]. Macular pigments also contribute to the antioxidant activity of the retina by quenching the photoexcited state of photopigments and singlet oxygen, and by scavenging free radicals[14]. The polyene chains of the macular pigments supply readily available electrons, which is what allows them to quench ROS[12].

The retina is a multilayered structure that contains two synaptic layers sandwiched between three nuclear layers containing somata (Fig. 1.2). Light entering the eye must pass through all of these layers to reach the photoreceptors. The primary vertical retinal pathway begins with light raining onto photoreceptors in the outer retina that synapse onto bipolar cells and then to retinal ganglion cells (RGCs) in the inner retina, which send their axons to the brain[7]. The retina consists of 5 broad classes of retinal neurons (photoreceptors, bipolar cells, horizontal cells, amacrine cells, and RGCs) and three types of glial elements (Müller cells, astrocytes, and microglia)[4]. The cell bodies of photoreceptors make up the outer nuclear layer (ONL) and are sandwiched between the RPE and the outer plexiform layer (OPL), which is a synaptic layer formed by connections between photoreceptors and second-order neurons (bipolar cells and horizontal cells)[9]. The cell bodies of bipolar, horizontal, amacrine, and Müller cells reside in the inner nuclear layer (INL). In the inner plexiform layer (IPL) synaptic connections are formed between second-order neurons (bipolar and amacrine cells), and third-order neurons, RGCs[9]. RGC somata comprise the most distal layer of the inner retina with their axons forming the connections of the retina to the brain[4,9].

## 1.2 Photoreceptors

Photoreceptors are the light sensitive cells that absorb photons to initiate phototransduction and begin the process of vision. The vertebrate retina contains two types of photoreceptors, rods and cones. Rods are more sensitive to light and are generally used for low-light or scotopic conditions[15]. They are responsible for our night vision. Cones, on the other hand, are less sensitive to light and are used in bright-light or photopic conditions[16]. Cone photoreceptors are responsible for color vision. Most vertebrate species have multiple subtypes of cones, which are distinguished from each other morphologically and spectrally.

Each rod and cone photoreceptor consists of four main parts: an outer segment, an inner segment, the cell body (or somata) containing the nucleus, and a synaptic terminal (Fig 1.4). The outer segment of a photoreceptor is roughly cylindrical in shape for rods and conical in shape for cones, which explains how the photoreceptors were named. In rods, the outer segment is filled with stacks of free-floating membranous discs enclosed in the outer segment membrane, but for cones the membranous discs are formed by invaginations or lamellae of the outer segment membrane[1]. The outer segment membrane and membranous discs are rich in polyunsaturated fatty acids.

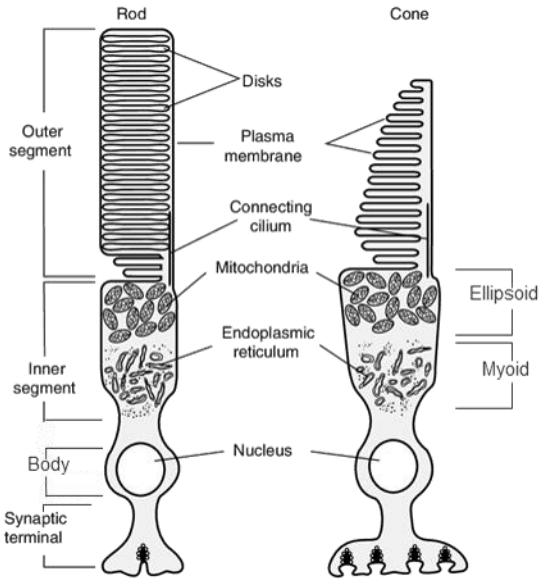


**Figure 1.4:** Schematic of basic rod and cone photoreceptor anatomy. See text for description. Adapted from Cote, 2006.

Docosahexanoic acid (DHA), the most highly polyunsaturated fatty acid, makes up approximately 50% of the phospholipids in the photoreceptor membrane[17]. The combination of constant light exposure with the high polyunsaturated fat content of the outer segments makes them prone to photooxidation and lipid peroxidation, leading to membrane damage[17]. As a result, the outer segment undergoes constant renewal, where the tips of the outer segments containing the most light damage are continuously shed and phagocytized by the cells of the RPE and the new membranous discs and lamellae are formed at the base of the outer segment in rods and cones, respectively[6].

Embedded within the membranous discs and lamella of the outer segment are the photopigments. Photopigments consist of a protein opsin bound to a light-absorbing chromophore known as 11-cis retinal, a derivative of vitamin A[18]. Opsins are G protein-coupled receptors and are referred to as rhodopsin in rods and photopsins in cones[1]. When 11-cis retinal absorbs a photon of light, it is isomerized to all-trans retinal, which activates the opsin and initiates the start of the phototransduction cascade[18].

While the outer segment is the site for phototransduction reactions, the inner segment contains the metabolic machinery that supports the cell. The inner segment is divided into an outer ellipsoid and an inner myoid portion and is connected to the outer segment by a non-motile cilium (Fig 1.4). The ellipsoid is packed with mitochondria to meet the high metabolic energy demands associated with phototransduction, while the myoid contains organelles dedicated to protein synthesis[19]. The inner segment is continuous with the cell body and an axon-like process connects the body to the synaptic terminal, the information transmitting end of the photoreceptor.

In rods the synaptic terminal is round and is referred to as the spherule and in cones the terminal is conical in shape and known as the pedicle (Fig. 1.4)[18]. Cone pedicles exist more or less side-by-side in the same plane, while the more numerous rod spherules lie packed between and above cone pedicles[18]. Rod and cone photoreceptors form ribbon synapses with the invaginated processes of second-order neurons (bipolar and horizontal cells). Unlike, conventional synapses, which respond to action potentials resulting in episodic release of neurotransmitter, photoreceptors tonically release neurotransmitter based upon varying light intensities. The synaptic ribbon in photoreceptors is a structural component that mediates the docking and fusion of synaptic vesicles at the terminal in response to graded changes in membrane potential and is capable of rapid and sustained neurotransmitter release[20]. The synaptic ribbon is a specialized plate-like structure that has numerous glutamate-containing synaptic vesicles tethered to its surface, and a subset of vesicles docked at the base of the ribbon at or near the plasma membrane[20]. The synaptic ribbon consists of a unique protein called ribeye[21]. The ribbon runs perpendicular to the plasma membrane at the site of synaptic vesical fusion and its base is attached to the presynaptic membrane by an electron-dense structure called the arciform density (Fig. 1.5)[22]. Synaptic ribbons are exclusive to synapses that utilize graded depolarization to drive sustained neurotransmitter release in

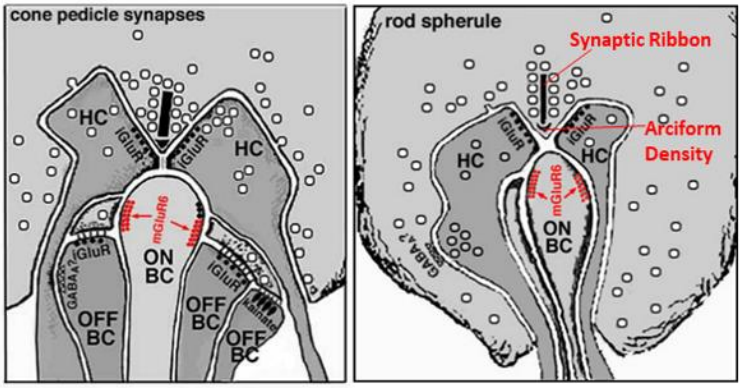


**Figure 1.5:** Illustration of the cone synaptic triad (left) and the rod ribbon synapse (right). Images adapted from Kolb, 2020 (Webvision: www.webvision.med.utah.edu).

cells. Typically, ribbon synapses are found in bipolar cells, photoreceptor-like cells in the pineal gland, electroreceptors, and auditory and vestibular hair cells[21]. They serve as a storehouse for large pools of readily releasable vesicles to support continuous neurotransmission and may aid in the transport of synaptic vesicles to the active zone[22]. Synaptic vesicle exocytosis in photoreceptors is mediated by the influx of $Ca^{2+}$ through voltage-gated L-type $Ca^{2+}$ channels (predominately $Ca_V1.4$), which are arrayed linearly parallel to the long axis of the ribbon, just beside the arciform density[20,22].

In the mammalian rod spherule, typically one synaptic ribbon is associated with one invaginating rod photoreceptor synapse containing three second-order neuron processes, two lateral horizontal cell dendrites and one centrally associated rod ON bipolar cell dendrite (Fig. 1.5)[18]. However, some lower vertebrates including fish have multiple synaptic ribbons on rods[21]. The arrangement of this invaginating synapse is known as the "synaptic triad." In the mammalian cone pedicle, although it is generally dependent upon the species, there are approximately 30 ribbons present and each ribbon is part of a synaptic triad. The cone terminal is comprised of multiple invaginating synapses or triads each with a synaptic ribbon, two lateral horizontal cell dendrites, and a centrally inserted dendrite of a cone ON bipolar cell, with OFF cone bipolar cells forming basal contacts at the base of the cone synaptic terminal (Fig 1.5)[18].

## 1.3 Phototransduction and the Visual Cycle

*Phototransduction in the vertebrate retina*

Phototransduction is the conversion of light into electrical signals in rod and cone photoreceptors. In the dark, there is a "dark current" or electrotonic spread of charge that

flows into the outer segment of the photoreceptor, throughout the cell to the synaptic terminal resulting in greater depolarization and release of L-glutamate[18]. In the dark, guanylyl cyclase (GC) present in the outer retina is actively converting guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). High levels of cGMP are able to bind to the intracellular side of cGMP-gated cation channels present on the outer segment membrane resulting in influx of cations, predominately $Na^+$ (80%) with smaller contributions from $Ca^{2+}$ (15%) and $Mg^{2+}$ (5%)[16,18]. The flow of ions entering through cGMP-gated cation channels in the outer segment is partially balanced by an outward leak current in the inner segment mainly due to $K^+$ efflux[23]. This results in a depolarizing electrotonic spread of charge radiating from the outer segment, through the inner segment and somatic regions to the synaptic terminal. This potential difference in charge across the membrane is the "dark current" and depolarizes the photoreceptor, resulting in the opening of voltage-gated $Ca^{2+}$ channels present on the cell body and synaptic terminal. This increase in voltage-dependent $Ca^{2+}$ influx results in the mobilization and fusion of synaptic vesicles at the photoreceptor terminal membrane and increases the release of the primary fast excitatory neurotransmitter, L-glutamate.

Exposure to light leads to closure of the cation channels and stops the "dark current", which causes the photoreceptors to hyperpolarize resulting in a decrease in L-glutamate release onto second-order neurons[23]. The hallmark of this mechanism involves the phototransduction cascade (see Fig. 1.6), which is initiated by the absorption of a single photon of light by the chromophore, 11-cis retinal, in the outer segment of the photoreceptor. Upon absorption, the 11-cis retinal is isomerized into all-trans retinal, which activates the opsin, the G protein-coupled receptor found in the membrane of the

lamellae or discs[19]. The activated opsin catalyzes the exchange of guanosine diphosphate (GDP) to GTP through the α-subunit of the G-protein, transducin. The α-subunit then dissociates from the β-subunit to bind with and inactivate cGMP-phosphodiesterase (PDE)[24]. Active PDE converts cGMP into GMP resulting in a decrease in cGMP below the levels necessary to keep the cGMP-gated cation channels open in the outer segment[23]. The cation channels close in the outer segment, removing the influx of charge which counters the outward leak current in the inner segment generated by the efflux of $K^+$. Therefore, this net negative charge generated by the efflux of $K^+$ hyperpolarizes the membrane leading to closure of voltage-gated $Ca^{2+}$ channels and a decrease in the fusion of synaptic vesicles with the synaptic terminal membrane, resulting in reduced L-glutamate release.

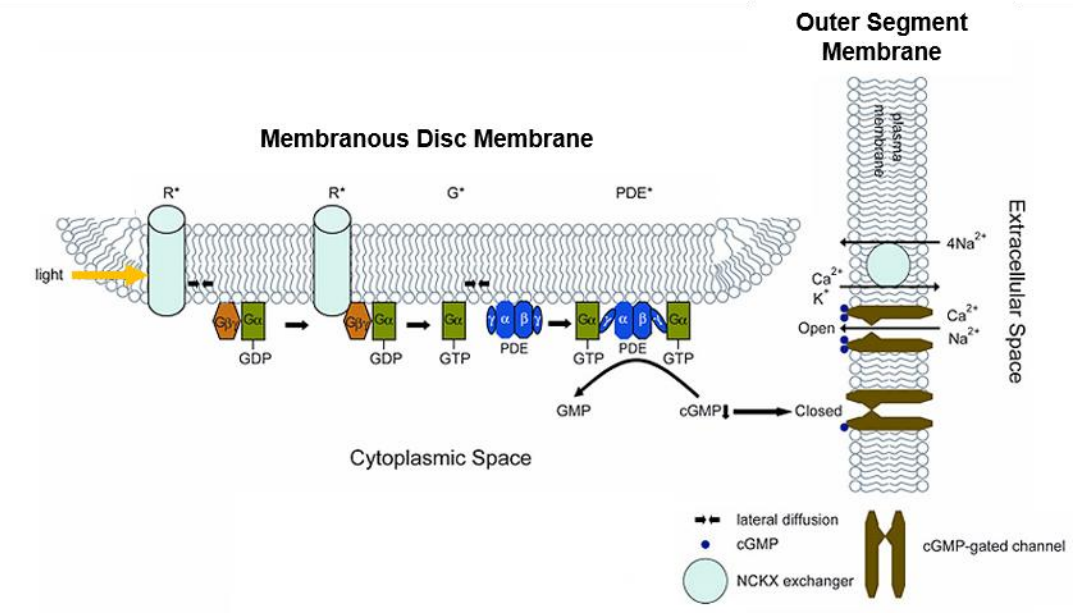


**Figure 1.6:** Schematic illustration of the phototransduction cascade in vertebrate photoreceptors. See text for description. Adapted from Fu, 2020 (Webvision: www.webvision.med.utah.edu).

*The vertebrate visual cycle*

Phototransduction begins with the isomerization of 11-cis retinal to all-trans retinal, but to sustain continuous vision the chromophore must be recycled in a complex sequence of enzymatic reactions, collectively known as the visual cycle. After prolonged or intense exposure to light the all-trans retinal separates from opsin and the opsin becomes bleached and inactive. In order for opsin to become active again, it needs another 11-cis retinal to replace the all-trans retinal. Within the outer segment the all-trans retinal is converted to all-trans retinol and is then transported to a second compartment, within the RPE, where it is esterified into retinyl esters[25]. In the RPE the retinyl esters are converted to 11-cis retinol, which is oxidized to 11-cis retinal again, and then shuttled back to the photoreceptor outer segment (Fig. 1.7)[25].

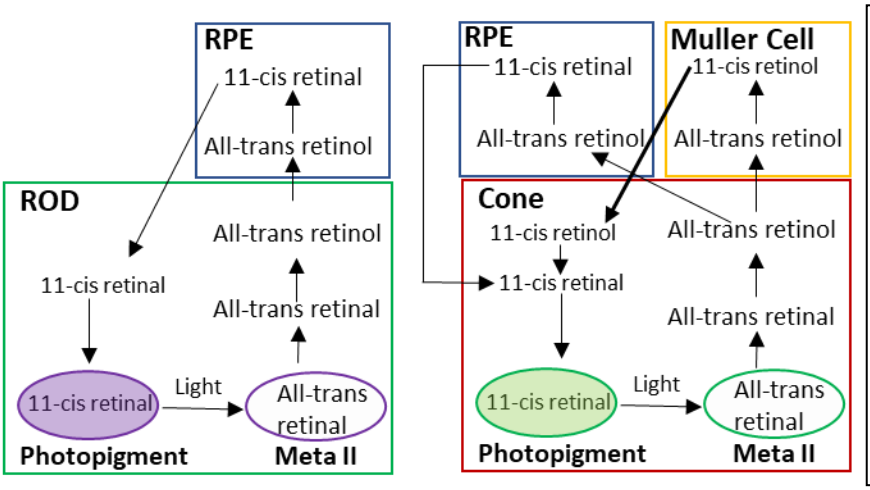


**Figure 1.7:** Schematic of the canonical visual cycle pathway (left) and the cone-specific visual cycle pathway (right). See text for description of both pathways. Adapted from Miyazono et al., 2008.

The canonical visual cycle pathway takes place in two compartments, the outer segment and the RPE, and has been described in detail for rods[25]. More recently, studies

have begun to look at the visual cycle in relation to cones. It is interesting to note that while rods become saturated under twilight or mesopic conditions, cones do not become saturated, even under bright-light conditions, and experience opsin activation and bleaching at a faster rate. This suggests that the 11-cis retinal chromophore is supplied at a substantially faster rate in cones than in rods in order to remain responsive under such conditions[26]. While it has been shown that the RPE supplies chromophores to both rods and cones, it has been revealed that the canonical RPE visual cycle is too slow to support cone function suggesting that another recycling pathway must exist to meet the high chromophore demands of cones[27].

Studies have found that in cone- and rod- dominated retina alike, an alternative cone-specific visual pathway does exist[28-30]. In this pathway, the second compartment is the Müller cells of the INL instead of the RPE (Fig. 1.7). So, in addition to receiving regenerated chromophores from the RPE, cones also receive chromophores from Müller cells in order to meet their functional needs[26,28]. In the cone specific pathway all-trans retinal is reduced to all-trans retinol and is then transported to Müller cells[26], where the all-trans retinal is isomerized by isomerase II. Unlike in the canonical visual cycle pathway, the chromophore that is supplied to cones from the Müller cells is 11-cis retinol. The 11-cis retinol is transported to the cone inner segment and is then transported to the outer segment where it is oxidized to 11-cis retinal[26,31].

*Differences in rod and cone sensitivity*

As mentioned previously, rod photoreceptors are more sensitive to light than cones. Rods are able to transduce light over a longer period of time and can operate at the

theoretical limit of a single photon, while cones decay more quickly, but are more adaptive and can detect varying light conditions when photon flux is less limited[32]. Variability in sensitivity between rods and cones can be attributed to differences in the rates of activation and inactivation of the phototransduction cascade[32].

Rods and cones have many of the same transduction proteins, but use different isoforms. In some cases, they may use the same isoform, but at different levels of expression. Rods are activated more quickly per photon absorbed and have a larger peak amplitude in response to the same stimulus in comparison to cones[32]. Differences in rates of activation seems to be produced by differences between the isoforms of rod and cone photopigments, transducin, and PDE6[32]. The inactivation rate of rods is much slower than that of cones, allowing rod responses to decay more slowly, contributing their increased sensitivity. The rate of inactivation is determined by the rates at which rhodopsin, transducin, and PDE return to their basal confirmations and also on the rate at which the cGMP concentration returns to "dark current" levels. The slower rate of inactivation in rods is attributed to differences in the species of transducin and PDE6, the expression levels of GC and GTPase-accelerating proteins, and PDE basal activity in rods and cones[32-35]. Rods sensitivity is due largely in part to the rapid activation rate and slower inactivation rate of the phototransduction of rods in comparison to cones.

### 1.4 The Zebrafish Retina

Zebrafish (*Danio rerio*) are a well-established vertebrate animal model that is widely used for the study of visual behavior, circuitry, and disease. The zebrafish retina is comparable to most classes of vertebrates in terms of development, structure, and

function. While the rodent model has been widely used to study vision and visual behavior, zebrafish retinas are similar to human retinas in many ways and are in some respects a better model for studying vision.

As mentioned previously, the human retina has a specialized cone-dense area responsible for central high-resolution color vision known as the macula. Although, the macula is absent from both the rodent and zebrafish retina, similar to humans, zebrafish are diurnal, meaning that they are active during the day and sleep at night. Not unsurprisingly, the retinas of zebrafish are cone-dominated[36]. Like humans, zebrafish really take advantage of their color vision[36,37]. During the day zebrafish rely on their color vision to locate food, avoid predators, and move around in their underwater environment. So, while zebrafish do not have a macula they do have cone-rich retinas akin to the human macula resulting in good color vision and a cone density similar to humans[36]. In contrast, rodents like mice and rats are nocturnal, meaning that they are most active during the night when it is dark. Rodent retinas are rod-dominated with rods making up about 97% of the photoreceptors and cones making up the remaining 3%, resulting in relatively poor color vision[24]. Mice rely more on olfactory, tactile, and auditory cues to move around their dark environment than they do on their vision.

Similar to the human eye, zebrafish have a retina that is composed of three nuclear layers (ONL, INL, GCL) sandwiched between two synaptic plexiform layers (OPL, IPL) and contains the same types of retinal neurons (photoreceptors, bipolar cells, horizontal cells, amacrine cells, RGCs) and Müller glia cells[37,38]. The ONL of the zebrafish contains a single type of rod and four spectral types of cones making them tetrachromatic. The rod cell bodies are located superficial to the cone nuclei and in the

light-adapted retina the rod inner and outer segments project beyond the cones to interdigitate with the microvilli of the RPE (Fig. 1.8)[38]. In a vertical retinal section, the cone photoreceptors are tiered within the ONL and can morphologically be organized into: short single cones (SWS1, ultraviolet-sensitive cones), long single cones (SWS2, blue-sensitive cones), and double cones, consisting of an accessory member, the middle wavelength sensitive cone (MWS, green-sensitive cone), and a principal member, the long wavelength sensitive cone (LWS1, red-sensitive cones) (Fig. 1.8)[37,39]. The four spectral cone subtypes can also be classified based on the peak sensitivities of the photopigments present in each type of cone: UV-cones ($\lambda_{max}$ = 362 nm), S-cones ($\lambda_{max}$ = 415 nm), M-cones ($\lambda_{max}$ = 480 nm), and L-cones ($\lambda_{max}$ = 570 nm)[39]. Humans lack UV-cones and have trichromatic vision, while mice are dichromatic and have single cones that co-express both S- and M-cone photopigments[24]. In the adult zebrafish, cones appear in a mosaic pattern where columns of alternating short single cones and long single cones alternate with columns of red-green double cones[40]. In mice, co-expressing cones show a gradient of mostly M-opsin (green) expression in the dorsal retina to mostly S-opsin (blue/UV) expression in the ventral retina[41]. Thus, the small percentage (~3%) of mouse cone photoreceptors co-expressing M- and S-cone opsins in a unique dorsal-ventral gradient likely confers very poor visual acuity in the visible light spectrum, and non-existent color vision.

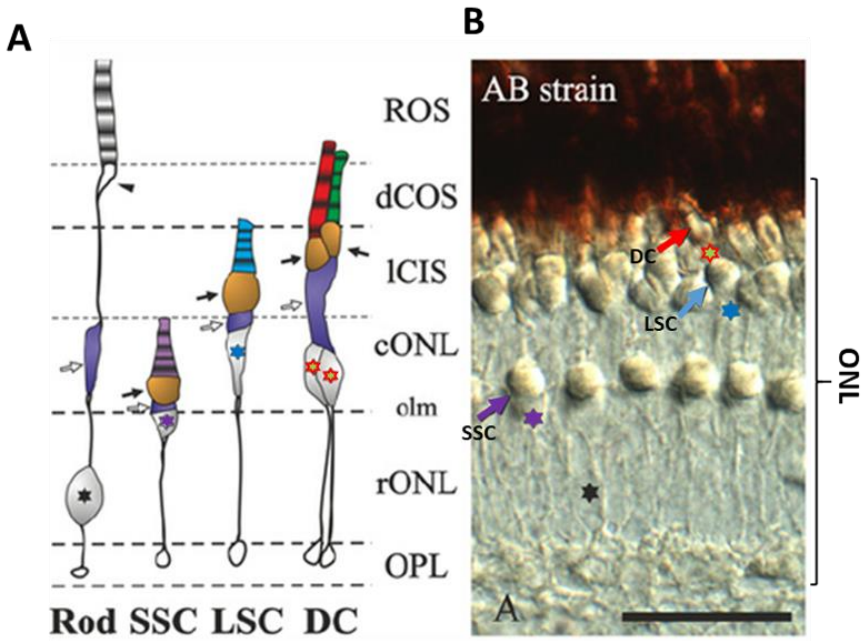


**Figure 1.8:** Adult zebrafish outer retina. **a)** Illustration of rod and cone subtypes in the outer nuclear layer (ONL). Black arrows indicate the ellipsoid portion of the inner segments and white arrows indicate the myoid portion. Stars denote nuclei. The outer limiting membrane (olm) divides the ONL into a rod ONL (rONL), where rod nuclei reside, and a cone ONL (cONL), where most of the cone nuclei reside. Note that the short single cones (SSC) have their nuclei in the rONL. **b)** Vertical retinal section of zebrafish outer retina (brightfield image). Arrows indicate inner segments. Stars denote nuclei. LSC, long single cone; DC, double cone; ROS, rod outer segment; dCOS, double cone outer segment; lCIS, long cone inner segment. Scale bar = 25 μm. Image adapted from Lagman et al., 2015.

In addition to good color vision, zebrafish also have relatively high visual acuity. In Caves et al. (2018), the authors define visual acuity as the ability of an animal to perceive static spatial detail[42]. The authors explain that visual acuity tends to be higher in diurnal animals than in nocturnal animals like rodents. This is because the retinas of nocturnal animals frequently exhibit spatial summation, a process where the photoreceptors pool together to collect light over a larger area making them functionally a single sampling unit. While this increases the light sensitivity of the cones, it comes at

the cost of visual acuity. Furthermore, the authors mention that visual acuity also tends to be higher in species that live in spatially complex habitats such as an underwater environment. Overall, the authors show that humans have exceptionally high visual acuity that is only surpassed by a few predatory bird species, and that fish have relatively high visual acuity, while nocturnal rodents have relatively poor visual acuity.

It is also important to note some of the issues surrounding visual acuity studies using rodents. First, contrast and overall luminance of the moving stimulus are not standardized throughout studies or corrected for, leading to variability of scores between studies[43]. This could explain why some studies report relatively high scores for rodents. Second, when testing for visual acuity, rodents often receive training to learn how to perform a certain task, while zebrafish typically do not receive training[43]. Training rodents requires a substantial amount of time and some type of deprivation to motivate them to perform. The preconditioning of rodents prior to testing can also skew visual acuity scores.

Visual acuity is typically measured using behavioral tests, but can also be measured by examining the density of RGCs in the retina[44,45]. Areas of the retina with a greater density of RGCs have been shown to have a higher visual resolution and animals with greater overall mean RGC density tend to have higher visual acuities[45-47]. The average RGC density in the zebrafish retina ranges from 11, 960 ± 4,095 cells/$mm^2$ to 37, 245 ± 7,055 cells/$mm^2$ [48]. Peak density of RGCs for zebrafish was found in the ventral-temporal retina, while the lowest density of RGCs occurred in the dorsal-nasal region of the retina resulting in a roughly threefold increase in RGCs from the peripheral to central retina. For rats, average RGC density ranges from 600 cells/$mm^2$ to of 3,600 cells/$mm^2$ [49].

Some studies for rats have reported a pattern in RGC density with a region of high density in the dorsal retina, while other studies have reported that the density distribution varies between individual rats with no visible pattern[49,50].

The visual behaviors of both larval and adult zebrafish are easy to assay and require no training for the fish. There are a variety of existing assays that can used to study the zebrafish visual system and test for visual deficits. These include the optokinetic response (OKR), the optomotor response (OMR), the visual startle response, the escape response, and the phototactic behavioral response. The OMR is the tendency of a fish to swim towards a moving stimulus and involves head and body movements[37]. The OKR is based on reflexive eye movements in response to a moving stimulus in order to help stabilize the image on the retina[38]. The OKR relies only on tracking eye movements of the zebrafish, so both larval and adult zebrafish are immobilized during these tests. The OKR develops between 73 and 80 hours post-fertilization (hpf) and persists throughout adulthood[51], whereas the OMR is first present at 5 dpf. The OMR can be reliably evoked at 7 dpf and will persist throughout adulthood[51]. Zebrafish with visual deficits will swim in random patterns in the case of the OMR assay, or will be unable to track the moving stimulus with their eyes in the case of the OKR assay. The OMR and OKR reflexes provide evidence of a fully functioning visual system, and serve as a measure of visual acuity and contrast detection.

Zebrafish display an overall general startle response when exposed to tactile, acoustic, or visual stimuli and will avoid dark areas, moving objects, and open spaces[52]. Specifically, detecting approaching objects well before a collision and providing rich information about a potential threat's location is central to a zebrafish's evasive

strategies. For example, in zebrafish larvae the visual startle response is characterized by a brief period of elevated activity and can be induced when a light is abruptly turned on or off[53]. This continues throughout later stages of life, with changes in locomotion and activity following abrupt changes in lighting[54]. The mechanism underlying this effect in zebrafish is dependent upon the size and speed of the stimulus, with brighter stimuli eliciting more robust responses[51,52]. These responses are orchestrated through the Mauthner cell system (M-system), which likely receives this startle-related information from the tectum[51]. Specialized RGCs innervate tectal superficial inhibitory neurons[55,56] that alter the activity of tectal neurons relaying their information directly or indirectly to the M-system, which triggers the response following light onset or light offset[51,52,56]. Thus, the visual startle reflex is based on sudden exposure of light that triggers body movement through the M-system and provides a mechanism for escape[37]. This visual startle response can be used to study the development and maturation of the zebrafish visual system.

Zebrafish as described above use sensory cues to navigate towards environments where they are more likely to avoid predators, obtain food, or find mates. Similar to the OMR, efficient goal-directed locomotion requires coordination between motor action and sensory perception. In the visual system of larval and adult zebrafish, this is accomplished through phototaxis or the phototactile behavioral response. This behavior drives zebrafish towards illuminated regions and is hard-wired in the zebrafish visual system at 5 dpf. The phototactic behavioral response utilizes the tendency of the zebrafish to move towards an illuminated chamber[37]. Lastly, the escape response is the tendency of a fish to escape an approaching predator[39]. In this assay, larval or adult zebrafish are

placed in a rotating white drum with a single black strip that mimics a threatening predator. Fish with visual deficits will swim in random patterns and not display the predator-avoidance response[37].

In addition to using behavioral assays to study the zebrafish visual system, retinal function and neurotransmission can be studied using electroretinograms (ERGs). The ERG is an electrophysiological method or field recording that can be used to measure electrical activity of cells in the retina in response to a brief flash of light (10-100 msec) by placing a recording electrode on the surface of the cornea or inserting the electrode through a non-traumatic slit in the cornea[57]. Unlike the OMR, which captures visual function across the CNS as a whole, the ERG assesses neural function specifically in the retina. When visual input is detected and processed in the retina, an electrical field potential is generated and transferred to the corneal surface, where it can be detected. In zebrafish, three major components are generally used to assay for visual deficits: 1) the a-wave, which is a measure of the photoresponse or activity of photoreceptors; 2) the b-wave or ON response, which is a measure of photoreceptor to ON bipolar cell neurotransmission; 3) the d-wave or OFF response, which is measured at light offset with longer light stimuli ($\geq$ 0.5 msec)[58]. The ERG first appears as early as 4 dpf with measurements of the a-wave, b-wave, and d-wave; however, only cones contribute to early larval ERG measurements with rod function appearing at later stages of development closer to adulthood[58]. Indeed, the cone-dominated nature of the zebrafish retina and the fact that cone visual function *in vivo* is easily detected throughout the life cycle of the zebrafish makes a compelling argument for using zebrafish to study retinal

cone dystrophies (e.g., age related macular degeneration) that impact the cone-dominated parts (e.g., macula and fovea) of the human retina.

In summary, zebrafish serve as an excellent model system to study the vertebrate visual system, including circuitry, behavior, and disease. Zebrafish have diurnal circadian rhythms, are cone-dominated, have good color vision, and relatively high visual acuity. In contrast, rodents, the other predominate animal model used in vision sciences, have a nocturnal circadian rhythm, rod-dominated retinas, poor or absent color vision, and relatively poor visual acuity. In addition to using the ERG to assess retinal function, zebrafish display robust visual behaviors including, optokinetic reflexes, optomotor guided visual responses, visual-motor startle responses, phototactic or light-driven visual behaviors, and a visually guided escape response. This makes testing for changes in vision and visual behavior in zebrafish relatively easy to perform, and highly accessible with little training for either the animal or the tester. Considering the structural and functional similarities shared with the human retina, zebrafish may be a more appropriate model for studying human ocular diseases, particularly those that affect cone photoreceptors such as age-related macular degeneration.

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# Chapter 2

## Age-related macular degeneration (AMD) and the light induced retinal degeneration (LIRD) model for AMD

## 2.1 Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness among our aging population[1,2]. AMD is characterized by the loss or impairment of central vision due to macular dropout of the retinal pigment epithelium and death of photoreceptors. This disease negatively impacts daily life activities which rely on central vision such as reading, writing, driving, and watching TV. AMD is a multifactorial disease that is caused by a combination of genetic, environmental, nutrition, and lifestyle factors with the biggest risk factor being age[3,4]. Individuals 65 years or older are at a much greater risk for developing AMD[5]. The risk of developing AMD increases from 2% between the ages of 50-59 to 30% for those over 75 years of age[6,7]. Other risk factors include: smoking, ultraviolet rays, family history, poor diet, and high blood pressure[8].

### *Types of AMD*

The progression of AMD occurs gradually over three stages: early, intermediate, and late AMD. The early and intermediate stages of AMD are characterized by sub-retinal deposits of lipid and protein accumulations called drusen, changes in vision, and pigmentary changes of the RPE[9]. Patients with these changes can go on to develop one of the two types of late stage AMD: wet AMD or dry AMD. Wet AMD or neovascular AMD is characterized by the growth of abnormal choroidal blood vessels into the retina[10]. The subsequent leakage of these vessels results in RPE and photoreceptor death[11]. However, the majority of patients, roughly 85%-90%, will eventually develop the dry form of AMD[12]. Dry AMD, or geographic atrophy as it is known among ophthalmologists, is characterized by large confluent regions of atrophied RPE in the macular area leading to

photoreceptor death[10]. The exact pathogenesis of dry AMD is poorly understood and is thought to be a result of a combination of complex interacting factors. Factors that contribute to the onset and progression of the disease most often involve oxidative stress, the thickening of Bruch's membrane, lysosomal lipofuscin accumulation and drusen formation, RPE damage and dysfunction, dysregulation of the immune system, and inflammation[11].

*Clinical significance*

Age-related macular degeneration is a global healthcare problem. It is one of the top three leading causes of blindness worldwide and the most common cause of blindness in developed countries[13,14]. In the United States alone, it has been estimated that by 2050, the number of people diagnosed with AMD is expected to double from 2.07 million to 5.44 million[7]. Worldwide, the projected number of people diagnosed with AMD is expected to increase from about 196 million in 2020 to 288 million in 2040[2].

Progress has been made in understanding wet AMD and has led to several Food and Drug Administration (FDA) approved therapies that allow for recovery of vision and prevention of further vision loss. Current treatments include anti-VEGF agents, and therapies that target vessel maturation and remodeling[15]. In contrast, there is no existing FDA approved therapy available to repair damaged RPE cells or photoreceptors for the dry form of AMD. The only available treatment for dry AMD is the daily intake of the Age-Related Eye Disease Study (AREDS) formulation, which is an oral supplementation of antioxidant vitamins and minerals[16]. The AREDS formulation includes beta-carotene, vitamin C, vitamin E, zinc, lutein, zeaxanthin, and omega-3 fatty acids, and has been

shown to slow down the progression of dry AMD[15]. It is clear that additional treatment therapies are needed in order to prevent the progressive loss of vision seen in dry AMD.

## 2.2 Age-Related Macular Degeneration and Oxidative Stress

The retina is a structure that is uniquely primed for the generation of reactive oxygen species (ROS) and oxidative damage. ROS are highly reactive molecules that act to modify proteins, nucleic acids, carbohydrates, and lipids, often times resulting in dysfunction of the biomolecule. Despite the potential to induce damage, ROS have been shown to function as signaling molecules in regulatory pathways that are essential for the normal functioning of cells[17]. It is when ROS levels are above physiological levels that they can have serious pathophysiological effects. There are many characteristics of the retina and its environment that are favorable for ROS production. The retina is one of the highest oxygen consuming tissues in the body with high metabolic demands and continuous daily light exposure. In addition, the photoreceptor membrane has a high polyunsaturated fatty acid content and the outer segments are full of photosensitizing chromophores[18]. Oxidative stress has long been thought to be an important contributing factor to the onset and progression of AMD[19-21].

The main endogenous sources of ROS in the retina are generated through mitochondria, photosensitizers, lipid peroxidation, and RPE phagocytosis of photoreceptor outer segments. Due to the high metabolic activity of photoreceptors and the RPE, the mitochondria of these cells represent a major source of ROS[22]. This is primarily from electron leakage of complexes I and III in the mitochondrial respiratory chain resulting in the generation of superoxide. Photosensitizers or chromophores are

light absorbing substances that initiate a photochemical reaction. When a chromophore absorbs light, this causes a change in the distribution of electrons and generates an excited singlet state. In this state, the chromophore interacts with other intracellular molecules and generates ROS via electron transfer or singlet oxygen[20,23]. The major chromophores in the retina are 11-cis retinal and lipofuscin[23]. Lipofuscin is a lipid-protein aggregate that accumulates in the RPE with age, and is mainly derived from the chemically modified residues of incompletely digested photoreceptor outer segments[15,23]. Photoreceptors are prone to lipid peroxidation because of the high polyunsaturated fatty acid content of their outer segment membranes. Polyunsaturated fatty acids are particularly susceptible to lipid peroxidation because their conjugated double bonds are sources of hydrogen atoms, which contain one electron[20]. DHA comprises up to 50% of the polyunsaturated fatty acid content in the outer segment membrane and contains 6 double bonds[20,24]. Lastly, RPE plays an important role in phagocytosing light-damaged photoreceptor outer segments, which results in an oxidative event leading to the generation of ROS[25].

In the normal non-diseased eye, the effects of oxidative stress are minimized by a range of antioxidant and repair systems. Antioxidant defense systems include non-enzymatic and enzymatic systems. Non-enzymatic antioxidants that provide protection against ROS include, but are not limited to, carotenoids (macular pigments), vitamin C, vitamin E, flavonoids, glutathione, and melanin[20,26,27]. There are several enzymes with antioxidant activity; these include superoxide dismutase, catalase, and glutathione peroxidase[28]. All three of these enzymes are found in photoreceptors and the RPE[20]. While these effective antioxidants exist to defend against ROS, oxidative damage can

still occur. Fortunately, cells have a number of nuclear and mitochondrial DNA repair pathways, with the base excision repair pathway being one of the most important pathways for repairing oxidative base lesions in DNA[28,29].

There is an inverse relationship between the levels of antioxidants and ROS in the ageing eye. With increasing age, there is a decline in antioxidants, both enzymatic and non-enzymatic, and an increase in ROS levels with subsequent oxidative damage[30]. This effect seems to be intensified in AMD. In patients with AMD, there is a significant increase in lipid peroxidation and protein oxidation, and a decrease in the antioxidants, glutathione peroxidase and superoxide dismutase in comparison to control patients[19,31,32]. In a clinical study by Yildirim et al. (2011), the authors showed that serum levels of malondialdehyde (MDA), a lipid peroxidation byproduct, and advanced oxidation protein products were significantly higher in the AMD patient group than in the control group[19]. They also found that there was a significant decrease in superoxide dismutase activity in the serum of the AMD patients. Another study demonstrated that patients with AMD had significantly higher MDA serum levels and low superoxide dismutase and glutathione peroxidase activity in comparison to control patients[32].

Furthermore, animal models also strongly support a role for oxidative stress in AMD[18]. Mice deficient in superoxide dismutase (SOD1) have been shown to develop an AMD-like phenotype that recapitulates key elements of the human pathology[33]. Imamura et al. (2006) demonstrated that the lack of SOD1 resulted in the development of drusen, choroidal neovascularization, and RPE dysfunction[33]. Additionally, markers of oxidative damage were discovered in the Bruch's membrane and RPE of these mice, strongly suggesting that oxidative stress may be one of the initial events in the AMD model. In

another rodent model, mice deficient in erythroid 2-related factor (Nrf2), a transcription factor that plays a central role in the regulation of oxidative stress and expression of several antioxidant enzymes, developed a retinal pathology similar to AMD[18,34]. Zhao et al. (2011) demonstrated that Nrf2 deficient mice developed drusen-like deposits, lipofuscin accumulations, choroidal neovascularization, and sub-RPE inflammatory proteins, supporting a causative role for oxidative stress in the pathogenesis of AMD[34]. The evidence provided by both human and animal studies shows that oxidative stress plays a significant role in the pathogenesis of AMD.

## 2.3 Light-Induced Retinal Degeneration as a Model of Age-Related Macular Degeneration

Light-induced models of retinal degeneration are used to recapitulate the retinal pathology of degenerative photoreceptor diseases such as age-related macular degeneration[35-38]. The light-induced retinal degeneration (LIRD) model has been shown to cause extensive photoreceptor damage and death while leaving the rest of the retina relatively intact[22,39,40]. LIRD is an effective non-invasive model that can be used to study the mechanisms of photoreceptor apoptosis, which is often the final pathway in many degenerative retinal diseases. The major advantages of LIRD in comparison to genetic models of degenerative photoreceptor diseases is that photoreceptor apoptosis in this model is synchronous and occurs within a relatively short amount of time[36,39-42]. The majority of photoreceptor apoptosis in the LIRD model occurs within the first 24 hours of light exposure[36,40,43]. Use of the LIRD model has generally been successful in a number of vertebrate species including albino mice, rats, and fish, and pigmented fish, but has

been less successful in the case of pigmented rodents[40-42,44,45]. In pigmented mammals, it has been proposed that the melanin in the RPE protects the photoreceptors by absorbing light resulting in damage that is inconsistent or non-existent, meaning that albino animals are generally employed as the only experimental option in LIRD studies[42].

The LIRD model is preformed using a prolonged period of dark-adaptation (7-14 days) followed by several days of exposure to constant high-intensity light[36,38]. The high-intensity light exposure in the LIRD model causes extensive photochemical damage, particularly photooxidative damage. It is important to note that oxidative stress is strongly associated with AMD onset and progression. The primary mechanisms of damage in the model are through the generation of ROS and an associated increase in intracellular calcium leading to photoreceptor apoptosis[43,46,47]. In the model a photosensitized reaction occurs where a chromophore (11-cis retinal in photoreceptors and lipofuscin in RPE) absorbs a photon of light and generates a photosensitizer molecule in an excited singlet state, eventually leading to the generation of ROS[46]. Damage in this model is first seen in the photoreceptor outer segments, which contain high concentrations of polyunsaturated fatty acids and are where photoreceptor chromophores are located. This is most likely one of the initial areas for photochemical damage to occur. The generation of ROS by excitation of chromophores in the retina leads to lipid peroxidation resulting in damage to the photoreceptor membrane and membrane dysfunction[46]. As discussed in a previous chapter, the generation of ROS and lipid peroxidation is something that occurs normally in the eye, where parts of the outer segment are normally shed due to lipid peroxidation damage, but the effect is minimized by antioxidants and cellular repair systems. With exposure to constant high-intensity

light, this effect is multiplied until it reaches a critical threshold where light becomes damaging[47].

Concurrent with increases in ROS, there is also an increase in intracellular calcium. Donovan et al. (2001) demonstrated that there were increased levels of superoxide occurring alongside increased intracellular calcium in the LIRD model[43]. Oxidants and lipid oxidation products are known to alter calcium homeostasis and oxidative stress can lead to disturbances in calcium signaling, which can result in neurodegeneration. Oxidants have been shown to cause an increase in calcium levels in the cytoplasm of many cell types including neurons[48]. Cytoplasmic calcium increases can occur as a result of calcium release from internal stores such as the endoplasmic reticulum (ER) or from influx from extracellular sources[48]. Increases in cytoplasmic $Ca^{2+}$ can lead to damaging enzymatic cascades within cells and creates problems with both mitochondrial and ER $Ca^{2+}$ handling. The strong relationship between mitochondrial and ER $Ca^{2+}$ homeostasis is tied to the close proximity of the mitochondrial surface to the ER. Thus, when calcium from the ER is released, elevated levels of $Ca^{2+}$ will rapidly be accumulated in the mitochondria, initiating a signaling cascade leading to apoptosis.

Donovan et al. (2001) has shown that in the LIRD model, nitric oxide synthetase (NOS) activity is required for intracellular increases in calcium[43]. A downstream effector of nitric oxide is guanylate cyclase, which converts GTP to cGMP eliciting a cGMP increase. In photoreceptors, cGMP increases can lead to the opening of cGMP-gated cation channels in the outer segment membrane leading to excessive calcium influx. In addition, there is swelling of the ellipsoid portion of the inner segment, due to mitochondrial swelling in the LIRD model. The authors' study revealed that

mitochondria are the initial target site in calcium-induced photoreceptor apoptosis. Calcium entering through cGMP-gated channels in the outer segment travels into mitochondria disrupting the mitochondrial membrane potential, possibly leading to the release of apoptotic factors and photoreceptor death.

It is unclear whether photoreceptor apoptosis in the LIRD model occurs through a caspase-dependent or caspase-independent pathway, but there is stronger evidence for a caspase-independent pathway. Donovan and Cotter (2002) revealed that caspases -1, -3, -7, -8, and -9 are not activated during light-induced photoreceptor apoptosis and that cytochrome c, which initiates activation of caspase-9, is not released from mitochondria following light-exposure[49]. Their experiments revealed that calpains, calcium-activated proteases, were activated the LIRD model and that increases in intracellular calcium were necessary to induce photoreceptor death. This suggests that a caspase-independent pathway involving calcium-dependent proteases promotes photoreceptor apoptosis in the LIRD model.

### 2.4 The LIRD Model and the Zebrafish Retina

Current light-induced damage models of AMD in zebrafish are performed using a prolonged dark-adaptation period of seven to fourteen days followed by several days of exposure to constant high-intensity light (> 20,000 lux)[36,37,44]. The LIRD model is characterized by damage and truncation of the rod and cone outer segments, swelling of the ellipsoid portion of the inner segment and cell bodies, followed by photoreceptor apoptosis, and thinning and disorganization of the ONL (Fig. 2.1)[36,37]. Photoreceptor death appears to peak at 24 hours of light exposure with apoptotic rods being more

prevalent than cones[36,37]. While the majority of rods appear to undergo apoptosis within that initial 24 hours of exposure, cones appear to be more resistant to light damage and degenerate more slowly over a period of several days[36].

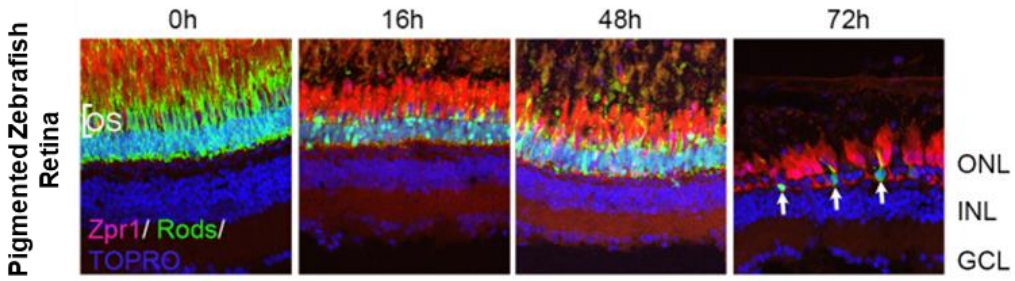


**Figure 2.1:** Vertical retinal sections of pigmented zebrafish at 0 hours prior to light exposure and over a period of 72 hours of light exposure. Rods express GFP (green), red-green double cones are immunolabeled with Zpr-1 antibody (red), and nuclei are counterstained with the nuclear stain TOPRO (blue). Truncation of the outer segments and swelling of the ellipsoids is apparent after 16 hours, as is the thinning of the ONL. OS, outer segment; ONL, outer nuclear layer; GCL, ganglion cell layer. Adapted from Rajaram et al., 2014.

The LIRD model has been well characterized in the zebrafish retina with damage occurring in the same progression and timing as described above. In zebrafish extensive photoreceptor degeneration occurs in the central and dorsal retina, while more minimal photoreceptor damage occurs in ventral retina[35]. This effect is possibly due in part to an additional reflective layer known as the tapetum lucidum in these regions of the retina. The tapetum lucidum reflects photons that were not initially absorbed back to the photoreceptors, and may produce a higher effective irradiance of the dorsal and central photoreceptors resulting in more damage to these regions[35]. Using zebrafish over rodents in this model is particularly advantageous for several reasons: 1) a relatively large number of fish can be run through this light lesioning paradigm simultaneously, 2) the

LIRD setup for zebrafish is inexpensive, 3) and tanks housing the fish do not need to be cleaned throughout the experiment. In the set up for the LIRD model using zebrafish, fish are free swimming in a tank with the high-intensity light source (halogen, florescent, LED) placed 10-50 cm away from the tank[35-37]. Two basic light lesioning paradigms have been described in the literature for free swimming zebrafish. In the first lesioning paradigm, zebrafish are exposed to high-intensity (20-21,000 lux) light over a long period of time (3-7 days), and in the second lesioning paradigm fish are exposed to an ultrahigh-intensity (>120,000 lux) light over a much shorter period of time, 30 minutes to a few hours[50]. The first lesioning paradigm results in more widespread photoreceptor degeneration, while the second paradigm causes photoreceptor degeneration in a relatively narrow naso-temporal band in the central retina[50]. The majority of zebrafish studies using LIRD, including this body of work, focus on the first lesioning paradigm. In zebrafish, the LIRD model has mainly been used to study retinal regeneration[35-37,39,44,51-54], but other studies have used the model to evaluate drugs and study photoreceptor apoptosis[55-57].

While there are many LIRD zebrafish studies that use albino fish[35,36,39,44,53,54,58], the advantage of using zebrafish for this model is that unlike rodents, the LIRD model can successfully produce retinal damage in pigmented zebrafish[37,51,52,55]. Rajaram et al. (2014) performed a thorough comparison of retinal damage and regeneration in albino vs. pigmented zebrafish using the LIRD model and found that the timing and progression of photoreceptor apoptosis and regeneration were nearly identical[37]. This is a huge advantage over using a rodent model, where LIRD can only be produced in albino animals.

There are many challenges and issues associated with using albino animals for vision studies. Albinism results in a lack of intraocular melanin, which renders normally pigmented structures in the eye [iris, ciliary body, choroid, and RPE] translucent[59,60]. In albino animals, light can also pass through the scleral wall to reach the photoreceptors, bypassing the optical image-forming apparatus of the eye. This increase in intraocular light scatter contributes to retinal image degradation and thus visual impairment in albino animals and humans[60,61]. Because these conditions prevail from early development, disruption of eye structure contributes to the refractive errors in albinism, which are consistent with poor visual acuity [61-63]. This means that albino animals including humans have reduced vision and significant refractive issues. Moreover, this leads to abnormalities in the number, development, and connections of retinal neurons in several albino animal species (cats, ferrets, wallabies, and rabbits) including humans[64-67]. For example, rod photoreceptor deficits[68,69] as well as abnormalities in the cone-dominated foveal regions of the retina have been described in some albino species, including humans[65,70]. Electroretinogram (ERG) studies also point to altered function of both the inner and outer retina as well as RPE in the mammalian albino retina[66,71,72], which is worsened by the presence of nystagmus (involuntary eye movement) in humans with albinism[73,74]. Taken together, these confounding factors argue heavily against any advantage attained by using albino animals in a LIRD model to study AMD. Also, most cases of dry AMD occur in the aging population in individuals that generally do not possess other eye diseases or confounding factors[5]. Therefore, this truly sets the LIRD model in pigmented zebrafish apart from other animal models performed in albino mammals.

*Development of a novel model of LIRD in pigmented zebrafish*

The dark-adaptation period in the LIRD model serves to increase the animal's susceptibility for retinal light damage[22]. As previously described, the current LIRD model uses a prolonged period of dark-adaptation of about 7-14 days prior to light exposure. However, utilizing such a long period of dark-adaptation is time consuming and presents issues with intermittent light exposure during feedings. As a critical change for my thesis, I proposed to modify the original LIRD model by reducing the dark-adaptation period to just 24 hours (see Fig. 2.2)[75]. The rationale for this change in the dark-adaptation period was based upon the following criteria: First, it has been shown that increases in sensitivity to light damage due to a dark-adaptation period required at least 12 days of dark-adaptation to reach a steady state[76,77], but we believe that a short dark-adaptation period would still increase retinal sensitivity enough to produce damage. Second, a short dark-adaptation period would prevent any light exposure in the dark-adaptation period due to feedings, which are necessary in a long period of dark-adaptation. Thirdly, we believe that this short dark-adaptation period would work in pigmented zebrafish, but perhaps not in pigmented mammals, due to the tapetum lucidum in the zebrafish retina increasing the vulnerability of specific retinal regions to damage[35]. Our study using pigmented zebrafish, details the progression, timing, and magnitude of photoreceptor damage in our modified model in comparison to the original LIRD model. Results of this study will be discussed in detail in chapter 4.

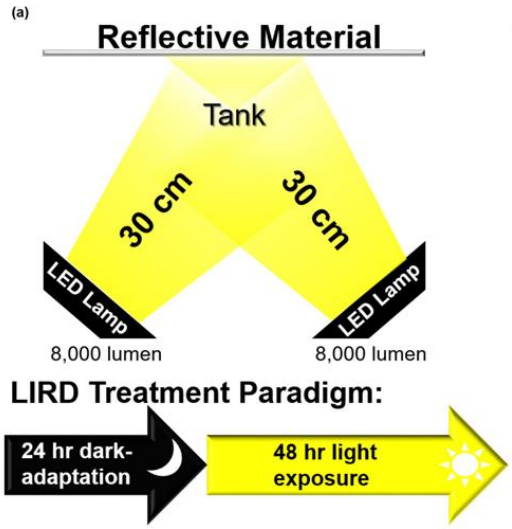


**Figure 2.2:** Illustration of modified LIRD model. Top shows illustration of experiment setup with tank used to house fish, reflective backing, and the high-intensity light sources. The bottom shows the light treatment paradigm progression.

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# Chapter 3

# Adenosine and its role as a neuroprotective molecule in the CNS and retina

### 3.1 Introduction to Adenosine and Adenosine Receptors

Adenosine is an endogenous purine nucleoside composed of an adenine attached to a ribose. It plays an essential role in physiological functions by being involved in the synthesis of nucleic acids and forming the backbone of ATP. In the central nervous system (CNS) adenosine acts as a neuromodulator and affects neural activity through multiple mechanisms, which include controlling neurotransmitter release presynaptically, hyperpolarizing or depolarizing neurons postsynaptically, and non-synaptically through regulatory effects on glial cells[1]. Adenosine is present in low concentrations in the extracellular space and is known to increase during cellular stress[2]. Adenosine can be formed intracellularly either through the dephosphorylation of AMP by 5′-nucleotidase or by hydrolysis of S-adenosylhomocysteine[2]. Extracellularly, adenosine can be formed from the release and degradation of precursor adenine nucleotides (ATP, ADP, AMP) by ectonucleotidases, but its accumulation in the extracellular space is limited by catabolism via adenosine deaminase (Fig. 3.1)[3]. The release and uptake of adenosine is mediated by bidirectional nucleoside transporters where the direction of transport depends on the concentration of adenosine between the cytoplasm and extracellular space[4]. The actions of adenosine can be terminated by an efficient reuptake system and a metabolizing system. After intracellular uptake adenosine can be metabolized by adenosine kinase, adenosine deaminase, and inosine (Fig. 3.1)[5].

The actions of adenosine are mediated through $P_1$ purinergic receptors, also called adenosine receptors. Adenosine receptors are seven transmembrane domain, G protein-coupled receptors and to date four adenosine receptor subtypes have been identified ($A_1$, $A_{2A}$, $A_{2B}$, $A_3$)[6]. $A_1R$ and $A_3R$ act through $G_{i/o}$ proteins to inhibit adenylyl cyclase and

decrease intracellular cyclic AMP (cAMP) concentrations, while $A_{2A}R$ and $A_{2B}R$ act through $G_s$ and $G_{s/q}$ proteins respectively to stimulate adenylyl cyclase and increase cAMP concentrations[7]. $A_1R$ (~70 nM) and $A_{2A}R$ (~150 nM) have a stronger affinity for adenosine, while $A_{2B}R$ (~5100 nM) and $A_3R$ (~5100 nM) have a lower affinity for adenosine[6]. All four adenosine receptor subtypes are present in the CNS with $A_1R$ being the most abundant and densely expressed throughout the CNS[8].

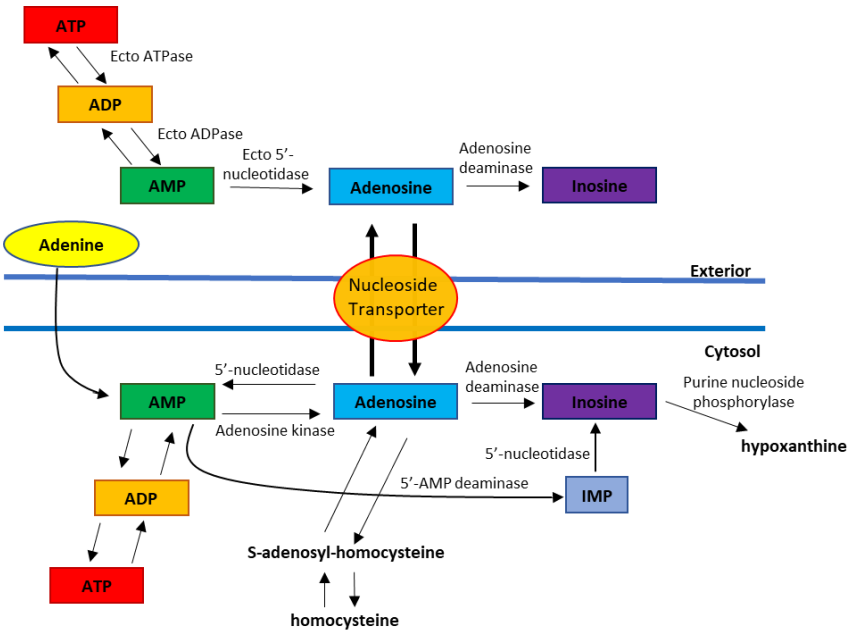


**Figure 3.1:** Adenosine formation and catabolism. See text for description. Adapted from Sachdeva and Gupta, 2012.

### 3.2 The Neuroprotective Effects of Adenosine in the Central Nervous System

The effect of adenosine receptor activation in nearly all regions of the brain is reduced excitability[6]. In addition to acting as an inhibitory neuromodulator, adenosine also plays a neuroprotective role in the CNS including the retina. Intracellular and

extracellular adenosine have been shown to increase in response to a number of pathological stimuli[4,9-12]. Acute protective effects of adenosine in the brain have been noted in instances of ischemia, hypoxia, hypoglycemia, and mechanical cell injury[13-16]. Stimulation of $A_1R$ and $A_3R$, in particular, have been shown to mediate some neuroprotective effects in the brain. Activation of $A_1R$ reduces neuronal activity by decreasing excitatory synaptic transmission[17-19] and/or through hyperpolarization via the activation of $K^+$ channels, leading to the inhibition of $Ca^{2+}$ channels[6,19,20]. $A_1R$ activation has been shown to reduce excitotoxicity by inhibiting presynaptic voltage-sensitive $Ca^{2+}$ channels and limiting $Ca^{2+}$ entry into cortical and hippocampal neurons[21]. Under stressful conditions, adenosine seems to provide negative feedback to maintain cellular preservation with the major proposed mechanism aimed at reducing neuronal excitability and firing rate[21].

### 3.3 The Neuroprotective Effects of Adenosine in the Retina

*The inner retina*

In the retina, both inner and outer retinal neurons are affected by adenosine. Much of the research concerning adenosine and the retina is focused on the inner retina with little attention given to the outer retina. The inner retina includes the INL, IPL, and the GCL, while the outer retina includes the OPL and the ONL to the RPE. Many of the studies looking at the neuroprotective effects of adenosine on the inner retina focus on glaucoma, retinal ganglion cells (RGCs), and optic nerve damage[22-26]. Glaucoma is a neurodegenerative disease that is associated with increased intraocular pressure and is characterized by the progressive degeneration of RGCs leading to optic nerve damage

and vision loss. In the inner retina, stimulation of $P2X_7$ receptors resulted in the elevation of intracellular $Ca^{2+}$ levels and RGC death[22]. This damage was reduced by $A_3R$ and $A_1R$ activation, which led to decreased levels of intracellular $Ca^{2+}$ and resulted in RGCs being protected from the negative consequences of elevated ATP[22]. In another study, $A_3R$ activation was found to have neuroprotective effects in a partial optic nerve transection injury model using rats[23]. The authors found that activation of $A_3R$ led to increased survival of RGCs and decreased numbers of apoptotic cells. In addition to the effects of $A_3R$ and $A_1R$, stimulation of $A_{2A}R$ played a protective role in traumatic optic nerve neuropathy (TON) by reducing TON-associated ROS release and MAPK-mediated inflammatory cytokine release in activated microglia[25]. In contrast with another study using a glaucoma model in which neuroinflammation-induced RGC death was caused by elevated pressure, the authors demonstrated that the blockade of $A_{2A}R$ significantly decreased RGC death by preventing microglia activation and the subsequent production of pro-inflammatory cytokines[26].

Other studies have shown that adenosine can protect against glutamate toxicity[23,27-30], which is a phenomenon characterized by overactivation of NMDA glutamate receptors leading to a massive increase in intracellular $Ca^{2+}$ resulting in calcium-dependent cell death. In a study using isolated rat RGCs, the authors found that stimulation of $A_3R$ significantly decreased the rise in intracellular $Ca^{2+}$ triggered by glutamate and that $A_1R$ contributed to this block[30]. A different excitotoxicity study looked at the effects of $A_1R$ stimulation alone using rat RGC cultures and retinal wholemounts and found that stimulation of $A_1R$ led to a significant decrease in glutamate-evoked calcium influx and voltage-gated calcium currents in RGCs[27]. Lastly,

an excitotoxicity study using rat RGC cultures and organotypic cultures demonstrated that activation of $A_3R$ increased RGC survival and decreased the number of apoptotic cells[23].

Adenosine has also been shown to be protective in ocular ischemia-reperfusion injury models[23,31,32]. In one study, it was reported that stimulation of $A_3R$ in a retinal ischemia-reperfusion injury model decreased RGC loss and decreased the number of apoptotic cells[23]. Other studies have shown that stimulation of $A_1R$ in an ischemia-reperfusion injury model can result in recovery of the b-wave, which reflects the health of the inner retina, including the ON bipolar cells and the Müller cells, after a short recovery period[31,32]. Interestingly, these same studies showed that stimulation of $A_{2A}R$ actually had a damaging effect on the retina during an ischemic event. Recently, it has been revealed that the blockade of $A_{2A}R$ in retinal ischemic events decreases activation of microglia, down-regulates the levels of pro-inflammatory cytokines, and increases retinal cell survival[33]. Yet in diabetic retinopathy models, activation of $A_{2A}R$ is protective against the death of retinal neurons[34-36]. In one diabetic retinopathy study, signaling triggered by $A_{2A}R$ on microglia prevented microglial activation and inhibited TNFα release[35]. The $A_{2A}R$ appears to have a dual role in retinopathies.

*The outer retina*

In the outer retina, the neuroprotective effects of adenosine have not been as thoroughly explored, especially in photoreceptors. The few studies concerning the outer retina focus on the neuroprotective effects of adenosine through the regulation of microglial activity and neuroinflammation[37,38]. The majority of studies looking at

adenosine and the outer retina are concerned with the effects of adenosine on photoreceptor neurotransmission[39-42].

A recent study by Solino et al. (2018) showed that in a LIRD model of AMD using albino rats, $A_1R$ stimulation prevented photoreceptor death, reduced microglial activation, decreased Müller glial reactivity, and prevented significant alterations in photoreceptor function and neurotransmission[37]. This lends support to the idea that adenosine acting at $A_1R$ is neuroprotective for photoreceptors. However, the authors did not attempt to test other adenosine subtypes, focusing solely on $A_1R$. Also, the authors used very high concentrations of an $A_1R$ selective agonist, N6-cyclopentyladenosine (CPA), in intravitreal injections negating the selectivity of the drug for $A_1R$ at the concentration tested (0.775 mM, $EC_{50}$ = 2.3 nM for $A_1R$, 790 nM for $A_{2A}R$, 43 nM for $A_3R$)[43,44]. Moreover, the authors blocked the effects of CPA with an antagonist, DPCPX, that is non-selective at the concentration tested (0.01 mM used, $IC_{50}$ = 1-1000 nM for all adenosine receptor subtypes and species)[44], which would likely block all adenosine receptor subtypes to some degree. Based on these findings the authors concluded that the protective effects of $A_1R$ stimulation were mediated through a reduction in microglial activation and a decrease in the release of pro-inflammatory cytokines. Additionally, the authors showed that $A_1R$ immunoreactivity was absent for photoreceptors but co-localized with microglial cells, which contradicts previous reports of functional responses, and retinal mRNA and immunoreactivity in the retina[45-50]. Furthermore, another study has shown that photoreceptor death was prevented due to the blockade of $A_{2A}R$ in microglia[38], and suggested that $A_{2A}R$ antagonism prevented microglial activity and the associated neuroinflammatory responses by protecting the photoreceptors from

neurotoxic microglial insults. Notwithstanding these discrepancies, there appears to be an important neuroprotective role for adenosine in the outer retina, and further studies are needed to clarify adenosine's actions at photoreceptors, especially in a LIRD model that can recapitulate the geographic atrophy observed in a disease like AMD.

Since it is not known which adenosine receptors are involved in neuroprotection, or whether the observed neuroprotective effects on photoreceptors are due to direct action on rod and cone photoreceptors or indirect signaling through microglial cells or macrophages, a clearer picture of adenosine's actions in the outer retina needs to be explored. A previous series of studies carried out in our lab on larval tiger salamander photoreceptors suggests that activation of adenosine receptors could potentially contribute to the observed neuroprotective effects of adenosine[39-42]. Adenosine receptor activation on rods and cones resulted in inhibition of voltage-gated $Ca^{2+}$ channels, which suppressed neurotransmission from cone and rod terminals. In tiger salamander rods, stimulation by selective $A_{2A}R$ agonists, but not $A_1R$, $A_{2B}R$ or $A_3R$ agonists, resulted in inhibition of L-type $Ca^{2+}$ channels and suppression of transmitter release[41,42]. For the cones, since there are multiple cone subtypes, it is possible $Ca^{2+}$ channel inhibition may occur through activation of more than one adenosine receptor subtype; however, the pharmacological actions of adenosine have not yet been determined for L-type $Ca^{2+}$ channels on cones[39]. Based on these observations it is possible that adenosine receptor-mediated inhibition of L-type $Ca^{2+}$ channels on photoreceptors could potentially contribute to photoreceptor-specific neuroprotection. Further studies are needed to clarify the neuroprotective mechanisms behind adenosine receptor activation and its actions directly on photoreceptors, especially in diseases like AMD.

In a recent study from our lab, we characterized the expression of adenosine receptors in the adult zebrafish retina using *in situ* hybridization and immunohistochemistry[45]. All four adenosine receptor subtypes were present and differentially expressed in the zebrafish retina. In particular, and as expected, the $A_{2A}R$ was present in the outer retina as puncta located within the ellipsoid region of the inner segment and somata of cones with lower expression around the rod somata (Fig. 3.2). Unexpectedly, strong $A_3R$ immunoreactivity was present within the ellipsoids of the cone photoreceptors (Fig. 3.2). It's possible that simulation of $A_{2A}R$ and $A_3R$ on photoreceptors could have neuroprotective effects in neurodegenerative diseases that affect photoreceptors like AMD.

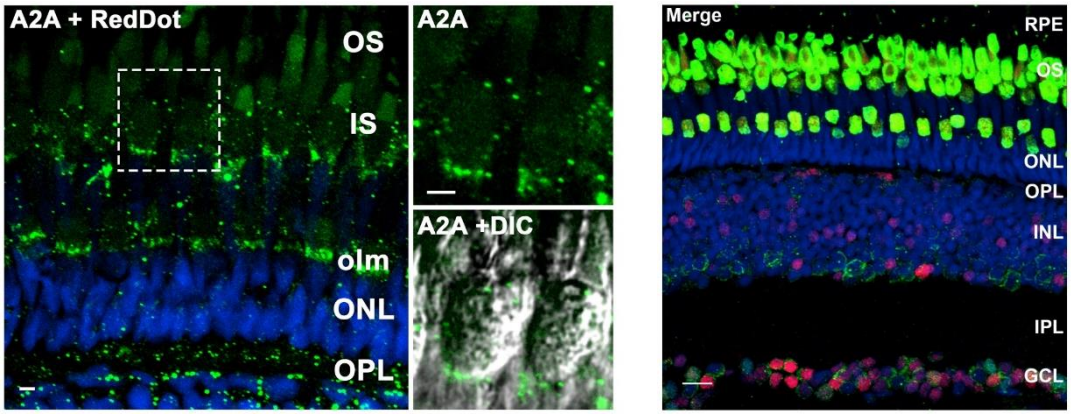


**Figure 3.2:** Vertical retinal sections of adult zebrafish. Nuclei are counterstained with DAPI (blue) **a)** $A_{2A}R$ present as green puncta localized to cone ellipsoids and somata and rod somata. **b)** $A_3R$ (green) present on ellipsoids of photoreceptors. RPE, retinal pigment epithelium; OS, outer segments; IS, inner segments; olm, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 20 µm. Image from Grillo et al., 2019.

### 3.4 Summary and Hypothesis

Age-related macular degeneration is a global healthcare problem that causes central vision loss due to macular dropout of the RPE and photoreceptors apoptosis. While there are treatments that can prevent vision loss in the wet form of AMD, there are currently no viable treatments for the more common dry form of AMD. Adenosine has already been shown to be neuroprotective in the inner retina in models of glaucoma, optic nerve injury, excitotoxicity, ischemia-reperfusion injuries, and diabetic retinopathy. In the outer retina, adenosine receptor activation has been shown to inhibit voltage-dependent $Ca^{2+}$ influx in photoreceptors, which could potentially protect these cells from neurodegeneration. This suggests that adenosine could provide a protective role in a disease like AMD, however, the effects of adenosine have not been explored in detail in AMD.

Since $A_{2A}R$ and $A_3R$ are present on zebrafish photoreceptors, perhaps activation of these receptors could be protective in a LIRD model of AMD, where the mechanism of damage is likely oxidative stress and is concurrent with alterations in calcium homeostasis. Oxidative stress is said to be a major contributing factor to the onset and progression of AMD, and oxidation products and reduced antioxidant activity have been implicated in both early and late forms of AMD[51,52].

The central hypothesis of this proposal is that adenosine is neuroprotective in a LIRD model of AMD in pigmented zebrafish. We will test this hypothesis by addressing two major questions as they relate to the role of adenosine in the zebrafish retina. 1) Does activation of adenosine receptors present on photoreceptors reduce the amount of damage observed in a LIRD model of AMD in pigmented zebrafish? 2) Will adenosine prevent

impairment of photoreceptor function in the outer retina and reduce alterations in visual behaviors in a LIRD model of AMD in pigmented zebrafish?

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# Chapter 4

# A short period of dark-adaptation is sufficient to generate light-induced photoreceptor degeneration in pigmented zebrafish

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## 4.1 Abstract

Light-induced retinal degeneration (LIRD) models are used to recapitulate the pathologies of retinal diseases that affect photoreceptors. Current LIRD models use a dark-adaptation period of 7–14 days followed by high-intensity light exposure. The purpose of this study was to determine whether photoreceptor damage and death would occur in pigmented zebrafish using a short period of dark-adaptation. Zebrafish were dark-adapted for 24h and then exposed to constant high-intensity light for 48h. Immunohistochemical analysis was performed on vertical retinal sections to assess damage and apoptosis. Photoreceptors exhibited structural damage, apoptosis, and cell loss after 24 and 48h of light exposure as previously reported in studies using 7–14 day dark-adaption. Also, photoreceptors lost following light damage were regenerated after 28 days. These results suggest that a short period of dark-adaptation is sufficient for a LIRD model in pigmented zebrafish.

## 4.2 Introduction

Light-induced retinal degeneration (LIRD) is a well characterized and efficient model for studying the mechanisms of photoreceptor apoptosis and for developing neuroprotective therapies[1-4]. In many retinal dystrophies and diseases, photoreceptor apoptosis is the final stage of cell death. The LIRD model has a number of advantages: first, this model can be utilized in a variety of vertebrate species including rats, mice, and zebrafish[1,2]. Second, this model selectively targets photoreceptors, while sparring other retinal neurons[3]. Finally, it has been shown to initiate synchronous photoreceptor death, followed by photoreceptor degeneration and loss in a relatively short period of time (e.g. 1–4 days)[1,4].

The primary insult in the LIRD model is achieved through constant exposure of high-intensity light (≥20, 000 lux). Damage to photoreceptors results in the generation of reactive oxygen species (ROS) through the excitation of chromophores (e.g. 11-cis retinal and lipofuscin) in the retina[5]. This LIRD model has been successfully used in albino animals[6,7]; however, in mammals, such as rodents, the pigmented retinal epithelium has been proposed to convey protection to the photoreceptors from high-intensity light exposure[8]. Unlike mammals, high-intensity light exposure generates retinal damage in pigmented teleosts, especially zebrafish[9-11], making this model better suited to recapitulate damage from ROS. Zebrafish are widely used for the study of vision and retinal diseases. Like humans, zebrafish have a cone-dominated retina with good visual acuity and color vision[12]. This makes zebrafish a more suitable model over rodents and other rod-dominated species for studying human retinal diseases that primarily affect cones [e.g. age-related macular degeneration (AMD)].

In the LIRD model, animals are dark-adapted before continuous high-intensity light exposure to produce retinal damage[1,4,7,13,14]. This model uses a prolonged period of dark-adaptation (7–14 days) before constant high-intensity light exposure over several days[13,14]. It is unclear why such a long period of dark-adaptation is necessary in the LIRD model. It has been proposed that the retinal sensitivity to high-intensity light damage is greatly influenced by the prior illumination level experienced by the animal[3]. This is supported by a previous report demonstrating that rats raised under normal light-dark cycles were less susceptible to light damage when compared to rats that were dark-adapted before light exposure, and the observed increase in retinal sensitivity to light damage required at least 12 days of dark-adaptation to reach a steady state[15]. The aim of this study was to test whether a short period of dark-adaptation (24h) before constant high-intensity light exposure would be sufficient to generate photoreceptor damage and apoptosis using pigmented zebrafish. Using our modified LIRD model, we demonstrated that photoreceptor damage and death occurred after 48h of light exposure. Based on our results, we conclude that the damage seen in our modified LIRD model using a short period of dark-adaptation was sufficient to achieve the level of photoreceptor damage reported in previous zebrafish LIRD studies[1,4,7,13,14].

## 4.3 Methods

*Animals*

Adult wild type (AB, Tu, or EKKwill) male and female zebrafish (*Danio rerio*) were used for this study. All zebrafish were maintained in 14-h light:10-h dark cycles at

28ºC. All experiments were performed according to the institutional guidelines approved by the Penn State College of Medicine Institutional Animal Care and Use Committee.

*Light-induced retinal degeneration model*

Zebrafish were placed in a triangular shaped aquarium (~4 l) with an air bubbler and heater. Two 8,000 lumen (100 W) LED lights (LEPOWER New Craft LED Flood Light) were placed 30 cm away from the tank. These are full spectrum lights (color temp: 5,500ºK with a peak near 450 nm) with a light intensity inside the tank of approximately 28, 000 lux. The tank was surrounded by reflective material (foil) and a small fan was used for cooling any heat generated by the LED lights (Fig. 4.1). Fish were dark-adapted for 24h in a light-tight box covering the tank, and then exposed to constant high-intensity light for 48h. Fish were collected at 0, 24, and 48h post dark-adaptation. A subset of fish were allowed to recuperate for 28 days after light treatment.

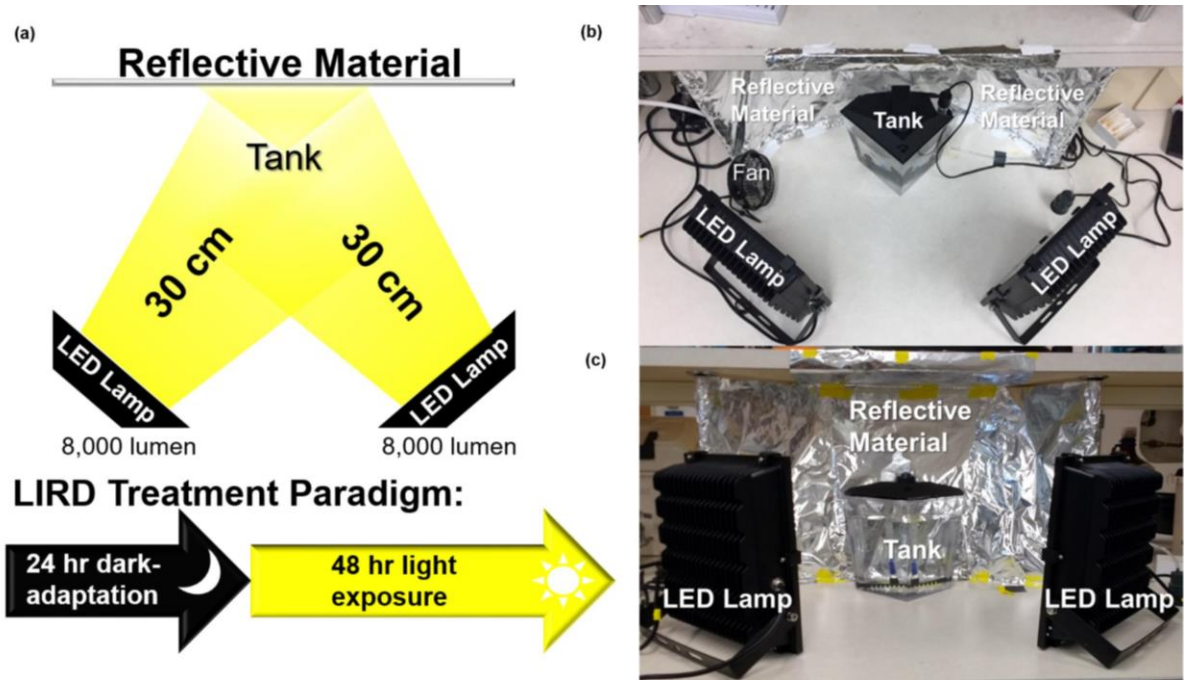


**Figure 4.1:** Light-induced retinal degeneration (LIRD) treatment paradigm. **a)** Illustration of the LIRD schematic setup from above, showing the distance of the LED lights from the tank and the sequence of dark-adaptation and light exposure. **b)** Image of the LIRD setup from an overhead view. **c)** Image of the LIRD setup from a side view.

*Tissue preparation and cryosectioning*

Zebrafish were euthanized with an overdose of Tricaine (0.32 mg/ml), and enucleated eyes (n = 24 retinas from 12 zebrafish, three zebrafish were used per group, with four groups: 0h, 24h, 48h, 28 days) were fixed in 4% paraformaldehyde for 15min and stored in 30% sucrose in PBS (pH 7.4) with 0.05% sodium azide at 4ºC. Eyes were embedded in optimal cutting temperature compound, frozen in isopentane, and cooled to −80ºC using liquid nitrogen. Frozen eyes were sectioned vertically on a cryostat at 12 µm on gelatin-coated slides and stored at −20ºC.

*Antibodies*

Primary antibodies used in this study were mouse antizpr-1 [1:200, Zebrafish International Resource Center (ZIRC), Eugene, Oregon], mouse anti-zpr-3 (1:1000, ZIRC, Eugene, Oregon), and mouse anti-proliferating cell nuclear antigen (PCNA) (1:200, MilliporeSigma, Cat P8825, Burlington, Massachusetts). Secondary antibodies used were goat anti-mouse conjugated to either CF 488A or CF 586 (1:1000, Biotium, Fremont, California). A nuclear fluorescent stain, RedDot1 (1:1000, Biotium, Fremont, California) was used for labeling cell nuclei.

*Immunohistochemistry and transferase-mediated dUTP nick-end labeling assay*

Vertical retinal sections were labeled using the indirect immunofluorescence technique[16]. Briefly, slides were washed three times for 5min with PBS and followed with a blocking step for 1h at room temperature in a solution containing 10% normal goat serum, 1% BSA, and 0.5% Triton-X-100 in PBS (pH 7.4). Sections were then incubated

in primary antibody solution overnight at 4ºC, followed by three washes in 0.1 M of PBS. Secondary antibodies were incubated in the dark at room temperature for 1h, followed by three washes in 0.1 M of PBS, air dried, and then coverslipped using Aqua Poly/ Mount (Polysciences Inc., Warrington, Pennsylvania). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays were performed on vertical retinal sections according to the manufacturer's instructions [In Situ Cell Death Detection Kit (Roche), MilliporeSIGMA, Burlington, Massachusetts]. Antigen retrieval was used for the PCNA antibody before immunolabeling as described previously in zebrafish[17].

*Confocal imaging and analysis*

Images were acquired using a Nikon C2+ confocal mounted to an Eclipse FN1 microscope with Nikon Elements Software (ver. 4.2) (Nikon Instruments, Melville, New York). All cell counts were performed from six regions per vertical retinal section (~50, 000 $\mu m^2$ area/ each) using a 40× objective, three peripheral and three central areas were averaged together. The differences between groups were analyzed using a one-way ANOVA with a Dunnett's post hoc test. Variance was reported as mean ± SEM and significance was chosen at $P < 0.05$ (Prism 5.0, GraphPad, San Diego, California).

## 4.4 Results

*Results short dark-adaptation is sufficient to generate light-induced photoreceptor damage and death*

The timeline of LIRD in zebrafish using prolonged periods of dark-adaptation has previously been described[1,4,7,18]. Our LIRD treatment paradigm (Fig. 4.1) uses a 24 h

period of dark-adaptation, followed by 48h of constant high-intensity light exposure to produce significant retinal damage. Retinas were collected at 0h (control retinas) before light exposure and after 24 and 48h of light exposure. Some fish were allowed to recuperate for 28 days post light treatment to assess their regeneration capacity. To assess structural damage of photoreceptors, red/green double cones were immunolabeled with zpr-1 (green, Fig. 4.2a) and rod outer segments were immunolabeled with zpr-3 (red, Fig. 4.2a). In comparison to the control retinas, we found that cone outer segments appeared truncated and exhibited swelling, with a complete loss of outer segments occurring after 48h of light exposure (Fig. 4.2a). After 48h of light, rod outer segments exhibited severe truncation similar to cones (Fig. 4.2a). Additionally, retinas displayed disorganization of nuclei in the outer nuclear layer (ONL). Fish allowed to recuperate for 28 days had cone and rod outer segment structure similar to controls with little damage (Fig. 4.2a).

A significant loss of photoreceptors occurred after 48h of light exposure (Fig. 4.2b), with both the number of red/green double cones and cells in the ONL decreasing significantly (0h cones: 53.54 ± 1.13 vs. 48h cones: 43.52 ± 0.99, n = 12, P = 0.0001; 0h ONL: 178.7 ± 3.45 vs. 48h ONL: 159.6 ± 3.71, n = 12, P = 0.0002; Fig. 4.2b). In contrast, photoreceptor numbers in retinas collected at 28 days were not significantly different from control retinas (0h cones: 53.54 ± 3.93 vs. 28 days cones: 55.72 ± 2.32, n = 12, P = 0.0001; 0h ONL: 178.9 ± 3.45 vs. 28 days ONL: 186.2 ± 3.12, n = 12, P = 0.0002; Fig. 4.2b). Also, the only significant differences in LIRD between central and peripheral retina were observed at 48h when counting all nuclei in the ONL. There were no statistically significant regional differences in the number of cones at either 24 or 48h

(cones: 24 h: central 56.6 ± 2.4 cells vs. peripheral: 55.5 ± 2.9 cells, n = 9, P = 0.994; 48 h: central 45.6 ± 1.4 cells vs. peripheral 41.4 ± 1.0 cells, n = 9, P = 0.5586; ONL: 24h central 171.5 ± 9.2 cells vs. peripheral 164.6 ± 3.3 cells; 48h central 169.9 ± 4.2 cells vs. peripheral 164.6 ± 3.4 cells, n = 9, P = 0.048), suggesting that slightly more damage occurs in the central retina vs. the peripheral retina as previously reported [19].

Constant high-intensity light exposure has been shown to induce photoreceptor apoptosis[2,19]. To measure apoptosis, we performed TUNEL assays on vertical retinal sections from retinas collected at 0, 24, and 48h after dark-adaptation, and after 28 days of recuperation (Fig. 4.3a). In control retinas, there were no TUNEL positive cells in the ONL. Retinas exposed to 24h of light had a significant increase in the number of TUNEL positive cells in the ONL in comparison to the control retinas (0 h: 0.0 ± 0.0 vs. 24 h: 50.57 ± 7.51, n = 12, P = 0.0002; Fig. 4.3b.) Interestingly, TUNEL positive cells were also present in the inner nuclear layer (INL) and ganglion cell layer (GCL) after 24h (Fig. 4.3a). After 48h of light, there was a decrease in the number of TUNEL positive cells, which were found almost exclusively in the ONL (Fig. 4.3). In the retinas of fish allowed to recuperate for 28 days, TUNEL labeling was similar to control retinas (Fig. 4.3).

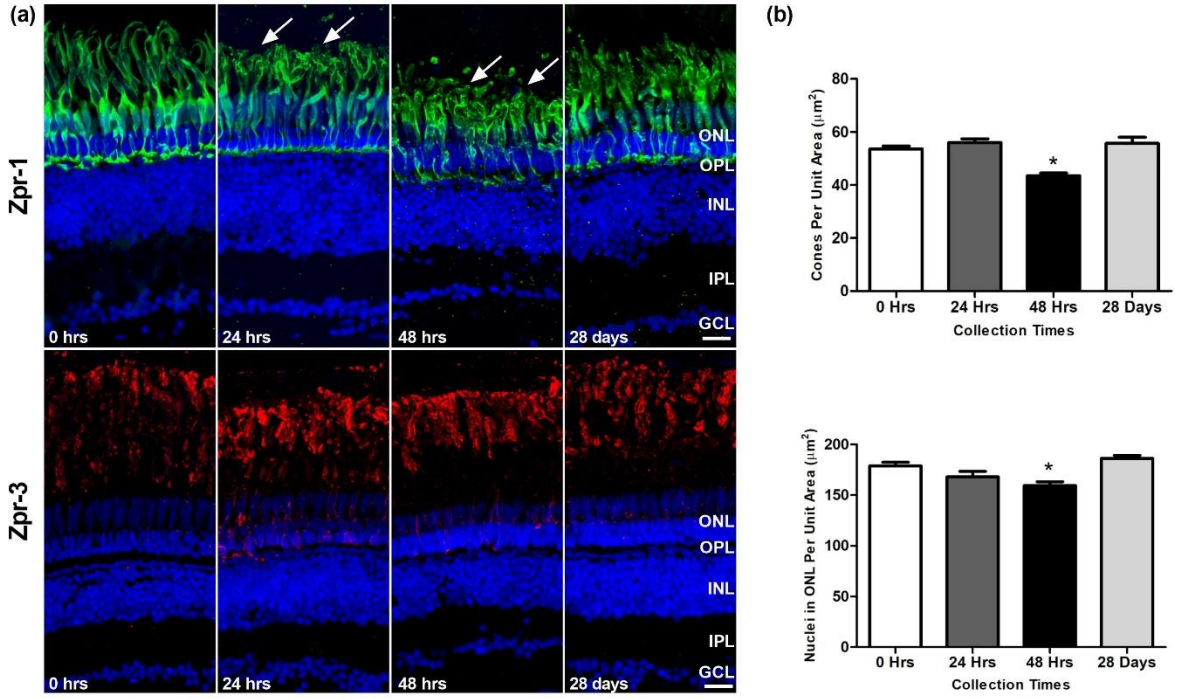


**Figure 4.2:** Confocal images and cell counts of vertical retinal sections of adult zebrafish at 0h, 24h, 48h, and 28 days after light treatment. **a)** Red/green double cones were labeled with zpr-1 (green), rod outer segments were labeled with zpr-3 (red), and nuclei were labeled with RedDot1 (blue). Animals treated with 24 and 48h of light exposure show truncation of outer segments (see arrows), and regenerate after 28 days. **b)** Quantification of red/green double cone photoreceptors (top) and all cells in the ONL (bottom) in a 50, 000 $\mu m^2$ area at 0 h, 24 h, 48 h, and 28 days after light treatment. Data represented as mean ± SEM (n = 24, nine retinal sections per collection time), and compared with a one-way ANOVA with a Dunnett's post hoc test ($P < 0.05$). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar is 15 μm.

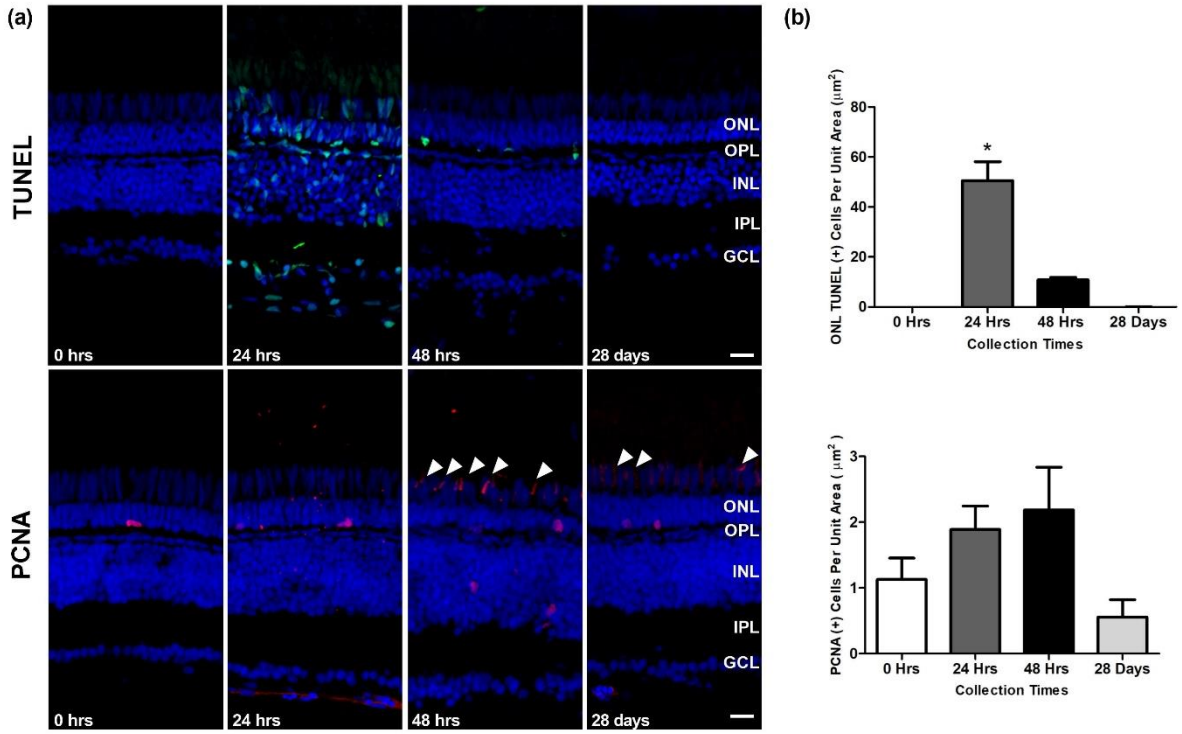


**Figure 4.3:** Transferase-mediated dUTP nick-end labeling (TUNEL) positive and proliferating cells in vertical retinal sections of adult zebrafish at 0h, 24h, 48h, and 28 days after light treatment. **a)** Top panel shows TUNEL positive cells (green) over 48h of light exposure and at 28 days after light treatment. Bottom panel shows PCNA positive cells (red) over 48h of light exposure and at 28 days after light treatment. Nuclei were counterstained with RedDot1 (blue). **b)** Quantification of TUNEL positive cells in the ONL (top) and PCNA positive cells in all retinal layers (bottom) in a 50 000 $\mu m^2$ area at 0h, 24h, 48h, and 28 days after light treatment. Data represented as mean ± SEM (n = 24, nine retinal sections per collection time), using a one-way ANOVA with a Dunnett's post hoc test ($P < 0.05$). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer (arrowheads = autofluorescence). Scale bar is 15 μm.

*Light-induced retinal degeneration following a short period of dark-adaptation stimulates regeneration of photoreceptors*

Injury to photoreceptors in the teleost retina can stimulate regeneration of retinal neuronal cells[20]. Müller glia in the INL are the source of photoreceptor progenitor cells following injury[21]. Light exposure lasting 48h induced photoreceptor damage and death in the zebrafish retina (Figs. 4.2 and 4.3). However, there was no significant difference in the number of cells present in the ONL between control fish and fish allowed to recuperate for 28 days (Figs. 4.2 and 4.3), suggesting that regeneration and proliferation of progenitor cells replaced lost photoreceptors. To visualize proliferating cells in the retina, zebrafish retinas were immunolabeled with a PCNA antibody (Fig. 4.3a)[1]. In control retinas and retinas collected 28 days post treatment, there were little to no PCNA positive cells in the ONL. Although not significantly different, we observed a slight increase in PCNA positive cells in the ONL after 24h of light exposure. The greatest numbers of PCNA positive cells were present in the INL at 48h (Fig. 4.3), suggesting the possibility that these could be Müller cells that re-entered the cell cycle and started to proliferate.

## 4.5 Discussion

This study shows that a shorter period of dark-adaptation before high-intensity light exposure is sufficient to recapitulate LIRD in a pigmented zebrafish retina. Moreover, this damage is comparable to photoreceptor damage observed in zebrafish that were dark-adapted for periods of 7–14 days before light exposure. We have shown that 48h of light exposure using our modified LIRD model induced structural damage in both

cone and rod photoreceptors, as well as, significant photoreceptor death. Additionally, the damage produced by our modified model was severe enough to stimulate a regenerative response.

The timeline of events for the original LIRD model, which uses a prolonged period of dark-adaptation, has been well established in zebrafish[1,11]. How does our modified LIRD model compare with the original model in terms of severity and timing of photoreceptor damage and death? In studies using a longer period of dark-adaptation in zebrafish, the greatest amount of structural damage occurred during the first 2–3 days of light exposure. Similar to other studies[1,11], rod and cone photoreceptors showed signs of structural damage after just 24h of light exposure (Fig. 4.2a). In addition, double cones displayed swelling of the cell bodies and ellipsoid region (Fig. 4.2a), as previously reported[1]. In our modified LIRD model, disorganization of the ONL occurred after 48h of light exposure, which was observed at 24–31h in other studies[7,11]. Structural damage observed in our modified model is congruent with the timing and severity of zebrafish treated using longer periods of dark-adaptation.

Vihtelic and Hyde (2000) described the timeline of events in the original LIRD model in three distinct phases in zebrafish[1]. In the initial phase, cell death occurs rapidly within the first 24h of light exposure, followed by photoreceptor loss. Following this stage is the proliferation and migration of progenitor cells from the INL to the ONL, and the final phase is differentiation of new progenitor cells and replacement of photoreceptors lost. In our model, TUNEL positive cells peaked at 24h of light exposure, which were then significantly reduced at 48h (Fig. 4.3), similar to what was previously reported using 7–14 days of dark-adaptation[11,14]. Interestingly, these TUNEL positive

cells were observed in all layers at 24h and were predominately restricted to the ONL after that time point (Fig. 4.3a). In contrast, Vihtelic and Hyde (2000) reported that the majority of the TUNEL positive cells were in the ONL and those labeled cells were rods. In addition, they found that apoptotic rods were more prevalent than cones, suggesting that cones are more resistant to LIRD or had a slower time course for apoptosis[1]. It was surprising to see TUNEL positive cells in both the INL and GCL. Other studies have shown similar diffuse TUNEL labeling in the inner retina of zebrafish and larval tench fish (*Tinca tinca*) after ~24h of light exposure[22,23]. Retinal ganglion cell (RGC) death in LIRD has been linked to pyruvate kinase isozyme type M2 (PKM2) in rodents, a glycolytic enzyme up regulated in AMD[24]. In addition, Müller cells have been shown to phagocytize debris from dying rod photoreceptors, which most likely contained fragmented DNA, explaining the appearance of TUNEL labeling in the Müller cell cytoplasm in the INL[23]. More importantly, Müller cells have been shown to phagocytize dying cells during retinal development or following injury[23,25]. Therefore, the TUNEL labeling observed in the inner retina at 24h (Fig. 4.3a) in the GCL and INL might be due to RGC apoptosis from upregulation of metabolic components like PKM2 or the phagocytosis of the apoptotic retinal neurons by Müller cells.

The teleost retina has a robust capacity for regeneration following injury[20] with Müller cells providing a major source of neuronal progenitors in response to damage[9,21]. A significant decrease in the number of cells in the ONL was observed in our modified LIRD model at 48h (Fig. 4.2b). However, retinas exposed to 24h of light showed a small increase in PCNA positive cells in the ONL over controls (Fig. 4.3b). The PCNA positive cells at 24h most likely represent dividing rod progenitors at the base of the ONL, which

have a finite number of divisions before they are replenished by proliferating Müller cells[21]. At 48h, we observed the appearance of PCNA positive cells in the INL (Fig. 4.3a), suggesting that some Müller cells have likely re-entered the cell cycle[1,13]. Although increases in proliferating cells in our modified model were NS, we predict that these events occurred due to the complete recovery of cell numbers in the ONL at 28 days after light treatment (Fig. 4.2b). In other zebrafish studies, PCNA positive Müller cells appeared in the INL around 35h of light exposure, and by 72h, clusters of proliferating cells had increased in the INL and began to migrate into the ONL[11]. One explanation for the low numbers of PCNA positive cells may be related to the shorter duration (48h) of light exposure in our study. Perhaps if we extended these studies beyond 48h, we would get a better picture of how the proliferation and migration process occurs in our modified LIRD model in zebrafish. Finally, these results suggest that our modified LIRD model using shorter periods of dark-adaptation (e.g. 24h) before continuous light exposure can be used to achieve a similar outcome to other LIRD studies using longer periods of dark-adaptation (7–14 days) in zebrafish retina.

### 4.6 Conclusion

A shorter period of dark-adaptation can recapitulate LIRD of photoreceptors in pigmented zebrafish. This damage is comparable to the original LIRD model with a prolonged period of dark-adaptation. The events occurred with a similar timing and sequence; however, we observed more cell death in the INL than previous studies[1,11,19]. The short period of dark-adaptation provides an advantage of time and avoids accidental

light exposure during feeding. Taken together, this modified model is a robust alternative to LIRD models that use extended periods of dark-adaptation.

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# Chapter 5

# Adenosine protects zebrafish photoreceptors from damage and apoptosis in a light-induced retinal degeneration (LIRD) model of age-related macular degeneration (AMD)

## 5.1 Abstract

Photoreceptors are the light sensing cells of the retina and the site where vision begins. Retinal diseases that damage photoreceptors, particularly cones, have dire consequences for vision. The most significant of these diseases being atrophic (dry) age-related macular degeneration (AMD), which results in macular dropout and vision loss due to dying cone photoreceptors. While there are some effective treatments for exudative (wet) AMD, the large majority of AMD cases (> 90%) are atrophic or present as the "dry" form, which currently has no treatment. Purines, most notably adenosine, have demonstrated neuroprotective properties at neurons within the central nervous system (CNS) and prevent neurodegeneration following a variety of insults (e.g., ischemia, stroke, etc.). One approach to treating AMD is to utilize a "druggable" therapy targeted to neuroprotective mechanisms in photoreceptors. Models of light-induced retinal degeneration (LIRD) have successfully recapitulated some of the damage observed in AMD. Here, we explored the pharmacological consequences of using adenosine as a neuroprotective molecule in a LIRD model in the zebrafish retina. We were able to measure the protective effects of adenosine using cone photoreceptor markers and a zebrafish transgenic model Tg(nrd:EGFP), which labels rods in the adult retina. Treatment of retinas with adenosine resulted in a dose-dependent survival of both rod and cone photoreceptors after LIRD, that was mediated by both $A_1$R and $A_3$R in the retina. Any damage observed in adenosine-treated retinas was not enough to stimulate a regenerative response, arguing in favor of a neuroprotective mechanism. Moreover, higher concentrations of adenosine prevented structural damage and apoptosis of rods and cones while preserving both visual acuity and contrast sensitivity in zebrafish retinas, suggesting that adenosine has the capacity to preserve photoreceptor

function. Taken together, these findings demonstrate that adenosine acting on $A_1R$ and $A_3R$ has a significant capacity for neuroprotection and provides evidence of a potential novel therapeutic target for the treatment of the dry form of AMD.

## 5.2 Introduction

Adenosine is an endogenous purine nucleoside composed of an adenine attached to a ribose. In the central nervous system (CNS) adenosine acts as a neuromodulator and affects neural activity through multiple mechanisms[1]. Adenosine acts on $P_1$ purinergic receptors, also called adenosine receptors. These receptors are seven transmembrane domain, G protein-coupled receptors and there are four molecular and pharmacological adenosine receptor subtypes ($A_1R$, $A_{2A}R$, $A_{2B}R$, $A_3R$)[2]. $A_1R$ and $A_3R$ typically act through $G_{i/o}$ proteins to inhibit adenylyl cyclase and decrease intracellular cyclic AMP (cAMP) concentrations, while $A_{2A}R$ and $A_{2B}R$ act through $G_s$ and $G_{s/q}$ proteins, respectively, to stimulate adenylyl cyclase and increase cAMP concentrations[3]. Adenosine has been shown to be neuroprotective in the CNS and models of glaucoma, optic nerve injury, diabetic retinopathy, and retinal ischemia by decreasing apoptosis and increasing retinal neuronal survival[4-10]

The presence of adenosine receptors in the outer retina and particularly on photoreceptors has been demonstrated previously in a variety of species[11-17]. Adenosine has actions that alter photoreceptor gap junctional coupling[14,15], stimulate melatonin synthesis[18] and myoid elongation in cone photoreceptors[13], and suppress neurotransmission and inhibit photoreceptor excitability by decreasing activity of L-type $Ca^{2+}$ channels on rods and cones[19-22]. Thus, there is strong evidence for a functional role for adenosine receptors on photoreceptors, which could be protective against various forms of neurodegeneration. However, the role of adenosine as a neuroprotective molecule at photoreceptors has only been recently explored.

One study has shown that activation of $A_1$Rs in albino rats resulted in neuroprotection and maintenance of the a-wave and b-wave of the electroretinogram (ERG) following light damage[23]. This study lends support to the idea that adenosine acting at $A_1$Rs is neuroprotective for photoreceptors. Unfortunately, the authors did not attempt to test other adenosine receptor subtypes, focusing solely on $A_1$R. In addition, the authors used very high concentrations of $A_1$R selective agonist, $N^6$-cyclopentyladenosine (CPA), in their intravitreal injections negating the selectivity of the drug for $A_1$Rs at the concentration tested (0.775 mM used, $EC_{50}$ = 2.3 nM for $A_1$R, 790 nM for $A_{2A}$R, 43 nM for $A_3$R)[24,25]. Moreover, the authors blocked the effects of CPA with an antagonist (DPCPX) that was non-selective at the concentration tested (0.01 mM) and would likely block all adenosine receptor subtypes to some degree[25]. Finally, these experiments were performed in albino animals, which have ocular issues related to retinal function and connectivity that can obscure results[26-28]. Notwithstanding these discrepancies, further evidence is needed to clarify which adenosine receptors contribute to the neuroprotective mechanisms at photoreceptors, especially in a light-induced retinal degeneration (LIRD) model that can recapitulate the geographic atrophy observed in a disease like AMD.

LIRD is an established and efficient model for studying mechanisms of photoreceptor apoptosis and developing retinal drug therapies[29,30]. This non-invasive model selectively targets photoreceptors, allowing for synchronous photoreceptor apoptosis, and recapitulates many of the retinal pathologies seen in diseases that affect photoreceptors like AMD[29,31,32]. The constant high-intensity light exposure in the LIRD model causes extensive photochemical damage, particularly photooxidative damage[33]. It is important to note that oxidative stress is strongly associated with AMD onset and

progression[34-36]. The primary mechanisms of damage in this model are through the generation of reactive oxygen species (ROS) by excitation of chromophores (e.g., 11-cis retinal and lipofuscin) and an associated increase in intracellular calcium leading to photoreceptor apoptosis[33,37,38]. Current models of LIRD utilize a prolonged period of dark-adaptation prior to constant high-intensity light exposure to induce retinal damage[31,39]. This study will use a novel modified version of the LIRD model developed in our laboratory that utilizes a short period of dark-adaptation prior to light exposure, saving valuable time and reducing issues related to intermittent light exposure during feedings[40]. More importantly, the LIRD model has been shown to produce consistent retinal damage in pigmented zebrafish, a phenomena that does not occur consistently or at all in the pigmented mammalian retina, possibly due to protection conveyed to photoreceptors by melanin in the retinal pigment epithelium (RPE)[39,41].

The aim of this study was to determine whether adenosine was neuroprotective in a pigmented animal model of LIRD, and to identify the pharmacological subtype of adenosine receptor that confers neuroprotection of photoreceptors from light-induced damage and apoptosis. Based on our results, we concluded that adenosine prevented structural damage and apoptosis of photoreceptors, and preserved visual function through the stimulation of $A_1R$ and $A_3R$.

## 5.3 Methods

*Animals*

Adult male and female (≥ 6 months) wild-type (AB or EKKwill) and Tg(nrd:EGFP) zebrafish were used for these studies. Tg(nrd:EGFP) were a generous gift

from Dr. Teresa Nicholson (Oregon Health Sciences University, Portland, OR)[42]. The NeuroD GFP zebrafish line expresses GFP in all rods of adult zebrafish, but not in cones[43]. The NeuroD line was generated using a BAC clone (dK33b12) that was isolated and contained 67 kilobase pairs (kb) of sequence upstream and 89 kb of sequence downstream of the NeuroD gene. Recombination resulted in EGFP positioned at the endogenous start site. All animals were cared for according to institutional guidelines and all procedures were approved by the Penn State University College of Medicine IACUC.

*Light-induced retinal degeneration (LIRD)*

A modified light-induced retinal degeneration (LIRD) procedure that utilizes a short period of dark-adaptation was used to produce retinal damage in zebrafish. The protocol and setup for this modified LIRD treatment paradigm has previously been described in detail[40]. Briefly, zebrafish were placed in a 3 L aquarium with two 8,000 lumen (100 W) LED lights (LEPOWER New Craft LED Flood Light) placed 30 cm away from the tank. These are full spectrum lights (color temp: 5,500 ºK with a peak near 450 nm) with a light intensity inside the tank of approximately 28,000 lux. Briefly, fish were dark-adapted for 24 hours using a light-tight box covering the tank and then exposed to constant high-intensity light over a period of 96 hours. Fish were collected at 0 hours prior to light exposure and then again after 96 hours of light exposure.

*Intravitreal injection protocol*

Intravitreal injections were performed between the dark-adaptation period and the period of light exposure. Zebrafish were anesthetized with Tricaine (0.16 mg/ml) and

then placed on their sides on a 35 ml petri dish filled with ice. Injections were performed under a dissection microscope and fish were placed on a wet Kimwipe or paper towel with solution containing Tricaine. A dropper filled with a solution containing Tricaine was used to keep the fish moist and anesthetized throughout the procedure. Intravitreal injections were performed by making a puncture in the cornea near the limbus with a 32-gauge lancet, and inserting a custom 33-gauge needle with a Hamilton syringe (Hamilton Company, Reno, NV). Each eye received ~ 0.5 µl of total volume. After injections, fish were allowed to recover in a separate tank for a period of 10-15 minutes.

To observe the effects of adenosine in a LIRD model of AMD, fish received injections of adenosine dissolved in isotonic saline in both eyes and a control group received injections of saline alone. The concentration-dependent effects of adenosine (MilliporeSigma, Burlington, MA) were assessed with doses ranging from 0.1 mM (final concentration: 1.67 µM), 1.0 mM (final concentration: 167 µM), and 5.0 mM (final concentration: 833 µM). Final vitreous concentrations were calculated taking into account the internal fluid volume of the eye and the injected volume. The total internal volume of the zebrafish eye was calculated based upon the available space in the globe when subtracting out the volume of the lens, $V_{Feye}$ (final volume of the eye, which is the internal volume of the eye minus the volume of the lens, $V_{Feye} = 4/3\pi r^3_{eye} - 4/3\pi r^3_{lens}$). All measurements are based upon reported optical coherence tomography (OCT) measurements for adults ≥ 4 months[44]. To determine which adenosine receptor is responsible for the neuroprotective effects of adenosine on photoreceptors, fish were injected with one of four adenosine receptor agonists dissolved in saline: CGS-21680 hydrochloride (CGS), an $A_{2A}R$ agonist; BAY 60-6583 (BAY-60), an $A_{2B}R$ agonist; $N^6$-

Cyclopentyladenosine (CPA), an $A_1R$ agonist; and HEMADO, an $A_3R$ agonist (Tocris Bioscience, Ellisville, MO). Injected concentrations are as follows: 3 μM CGS (final concentration in the eye: 500 nM) and 0.3 μM BAY-60, 0.3 μM CPA, and 0.3 μM HEMADO (final concentration in the eye: 50 nM). All final concentrations in the eye were calculated based upon the moles injected (moles)/ $V_{Feye}$ (L).

*Tissue preparation and cryosectioning*

Zebrafish were euthanized with an overdose of Tricaine (0.32mg/ml) followed by decapitation. Eyes were dissected out and fixed in 4% paraformaldehyde for 15 minutes, washed in 0.1 M PBS, and then stored in 30% sucrose in PBS (pH 7.4) with 0.05% sodium azide at 4 ºC. Eyes were embedded first in gelatin (7.5%) and then frozen in optimal cutting temperature (OCT) compound in isopentane, and cooled to -80 ºC using liquid nitrogen. Frozen eyes were sectioned vertically on a cryostat at 12 μm on gelatin-coated slides.

*Immunohistochemistry and transferase-mediated dUTP nick-end labeling (TUNEL)*

Vertical retinal sections were labeled using the indirect immunofluorescence technique[45]. Slides were warmed on a heating block at 37 ºC and then washed initially for 5-10 minutes at 37ºC to remove the gelatin, and washed two more times for 5 minutes with PBS at room temperature (~25 ºC) to remove the OCT. All sections were placed in a blocking solution [containing 10% normal goat serum, 1% BSA, and 0.5% Triton-X-100 in PBS (pH 7.4)] for 1 hour at room temperature. Sections were incubated in primary antibody solution overnight at 4 ºC. The next day slides were washed three times in 0.1

M PBS followed by incubation of secondary antibodies in the dark at room temperature for 1 hour. Slides were then washed three times in 0.1 M PBS, air dried, and coverslipped using Aqua Poly Mount (Polyciences Inc., Warrington, PA). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays were performed on vertical retinal sections according to the manufacturer's instructions [In Situ Cell Death Detection Kit (Roche), MilliporeSigma, Burlington, MA]. Antigen retrieval was performed prior to immunolabeling in experiments that used the Sox2 antibody as described previously in zebrafish[46]. Additionally, in experiments using Sox2 antibody, the blocking solution and primary and secondary antibody solutions contained normal donkey serum instead of normal goat serum.

*Antibodies*

Primary antibodies used in this study include mouse anti-zpr-1(1:200, Zebrafish International Resource Center (ZIRC), Eugene, OR), mouse anti-glutamine synthetase (1:5,000, Millipore, Billerica, MA), and goat anti-hSox2 (1:500, R&D Systems, Minneapolis, MN). Secondary antibodies used in this study were goat anti-mouse conjugated to either CF 488A, CF 586, or CF 644 (1:1000, Biotium, Fremont, CA) or donkey anti-goat conjugated to either CF 568 or CF 644 (1:1000, Biotium, Fremont, CA). RedDot (1:1000, Biotium, Fremont, CA) and dapi (1:10,000, Biotium, Fremont, CA) florescent nuclear stains were used for labeling cell nuclei.

*Confocal imaging and analysis*

Images were acquired using a Nikon C2+ confocal mounted to an upright fixed stage Eclipse FN1 microscope with Nikon Elements Software (ver. 4.2) (Nikon Instruments, Melville, NY). Cell counts were performed from six regions per vertical retinal section (each region was ~50 000 $\mu m^2$ area) using a CFI Plan Fluor 40X 1.3 NA oil objective. Images were taken from three peripheral and three central regions for each section and cell counts were averaged together for the section. For each animal, cell counts from 3 sections were averaged together. The differences between groups were analyzed using a one-way ANOVA with a Tukey's post hoc test. Variance was reported as mean ± SEM and significance was chosen at $P < 0.05$ (Prism 5.0, GraphPad, San Diego, CA).

*Analysis of visual acuity and contrast sensitivity using the optomotor response (OMR)*

An optomotor response (OMR) test was used to measure the visual acuity and contrast sensitivity of control (saline) and adenosine-treated fish (0.1 mM, 1.0 mM, 5.0 mM). The OMR tests were performed using the OptoMotry System (Cerebral Mechanics Inc., Lethbridge, AB, Canada) as previously described[47]. A virtual cylinder composed of moving bar stimuli was generated using a 3-D sine wave grating displayed on monitors forming a cube. A transparent round tank (~13 cm in diameter) filled with 5-8 cm of water was placed on a raised platform located in the center of the cube. A column (~3 cm in diameter) was placed in the center of the tank to prevent the fish from swimming in the center. Fish were free-swimming for the OMR tests and generally swam between the sides of the tank and the column to follow the grating stimulus. Spatial frequency

threshold (SFT), a measure of visual acuity, was established at maximum contrast (100%) with an angular velocity of 12°/sec. The staircase method was used to determine SFT. In this method, the spatial frequency of the grating was increased until the fish no longer responded to the stimuli and a SFT was established. Contrast sensitivity threshold (CST) was determined using the staircase method, where luminance contrast of the grating began at 100% and was systematically reduced until the fish no longer responded. CST was determined at four spatial frequencies (0.014, 0.050, 0.103, 0.300 c/d) and the threshold at a spatial frequency was calculated as a Michelson contrast [$M_c = (L_{max} - L_{min})/(L_{max} + L_{min})$] from the screen luminesces. Contrast sensitivity (CS) is the reciprocal of CST and was converted from CST by calculating CS = 100/CST. CS was then plotted against spatial frequency in a log-log graph. Differences between groups were analyzed using a one-way ANOVA with a Tukey's post hoc test. Variance was reported as mean ± SEM and significance was chosen at $P < 0.05$ (Prism 8.0, GraphPad, San Diego, CA).

## 5.4 Results

*Adenosine protects against photoreceptor damage and apoptosis in a concentration-dependent manner in a LIRD model of AMD*

Zebrafish were dark-adapted for 24 hours before receiving intravitreal injections of saline (control) or adenosine (concentrations: 0.1 mM, 1.0 mM, 5.0 mM) dissolved in saline. Zebrafish were then exposed to constant high-intensity light for a period of 96 hours to produce retinal damage. Retinas were collected at 0 hours prior to light exposure and after 96 hours of light exposure. To assess structural damage of the photoreceptors, red-green double cones were immunolabeled with zpr-1 antibody (yellow, Fig. 5.1a), and

rods of Tg(nrd:EGFP) zebrafish express GFP (green, Fig. 5.2a). For all groups (control, and 0.1 mM, 1.0 mM, 5.0 mM adenosine-treated), retinas collected at 0 hours displayed normal cone (Fig. 5.1a) and rod morphology (Fig. 5.2a). In control retinas after 96 hours of light exposure evidence of damage was apparent. Cones of 96 hour control retinas exhibited swelling, especially of the ellipsoid, and severe truncation or complete loss of the outer segment. In 96 hour 0.1 mM adenosine-treated retinas, cones displayed some swelling of the cell body and ellipsoid, and severe truncation or complete loss of the outer segment. In contrast, 96 hour retinas treated with 1.0 mM and 5.0 mM adenosine appeared to have relatively normal cone morphology with some outer segment truncation.

A similar pattern of damage occurred in the rods after 96 hours of light, with control retinas experiencing the most severe damage. Both control and 0.1 mM adenosine-treated retinas displayed swelling of the cell body and severe truncation of the rod outer segment. When measured, rod outer segment length was significantly reduced in both control and 0.1 mM adenosine-treated retinas at 96 hours (0 hr control: 55.11 ± 2.60 vs. 96 hr control: 20.76 ± 2.36, n = 8, P = 0.0001; 0 hr 0.1 mM: 58.90 ± 2.20 vs. 96 hr 0.1 mM: 27.18 ± 4.71, n = 8, P = 0.001) (Fig. 5.2b). As with cones, the rods of 1.0 mM and 5.0 mM adenosine-treated retinas after 96 hours of light appeared relatively normal with some truncation of the outer segment. Additionally, outer nuclear layer (ONL) thickness significantly decreased after light exposure in control retinas (0 hr control: 31.43 ± 1.22 vs. 96 hr control: 21.03 ± 0.90, n = 11, P = 0.0001), but not in the adenosine-treated retinas (Fig. 5.3b), suggesting a reduction in the loss of photoreceptors in the ONL of adenosine-treated retinas.

There was a significant decrease in the number rod and cone photoreceptors, and the total number of nuclei in the ONL in control retinas after 96 hours of light in comparison to control retinas at 0 hours prior to light exposure (0 hr cone: 114 ± 2.21 vs. 96 hr cone: 74.0 ± 3.58, n = 6, P = 0.001; 0 hr rod: 151 ± 10.77 vs. 96 hr rod: 66.39 ± 4.74, n = 6 , P = 0.001; 0 hr ONL: 247 ± 10.31 vs. 96 hr ONL: 152 ± 6.05, n = 12, P = 0.001) (Fig. 5.1b, 5.2b, and 5.3a). In the adenosine-treated retinas at 96 hours, there was a significant reduction in the loss of rod and cone photoreceptors, and the total number of nuclei in the ONL in comparison to the control retinas at 96 hours.

To assess apoptosis, TUNEL assays were performed on vertical retinal sections from control and adenosine-treated retinas (Fig. 5.4a). In 96 hour control and 0.1 mM adenosine-treated retinas, there was a significant increase in TUNEL positive cells in both the ONL (0 hr control: 0.56 ± 0.02 vs. 96 hr control: 107.1 ± 12.87, n = 11, P = 0.0001; 0 hr 0.1 mM: 0.34 ± 0.18 vs. 96 hr 0.1 mM: 111.7 ± 9.47, n = 10, P = 0.0001) and whole retina (0 hr control: 1.57 ± 0.51 vs. 96 hr control: 196.5 ± 10.65, n = 11, P = 0.0001; 0 hr 0.1 mM: 3.25 ± 1.70 vs. 96 hr 0.1 mM: 202.2 ± 12.28, n = 10, P = 0.0001) in comparison to control and 0.1 mM adenosine-treated retinas collected at 0 hours (Fig. 5.4). There were no significant differences in the number of TUNEL positive cells between the control and 0.1 mM adenosine-treated retinas at 96 hours, suggesting that there was a similar amount of cell death occurring in both groups after light exposure. However, retinas treated with 1.0 mM or 5.0 mM adenosine exhibited a significant reduction in TUNEL positive cells after 96 hours of light exposure in comparison to control retinas at 96 hours in both the ONL and whole retina. Adenosine, particularly the 1.0 mM and 5.0 mM concentrations, reduced photoreceptor damage, loss, and apoptosis.

Altogether, this data suggests that adenosine has a protective effect on photoreceptors in a LIRD model of AMD in a dose-dependent manner.

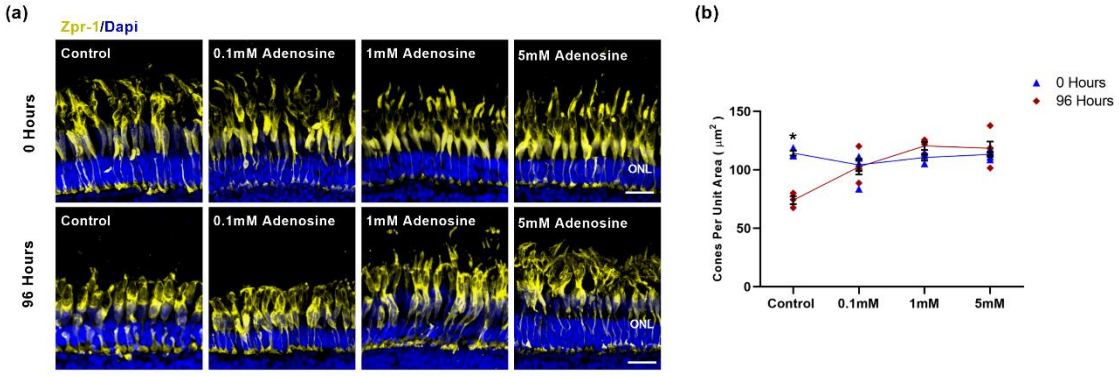


**Figure 5.1:** Confocal images, ONL measurements, and cell counts of vertical sections of control and adenosine-treated retinas of adult zebrafish. **a)** Red-green double cones were labeled with zpr-1 (yellow) and nuclei were labeled with dapi (blue). Retinas treated with 1.0 mM and 5.0 mM adenosine show relatively normal cone morphology. **b)** Quantification of all cone types in a 50, 000 $\mu m^2$ area in control and adenosine-treated retinas at 0 hours prior to light exposure and after 96 hours of light exposure. Data represented as mean ± SEM (n = 4-6 animals per group), using a one-way ANOVA with a Tukey's post hoc test ($p < 0.05$). ONL, outer nuclear layer. Scale bar = 20 μm.

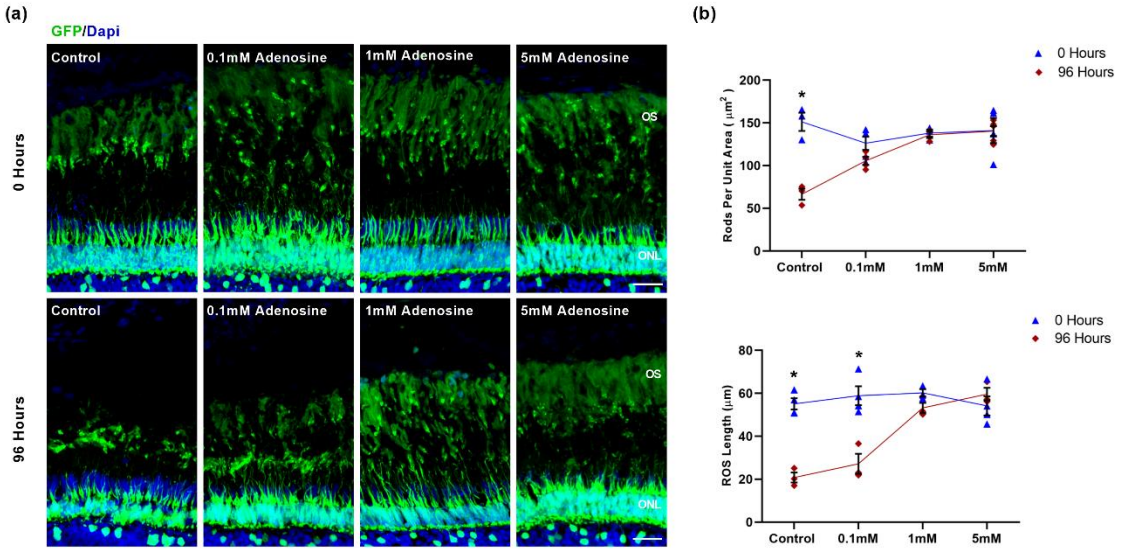


**Figure 5.2:** Confocal images, rod outer segment measurements, and cell counts of vertical sections of control and adenosine-treated retinas of adult zebrafish. **a)** Rods express GFP (green) and nuclei were labeled with dapi (blue). Retinas treated with 1.0 mM and 5.0 mM adenosine exhibit relatively normal rod morphology. **b)** Quantification of rods (top) in a 50,000 $\mu m^2$ area in control and adenosine-treated retinas at 0 hours prior to light exposure and after 96 hours of light exposure (n = 5-6 animals per group). Measurements of rod outer segment length (bottom) in the central retina of control and adenosine-treated fish at 0 hours and 96 hours (n = 4-5 animals per group). Data represented as mean ± SEM, using a one-way ANOVA with a Tukey's post hoc test ($p < 0.05$). OS, outer segment; ONL, outer nuclear layer; ROS, rod outer segment. Scale bar = 20 μm.

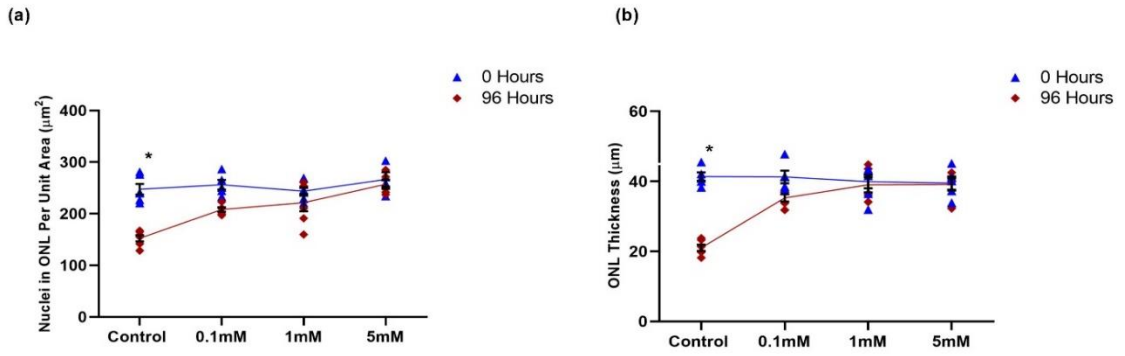


**Figure 5.3:** Photoreceptor counts and the overall changes in thickness of the ONL following LIRD. **a)** Comparison of the total number of nuclei in the ONL after 96 hours of light damage versus 0 hours in the presence of increasing concentrations of adenosine (0.1, 1.0, 5.0 mM). **b)** Comparison of the total thickness of the ONL after 96 hours of light damage versus 0 hours in the presence of increasing concentrations of adenosine (0.1, 1.0, 5.0 mM). Data represented as mean ± SEM (n = 5-6 animals per group), using a one-way ANOVA with a Tukey's post hoc test ($p < 0.05$). ONL, outer nuclear layer.

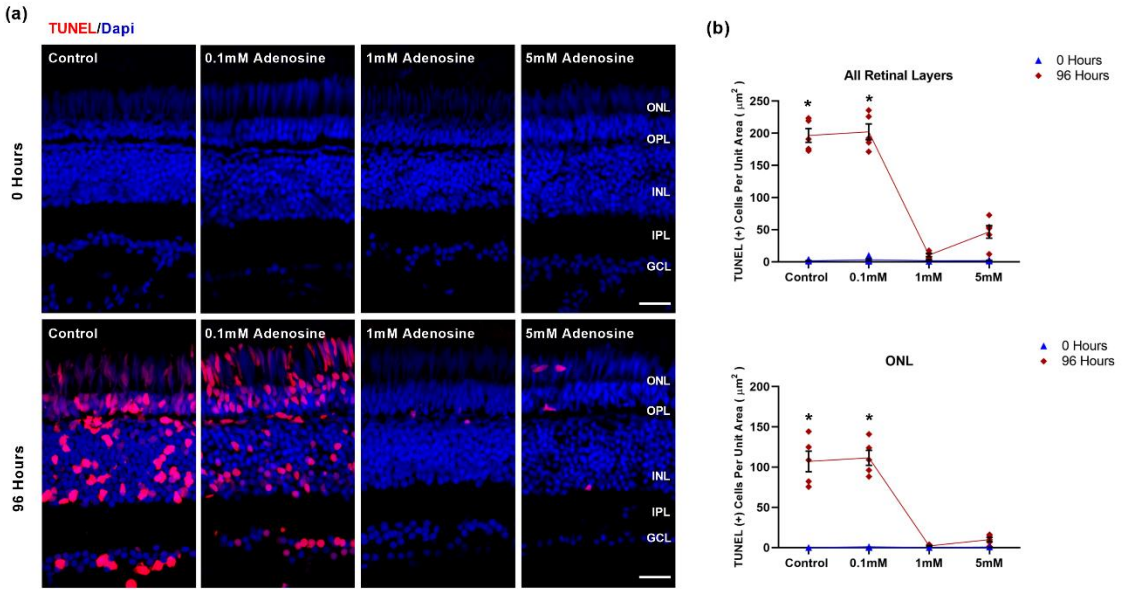


**Figure 5.4:** Transferase-mediated dUTP nick-end labeling (TUNEL) positive cells in vertical sections of control and adenosine-treated retinas of adult zebrafish. **a)** Apoptotic cells were labeled with TUNEL (red) and nuclei were counterstained with dapi (blue). Retinas treated with 1.0 mM and 5.0 mM adenosine show a significant reduction in the number of TUNEL positive cells. **b)** Quantification of TUNEL positive cells in all retinal layers (top) and in the ONL (bottom). Data represented as mean ± SEM (n = 4-5 animals per group), using a one-way ANOVA with a Tukey's post hoc test ($p < 0.05$). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell

*Adenosine prevents damage-induced regeneration in the zebrafish retina in a LIRD model of AMD*

The adult zebrafish retina is capable of regeneration in response to photoreceptor injury[32,48]. Retinal damage stimulates Müller cells in the inner nuclear layer (INL) to become active and re-enter the cell cycle, where they proliferate to produce progenitor cells for all retinal neuronal cell types[49,50]. Cone and rod progenitor cells produced by Müller cells migrate from the INL to the ONL to replace photoreceptors lost[39]. To assess retinal regeneration, zebrafish received intravitreal injections of either 1.0 mM adenosine or saline (control) and were then exposed to constant high-intensity light over 96 hours. Retinas were collected at 0 hours prior to light exposure and after 96 hours of light exposure. To visualize regeneration, vertical retinal sections were double-labeled with the antibodies, Sox2 and glutamine synthetase, a marker for Müller cells. Sox2 is a transcription factor that is required for the damage-induced reprograming of Müller cells to produce pluripotent cells[51]. In the undamaged adult zebrafish retina, Sox2 is expressed at low levels in the Müller cells and in a subset of amacrine cells in the INL and the ganglion cell layer (GCL)[51]. Prior to light exposure, Müller cells in both the control retinas (Fig. 5.5a A-D, arrowheads) and the adenosine-treated retinas (Fig. 5.5a I-L, arrowheads) show basal levels of Sox2 expression. In the damaged retina, Sox2 expression is upregulated in reprogramed or active Müller cells and is also expressed in the resultant progenitor cells[51]. There was a significant increase in Sox2 expressing cells after 96 hours of light in control retinas in comparison to control retinas at 0 hours prior to light exposure (0 hr control: 23.06 ± 1.96 vs. 96 hr control: 50.26 ± 2.19, n = 6, P = 0.0001). This suggests that some Müller cells have reentered the cell cycle and that the

clusters of Sox2 labeled cells surrounding the Müller cells are progenitor cells (Fig. 5.5a E-H, arrows). In contrast, adenosine-treated retinas after 96 hours of light exposure did not show a significant increase in Sox2 labeled cells. While regeneration appeared to have occurred in the control retinas after high-intensity light exposure, the results suggest that adenosine can prevent damage-induced Müller cell activation in a LIRD model of AMD.

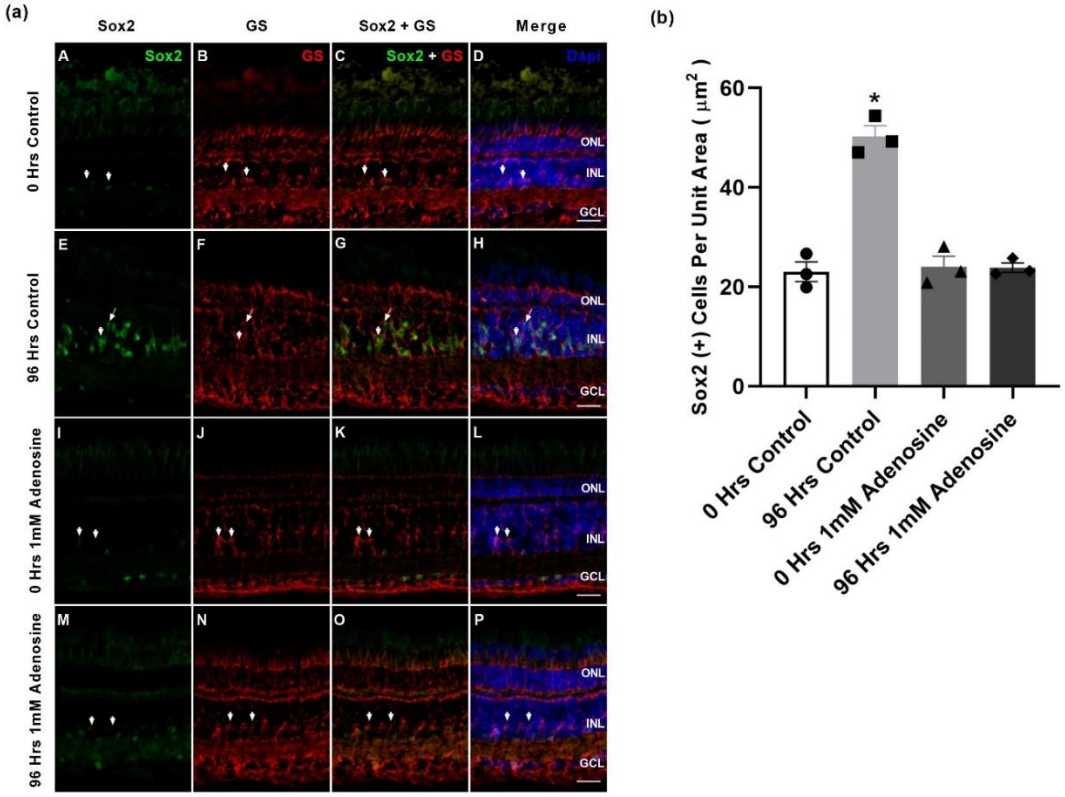


**Figure 5.5:** Confocal images and Sox2 positive cell counts of vertical sections of control and adenosine-treated retinas at 0 hours prior to light exposure and 96 hours after light exposure. **a)** Müller cells were double-labeled with Sox2 (green) and glutamine synthetase (red) antibodies, progenitor cells were labeled with Sox2, and nuclei were labeled with dapi (blue). Arrowheads indicate Müller cells, while arrows indicate progenitor cells. Adenosine-treated retinas after 96 hours show no increase in Sox2 labeling. **b)** Quantification of Sox2 labeled cells in a 50, 000 μm$^2$ area at 0 hours and 96 hours in control and adenosine-treated retinas. Data represented as mean ± SEM (n = 3 animals per group), using a one-way ANOVA with a Tukey's post hoc test ($p < 0.05$). GS, glutamine synthetase; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar = 20 μm.

*Activation of $A_3R$ and $A_1R$ prevents photoreceptor damage and loss in a LIRD model of AMD*

All four subtypes of adenosine receptors are expressed in the adult zebrafish retina, but only two of the four are found specifically on photoreceptors. $A_{2A}R$ are present as puncta around the ellipsoids and cell bodies of cones and less prominently in rod cell bodies[16]. $A_3R$ are strongly expressed in the ellipsoids of zebrafish photoreceptors[16]. To determine which adenosine receptor conveys the protective effects seen in the adenosine-treated retinas after LIRD, zebrafish received intravitreal injections of either saline (control) or an adenosine receptor agonist: CGS-21680 hydrochloride (CGS), an $A_{2A}R$ agonist; BAY 60-6583 (BAY-60), an $A_{2B}R$ agonist; $N^6$-Cyclopentyladenosine (CPA), an $A_1R$ agonist; and HEMADO, an $A_3R$ agonist. Zebrafish were then exposed to 96 hours of high-intensity light and retinas were collected at 0 hours prior to light exposure and after 96 hours of light exposure.

To assess structural damage of the photoreceptors, red-green double cones were immunolabeled with zpr-1 (yellow, Fig. 5.6a) and rods of Tg(nrd:EGFP) zebrafish express GFP (green, Fig. 5.7a). At 0 hours prior to light exposure, the rods and cones of the control and agonist-treated groups showed normal photoreceptor morphology. After 96 hours of light exposure, the cones of the control retinas and retinas treated with BAY-60 and CGS exhibited severe truncation or complete loss of the outer segment (Fig. 5.6a). Cones of control retinas and BAY-60-treated retinas at 96 hours also displayed swelling of the cell body and inner segment, while cones of CGS-treated retinas experienced minimal swelling. In addition, both the 96 hour control and BAY-60-treated retinas exhibited swelling of cone synaptic terminals, which was mostly absent in CGS, CPA,

and HEMADO-treated retinas. Remarkably, cones from retinas treated with CPA and HEMADO retained relatively normal morphology with minor truncation of the outer segment.

When looking at rods at 96 hours (see Fig. 5.7a), control retinas and retinas treated with BAY-60 and CGS exhibited swelling of the rod cell body and severe truncation or loss of the outer segment, while retinas treated with CPA and HEMADO exhibited relatively normal rod morphology with some truncation of the outer segment. Rod outer segment length was significantly reduced in control retinas and retinas treated with BAY-60 and CGS at 96 hours (Fig. 5.7b, 0 hr control: 57.21 ± 3.07 vs. 96 hr control: 24.49 ± 0.86, n = 6, P = 0.0001; 0 hr BAY-60: 57.95 ± 6.78 vs. 96 hr BAY-60: 26.85 ± 2.60, n = 6, P = 0.0001; 0 hr CGS: ± vs. 96 hr CGS: 55.25 ± 1.82, n = 6, P = 0.0001). Additionally, ONL thickness significantly decreased after light exposure in control retinas and retinas treated with BAY-60 and CGS (0 hr control: 40.14 ± 1.56 vs. 96 hr control: 19.42 ± 0.84, n = 6, P = 0.0001; 0 hr BAY-60: 39.30 ± 1.40 vs. 96 hr BAY-60: 21.23 ± 1.40, n = 6, P = 0.0001; 0 hr CGS: 42.04 ± 1.09 vs. 96 hr CGS: 21.27 ± 1.44, n = 6, P = 0.0001), but not in the CPA and HEMADO-treated retinas, suggesting that stimulation of $A_1R$ and $A_3R$ reduced photoreceptor loss in the ONL (Fig. 5.8b).

To assess cell loss, counts of cones (all subtypes), rods, and nuclei in the ONL were performed. There was a significant loss of cones, rods, and nuclei in the ONL in control retinas (Fig. 5.6b, cones: 127.9 ± 6.37; Fig. 5.7b, top panel, rods: 98.31 ± 3.71; Fig. 5.8a, ONL: 221.1 ± 3.34, n = 9), and BAY-60 (Fig. 5.6b, cones: 137.2 ± 5.6; Fig. 5.7b, top panel, rods: 99.15 ± 8.18; Fig. 5.8a, ONL: 234.2 ± 9.20, n = 9) and CGS-treated (Fig 5.6b, cones: 135.7 ± 3.31; Fig. 5.7b, top panel, rods: 95.49 ± 4.10; Fig. 5.8a, ONL:

230.4 ± 3.538, n = 9) retinas at 96 hours in comparison ($p < 0.05$) to control retinas (Fig. 5.6b, cones: 170.1 ± 5.12; Fig. 5.7b, top panel, rods: 147.1 ± 2.87 ; Fig. 5.8a, ONL: 318.1 ± 3.03, n = 9), and retinas treated with BAY-60 (Fig. 5.6b, cones: 171.3 ± 5.32; Fig. 5.7b, top panel, rods: 142.7 ± 11.08; Fig. 5.8a, ONL: 308.8 ± 9.64, n = 9) and CGS (Fig. 5.6b, cones: 177.5 ± 4.12; Fig. 5.7b, top panel, rods: 138.5 ± 7.90 ; Fig. 5.8a,ONL: 315.9 ± 11.11, n = 9) at 0 hours. Interestingly, retinas treated with CPA and HEMADO showed no significant loss of cones, rods, or nuclei in the ONL and although not significant, there appeared to be a slight decrease in the number of rods for both of these groups. This data suggests that stimulation of both $A_3R$ and $A_1R$ conveys a neuroprotective effect in a LIRD model of AMD, and that perhaps the combination of both receptors is necessary for a more complete protection of the rod photoreceptors.

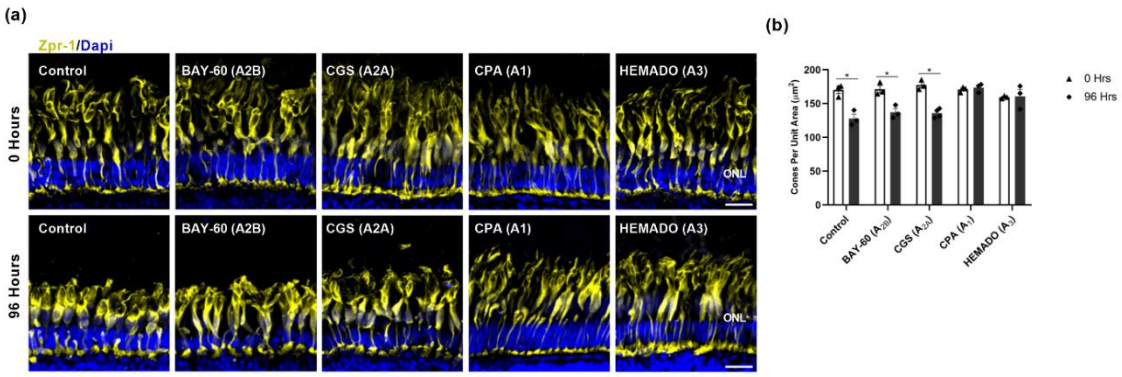


**Figure 5.6:** Confocal images and cell counts of vertical sections of control and adenosine receptor agonist-treated retinas of adult zebrafish. **a)** Red-green double cones were labeled with zpr-1 (yellow) and nuclei were labeled with dapi (blue). Retinas treated with CPA and HEMADO show relatively normal cone morphology with some truncation of the outer segment. **b)** Quantification of all cone types in a 50, 000 μm$^2$ area in control and agonist-treated retinas at 0 hours prior to light exposure and after 96 hours of light exposure. Data represented as mean ± SEM (n = 3 animals per group), using a one-way ANOVA with a Tukey's post hoc test ($p < 0.05$). ONL, outer nuclear layer. Scale bar = 20 μm.

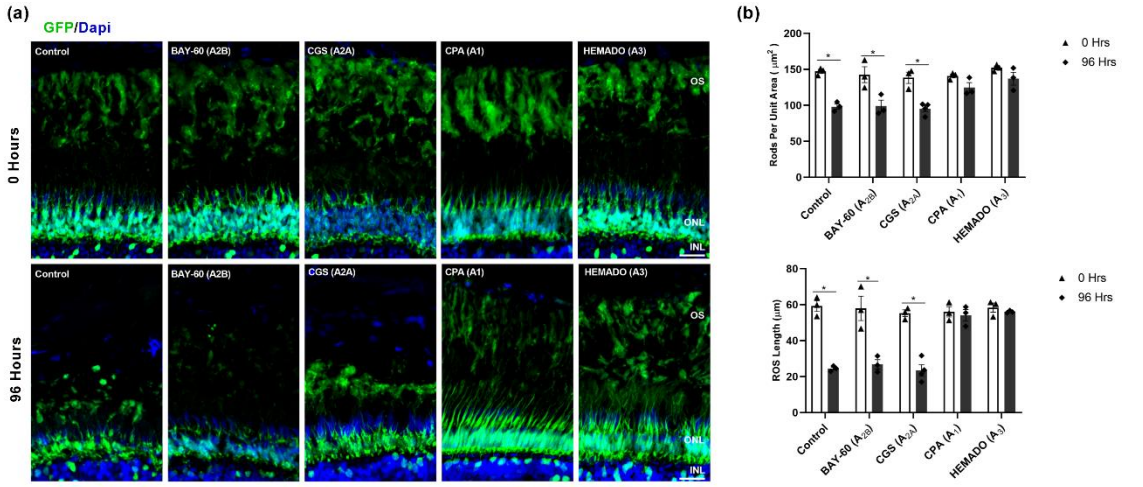


**Figure 5.7:** Confocal images, rod outer segment measurements, and cell counts of vertical sections of control and adenosine receptor agonist-treated retinas of adult zebrafish. **a)** Rods express GFP (green) and nuclei were labeled with dapi (blue). Retinas treated with CPA and HEMADO show relatively normal rod morphology with some truncation of the outer segment. **b)** Quantification of rods (top) in a 50,000 μm$^2$ area in control and agonist-treated retinas at 0 hours prior to light exposure and after 96 hours of light exposure. Measurements of rod outer segment length (bottom) in the central retina of control and agonist-treated fish at 0 hours and 96 hours. Data represented as mean ± SEM (n = 3 animals per group), using a one-way ANOVA with a Tukey's post hoc test ($p < 0.05$). OS, outer segment; ONL, outer nuclear layer. Scale bar = 20 μm.

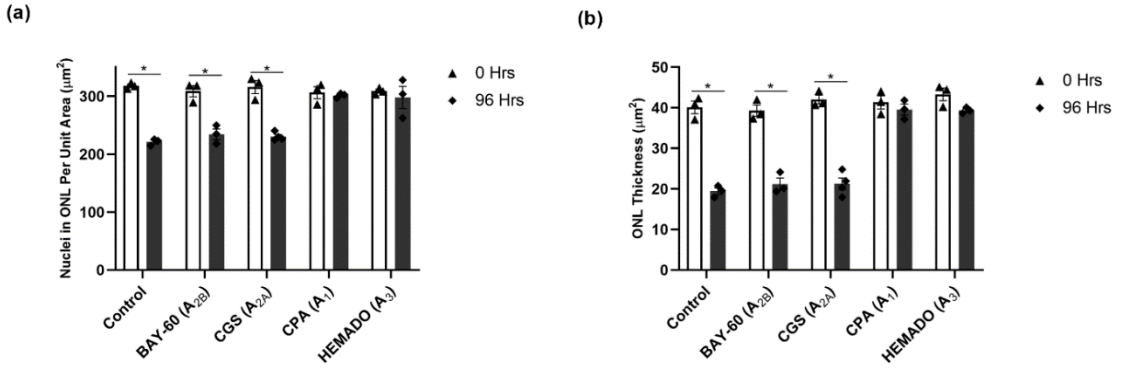


**Figure 5.8:** Photoreceptor counts and the overall changes in thickness of the ONL following LIRD in adenosine receptor agonist-treated retinas. **a)** Comparison of the total number of nuclei in the ONL after 96 hours of light damage versus 0 hours in the presence of adenosine receptor agonists. **b)** Comparison of the total thickness of the ONL after 96 hours of light damage versus 0 hours in the presence of adenosine receptor agonists. Data represented as mean ± SEM (n = 3 animals per group), using a one-way ANOVA with a Tukey's post hoc test ($p < 0.05$). ONL, outer nuclear layer.

*Adenosine preserves visual acuity and contrast sensitivity in a LIRD model of AMD in zebrafish*

To assess vision and visual behavior, visual acuity and contrast sensitivity were measured using the optomotor response. The OMR tests were performed using the OptoMotry System from Cerebral Mechanics Inc., as described in the methods. The setup utilizes a virtual cylinder composed of moving bar stimuli that was generated using a 3-D sine wave grating displayed on a set of monitors surrounding the test subject. A transparent round tank (~13 cm in diameter) filled with 5-8 cm of water was placed on a raised platform located in the center. A column (~3 cm in diameter) was placed in the center of the tank to prevent the fish from swimming in the center. Fish were free-swimming for the OMR tests and generally swam between the sides of the tank and the column to follow the grating stimulus (see Fig. 5.9a). Fish received intravitreal injections of saline or adenosine (concentrations: 0.1 mM, 1.0 mM, 5.0 mM) and were then exposed to 96 hours of light prior to testing. A control group tested at 0 hours prior to light exposure was dark-adapted for 24 hours but did not receive injections. Visual acuity is described as the ability to distinguish two objects (spatial resolution) at given angular distance[52]. Visual acuity or the spatial frequency threshold (SFT) was determined at maximum contrast (100%) with an angular velocity of 12°/sec. SFT was established using the staircase method, by increasing the spatial frequency of the grating until the fish no longer responded to the stimuli. A significant reduction in visual acuity was observed in control fish after 96 hours of light exposure in comparison to the control fish at 0 hours prior to light exposure (Fig. 5.9b, 0 hr control: 0.701 ± 0.01 vs. 96 hr control: 0.080 ± 0.05, n = 10, P = 0.0001). There was a significant reduction in the loss of visual acuity at

96 hours in fish treated with 1.0 mM and 5.0 mM adenosine in comparison with the 96 hour control group (Fig. 5.9b, 96 hr control: 0.080 ± 0.05 vs. 96 hr 1.0 mM: 0.432 ± 0.05, n = 10, P = 0.0001; 96 hr control: 0.080 ± 0.05 vs. 96 hr 5.0 mM: 0.505 ± 0.03, n = 10, P = 0.0001). While not significant, we also observed a slight recovery of visual acuity in 0.1 mM adenosine-treated fish at 96 hours in comparison to the 96 hour control fish. These results suggest that adenosine can preserve visual acuity following light damage in zebrafish in a concentration-dependent manner.

Spatial contrast refers to the light-dark transition of a border or an edge in an image that demarcates the existence of a pattern or a target object[53]. Contrast sensitivity is a measure of how much contrast a person requires to distinguish objects from each other or from the background[53]. To calculate contrast sensitivity, contrast sensitivity threshold (CST) must first be established. CST is the percent contrast at which a fish can track the stimulus reliably[54]. The lower the contrast percentage, the more sensitive the retina is to contrast differences. CST was established with an angular velocity of 12°/sec beginning with a maximum contrast of 100% and then gradually reduced until the fish stopped responding. The contrast sensitivity threshold was identified at 4 different spatial frequencies: 0.014, 0.050, 0.103, 0.300 c/d. CST increased significantly at all spatial frequencies in control fish after 96 hour of light exposure in comparison to 0 hour control fish tested prior to light exposure (Fig. 5.9, 0.014 c/d: 0 hr control: 21.72 ± 2.17 vs. 96 hr control: 82.74 ± 3.99; 0.050 c/d: 0 hr control: 1.26 ± 0.47 vs. 96 hr control: 73.63 ± 9.59; 0.103 c/d: 0 hr control: 1.76 ± 0.59 vs. 96 hr control: 72.82 ± 4.42; 0.300 c/d: 0 hr control: 16.56 ± 2.03 vs. 96 hr control: 88.80 ± 2.83, n = 5 per group, P = 0.0001), suggesting that contrast sensitivity worsened in 96 hour control fish. Contrast sensitivity

(CS: dimensionless) is the reciprocal of CST. Plotting CS against spatial frequency revealed a U-shaped contrast sensitivity curve for each group (Fig. 5.9c). There was a significant reduction in contrast sensitivity in the 96 hour control group at all spatial frequencies in comparison to the 0 hours control group (Fig. 5.9a, 0.014 c/d: 0 hr control: 21.71 ± 2.17 vs. 96 hr control: 82.74 ± 4.00; 0.050 c/d: 0 hr control: 1.26 ± 0.48 vs. 96 hr control: 73.62 ± 9.59; 0.103 c/d: 0 hr control: 1.76 ± 0.60 vs. 96 hr control: 72.82 ± 4.14; 0.300 c/d: 0 hr control: 16.56 ± 2.03 vs. 96 hr control: 88.80 ± 2.83, n = 5 per group, P = 0.0001). Although not significant, there was a small increase in contrast sensitivity in the adenosine-treated groups for all spatial frequencies.

CST was partially preserved in adenosine-treated fish in comparison to the 96 hour control fish, with the 1.0 mM and 5.0 mM concentrations showing significant improvement at all spatial frequencies (Fig 5.10, 0.014 c/d: 96 hr control: 82.74 ± 3.99 vs. 96 hr 5.0 mM: 67.88 ± 7.34, P = 0.0001; 0.050 c/d: 96 hr control: 82.74 ± 3.99 vs. 96 hr 1.0 mM: 48.64 ± 3.48, P = 0.05, 96 hr control: 82.74 ± 3.99 vs. 96 hr 5.0 mM 34.28 ± 3.46, P = 0.01; 0.103 c/d: 96 hr control: 82.74 ± 3.99 vs. 96 hr 1.0 mM: 45.06 ± 4.81, P = 0.01, 96 hr control: 82.74 ± 3.99 vs. 96 hr 5.0 mM 48.15 ± 8.17, P = 0.05; 0.300 c/d: 96 hr control: 82.74 ± 3.99 vs. 96 hr 1.0 mM: 74.42 ± 2.57, P = 0.05, 96 hr control: 82.74 ± 3.99 vs. 96 hr 5.0 mM 73.00 ± 2.28, P = 0.05; n = 5 per group) and the 0.1 mM trending towards an improved CST at all spatial frequencies. Fish treated with the 5.0 mM adenosine concentration did not show a significant improvement at 0.014 c/d, but were trending towards improvement as well.

The results for CST and CS suggest that adenosine protects against the impairment of contrast sensitivity threshold and contrast sensitivity. The overall

behavioral data suggests that adenosine protects against functional impairments in vision and visual behavior and is in line with the preservation of rod and cone photoreceptors following light damage.

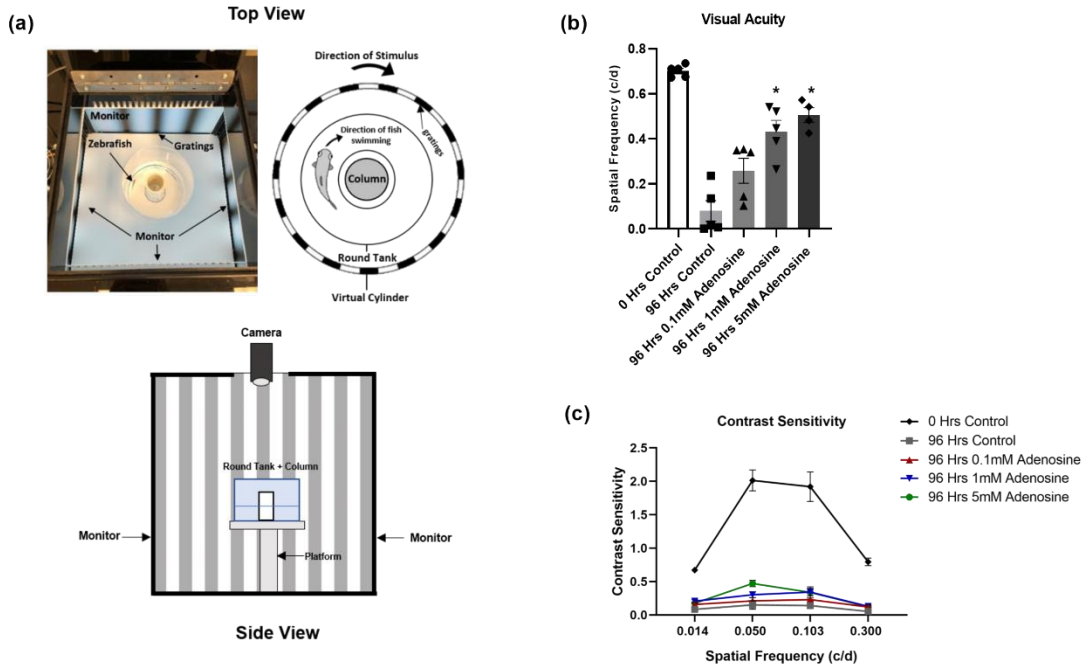


**Figure 5.9:** Optomotory setup, mean visual acuity, contrast sensitivity (CS) vs. spatial frequency. **a)** Schematic view of the OptoMotry System setup with round tank. (Top left) Image of OMR setup from above showing round tank with column and grating on monitors. (Top right) Illustration of OMR setup with the virtual cylinder projected in 3-D coordinate space on the monitors. The fish in the round tank is depicted as swimming in the direction of the grating stimulus around the center column. Side view illustration (bottom) of the OptoMotry System showing raised platform with tank in the center. **b)** Visual acuity was established at 100% contrast with an angular velocity of 12°/sec. A significant reduction in visual acuity was observed after 96 hours of light exposure that was recovered with adenosine treatment. **c)** There was a significant reduction in CS in control fish after 96 hours of light and while not significant, there was a trend towards increased CS in the adenosine-treated fish. Data represented as mean ± SEM (n = 5 animals per group), using a one-way ANOVA with a Tukey's post hoc test ($p < 0.05$).

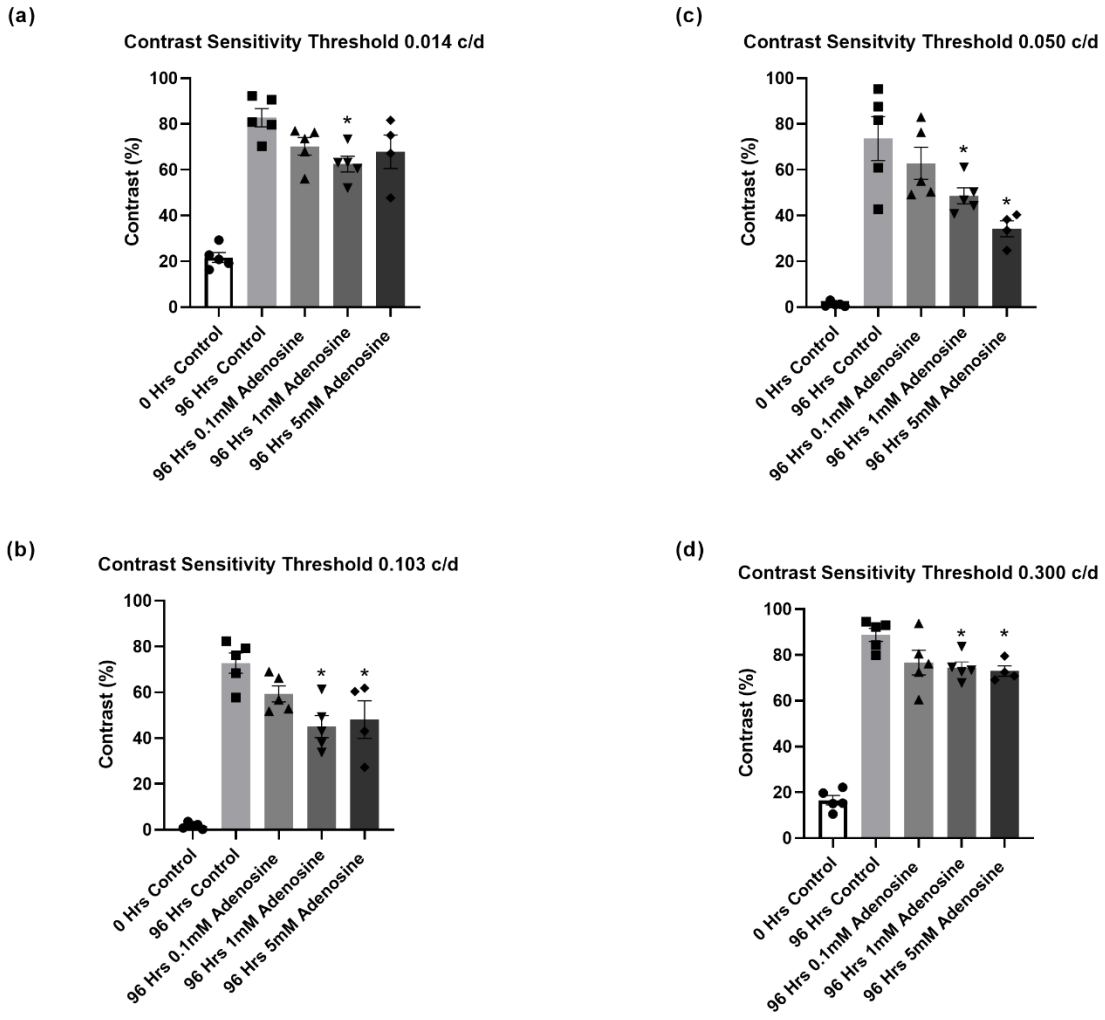


**Figure 5.10:** Contrast sensitivity threshold (CST) in control and adenosine-treated fish. CST was measured at four spatial frequencies (0.014, 0.050, 0.103, 0.300 c/d) with an angular velocity of 12°/sec. There was a significant increase in CST in control fish after 96 hours, which was improved in adenosine-treated fish. Data represented as mean ± SEM (n = 5 animals per group), using a one-way ANOVA with a Tukey's post hoc test ($p < 0.05$).

## 5.5 Discussion

In the present study, we looked at the effects of adenosine on photoreceptors in a LIRD model of AMD in zebrafish, and then determined which adenosine receptor subtypes ($A_1R$, $A_{2A}R$, $A_{2B}R$, $A_3R$) conveyed these effects. We showed that adenosine prevented light-induced structural damage to both cone and rod photoreceptors and reduced the amount of photoreceptor loss and apoptosis in a concentration-dependent manner. Adenosine concentrations above 1.0 mM (final concentration in the eye: 167 μM) prevented photoreceptor damage and apoptosis, while lower concentrations of adenosine failed to prevent significant damage and death. To determine which adenosine receptor conveyed this neuroprotective response, we utilized four potent and selective adenosine receptor agonists for each adenosine receptor subtype. As mentioned previously, $A_{2A}R$ and $A_3R$ were present on zebrafish photoreceptors in the outer retina[16]. We had hypothesized that adenosine might act directly on one or both of these receptors in order to convey protection in a LIRD model of AMD. Surprisingly, we uncovered that retinas treated with CGS, an $A_{2A}R$ agonist, did not prevent structural damage and apoptosis following light damage, suggesting that this receptor subtype does not play a role in photoreceptor neuroprotection. In contrast, both the $A_3R$ agonist, HEMADO, and the $A_1R$ agonist, CPA, prevented photoreceptor structural damage and loss. Though not significant, there was a decrease in the number of rods in retinas treated with these agonists at 96 hours, which was not seen in the retinas treated with higher concentrations of adenosine after LIRD. This pointed to the neuroprotective effects of adenosine on photoreceptors being conveyed by a combination of $A_3Rs$ and $A_1Rs$. This study provides the first extensive pharmacological evidence for the emerging role of adenosine as a

neuroprotective agent at both rod and cone photoreceptors and will be considered further below. It is conceivable that adenosine might provide a novel and unique pathway for exerting neuroprotection at photoreceptors and preserving vision.

Since $A_3$Rs are expressed on photoreceptors, it is likely that stimulation of this receptor has a direct effect on the photoreceptors themselves. Previously, our lab studied the effects of adenosine on neurotransmission in larval tiger salamander photoreceptors and showed that $A_{2A}$R activation resulted in the inhibition of L-type voltage-gated calcium channels, which suppressed L-glutamate release by inhibiting depolarization-evoked calcium influx into the photoreceptor terminals[19-22]. Adenosine is known to convey neuroprotection under a number of different pathological conditions by altering the metabolism of cells and reducing neuronal excitability[2,55-57]. Furthermore, it has been shown that increases in intracellular calcium are necessary to induce photoreceptor apoptosis in the LIRD model, and while it is still debated whether or not photoreceptor apoptosis is caspase-dependent or independent, there is strong evidence supporting a caspase-independent pathway involving calcium-dependent proteases[38,58,59]. Donovan and Cotter (2002) demonstrated that calpains, calcium-activated proteases, were activated in the LIRD model providing evidence for calcium-induced photoreceptor apoptosis and a caspase-independent pathway[58]. One possibility is that $A_1$R and $A_3$R are protecting photoreceptors from increases in intracellular calcium that are tied to light-induced generation of ROS and oxidative stress[38]. Donovan et al. (2001) demonstrated that both increases in intracellular calcium and oxidative stress play a significant role in photoreceptor apoptosis in the LIRD model, so it is possible that stimulation of at least $A_3$R is indirectly protecting photoreceptors from the effects of ROS activity on calcium

homeostasis. It is important to note that oxidative stress has long been implicated as a major contributing factor to the onset and progression of AMD[34-36].

Stimulation of $A_1$Rs on retinal ganglion cells has been shown to be neuroprotective in rodent models of glaucoma, optic nerve injury, and glutamate excitotoxity[5,60,61]. In the zebrafish retina, $A_1$R is predominantly expressed in the plexiform layers[16]. A recent study by Solino et al. (2018), identified $A_1$Rs on retinal microglia cells in rats, which may be a more likely target of stimulation[23]. However, the authors' immunolabeling was poor at best and the tissue had significant autofluorescence making this interpretation difficult. Yet this idea of $A_1$R mediated activity of microglial cells could be a potential pathway to explore the neuroprotection of photoreceptors. It is well established that microglia are resident immune cells of the retina that perform immune surveillance and are activated upon pathologic changes. In a healthy retina, they reside in the inner retina and release neuroprotective and anti-inflammatory factors, but under pathological conditions activation of microglia promotes phagocytosis of degenerated cells and waste, and secretion of neuronal survival factors[62]. Chronic activation of microglia causes these cells to upregulate their expression of inducible nitric oxide synthase (iNOS) and the production of pro-inflammatory cytokines leading to chronic inflammation and neuronal cell loss[63]. Studies have shown that microglial activation often precedes retinal degeneration and photoreceptor apoptosis[64,65]. In the eyes of patients with the dry form of AMD, activated microglia were found to have migrated to the ONL suggesting a role for microglia in the pathogenesis of this disease. Additionally, oxidative stress present in both the aging and AMD retina can lead to a low grade inflammatory response, known as a para-inflammatory response, that occurs in

order to repair and remodel the tissue, usually through microglial, macrophage, and complement activation[62]. However, where these para-inflammatory responses in the normal aging retina are controlled and necessary to restore homeostasis in the tissue, in the AMD eye these responses are abnormal and contribute to the pathogenesis of AMD[62]. Studies using the LIRD model have shown increases in activated microglia and pro-inflammatory cytokines, migration of microglia to the ONL, and microglial phagocytosis of apoptotic photoreceptors in response to light exposure, recapitulating the activity of microglia and inflammation seen in AMD[23,66-68]. Taken together, it is possible that adenosine acting through $A_1$Rs expressed on microglial cells could modulate the apoptotic driven pathway that damages photoreceptors following light damage.

Support for this hypothesis comes from a recent LIRD study in albino rats that demonstrated rats treated with intraocular injections of CPA, the same $A_1$R agonist used in these studies, exhibited a reduction in the number of activated microglia cells and a decrease in the subsequent release of pro-inflammatory cytokines, TNFα and iNOS[23]. This finding is supported by other studies that show $A_1$R stimulation inhibits morphological activation of microglia and the subsequent pro-inflammatory responses[69-71]. These studies also found that under pathological conditions, expression of $A_1$R was upregulated in microglial cells[69,70]. Stimulation of $A_{2A}$Rs on microglia has been shown to prevent microglial activation and subsequent pro-inflammatory effects, resulting in increased photoreceptor survival[72]. However, in our study, we saw no significant increases in photoreceptor survival following application of CGS, a $A_{2A}$R agonist, arguing against any beneficial effects of the $A_{2A}$R pathway in mediating neuroprotection of photoreceptors. In the current study, the significant reduction in photoreceptor

structural damage and loss indicates that stimulation of $A_1R$ had a protective effect in a LIRD model of AMD in zebrafish, which may be mediated through modulation of the microglia immune response, and is currently being explored as a possibility in our laboratory.

Like $A_{2A}R$, $A_{2B}R$ is also present in the zebrafish retina, and predominantly localized to Müller cells[16]. Less is known about the effects of this receptor. $A_{2B}R$ is a low affinity receptor expressed at low levels in the CNS, suggesting that it may be recruited under pathological conditions[73]. Yet we found that the $A_{2B}R$ agonist, BAY-60, was without any effect on rods or cones, suggesting that it likely does not play a role in photoreceptor neuroprotection. It is possible that stimulation of $A_1R$ and $A_3R$ on other immune cells could have contributed to the protective effects induced by CPA and HEMADO respectively, since adenosine is recognized for modulating numerous immune functions[74], and has reduced the damaging pro-inflammatory response of the microglia in other preparations.

The zebrafish retina is capable of regeneration of all retinal neuronal cell types[48,50]. In response to photoreceptor damage, Müller cells are activated and proliferate to produce progenitor cells, which will migrate to the ONL to replace photoreceptors lost[49]. In LIRD studies using zebrafish, Müller cells labeled with proliferating cell nuclear antigen (PCNA) antibody appear in the INL after 34 hours of light exposure, indicating that these cells had reentered the cell cycle, and clusters of proliferating cells appeared in the INL after 72 hours of light[39]. Sox2 is a transcription factor that is required for the reprograming of Müller cells to produce progenitor cells for retinal regeneration in zebrafish. In the normal adult zebrafish retina, Sox2 is expressed at low levels in Müller

cells, but in the damaged retina Sox2 is upregulated in the active Müller cells and progenitor cells[51]. In control retinas after 96 hours of light, the number of Sox2 cells increased significantly. The damage these retinas sustained after LIRD was strong enough to evoke a regenerative response, which is not surprising considering the amount of photoreceptor damage and apoptosis these retinas experienced. In contrast, 1.0 mM adenosine-treated retinas had significantly less Sox2 labeling, suggesting that any damage to the ONL in these retinas was not enough to stimulate a regenerative response, strengthening the argument that adenosine is neuroprotective in the outer retina.

Since adenosine has a protective effect on the structure of photoreceptors and the outer retina in a LIRD model of AMD, it is necessary to establish whether or not adenosine also has a functional effect on the retina and visual behavior. The optomotor response (OMR) was used to measure the coordinated visual function of zebrafish following our light damage paradigm. This is a robust behavior that argues strongly in favor of a functional intact visual system from the photoreceptors in the outer retina to the central processing in the tectum[41]. The OMR response involves eye, head, and whole-body movements that help fish to compensate for movements of the environment in order to stabilize its image on the retina. This is an excellent approach for deciphering the impact of visual defects in zebrafish. In short, the OMR response can be used to measure visual acuity and contrast sensitivity. In patients with AMD, central vision is severely impacted by damage to the RPE and photoreceptors of the macula resulting in the progressive impairment of both visual acuity and contrast sensitivity[75,76]. We found that in fish treated with higher concentrations of adenosine, visual acuity was partially preserved at 96 hours, contrasting control fish at 96 hours who experienced a severe loss

of visual acuity. We saw a similar pattern when testing for contrast sensitivity threshold. At each spatial frequency tested, fish treated with higher concentrations of adenosine showed a significant improvement and were able follow the stimulus at lower percent contrast than the 96 hour control fish. This preservation of vision in adenosine-treated fish reflects the maintenance of photoreceptor function and neurotransmission by adenosine receptor activation on or near photoreceptors.

### 5.6 Conclusion

Our findings support the hypothesis that adenosine can prevent light-induced structural damage, loss, and death of photoreceptors as well as preserve visual processing by protecting photoreceptors from damage in a concentration-dependent manner. Protection is likely conferred by stimulation of $A_3$Rs present on photoreceptors, and possibly through the stimulation of $A_1$Rs on resident microglia cells following initial damage, resulting in protection from some of the damaging pro-inflammatory signaling present following LIRD. Taken together, understanding how adenosine mediates neuroprotection in photoreceptors during LIRD holds significant promise for the identification of a “druggable” target, which could be transformational for treating AMD and other diseases that affect photoreceptors.

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# Chapter 6

# Discussion

## 6.1 Age-Related Macular Degeneration (AMD), Oxidative Stress, and Neuroinflammation

The hypothesis of this work was driven by a long-standing, unresolved question regarding the mechanisms of cone-targeted degeneration or age-related macular degeneration. Many studies have focused on a variety of approaches to address the underlying mechanisms tied to AMD. As mentioned previously, AMD is a complex disease caused by a combination of genetic, environmental, nutrition, and lifestyle factors, but the biggest risk factor is age[1,2]. AMD has a slow onset and progression with two late stage forms: wet AMD and dry AMD. This work focuses on the dry form of AMD, which is characterized by large confluent regions of atrophied RPE in the macular area leading to mostly cone photoreceptor death[3].

The primary factor in this disease is ageing, and thus with an ever expanding aging population worldwide, an exigent need exists for a robust, approachable, and tractable animal model to explore potential targets for therapeutic intervention. Since age itself remains by far the most important risk factor, making it an urgent priority to understand the connections between underlying aging mechanisms and pathophysiology of AMD. It is important to note that as we age so does our visual system, i.e., an individual's perception of the visual scene at 20 years of age is far different than that of someone in their 80s. This framework extends to the perception of colors (color vision), dark and light adaptation (sensitivity), and most importantly visual acuity (discrimination of fine details). All of these deficits in humans lie in retinal changes, in particular, changes that impact photoreceptors or more specifically cones. However, one highly significant feature of biological aging is inflammation and molecular damage involving

oxidative stress. Oxidative stress is the cornerstone of the damage that occurs in cones within the macula of humans with AMD, and posits that a constellation of these "bad actors" causing oxidative stress contribute to the pathophysiology or sequelae of degenerative vision loss. The pathogenesis of dry AMD is still largely unknown, and factors that contribute to the onset and progression of the disease most often involve oxidative stress, the thickening of Bruch's membrane, lysosomal lipofuscin accumulation and drusen formation, RPE damage and dysfunction, dysregulation of the immune system, and inflammation[4].

There are many studies that have explored oxidative damage as it relates to AMD in the retina using a variety of animal models (e.g., drosophila, fish, mice, rats, guinea pigs, and monkeys) [5-9]. While the primate models may be preferable due to their similarities in retinal structure and AMD-related changes (e.g., drusen formation and composition) with humans, the cost, lack of genetic tools for primates, longer time required for inducement, and challenges associated with breeding and fecundity make other animal models more attractive. Thus, the ideal animal model for studying AMD should involve: 1) a cone-dominated animal with relatively robust visual acuity; 2) possess robust visual behaviors that can be used to easily measure visual function; 3) have a lower cost; 4) faster disease progression; 5) high fecundity; and 6) easy and approachable genetic manipulation. However, no existing animal model fully recapitulates the retinal changes found in human AMD. Notwithstanding, the rodent models have shown retinal changes including subretinal deposits, thickening of the Bruch's membrane, loss of retinal pigment epithelium and photoreceptors, and choroidal neovascularization, which are the characteristics of AMD[5-7]. However, mice have few

cones and are rod-dominated, and possess poor visual acuity[10,11]. In addition, one can argue that rodents hardly utilize their visual system for navigation in a 3D space, meaning olfactory and somatosensory cues, like smell and touch are much more important than vision for navigation, which makes assessing visual behavioral endpoints very challenging. Indeed, zebrafish have the potential to serve as this model for AMD which is outlined in chapter 1.

Oxidative stress has been shown to play a significant part in the pathogenesis of dry AMD[8,12,13]. The retina is particularly susceptible to oxidative stress for a number of reasons, but the eye has evolved antioxidant defense and repair systems to minimize oxidative damage[14,15]. In the ageing eye, there is a decrease in antioxidants and an increase in ROS levels and oxidative stress. This inverse relationship appears to be intensified in the AMD eye[9,12,13,16-18]. Inflammation is a defensive process which protects tissue against dangerous stimuli and restores tissue homeostasis[19]. The eye is an immune privileged organ, which has a number of regulatory molecules and cells that actively suppress or limit immune responses that lead to ocular inflammation in order to preserve vision[20]. Ocular inflammation is thought to play a role in many retinal degenerative diseases including AMD[21]. In the ageing eye, cells of the innate immune system can evoke a low-grade inflammatory response, known as para-inflammation, in order to restore homeostasis in stressed cells and tissues[19]. The para-inflammatory response is an intermediate inflammatory response between the basal homeostatic state and a classic inflammatory response that relies mainly on tissue resident macrophages[22]. In the normal aging retina, oxidative stress is the main initial cause for retinal para-inflammatory responses[23]. In the AMD retina, with increased oxidative stress, this response becomes

chronic and the excessive activation of the inflammatory response and persistent activation of resident immune cells has adverse effects on the retina[19]. This chronic oxidative damage-induced inflammatory response in the AMD eye likely contributes to the pathogenesis of the disease.

There are a number of therapies to treat the wet (vascular) form of AMD, but none to date for the treatment of the dry form of AMD, which afflicts the majority of patients in the world (~90%). To date, there is only the daily intake of the Age-Related Eye Disease Study (AREDS) formulation, which is an oral supplementation of antioxidant vitamins and minerals[24]. This has only been shown to be minimally effective at best, and functions to slow down the progression of the disease, but is not a viable long-term treatment and does not stop the disease or allow for recovery of vision. Clearly, there is a huge unmet need for the development of more therapies to treat dry AMD.

### 6.2 Light-Induced Retinal Denegation as a Model of AMD: Oxidative Stress and Neuroinflammation

Light-induced models of retinal degeneration are used to recapitulate the retinal pathologies of diseases that primarily affect photoreceptors like AMD[25-28]. Exposure of the retina to constant high-intensity full spectrum light results in photoreceptor apoptosis and thinning of the ONL due to photooxidative damage[29,30]. The main mechanism of damage occurs through excitation of chromophores (e.g., 11-cis retinal and lipofuscin) resulting in the generation of ROS and oxidative stress with a concurrent rise in intracellular calcium via nitric oxide synthase (NOS) activation[29-31]. Photoreceptor

apoptosis in this model seems to be calcium-dependent and occurs through a caspase-independent pathway involving calpains, calcium-activated proteases[31,32].

The oxidative stress seen in the model also appears to elicit a neuroinflammatory response[33,34]. Oxidative stress is a key mechanism of tissue stress triggering the inflammatory response in the retina[23,34]. Bian et al. (2016) revealed an increase in the expression of pro-inflammatory genes: interleukin 1β (IL-1β), chemokine (C-C motif) ligand 2 (Ccl2), cyclooxygenase-2 (COX2), and tumor necrosis factor-α (TNFα) after LIRD in mice[34]. During retinal inflammation, oxidative stress is required for Ccl2 production in response to inflammatory stimuli, and Ccl2 is necessary for promoting the inflammatory response by recruiting and activating immune cells including monocytes, macrophages, and lymphocytes[35]. Activated microglia produce pro-inflammatory cytokines like IL-1β and TNFα and play an important role in modulating inflammatory responses in neurodegenerative diseases[36]. Interestingly, microglial activation is believed to play a role in the pathogenesis of dry AMD[37]. Solino et al. (2018) demonstrated an increase in activated microglia and increased levels of pro-inflammatory cytokine TFNα in rats after LIRD[33]. Bailey et al. (2010) revealed an increase in activated microglia after LIRD in the zebrafish retina[38]. Using tench fish, Benjarano-Escobar et al. (2012) demonstrated that activated microglia were proliferative, suggesting the increase in microglia after LIRD was from local proliferation[39]. Joly et al. (2009) showed that in mice activated resident microglia and blood-derived microglia precursors that migrated from adjacent tissues like the choroid were both present in the retina after LIRD[40]. All together, this evidence suggests that LIRD models the oxidative stress-induced neuroinflammatory response seen in AMD.

## 6.3 A Short Period of Dark-Adaptation Prior to Light Exposure Induces Retinal Degeneration

In chapter four, we investigated the hypothesis that a short period of dark-adaptation before constant high-intensity light exposure would be sufficient to generate photoreceptor damage and apoptosis using pigmented zebrafish. Current LIRD models use a prolonged period (7-14 days) of dark-adaptation to increase the retina's sensitivity to light-induced damage[26,41,42]. Our rationale for this study was that the prolonged dark-adaptation in the original LIRD model was unnecessarily long, and that the intensity of the full spectrum LED lights (~28,000 lux) in our model was damaging enough without such a long preconditioning period of dark-adaptation. In this study, we found that using a short period of dark-adaptation of 24 hours was enough to produce retinal damage similar to the damage seen in other zebrafish LIRD studies using a prolonged period of dark-adaptation. The timing, progression, and severity of photoreceptor damage, apoptosis, and loss was similar to the timeline of events seen in the original model. Vihtelic and Hyde (2000), noted TUNEL labeling of apoptotic cells in the ONL only[26]. However, in our study we found that we had some TUNEL positive cells in both the INL and GCL. Other LIRD studies using teleosts also showed diffuse TUNEL labeling in the inner retina. The apoptotic cells seen in these layers might be from RGC apoptosis via the upregulation of metabolic components like pyruvate isozyme type M2, and/or the labeling of fragmented DNA in the cytoplasm of Müller cells and microglia post-phagocytosis of apoptotic photoreceptors[38,43,44].

Retinal damage produced by our modified LIRD model was enough to stimulate a regenerative response as was seen in zebrafish studies using the original LIRD model.

Proliferating cells were present in the INL after 48 hours in our modified model, suggesting that some Müller cells had reentered the cell cycle and were reprogramed to produce progenitor cells. The length of our light treatment was not enough to observe the pattern of proliferation seen in the original model using zebrafish, but fish allowed to recuperate for 28 days had a full recovery of their photoreceptors, indicating that similar regenerative events had occurred in our modified model. The results of this study showed that our modified model with a short period of dark-adaptation is a robust alternative to the original and saves on time, while avoiding intermittent light exposure issues related to feedings.

### 6.4 Adenosine is Neuroprotective in Photoreceptors in a LIRD Model of AMD in Zebrafish

The central hypothesis of Chapter 5 was to test whether adenosine is neuroprotective in a LIRD model of AMD in pigmented zebrafish. In this chapter we addressed two major aims related to the role of adenosine in the zebrafish retina. 1) Does activation of adenosine receptors present on photoreceptors reduce the amount of damage observed in a LIRD model of AMD in pigmented zebrafish? 2) Will adenosine prevent impairment of photoreceptor function in the outer retina and reduce alterations in visual behaviors in a LIRD model of AMD in pigmented zebrafish? The rationale for this study was that adenosine has already been shown to be neuroprotective in the CNS and the inner retina by modulating the metabolism of stressed cells and reducing cell excitability through the reduction of excitatory synaptic transmission and hyperpolarization[45,46]. In a previous study from Stella and colleagues, it was discovered that activation of adenosine

receptors on larval tiger salamander photoreceptors inhibited voltage-gated calcium channels, which suppressed glutamate release by inhibiting depolarization-evoked calcium influx into photoreceptor terminals[47-50]. Also from Stella and colleagues, it was discovered that photoreceptors in the adult zebrafish retina expressed $A_{2A}R$ and $A_3R$. Taken together, we believed that direct stimulation of the adenosine receptors on zebrafish photoreceptors would have a protective effect in a LIRD model of AMD.

The results of the study revealed that concentrations of adenosine 1.0 mM or higher prevented photoreceptor structural damage, apoptosis, and loss in retinas after LIRD. In retinas treated with 0.1 mM adenosine, there was significant photoreceptor structural damage and apoptosis. Adenosine was protective in a LIRD model of AMD in zebrafish in a dose-dependent manner. In the zebrafish retina, the LIRD model has been shown to generate enough damage to elicit a regeneration response where Müller cells become active and produce progenitors to replace the photoreceptors lost[26,27]. In retinas treated with 1.0 mM adenosine, the regenerative response was not observed, meaning that any damage produced by LIRD in adenosine-treated retinas was not great enough to stimulate regeneration. In addition to having a protective effect on the structure of the ONL, adenosine also preserved photoreceptor function and vision in the zebrafish retina. In our study, the optomotor response (OMR) was tested and used as a measure of visual acuity and contrast sensitivity. Impairment of visual acuity and contrast sensitivity threshold (CST) were significantly reduced in fish treated with higher concentrations of adenosine, with the 0.1 mM adenosine-treated fish trending towards improvement as well. Contrast sensitivity, the reciprocal of CST, although not significant, showed a small improvement in adenosine-treated fish after LIRD.

Looking at the effects of adenosine receptor agonists on each of the adenosine receptor subtypes, we discovered that stimulation of $A_3R$ and, surprisingly, $A_1R$ conveyed the protective effects of adenosine in a LIRD model of AMD in zebrafish. The effects of the $A_3R$ agonist were likely conferred through direct stimulation of the receptors on photoreceptors resulting in a decrease in intracellular calcium and calcium-dependent photoreceptor apoptosis. The effects of the $A_1R$ agonist may have been conferred through the activation of $A_1Rs$ on microglia, which likely prevented the activation of microglia and microglial secretion of pro-inflammatory cytokines, reducing neuroinflammation. Adenosine protects zebrafish photoreceptors in a LIRD model of AMD by reducing photoreceptor excitability through inhibition of voltage-gated calcium channels, and influencing immune cells and the immune response. This study provides strong evidence of a potential novel therapeutic target for the treatment of dry AMD.

### 6.5 Adenosine and the Retinal Pigment Epithelium

The retinal pigment epithelium (RPE) lies between the choroid and the retina and has a close relationship with the photoreceptors both physically and metabolically. The RPE absorbs extraneous light and protects the photoreceptors from photooxidation, transports nutrients from the choroid to the photoreceptors and waste in the opposite direction, and phagocytizes the shed outer segments of photoreceptors[51]. Dysfunction and death of the RPE is thought to proceed photoreceptor apoptosis in dry AMD[52]. Furthermore, the RPE also contributes to the immune privilege status of the eye through the secretion of immune suppressive factors and as a contributing structure to the blood-retinal barrier[51]. Some of the RPE-secreted cytokines can prevent or downregulate the

immune/inflammatory response, while others initiate or augment it[53]. Expression of adenosine receptors in the RPE has been noted in humans and rodents[54-56]. An alternative avenue of investigation could be to look at the effects of adenosine on the RPE in a LIRD model of AMD. Due to the close relationship the RPE has with the photoreceptors, if adenosine has a protective effect on the RPE, it would most likely benefit the photoreceptors. Investigating the effects of adenosine on the RPE would give us a better, more complete picture of how adenosine protects the outer retina in a LIRD model of AMD.

**6.6 Limitations**

A relatively unknown variable that we did not address in this study is the duration of adenosine following injection in the eye, and the length of time adenosine remains in the retina and eye. What are the pharmacokinetic parameters of adenosine following an intraocular injection? We can extrapolate or infer form our studies that based on the relatively high concentrations used that adenosine persists at least for the duration of the high intensity light exposure (e.g., 96 hours). If we had performed the intravitreal injections of adenosine several days prior to high-intensity light exposure would we still have observed similar neuroprotective effects of adenosine as seen in our study? The eye has a blood-retinal barrier (BRB) which includes an inner BRB formed by retinal capillary endothelial cells, and an outer BRB formed by the RPE and Bruch's membrane[57]. The BRB regulates fluid and molecular transport between the vasculature of the eye and the retina, prevents infiltration of macromolecules and other potentially harmful agents into the retina, and maintains the microenvironment of the retina[57].

There are few studies that look at the transport of adenosine across the BRB, and they tend to focus on the inner BRB[57-60]. In the rat eye, adenosine nucleoside transporters have been identified at the inner BRB, predominately ENT2, a bi-directional equilibrative transporter[57,58]. ENT2 likely mediates adenosine transport at the inner BRB and may play a role in regulating the adenosine concentration in retinal interstitial fluid[59]. The focus of these studies is on adenosine uptake into the retina, and on ENT2 as a potential route for delivering nucleoside drugs from the circulating blood to the retina[58,60]. Not much research has been conducted on adenosine efflux from the eye, which would also be useful for our studies. More research on adenosine transport across the BRB is needed, especially in the zebrafish BRB.

Another possible limitation of our adenosine study was the duration of high-intensity light exposure that we chose. In the LIRD model, the majority of the photoreceptor damage and apoptosis occurs in the first 24 hours of light exposure; however, cones appear to be more resistant to light damage and will degenerate more slowly over several days[26,27]. In our LIRD experiments, light exposure occurred over a period of 96 hours encapsulating the period of highest photoreceptor damage. If we had extended the duration of our light exposure beyond 96 hours, would we have seen the continued protective effects of adenosine or would another dose of adenosine need to be administered?

While the zebrafish model has many advantages, particularly in retinal and ocular disease research, there were some limitations in using this animal model in our adenosine experiments. The focus of our adenosine study has been on adenosine receptor activation as a means to prevent retinal damage and apoptosis in a LIRD model of AMD. Although,

adenosine and adenosine receptor activation seem to play more of a neuroprotective role in the retina, it has been reported in other tissues and organs that adenosine receptor activation promotes wound repair through neovascularization in damaged tissue and by promoting vasodilation to increase blood flow and the delivery of oxygen to the lesioned area[61-63]. Is it possible that adenosine can be used as a therapeutic to aid in the repair of pre-existing damage in the retina as well? As stated previously, the adult zebrafish retina is capable of regeneration in response to photoreceptor injury[64,65]. While the results of our adenosine study suggest that adenosine can prevent stimulation of the regenerative response in the zebrafish retina in a LIRD model, it is possible that the zebrafish's robust capacity for regeneration could complicate studying the effects of adenosine on retinal tissue repair. It is also likely that adenosine's protective action is the underlying mechanisms that prevented stimulation of a regeneration response in the zebrafish retina. Therefore, we might not be able to examine the full scope of adenosine's actions on retinal damage in a LIRD model of AMD in zebrafish because of this.

Another limitation to using the zebrafish model is that zebrafish have undergone a gene duplication event in evolution[66]. This makes performing loss-of-function studies difficult for certain genes, meaning there is a great deal of redundancy or overlap present. We would need to look very carefully at the expression of each of the genes using in situ hybridization. Comparative studies have shown that a whole-genome duplication event occurred early in the teleost lineage after separation of the fish and tetrapod lineages[66,67]. However, not all of these gene duplicates have been preserved in evolution. In teleost, many genes seen in mammalian species are duplicated and occur on separate chromosomes[68]. It has been estimated that for at least 20% of human genes, the zebrafish

have persevered two orthologues[66]. In zebrafish, there appears to be one or more orthologs for the genes that encode each adenosine receptor subtype[69,70]. This will make generating knockout models more challenging for each adenosine receptor subtype.

### 6.7 Adenosine as an Immunomodulator and Future Directions

Adenosine also functions as an endogenous modulator of the immune system and plays a pivotal role in the regulation of the inflammatory response[33,71-73]. Adenosine receptors are expressed on most innate immune cells (neutrophils, macrophages, dendritic cells, mast cells, natural killer cells) and activation of these receptors regulates immune/inflammatory cell functions[72-74]. Under normal conditions, adenosine is kept at low concentrations in the extracellular matrix (< 1 μM), but under metabolically stressful conditions, the concentration rises acting like a sensor to provide information to the immune system about tissue stress and inflammation[74]. Recently, much attention has been given to adenosine receptor activation as a therapeutic target in various inflammatory diseases[72,73]. In the retina, a particular focus has been given to adenosine receptors on microglia and the microglial inflammatory response[33,71,75-78]. AMD is not a classic inflammatory disease, but chronic inflammation does play a role in its pathogenesis[19].

As described at length in chapter 5 important questions remain regarding the role of adenosine as a potential therapeutic target. Previous studies show that both A3 and A2A adenosine receptors are expressed on rod and cone photoreceptors in zebrafish[70]. However, we found that the A3 adenosine receptor agonist, HEMADO, was able to confer more protection of photoreceptors than the A2A agonist, CGS-21680, and was equivalent to the A1 receptor agonist, R-PIA, suggesting that there is an additional

unknown mechanism involved. We have hypothesized that it likely involves microglia, but this still needs to be demonstrated. Since survivability of photoreceptors remains a crucial problem in AMD, it is essential to decipher the exact role that adenosine receptors may play in preventing the apoptosis of photoreceptors.

Initially, we were interested in the effects of adenosine receptor activation on the photoreceptors themselves. The next step of this project should shift focus to looking at the effects of adenosine on zebrafish immune cells and oxidative damage-induced neuroinflammation in a LIRD model of AMD. Further studies that will need to be conducted are: 1) Identify the expression of adenosine receptor subtypes on microglia in the zebrafish retina, 2) investigate the effects of adenosine receptor antagonists to confirm that activation of $A_1R$ and $A_3R$ is protective in the zebrafish retina, and 3) measure the expression of pro-inflammatory cytokines secreted by microglia during $A_1R$ and $A_3R$ activation and blockade to observe their effects on neuroinflammation and degeneration in the zebrafish retina. Since most innate immune cells express adenosine receptors, other future studies may include investigating the effects of adenosine receptor activation on other innate immune cells in the zebrafish retina in a LIRD model of AMD.

Key questions still remain, for example, how might certain adenosine receptors confer neuroprotection. What downstream steps must occur to prevent photoreceptor apoptosis? Moreover, is there an oxidative stress dependent response in photoreceptors that reduces the formation of reactive oxygen species or the formation of damaging free radicals generated by constant light exposure? How does circadian modulation of light and dark signaling between dopamine and adenosine influence the survival of photoreceptors following light damage? Therefore, is it possible that activation of

adenosine receptors promotes lasting changes in photoreceptor antioxidant activity that could help prevent light-induced retinal degeneration, similar to what we have observed in chapter 5?

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|---|---|
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## PUBLICATIONS

**Khan AS,** Friemel TD, Grillo SL, SL Stella. (2020). Adenosine protects zebrafish photoreceptors from damage and apoptosis in a light-induced retinal degeneration model of age-related macular degeneration. (In preparation).

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