

**MANIPULATION OF PHOTORECEPTOR ENERGY
METABOLISM FOR CLINICAL ADVANTAGE**

A thesis submitted in fulfilment of the
DEGREE OF MASTER OF PHILOSOPHY
in

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ABSTRACT

Photoreceptors are metabolically active cells that account for the majority of the energy consumption in the retina. Photoreceptors also display high rates of aerobic glycolysis which is unusual in non-proliferating cells. Many diseases of the retina involve degeneration of photoreceptors. Retinitis pigmentosa is the most commonly encountered hereditary retinal dystrophy, featuring degeneration of rod photoreceptors followed by cone photoreceptors. The genetic mutations in retinitis pigmentosa are exclusively expressed in rods, and the secondary death of cones likely involves a component of energy failure. The overriding premise of this thesis was to further our understanding of photoreceptor energy metabolism, then manipulate photoreceptor energy metabolism for clinical advantage using an *in vivo* model of retinitis pigmentosa.

The current thesis comprises three main experiments. In the first experiment, we investigated the role of glucose in the mouse retina and sought to provide an explanation for the high rate of aerobic glycolysis. This was performed by observing the distribution of carbon-14 glucose in the mouse retina using autoradiographic methods. In the second experiment, we characterized the temporal and spatial distribution of cone degeneration in a mouse model of retinitis pigmentosa. Cone quantification was performed using immunohistochemistry and reverse transcription polymerase chain reaction. In the third experiment, we tested two novel bio-energetic agents on the previously characterized mouse model of retinitis pigmentosa. Mice received either systemic creatine, delivered orally, or local glucose, delivered via repeated subconjunctival injection, and the effect on cone survival was measured using immunohistochemistry and neuro-behavioral responses. The results revealed that local glucose injections had no protective effect on cone survival. However, oral creatine treatment preserved cones and improved neuro-behavioral responses in retinal degeneration mice.

Therefore, we demonstrated that cone degeneration in retinitis pigmentosa likely occurs due to a mechanism that involves, at least in part, energy failure. Bio-energetic treatments, such as creatine, may play a role as future therapies for retinitis pigmentosa. Further studies will be performed to elucidate the precise mechanisms through which creatine exerts its protective effects, prior to developing a pathway to clinical translation in human patients.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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January 2018

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Daniel S. Narayan

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1. INTRODUCTION

Photoreceptors are the first order neurons of the visual pathway. There are three known types of photoreceptors in the mammalian retina: rods and cones (the classical photoreceptors), and photosensitive retinal ganglion cells. Rods have low spatial resolution but are extremely sensitive to light. They are specialized for sensitivity at the expense of resolution and are responsible for vision in dimly lit conditions. Cones have high spatial resolution but are relatively insensitive to light and are responsible for high acuity central vision and colour vision. Photosensitive retinal ganglion cells mainly have subcortical targets, with minimal visual processing.

Many diseases of the retina involve degeneration of photoreceptors. These include inherited diseases such as retinitis pigmentosa (RP), as well as acquired diseases such as diabetic retinopathy, age-related macular degeneration, central retinal artery occlusion and central retinal vein occlusion. These diseases currently have limited treatment options and are responsible for a large portion of visual impairment worldwide.

Photoreceptors are highly metabolically active cells and account for much of the energy consumption in the retina¹⁻¹⁴. The major photoreceptor energy substrate *in vivo* is glucose⁴⁻¹⁶. Glucose metabolism also performs many non-energetic roles in photoreceptors^{4,17-20}. Additionally, photoreceptors have the capacity to utilize alternative energy substrates such as phosphocreatine, lactate and amino acids^{4,13,21}. Photoreceptors are difficult to isolate in experimental models, making them challenging to study. Hence, the majority of data on photoreceptor metabolism is derived from studies on whole retinas²²⁻²⁵.

Energy failure, which refers to the relative deficiency of substrates required to make energy for cellular processes, has been suggested to play a role in a number of diseases of photoreceptor degeneration^{26,27}. Recent studies suggest that bio-energetic therapies may help preserve photoreceptors in many models of photoreceptor degeneration²⁷⁻²⁹. However, the

pathways by which photoreceptors meet their energy demands remain incompletely understood. The aim of this thesis was to investigate retinal energy metabolism by examining the role of glucose in the retina and to manipulate retinal energy metabolism for clinical advantage by examining the effect of bio-energetic therapies on an *in vivo* model of retinal degeneration.

1.1. Structure and Function of Photoreceptors

The mammalian retina, like all vertebrate retinas, comprises two synaptic layers intercalated between three neuronal body layers³⁰ (Fig. 1.1). In vertebrates, photoreceptors are situated in the outer retina. The percentage of rods and cones varies between different species. For example, in the human retina rods account for approximately 95% of photoreceptors and cones account for the remainder^{31,32}. Furthermore, the density of rods and cones varies with retinal eccentricity in the human: rods dominate the peripheral retina in humans; cone density increases towards the macula and the foveola is exclusively cones.

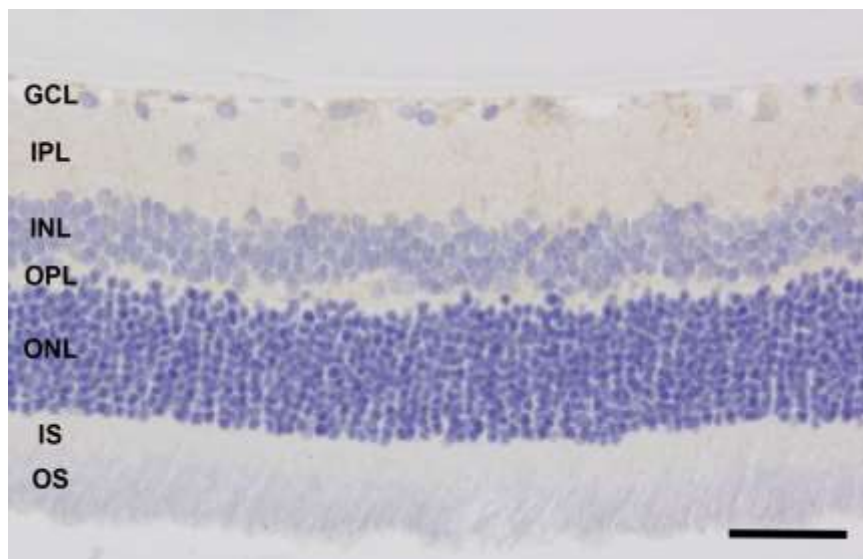


Figure 1.1. Photomicrograph of a cross section of the rodent retina. Hematoxylin counterstain has been used to highlight the cell nuclei. There are three neuronal cell body layers (GCL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer) and two synaptic layers (IPL = inner plexiform layer; OPL = outer plexiform layer). The photoreceptor layer is situated in the outer retina and is composed of the inner and outer

segments of the rods and cones (IS = inner segments; OS = outer segments) together with the cell bodies of the photoreceptors that are located in the ONL. Scale bar 50 μm .

Photoreceptors are elongated narrow cells that consist of four primary structural and functional regions: outer segment, inner segment, cell body, and synaptic terminal (Fig. 1.2). Most of the transporters, ion pumps and glycolytic enzymes are located in the inner segment^{33,34}. Photoreceptor outer segments contain the visual pigments responsible for absorption of light and initiation of neuro-electrical impulses. The inner and outer segments are connected by a stalk of modified cilium and spatially separated from the cell body by the outer limiting membrane. Photoreceptor cell nuclei are situated in the outer nuclear layer of the retina and their axons pass into the outer plexiform layer. The photoreceptor synaptic terminal encapsulates the dendritic tips of downstream bipolar and horizontal cells. The synaptic terminals exhibit dense structures known as synaptic ribbons. Mitochondria are distributed throughout the inner segment and synaptic terminal^{6,9,11,35}.

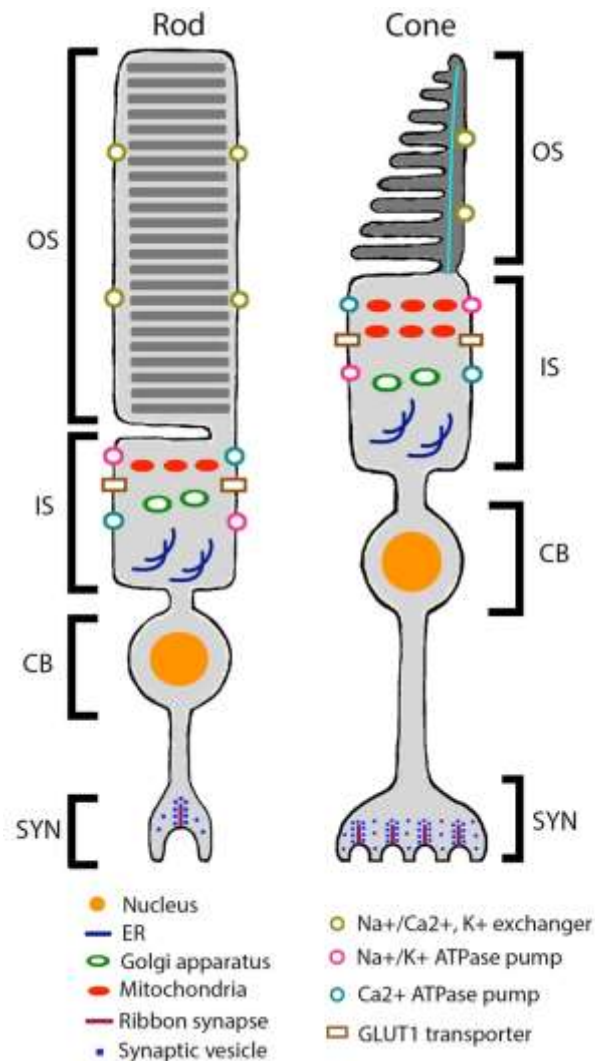


Figure 1.2. Diagram outlining the structural differences between rods and cones. Photoreceptor cells are composed of four structural regions: outer segment (OS), inner segment (IS), cell body (CB), and synaptic terminal (SYN). The Cone OS is conical shaped and shorter than the rod OS. The cone OS is formed by invaginations of the plasma membrane into stacks of membranous discs (dark grey). Cone visual pigments exist as transmembrane proteins in these membranous discs. Cone OS discs are connected to a ciliary membrane (orange) that extends the length of the OS. A ciliary membrane also extends the length of rod OS; however, rod OS discs are not connected to the ciliary membrane. The cone IS contains twice as many mitochondria (pink) as the rod IS. GLUT-1 receptors and ion pumps (green) are heavily concentrated in the IS of both types of photoreceptors.

The different architectures of rod and cone outer segments represent a major distinctive feature of the two photoreceptor cell types. Cones are conical shaped cells and their

outer segments are generally shorter than rods. Like rods, the visual pigments in cones are arranged as discs in the outer segments. Unlike rods, however, the discs in cones are not surrounded by a plasma membrane ³⁶. Instead, they are in free communication with the intracellular space. In addition, rods and cones express different visual pigments. Rhodopsin, which is a G-protein coupled receptor, is the primary light-sensitive visual protein found in rods. Cone visual pigments, however, can be divided into a number of subgroups, which correspond to their absorption spectra, e.g. long wavelength, middle wavelength and short wavelength. Additionally, the structure of synaptic terminals represents another feature that distinguishes rods from cones. Rod synaptic terminals (spherules) contain a single synaptic ribbon that contacts no more than seven or so processes of horizontal or bipolar cells. Each cone synaptic terminal (pedicle) contains many synaptic ribbons and contacts hundreds of such processes.

In all mammalian species, a rich vascular bed lies behind the retina. However, the extent of vascularisation within the retina varies considerably between species ^{37,38}. Mammalian species that contain richly vascularized retinas include humans, monkeys, pigs, cats, cows, mice and rats. Species that contain partially vascularized or avascular retinas include rabbits and guinea pigs. In all species, the retina receives blood supply from two different vascular beds. The inner two-thirds of the retina is nourished by branches from the central retinal vessels. In species with avascular retinas these vessels do not penetrate the inner layers of the retina. The outer third of the retina, including the photoreceptors, is nourished by the choroidal circulation. Nutrients from the choroidal vessels traverse an outer and an inner blood-retinal barrier before reaching the photoreceptors. The outer barrier is the retinal pigment epithelium (RPE) interposed between the choriocapillaries and the outer retina, and the inner barrier is composed of endothelial cells within retinal capillaries ³⁹. Both barriers are mediated by tight junctions, but both cell types have facilitated glucose transporters that permit passive movement of glucose across their plasma membranes ^{40,41}. Vascularization of

the retina influences the distribution of mitochondrion in photoreceptor cells. In avascular retinas, mitochondria are confined to the inner segment of the photoreceptor. In vascularized retinas, mitochondria are also present at the photoreceptor synaptic terminals ^{6,11,35,42-44}.

1.2. Energy Consumption in Photoreceptors

The retina has one of the highest energy demands of any tissue in the human body ^{22,38,45}. Photoreceptors are the most metabolically active cells in the retina and account for the majority of the retinal energy consumption ¹⁻³. Active transport of ions against their concentration and electrical gradients is the largest energy consuming function in all neurons, including photoreceptors ^{2,46-48}. Energy consumption within photoreceptors is compartmentalized and light-dependent (Fig. 1.3).

During illumination, photo-transduction and light adaptation consume energy primarily in the outer segment. In darkness, energy is primarily consumed by ion pumps in the inner segment and by glutamate release at the synaptic terminal. The remainder of energy expenditure is used for the synthesis, recycling and transport of molecules such as visual pigments and neurotransmitters ².

Energy demands and oxygen consumption in photoreceptors are greater in darkness than in light, which is logical since there is an active dark current ^{14,49}. Okawa *et al* ¹ calculated that a single mouse rod consumes 1×10^8 adenosine triphosphate (ATP) per second in darkness and 2×10^7 ATP per second in light, which equates to a four-fold increase in energy consumption in darkness vs. in light. The absolute number of ATP molecules consumed varies among species, age, lifestyle, and time of the day ^{1,2,22}.

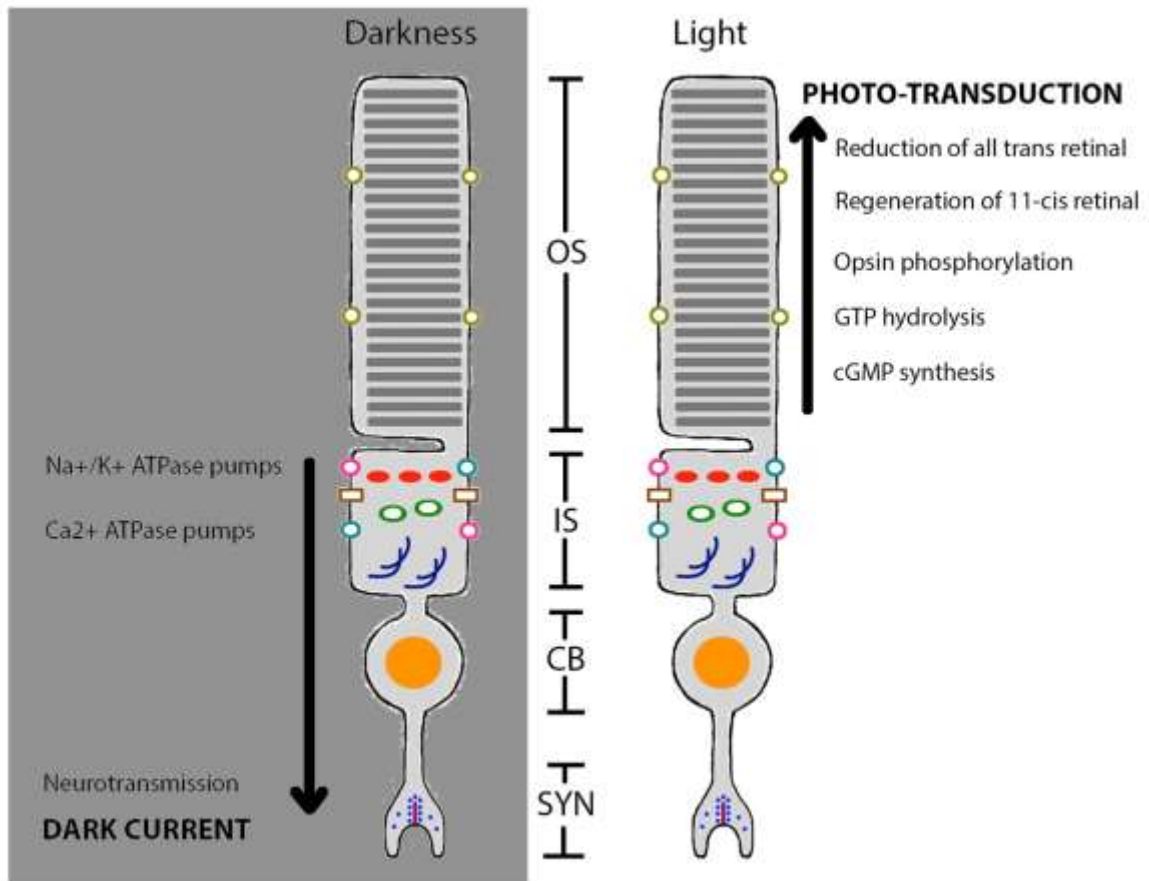


Figure 1.3. Diagram used to demonstrate the compartmentalized model of energy consumption in cones. In darkness, energy is consumed mainly by Na^+/K^+ ATPase pumps and Ca^{2+} ATPase pumps. These ion pumps are present throughout the entire cell and are mostly concentrated in the inner segment. ATP from oxidative phosphorylation is produced by mitochondria in the inner segment to support the ion pumps. In addition, small amounts of ATP are consumed to produce glutamate for release at the synaptic terminal. In light, energy consumption increases in the outer segment to support the reactions involved in photo-transduction. The main ATP consuming processes of photo-transduction are outlined. ATP from glycolysis is produced in the outer segment to support photo-transduction. ATP is also transported from the inner segment to the outer segment via the cilium and also through a phosphocreatine shuttle system to support photo-transduction. OS: outer segment, IS: inner segment, CB: cell body, SYN: synaptic terminal.

In the dark, a steady current flows into open channels in the photoreceptor cell membrane, which partially depolarizes the photoreceptor cell. This “dark current” is composed mainly of the influx of sodium ions (Na^+) and calcium ions (Ca^{2+}) which enter via cyclic guanosine monophosphate (cGMP) gated channels in the photoreceptor outer segment.

Additionally, the photoreceptor inner segments allow efflux of potassium ions (K^+) through K^+ channels, establishing a circular flux of current. Energy required for cGMP synthesis changes as a function of light intensity and totals $< 1 \times 10^7$ ATP per second in mouse rods ¹. The depolarization of the photoreceptor membrane opens voltage gated Ca^{2+} channels in the synaptic terminal. The increased intracellular concentration of Ca^{2+} causes neurotransmitter containing vesicles to merge with the cell membrane releasing glutamate into the synaptic cleft.

In the dark, excess Na^+ ions are removed via Na^+/K^+ ATPase pumps in the inner segment. Excess Ca^{2+} ions are removed by Na^+/Ca^{2+} , K^+ exchangers in the outer segment and Ca^{2+} ATPase pumps in the inner segment and synaptic terminal ⁵⁰. Na^+/K^+ ATPase consumes 1 ATP for every 3 Na^+ pumped out in exchange for 2 K^+ brought into the cell. Ca^{2+} ATPase consumes 1 ATP for every Ca^{2+} pumped out of the cell. Na^+/Ca^{2+} , K^+ exchanges 1 Ca^{2+} and 1 K^+ in exchange for 4 Na^+ out of the cell. In mouse rods, Na^+ pumping consumes 5.7×10^7 ATP per second and Ca^{2+} pumping consumes 3×10^7 ATP per second during darkness ¹. The rate of ATP consumption required for the synthesis of glutamate and recycling into synaptic vesicles is $2 - 4 \times 10^6$ ATP per second in darkness and decreases in light ^{1,51}. This is only a small fraction of the ATP needed for ion pumping.

Although the amplitude and voltage-dependence of the dark current is similar in rods and cones ^{52,53}, cone synapses expend more energy than rod synapses ⁶. Mammalian cone pedicles contain several times more ribbons than rod spherules. For example, cone pedicles contain 10 times more ribbons than rod spherules in mice and 50 times more in primates. Accordingly, cone pedicles contain more vesicles for glutamate uptake and priming and more Ca^{2+} ATPase pumps for removing Ca^{2+} ⁶.

During illumination, photoreceptors are actively engaged in photo-transduction. The process of photo-transduction occurs in a stepwise process shared by rods and cones ⁵⁴. The

visual pigments in photoreceptors consist of two parts, a membrane protein called an opsin, and a chromophore (11-cis-retinal). Light induces the isomerisation of 11-cis-retinal to all-trans-retinal. The photo-activated visual pigment molecule catalyses the activation of the G-protein transducin. Phosphorylation of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) activates the α -subunit of transducin which then dissociates and activates phosphodiesterase (PDE) by removing two regulatory (γ) subunits. The activated PDE then hydrolyses cGMP to 5'-GMP. A decrease in cGMP concentration leads to the closing of ion channels in the outer segments meaning that photoreceptor cells hyperpolarize in response to light. This leads to inhibition of neurotransmitter release, and signals the adjacent neurons of the light stimulus. After the photo-transduction cascade is underway, the photo-activated visual pigment molecule undergoes decay, releasing all-trans-retinal which is reduced by retinol dehydrogenase and its cofactor nicotinamide adenine dinucleotide phosphate (NADPH) to all-trans-retinol. The formation of all-trans-retinol proceeds at a rate over 100 times faster in mouse cones compared to rods⁵⁵. All-trans-retinol is transported to the RPE, where it is isomerised and oxidized back to 11-cis-retinal, which is recycled to photoreceptor cells. Additionally, GTP is hydrolysed back to GDP in preparation for the next cycle, and opsin is phosphorylated by opsin kinase allowing its association with arrestin which causes its own inactivation.

All of these processes involve GTP or ATP. The total amount of ATP consumed during photo-transduction is still relatively small compared to that of the dark current¹. For example, in mouse rods the conversion of GTP to GDP consumes $< 3 \times 10^6$ ATP per second and opsin phosphorylation consumes 6×10^5 ATP per second¹. The energy consuming processes in photo-transduction occur primarily in the outer segments where the visual pigments are located.

Cones consume more energy than rods during photo-transduction. Na^+ influx through cGMP-gated channels in cones does not fall below half that in rods during darkness^{1,56}. Also,

the turnover number of transducin is at least twice as great in cones as that in rods, opsin kinase activity is also much higher in cones, and cones depolarize more frequently than rods^{1,56}. The higher energy requirements of cones are also facilitated by a greater quantity of oxidative machinery. For example, mouse cones possess twice as many mitochondria as rods⁵⁷, and monkey cone inner segments contain approximately 10 times more mitochondria than monkey rod inner segments⁵⁸.

In addition to the dark current and the process of photo-transduction, ATP in photoreceptors is also used for active transport of intracellular cargo by kinesin and dynein from the inner segments to the outer segments. Kinesin cycles its conformation approximately 100 times per second, hydrolysing 1 ATP per cycle^{59,60}. Additionally, the phosphocreatine shuttle system is important in reversibly converting creatine and ATP to phosphocreatine and ADP which is transported to the photoreceptor synaptic terminal to provide energy for neurotransmission^{2,5,44,61}.

1.3. Glucose Metabolism in Photoreceptors

1.3.1 Glucose Delivery to the Retina

Glucose is the preferred energy substrate for the retina and is catabolized intracellularly to generate energy for metabolism in the form of ATP^{15,16,62}. When glucose is scarce, photoreceptors are known to have the capacity to take up and metabolize lactate, which is itself a product of glucose catabolism^{21,63}. In avascular guinea pig retinas, the neighbouring Muller glial cells represent a potential source for this lactate²¹. However, there is controversy as to whether photoreceptors in vascularized retinas metabolize significant amounts of lactate under normal physiological conditions⁶⁴⁻⁶⁶.

Glucose entry into the retina is mediated by a facilitated transport process involving members of the sodium-independent glucose transporter (GLUT) family. The various glucose

transporter isoforms demonstrate, to a certain degree, tissue specific localisation. Glucose entry into photoreceptors is crucially dependent on GLUT-1, which is the most widely expressed member of the GLUT family^{41,67-71}. GLUT-1 transports glucose from the choroidal vasculature to the outer retina across the blood–retina barrier formed by the RPE. The apical and basolateral membranes of RPE cells and the luminal and contraluminal plasma membranes of retinal vascular endothelial cells contain GLUT-1 transporters^{72,73}. GLUT-1 also mediates glucose uptake by the photoreceptors themselves^{13,41,68}. GLUT-1 transporters are concentrated in the inner segments in rat and mouse photoreceptors^{13,68}. GLUT-1 has also been found in human photoreceptor outer segments⁴¹. In cones, GLUT-1 function is dependent on a trophic factor produced by rods²⁸. Rod-derived cone viability factor (RdCVF) is released by rods and interacts with a complex formed by basigin-1 and GLUT-1 on the cell surface of cones, which accelerates intracellular glucose uptake in the latter cells²⁸. In addition to GLUT-1, GLUT-2 is present at the apical ends of Muller cells and appears to be involved in transport of glucose within the retina. GLUT-3, which is the primary neuronal GLUT in the mammalian central nervous system (CNS), is located in the inner and outer plexiform layers, as well as the inner nuclear layer and the ganglion cell layer of the human retina and rat retina^{41,74}. Additionally, rat photoreceptors possess GLUT-4 transporters which are regulated by insulin⁷⁵. However, GLUT4 has not yet been detected in the human retina^{41,76}.

1.3.2 Pathways of Glucose Metabolism in Photoreceptors

The metabolism of glucose in photoreceptors supports several key activities that are essential for photoreceptor survival. ATP production is an important outcome of glucose metabolism. Intracellular ATP is generated via two related metabolic pathways: oxidative phosphorylation and glycolysis^{22,77}. The energetic production advantage of oxidative phosphorylation in the mitochondria far outweighs that of glycolysis in the cytosol: one molecule of glucose yields only two net molecules of ATP via the glycolysis pathway, but 34 net molecules of ATP via

the oxidative pathway. In most cells, pyruvate, which is the end product of glycolytic catabolism of glucose, is converted to lactate when oxygen is scarce (anaerobic glycolysis). However, cultured retinal explants produce large amounts of lactate even under aerobic conditions ⁷⁸. This phenomenon is known as aerobic glycolysis and eponymously as the Warburg effect.

Oxygen consumption generally parallels ATP consumption in photoreceptors. Like ATP consumption, photoreceptor oxygen consumption is greater in the dark than in light ^{8,79}. In pigs, the inner retina metabolises 21% of glucose via glycolysis and 69% via oxidative phosphorylation ^{24,25}. In contrast, the pig outer retina metabolises 61% of glucose via glycolysis and 12% via oxidative phosphorylation ^{24,25}. In the cone-dominant ground squirrel retina, up to 95% of glucose is metabolised via glycolysis ⁸⁰. Despite the high rates of glycolysis, ATP production from this pathway constitutes only 16% of available ATP levels in rabbit photoreceptors in both darkness and light ^{2,22}. ATP production from oxidative phosphorylation constitutes 84% of the available ATP in rabbit photoreceptors in darkness and 61% in the light ^{2,22}.

Photoreceptors are thought to be responsible for the majority of glycolysis in the mammalian retina. This is supported by the fact that photoreceptors are particularly susceptible to glycolytic inhibition, and, in rat retinas that lack photoreceptor cells, there is greater than 50% reduction in the overall glycolytic activity compared to the normal rat retina ⁸¹. Additionally, expression of lactate dehydrogenase-A (LDH-A), which catalyses the conversion of pyruvate to lactate and serves as a biomarker for glycolysis, is also reduced in rat retinas that lack photoreceptors ⁸².

Photoreceptors are highly polarized cells that sequester key biochemical reactions in anatomically distinct compartments ¹. For example, the Na⁺/K⁺ ATPase pumps are the most metabolically active ion pumps involved in maintaining the dark current and they are

concentrated in the photoreceptor inner segment. The inner segments maintain the highest capacity for glycolysis, oxidative phosphorylation and ATP buffering. Glycolytic enzymes, such as hexokinase, are mostly localised to the photoreceptor inner segment ⁵. The inner segment also contains the largest number of mitochondria compared to other photoreceptor compartments ^{13,83}.

In the dark, energy is also required for glutamate synthesis and recycling in the synaptic terminal. In species with vascularized retinas, mitochondria are present at the synaptic terminal to provide ATP for neurotransmission ⁴²⁻⁴⁴. In contrast, species with avascular retinas have all their mitochondria in the inner segment ⁸⁴. In avascular retinas, ATP made in the inner segment may not reach the synapse because, during transit, it is exposed to the highly active Na⁺/K⁺ ATPase pumps in the plasma membrane between the mitochondria and the synaptic terminal ^{5,34}. Photoreceptors in avascular retinas use a unique type of energy shuttle to solve this problem ⁴⁴. They use phosphocreatine rather than ATP to deliver high-energy phosphate from the centrally located mitochondria to the synaptic terminal. ⁵.

Many of the energy-consuming reactions that occur in light take place in the photoreceptor outer segment. Since the outer segment is devoid of mitochondria and lacks GLUT-1 transporters, glucose metabolites must be transported from the inner segment to the outer segment to sustain energy-consuming reactions. However, there is limited experimental data to explain this process. One possibility is that ATP molecules generated in the inner segments diffuse to the outer segments via the connecting cilium. Another possibility is provided by the phosphocreatine shuttle pathway, which may transfer high energy phosphate groups to the outer segment. Additionally, Hsu and Molday ^{69,85,86} have suggested that outer segments might possess the ability to meet some of their own energy demands. Their studies show outer segment lactate production and suggest the presence of glycolytic enzymes in outer segments ^{69,85,86}. However, their outer segment preparations were likely contaminated with inner segments, diminishing the validity of results.

The reliance of photoreceptors on aerobic glycolysis is highly unusual. The metabolism of photoreceptors more closely resembles that of rapidly proliferating or dividing cells instead of other neurons ^{5,13,77}. Since photoreceptors have high-energy demands, it is unclear why they metabolise the majority of available glucose using aerobic glycolysis, which produces less net energy than oxidative phosphorylation. One possible explanation for this phenomenon is that, apart from using glucose for energy production, photoreceptors may also use glucose to produce metabolic intermediates for amino acid biosynthesis ^{13,87,88}. Rapidly proliferating neoplastic cells also use this strategy. Provided that the energy supply is sufficient, glycolytic metabolites become diverted towards biosynthesis rather than towards oxidative phosphorylation ⁸⁹. The molecular switch controlling glycolytic flow is thought to be the M2 isoenzyme of pyruvate kinase (PKM2). PKM1 and PKM2 are both present in the mouse retina. However, the outer retina contains only PKM2. This implicates photoreceptors as a primary site of glycolysis in the retina ^{13,84,90}.

Adult mammalian photoreceptors are non-proliferative; however, they possess high biosynthesis requirements due to the prodigious turnover of the visual pigments in the disc membranes of the outer segments ^{77,91,92}. Both rod and cone outer segments are renewed in an orderly fashion, as first revealed by autoradiographic studies in which radioactive amino acids became trapped in new membranous discs generated at the outer segment base, then moved towards the photoreceptor tip, and were finally phagocytosed by the RPE ^{91,92}.

The visual pigments in photoreceptor outer segments share several common amino acid motifs. Their distinct molecular properties arise from differences in the residues at positions 122 and 189 of the amino acid sequence ⁹³⁻⁹⁵. Many of the amino acids used to form visual pigments, such as serine and glycine, can be produced through glycolysis. Glucose can be directed into the pentose phosphate pathway to generate nucleotides and NADPH. We believe that glucose metabolism might be used to synthesize the amino acids, serine and glycine, branching from glycolysis at phosphoglycerate ^{89,96-98}. Hence, glycolytic

intermediates may be incorporated into the production of visual pigments. This hypothesis is tested in Experiment One.

Glucose metabolism in photoreceptors serves a number of other functions in addition to ATP production and visual pigment synthesis. Glucose contributes to synthesis of N-acetylglucosamine, which is required for asparagine N-linked protein glycosylation and subsequent trafficking to the cell surface ^{4,17}. Proper trafficking of transporters and growth factor receptors is required for glucose uptake and cellular responses to pro-survival signals ^{17,20}. Glucose is also needed for production of cytosolic NADPH which is required for the reduction of 11-cis-retinal to all-trans-retinol. NADPH also helps to inhibit caspase-mediated apoptosis, support anabolic activity, and maintain appropriate levels of reactive oxygen species ^{12,18,19,99}.

1.4. Creatine Metabolism in Photoreceptors

Another source of energy for photoreceptors is provided by the metabolism of creatine ^{5,44}. Creatine is synthesized in the kidney and liver from L-arginine and glycine. Additional creatine can be absorbed from diet via the intestine. Creatine exists intracellularly both as free creatine and phosphocreatine which together comprise the total creatine pool. The highest levels of creatine and phosphocreatine are found in skeletal muscle, cardiac muscle, spermatozoa and photoreceptors ¹⁰⁰.

Creatine entry across the blood–retina barrier is mediated by a specific plasma membrane creatine transporter (CRT) ¹⁰¹⁻¹⁰³. CRT is related to the gamma-aminobutyric acid and norepinephrine transporters and is part of a large gene family known as solute carrier family 6. Like GLUT-1, CRT is expressed in the luminal and contraluminal membranes of retinal vascular endothelial cells ^{102,103}. CRT is also expressed by most neurons in the mammalian retina, including photoreceptors ^{104,105}.

Intracellular creatine is reversibly phosphorylated to produce phosphocreatine in an anaerobic reaction catalysed by creatine kinase (CK) (Fig. 1.4). Phosphocreatine can then be converted back to creatine which liberates high-energy phosphate groups to regenerate ATP from ADP ^{106,107} (Fig. 1.4). CK is abundantly expressed in cells with high energy requirements such as skeletal muscle, cardiac muscle, spermatozoa and photoreceptors ^{100,108}. CK exists in a number of isoforms which are encoded by separate nuclear genes. In most tissues, a single cytosolic CK isoform is co-expressed with a single mitochondrial CK isoform. The mitochondrial CK isoform synthesizes phosphocreatine from ATP made by mitochondria. The cytosolic CK isoform transfers the high energy phosphate from phosphocreatine back to ATP at sites where ATP is needed. CK isoforms are localized differentially on a subcellular level and these specific locations are essential for the functioning of the CK network.



Figure 1.4. Creatine is phosphorylated to phosphocreatine in a reversible chemical reaction catalyzed by the enzyme creatine kinase (CK). ATP: adenosine triphosphate, ADP: adenosine diphosphate.

Cytosolic CK consists of two subunits, which can be either *B* (brain type) or *M* (muscle type). There are, therefore, three different cytosolic CK isoforms: CK-MM, CK-MB and CK-BB. CK-BB is the main isoform expressed in the retina ¹⁰⁹. In photoreceptors, CK-BB is present in inner segments and synaptic terminals and mitochondrial CK is localized to inner segments ⁵. As previously outlined, it has been suggested that a phosphocreatine shuttle pathway transports high-energy phosphate groups from the inner segment to the synaptic terminal in avascular retinas ^{13,44,104}. Mitochondrial CK can use ATP to phosphorylate creatine to generate phosphocreatine in the inner segment. After the phosphocreatine reaches the synapse, CK-BB can transfer the stored energy from phosphocreatine back to ATP to fuel

neurotransmission ⁵.

The phosphocreatine and CK energy pathway represents an efficient energy buffering system. Phosphocreatine has a slightly higher diffusion capacity than ATP making phosphocreatine transport a more efficient energy delivery system to different cellular locations. Also, subcellular localization of cytosolic and mitochondrial CK couples areas of energy generation with energy production. Thus, the phosphocreatine and CK energy pathway serves as a spatial energy circuit within the cell ¹⁰⁸. This is particularly important in photoreceptors where energy consumption is compartmentalized and light-dependent. Additionally, the phosphocreatine and CK energy pathway allows reduction of peak rates of ATP synthesis. In cells that alternate between periods of high and low energy consumption, this facilitates repayment of energy “debt” incurred during periods of high energy demand to occur during periods of low energy consumption.

1.5. Bio-energetic Neuroprotection in the Retina

Bio-energetic based neuroprotection refers to the concept of protecting injured and threatened neurons by increasing their available energy supply. This strategy is based on the notion that if energy failure is part of the pathogenesis of a disease, then energy delivery may be a potential therapy. Bio-energetic based neuroprotection has achieved considerable success in experimental models of several common debilitating neurological conditions, including Huntington’s disease ^{110,111} and Parkinson’s disease ¹¹¹⁻¹¹⁴.

Previous studies from our research group have demonstrated that providing glucose to retinal neurons can afford a robust neuroprotective effect in models of injury featuring oxygen and/or nutrient deprivation ¹¹⁵⁻¹²¹. Specifically, chronically elevating vitreous glucose levels *in vivo* produced a substantial degree of protection against acute ischemic injury ¹¹⁶, chronic ischemic injury ¹¹⁵ and experimental glaucoma ¹¹⁷. Local delivery of glucose to the eye by

subconjunctival injection also protected against acute ischemic injury ¹¹⁹. Further, glucose exerts a dose-dependent protective effect against retinal neuronal death in a model of respiratory inhibition *in vitro* ^{120,121}. Excitingly, in two recent double blind randomized human clinical trials, Casson and colleagues ^{118,119} showed that local delivery of glucose, either topically or by subconjunctival injection, temporarily recovered contrast sensitivity in patients with severe primary open-angle glaucoma.

Creatine supplementation has also been shown to be efficacious in a variety of animal and cellular models ¹²² of neurodegenerative diseases including Alzheimer's disease ¹²³, Parkinson's disease ¹²⁴, amyotrophic lateral sclerosis ¹²⁵, and Huntington disease ¹²⁶⁻¹²⁸. In these studies, it was argued that the beneficial effects of creatine treatment were mediated through improvement in the overall bioenergetics by increasing the phosphocreatine pool, enhancing mitochondrial function and reducing susceptibility to mitochondrial-mediated apoptosis that is associated with each particular neurodegenerative disease ¹²⁹.

Since photoreceptors account for a large portion of the energy consumption in the retina, it seems logical that diseases of photoreceptor degeneration likely involve a component of energy failure. Therefore, in Experiment Three we examine the effect of local glucose treatment and systemic creatine treatment in an animal model of photoreceptor degeneration.

1.6. Retinitis Pigmentosa

Photoreceptor degeneration causes blindness in a variety of human retinal diseases including RP, diabetic retinopathy and age-related macular degeneration ¹³⁰⁻¹³². RP comprises a complex group of hereditary retinal dystrophies that feature degeneration of rod and cone photoreceptors. RP is the most commonly encountered hereditary retinal dystrophy with a worldwide prevalence of approximately 1 in 4000 and affects a total of more than 1 million individuals ¹³³⁻¹³⁷. The disease can be inherited as an autosomal-recessive (about 50 – 60% of

cases), autosomal-dominant (30 – 40%), or X-linked (5 – 15%) trait ¹³⁸⁻¹⁴⁰. Investigators have identified at least 45 loci at which mutations cause the disorder, and these genes collectively account for disease in approximately half of all patients ¹³⁰. At present, RP is an incurable disease and all affected patients develop blindness. Vitamin A supplementation is the only current therapy that has been shown to delay visual decline in a human clinical trial ¹³⁷.

An early clinical feature of RP is night blindness, often starting in adolescence, followed by progressive loss of peripheral vision and in many cases culminating in loss of central vision and complete blindness in mid-life. These visual symptoms reflect the gradual degeneration of the two photoreceptor types: rods, which mediate achromatic night vision, followed by the tightly-packed cones, which are critical for high acuity central vision. In all forms of RP the genetic mutations are exclusively expressed in rods; however, the disease is characterized by the death of both rods and cones. In contrast, mutations in genes that are expressed in cones typically only lead to degeneration of cones without affecting rods ¹⁴¹⁻¹⁴⁷. Thus, the secondary death of cones in RP remains somewhat mysterious.

In all diseases of photoreceptor degeneration, the main cause of clinically significant vision loss is cone cell degeneration rather than rod cell death ³⁶. People with night blindness can lead a normal life, especially in industrialised countries, because they can still see satisfactorily despite the loss of rods. Thus, prevention of cone cell loss is a major goal of therapeutic strategies in RP ¹⁴⁸. Knowledge of the causal genes in RP and their associated biochemical defects is increasing, which has led to optimism that novel treatments will soon be developed ¹³⁰. Many mechanistically diverse approaches to treat RP are being investigated. These include gene-specific approaches ¹⁴⁹⁻¹⁶⁰ as well as interventions in secondary biochemical pathways ^{27-29,161-163}. Gene-specific approaches are dependent on the type of mutation, and require early disease detection. Interventions in secondary biochemical pathways might benefit groups of patients with various gene defects and could remain efficacious even when commenced later during the disease process. A better understanding of

the mechanisms that cause cone degeneration in RP could lead to novel treatments that spare cones and revolutionize the devastating natural history of the disease ^{161,162}.

Several animal models are available for studying RP ¹⁶⁴. Many of the genetic mutations that result in retinal degeneration in animals have been found to be similarly abnormal in the human. Therefore, these animal models both mimic the human disease genetically and produce a similar functional phenotype ¹⁶⁵. As a consequence, much of our understanding of the mechanisms leading to cone degeneration in RP has been derived from animal models. In Experiment Two, we characterize the temporal and spatial distribution of cone degeneration in a commonly used animal model for RP.

1.7. Mechanisms of Cone Degeneration in Retinitis Pigmentosa

1.7.1 Trophic Factors

Trophic factors can broadly be defined as endogenously synthesized molecules that stimulate growth, maturation and nourishment of developing and mature neurons. Numerous studies, using *in vivo* and *ex vivo* (organotypic/explant cultures) models of retinal degeneration, have demonstrated that trophic factor supplementation can delay or prevent photoreceptor cell death ¹⁶⁶. Yet, the overwhelming majority of studies have focused on survival of rods rather than cones. Assessment of cone viability in commonly used retinal degeneration models is more problematic because cone loss, as compared to that of rods, evolves over a much longer period of time. Accordingly, appreciably less is known about the effectiveness of different trophic factor therapies on the rate of cone degeneration.

Early work by LaVail and colleagues ¹⁶⁷ showed that classical neuroprotective substances, including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), fibroblast growth factor-2 (FGF-2), neurotrophin-4, and combinations thereof, all failed to promote cone survival in the retinal degeneration-1 (Rd1) mouse. The authors

postulated that the long post-injection time period prior to analysis may have contributed to the negative results. Other *in vivo* studies have shown more positive results; for example, long-term expression of glial-derived neurotrophic factor (GDNF) increased cone density and the photopic electroretinogram (ERG) b-wave amplitude in Rd10 mice compared with controls¹⁶⁸, while subretinal transplantation of neurotrophin-secreting Schwann cells preserved cone density in rhodopsin knockout mice¹⁶⁹. Promising data have also been generated using *ex vivo* models. Thus, FGF-2 and epidermal growth factors boosted cone survival in adult porcine photoreceptor cultures¹⁷⁰, while, in retinal explants from rhodopsin knockout mice, GDNF and CNTF likewise increased cone viability¹⁷¹.

Arguably the most exciting development in unravelling the role that trophic factors play in cone survival has been the discovery that normal rods secrete survival factors that are essential for the maintenance of healthy cones^{28,161,172-175}. Cone cell death ensues when these factors are no longer available as a result of the progressive loss of rods^{172,173,175}. Mohand-Said *et al* presented the first evidence that cone survival in RP depends specifically on rods and could potentially be mediated by diffusible trophic factors. They demonstrated that cone loss in Rd mice, whose retinas were devoid of rods, was significantly diminished *in vitro* by co-culture with rod-rich retinal tissue from normal-sighted mice¹⁷². Further study showed that rods removed from normal-sighted mice and transplanted into the subretinal space of Rd mice promoted the rescue of a large percentage of cones, which otherwise were destined to die in the two-week period following surgery¹⁷⁴.

Subsequently, Leveillard *et al*¹⁶¹ identified a trophic factor produced by healthy rods that promotes survival of cones. This factor has been named rod-derived cone viability factor (RdCVF). Two isoforms of rod-derived cone viability factor have been identified: RdCVF and RdCVFL. The truncated form (RdCVF) is a thioredoxin-like protein that lacks thioredoxin reductase activity and promotes cone survival, while the full-length isoform (RdCVFL) contains a thioredoxin fold and is involved in oxidative signaling and protection

against hyperoxia, but does not promote cone survival ¹⁷⁶. RdCVF was identified by high content screening of a mouse retinal complementary deoxyribonucleic acid (cDNA) library on cone-enriched cultures from chicken embryos. RdCVF slowed cone degeneration in a dose-dependent manner in both organotypic mouse and chicken models. Furthermore, subretinal RdCVF administration in retinal degeneration mice decreased cone loss by 40% compared to control injected eyes over a 2-week period. Reverse-transcription polymerase chain reaction (RT-PCR) showed RdCVF expression was decreased in rod-deficient mice and immunohistochemistry with antibodies to RdCVF showed specific staining on cone outer segments and the inter-photoreceptor matrix in normal-sighted mice, suggesting the presence of an RdCVF cell surface receptor on cones.

Ait-Ali *et al* ²⁸ recently discovered that the cell surface receptor for RdCVF is Basigin-1 (BSG-1), a transmembrane protein expressed by cones. Using co-immunoprecipitation followed by mass spectrometry and fluorescence resonance energy transfer assay, they found that BSG-1 binds to GLUT-1, which is the main glucose transporter in the retina ¹⁷⁷. This allows increased glucose entry into cones and promotes their survival by stimulating aerobic glycolysis. Thus, the downstream effects of the trophic factor RdCVF are to increase the availability of energy substrates to cones. Depriving cones of this trophic factor can result in energy failure and cellular death (Fig. 1). Interestingly, the idea that cones suffer from energy failure after rods have degenerated was previously proposed as a separate mechanism for cone degeneration in RP (see below); however, it now seems the two theories are closely related. The studies on RdCVF provide a new explanation for the biochemical basis for the previously described paracrine interaction between rods and cones ¹⁷². One of the implications of the identification of the RdCVF receptor BSG-1 and its binding partner GLUT-1 is the potential for developing therapies that could activate them and consequently preserve cones in RP ¹⁷⁸.

1.7.2. Cone Starvation

The recent discovery of RdCVF and its mechanism of action provides support for an alternative hypothesis to explain cone degeneration in RP advocated by Punzo *et al* ²⁷, which argues that cone degeneration in RP occurs secondary to nutrient deficiency. They found that genes involved in cellular metabolism, in particular those belonging to the mechanistic target of rapamycin (mTOR) signaling pathway, were downregulated in numerous mouse models of RP. mTOR is a serine-threonine kinase that integrates intracellular responses to trophic factors, mitogens, hormones, stress, nutrients and the energy status of the cell. Under normal conditions, activated mTOR phosphorylates a number of substrates that potentiate anabolic processes such as messenger ribonucleic acid (mRNA) translation and lipid synthesis, and also limit catabolic processes such as autophagy. Under stress conditions, such as diminished nutrient availability or hypoxia, mTOR shifts the balance in the opposite direction. mTOR is the catalytic subunit of two structurally distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Punzo *et al* ²⁷ suggested that reduced levels of active mTOR in RP reflect poor nutrient conditions, leading to activation of autophagy within cones, a process whereby intracellular proteins and organelles are reabsorbed in vacuole-like structures to retrieve cellular nutrients. In this way, nutrient deficiency results in cone death. The hypothesis is supported by the findings from another study by the same group where it was observed that constitutive activation of mTORC1 in cones maintained cone function and promoted long-term cone survival ²⁹.

The “cone starvation” hypothesis further attempts to provide an explanation for the progressive and delayed nature of cone degeneration in RP. In the healthy retina, the RPE facilitates nutritional support and oxygen to the retina, while continually phagocytosing the shed outer photoreceptor segments. This process is made possible by the large number of contacts that are established between the RPE and the outer segments of the rods. When rod loss exceeds a critical threshold, RPE-cone outer segment interactions are irreversibly

disrupted, compromising the uptake of nutrients to cones. This explanation is supported by the observation in four different mouse models of RP that cone death starts when rods are reduced to a single row of cells ²⁷. The hypothesis also predicts that if cones dominated the retina and were primarily genetically affected (i.e. the reverse of the human situation), then initial cone degeneration would eventually be followed by late-stage rod degeneration. In fact, in zebrafish, where the overall ratio of cones versus rods is reversed, a mutation in a cone-specific phosphodiesterase leads to cone degeneration that is associated with secondary rod loss in regions of low rod concentration ¹⁷⁹.

Punzo *et al* ²⁷ also discovered that glucose depletion in cones was associated with upregulation of GLUT-1 and hypoxia inducible factor 1-alpha (HIF-1 α). The upregulation of both factors is consistent with a response in cones to overcome a shortage of glucose. Given the recent observations of Ait-Ali *et al* ²⁸, the upregulation of GLUT-1 likely reflects a compensatory response to decreased levels of RdCVF that occurs secondary to rod degeneration. In this regard, it is important to note that although BSG-1 increased the function of GLUT-1, it did not increase the concentration of GLUT-1 receptors on the cell surface ²⁸.

Overall, the theories proposed by Punzo *et al* ²⁷ and Ait-Ali *et al* ²⁸ both support the idea that in RP, cones ultimately die from starvation via a mechanism that involves compromised glucose uptake (Fig. 1.5). The combined results of these studies suggest that accelerating glucose entry into the cell is a convergence point for different pathways, such as RdCVF and mTOR, which protect cones.

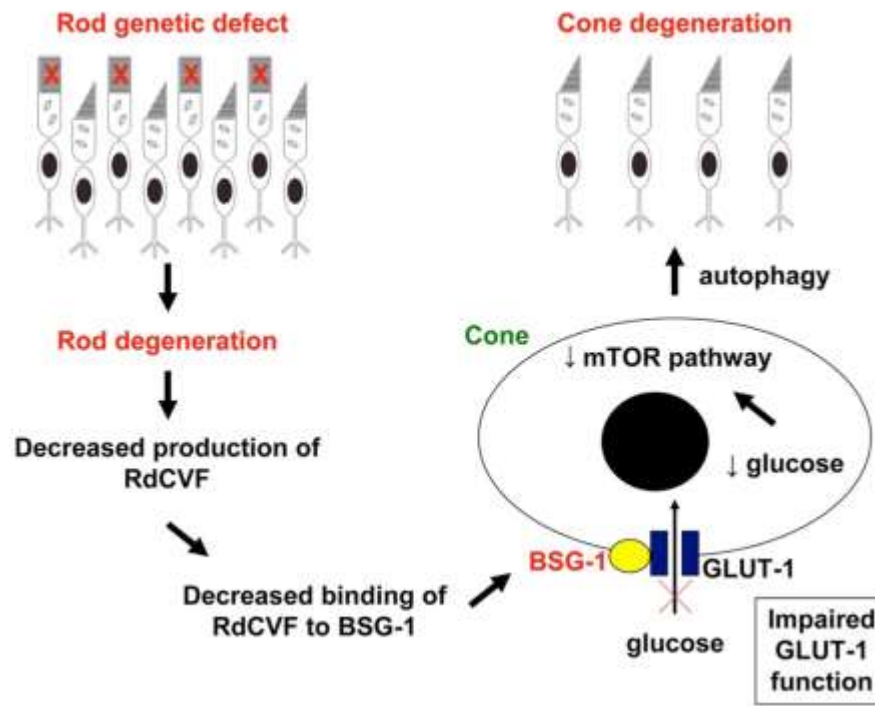


Figure 1.5. Diagram of the proposed mechanism that explains the relationship between RdCVF deficiency and nutrient deficiency causing cone death in RP. The decreased production of RdCVF that occurs after rod degeneration leads to decreased binding of BSG-1 to cones and impaired functioning of GLUT-1. This results in decreased entry of glucose into cone cells. The resulting intracellular nutrient deficiency causes reduced activation of mTOR, leading to autophagy of cones.

1.7.3. Oxidative Stress

Oxidative stress, which represents an imbalance between the production and elimination of reactive oxygen species (ROS), has also been suggested to play a role in cone degeneration in RP¹⁸⁰. Oxidative stress has a potentially pathogenic role in many neurodegenerative CNS diseases¹⁸¹⁻¹⁹⁰. Rods comprise approximately 95% of the photoreceptor population and consume the majority of oxygen delivered to the outer retina. The choroidal vessels that supply the outer retina are not subject to autoregulation by tissue oxygen levels, hence, as death of rods occurs in RP, the level of oxygen within the retina increases^{147,191}. It follows that the oxygen level per cone increases sharply as rods degenerate. The high levels of oxygen cause activation of NADPH oxidase in cones, which increases the production of superoxide

radicals ¹⁹². The excess superoxide radicals generate other ROS, for example peroxynitrite, which attacks lipids, proteins, and DNA ¹⁸⁰. Normally, photoreceptor inner segments utilize endogenous antioxidant pathways, such as superoxide dismutases (SODs), to clear ROS ¹⁹³. Additionally, photoreceptor outer segments clear oxidized proteins and lipids through daily outer disc shedding and renewal ¹⁹⁴. However, when rods degenerate, cones are faced with increasing production of ROS. As a consequence, their antioxidant defense systems gradually become overwhelmed leading to cellular damage.

In addition to the hazard presented by surplus oxygen, cones are under constant environmental challenges that make them highly susceptible to oxidative stress. Their function as light sensors necessitates that they are constantly exposed to ultraviolet radiation from sunlight, which can induce formation of ROS ^{195,196}. Also, the high metabolic rate of cone photoreceptor cells *per se* ²² presents an intrinsic risk factor for oxidative damage, since ROS are formed as a natural by-product of mitochondrial metabolism. Cones contain twice as many mitochondria as rods in the mouse retina and ten times as many in the primate retina ^{57,58}, which renders them particularly susceptible to oxidative stress.

The oxidative stress theory (Fig. 1.6) proposes that the progressive death of cones in RP, which occurs secondary to rod loss, is due to accumulated oxidative damage. The hypothesis is consistent with observations that relative cell density is a crucial determinant of cone death ^{145,179,197}. Irrespective of whether oxidative stress is the principal trigger of cone loss, there is an increasing body of data suggesting that oxidative damage contributes to cone death in animal models of RP. Shen *et al* ¹⁸⁰ observed strong immunolabelling of acrolein and 4-hydroxynonenal-adducts, which are biomarkers of oxidative damage, in a transgenic pig model of RP. Recently, Campochiaro *et al* ¹⁹⁸ found that intraocular levels of the antioxidant protein glutathione were decreased, and the oxidized form, glutathione disulfide, were elevated in human patients with RP. This indirectly suggests the presence of high levels of intraocular ROS in patients with RP.

A number of recent studies have also reported cone-preserving effects of exogenous antioxidant treatment in animal models of RP^{163,199-202}. These findings lend weight to the oxidative stress hypothesis as a cause of cone death in RP. Komeima *et al*^{163,199} demonstrated that daily systemic administration of a mixture of exogenous antioxidants slowed cone death in several mouse models of RP. Lee *et al*²⁰⁰ also demonstrated that oral and topical administration of N-acetylcysteine preserved cones by reducing oxidative damage in Rd1 and Rd10 mice. Usui *et al*²⁰¹ found that inducible co-expression of SOD2 and catalase in transgenic Rd1 and Rd10 mice delayed cone death. However, overexpression of SOD1 accelerated cone death, suggesting a possible deleterious effect of some types of antioxidant proteins in RP. Xiong *et al*²⁰² used adeno-associated virus (AAV) vectors to deliver SOD2, catalase, peroxisome proliferator-activated receptor γ coactivator 1- α (PGC1 α) and nuclear factor erythroid-derived 2-like 2 (NRF2) in combination or separately in multiple mouse models of RP. PGC1 α and NRF2 are transcription factors that regulate the expression of antioxidant proteins^{203,204}. Overexpression of NRF2 alone exhibited the most potent rescue effect, while overexpression of PGC1 α had an unexpected detrimental effect.

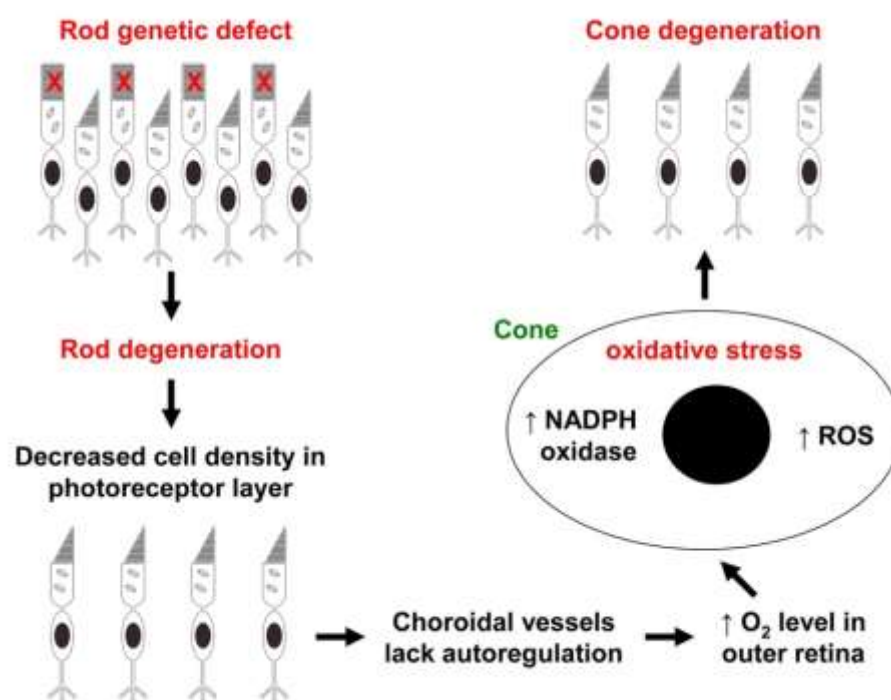


Figure 1.6. Diagram of the suggested mechanism to explain the role of oxidative stress on cone death in RP.

1.7.4. Microglial Activation

Microglia comprise the resident immunocompetent cells of the CNS. They are present as perivascular cells in the inner layers of healthy retinas²⁰⁵. Microglia respond rapidly to disruptions of tissue homeostasis by proliferating, assuming an amoeboid morphology, migrating to the site of injury and phagocytosing cellular debris. Activated microglia possess both neuroprotective and neurodestructive properties. The neurodestructive properties of activated microglia are believed to occur due to loss of the beneficial functions and/or a shift to a pro-inflammatory phenotype²⁰⁶⁻²⁰⁸. In neurodegenerative diseases, proteins and other signaling factors released from dying cells, as well as environmental toxins, can stimulate a pro-inflammatory microglial response²⁰⁹⁻²¹². In response to these triggers, activated microglia migrate to the site of the disease process and produce a large array of pro-inflammatory cytokines, including tumour necrosis factor-alpha (TNF- α) and nitric oxide synthase-2 (NOS-2)^{213,214}. TNF- α binds to cell walls and can trigger apoptotic pathways²¹⁵, while NOS-2 can induce oxidative stress leading to caspase activation and mitochondrial dysfunction resulting in cell death²¹⁶.

Early rod degeneration is thought to trigger microglial activation in RP, which may contribute to the subsequent death of cones (Fig. 1.7). Microglial activation has been observed in the inner and outer nuclear layers and subretinal space in human patients with RP²¹⁷. Moreover, in animal models of RP, studies have similarly demonstrated early retinal microglial migration and activation^{218,219}, the timing of which coincided with the onset of photoreceptor degeneration. Additionally, increased expression of numerous microglial-derived toxic factors, including monocyte chemoattractant proteins-1 and 3, macrophage inflammatory proteins 1a and 1b, chemokine ligand 5, and TNF- α has been shown²¹⁹.

A causal relationship between microglial activation and photoreceptor degeneration in RP has also been established. Roque and Caldwell²²⁰ developed an isolated culture of retinal microglia from Royal College of Surgeons (RCS) rats, which express the retinal degeneration

phenotype. Roque *et al*²²¹ found that co-culture of these microglia with an immortalized cone photoreceptor line resulted in an increase in apoptosis-related cell death in the photoreceptor cells. Also of note, Peng *et al*²²² backcrossed Rd10 mice with mice that expressed the chemokine receptor Cx3cr1 knockdown gene. Cx3cr1 is specifically expressed by microglia and, in some disease models, Cx3cr1 deficiency dysregulates microglial activation and promotes neurodegeneration^{223,224}. Peng *et al*²²² observed that Cx3cr1 deficiency exacerbated rod loss without affecting cone density in Rd10 mice. Peng *et al*²²² also treated Rd10 mice with minocycline, which is an inhibitor of microglial activation, and found reduced expression of pro-inflammatory cytokines and preservation of rod photoreceptors.

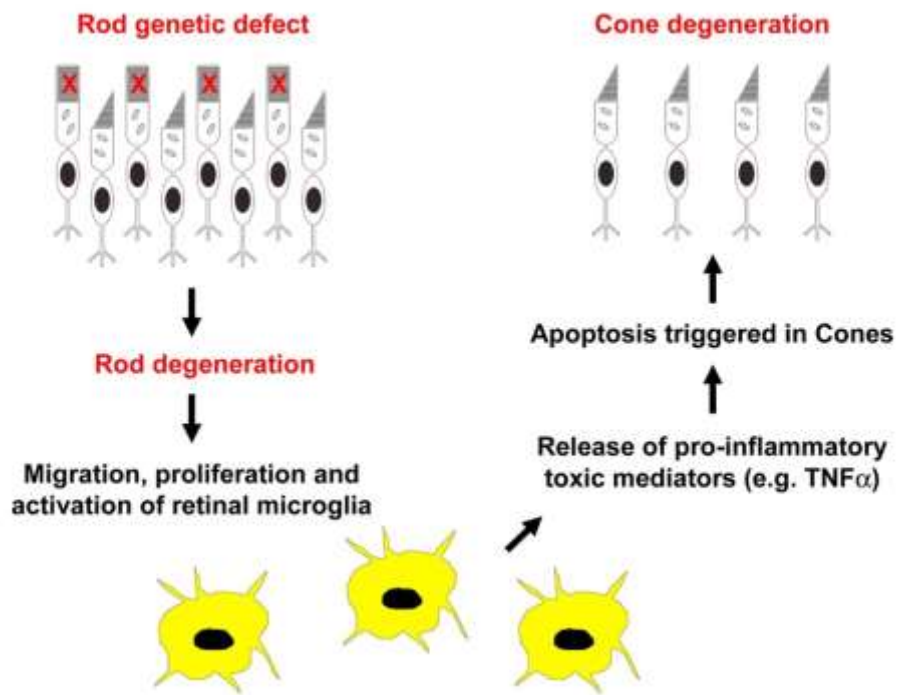


Figure 1.7. Diagram of the mechanism to describe how microglial activation may contribute to cone death in RP.

1.7.5. Rod-Derived Toxins

A fifth explanation for cone degeneration in RP is that dying rods release toxic substances into the extracellular space between photoreceptors or via gap junctions between rods and cones that ultimately cause cone death²²⁵⁻²²⁷. In this “rod-produced toxin” theory, the rapid

phase of rod degeneration results in a large release of toxic factors, such as glutamate or the toxic products of apoptosis and necrosis, that account for the initial degeneration of cones. Surviving cones then die more slowly because of reduced generation of these toxic factors. Although an attractive hypothesis, there is very limited experimental data to support this hypothesis.

1.8. Aims of the Project

This thesis is divided into three experiments. The overriding aim of the thesis is to further our understanding of photoreceptor energy metabolism and manipulate photoreceptor energy metabolism for clinical advantage using an *in vivo* model of RP.

1.8.1. Experiment One

In the first experiment we investigate the role of glucose in the retina and seek to provide an explanation for the high rate of aerobic glycolysis. We hypothesized that radiolabeled glucose would be incorporated, to a high degree, into the photoreceptor outer segments of mice, which are composed mainly of visual pigment proteins, and move in a scleral direction as rhodopsin is turned over and phagocytosed by the RPE. This finding would suggest that the mouse retina shunts glucose away from energy production and instead uses glucose for biomass synthesis, which would provide an explanation for the high rate of aerobic glycolysis in the retina.

1.8.2. Experiment Two

In the second experiment we characterize an animal model of retinitis pigmentosa. Using the Rd1 mouse, we describe the temporal and spatial distribution of cone cell body degeneration and cone outer segment degeneration. Understanding natural history of cone degeneration is vital for evaluating potential treatments of RP and can be widely applied to studies that use the Rd1 mouse as a model for RP.

1.8.3. Experiment Three

In the third experiment we build on the data obtained in second experiment and test two novel bio-energetic agents in the Rd1 mouse model of RP. Systemic oral creatine and local glucose treatment were administered to Rd1 mice and their effects of cone survival were measured using immunohistochemical methods and neuro-behavioral responses. We hypothesized that bio-energetic treatment would prolong cone survival in this *in vivo* model of photoreceptor degeneration.

2. MATERIALS AND METHODS

2.1. Ethics Approval

All experiments were approved by the Animal Ethics Committees of SA Pathology and the University of Adelaide. Approval for the use of radiation in Experiment One was obtained from the Radiation Safety Committee at the Hanson Institute, South Australia. All experiments conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2013) and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Animal Handling

C3H/HeJArc mice and C57BL/6J mice were obtained from Animal Resources Centre, Perth, WA. The C3H/HeJArc mouse is an inbred strain homozygous for the PDE6b mutation. This mutation results in failure of PDE which impairs cGMP hydrolysis leading to rod degeneration. The PDE6b mutation can also affect humans, causing autosomal recessive RP. C3H/HeJArc mice were inbred to produce offspring. All mice were housed and all procedures were performed at the Animal Care Facility, Hanson Institute. Mice were kept in temperature and humidity controlled rooms with a 12-hour light and 12-hour dark cycle, and provided with food and water ad libitum. All mice used in the experiments were killed by transcardial perfusion with physiological saline under intraperitoneal anaesthesia (ketamine 100 mg/kg and xylazine 10 mg/kg). Eyes were enucleated and prepared for analysis. In eyes taken for whole mount immunohistochemistry, the superior aspect of each cornea was marked using a scalpel blade before globes were enucleated. The animal procedures are outlined in the experimental design sections of the following chapters.

2.3. Histology and Immunohistochemistry

2.3.1. Whole-Mount Retinas

Eyes were fixed in 4% (w/v) neutral buffered formalin for 24 hours and dissected into posterior eye-cups. The dissection was performed with the aid of a high magnification operating microscope. Vannas spring scissors were used to make a small sagittal incision in the eye behind the ora serrata, which is located posterior to the conjunctival fornix. The incision was extended circumferentially around eye until the anterior eye segment was separated from posterior eye segment. The previously made corneal mark was used to orient the eye and a small radial cut was made in the superior retina while it was still attached to the RPE in the eye-cup. The retina was then detached from the underlying RPE and dissected off its attachment at the optic nerve head. Retinas were removed and prepared as flattened whole-mounts by making another four radial cuts extending > 50% of the radius of the retina. Retinal whole-mounts were kept in phosphate buffered saline (PBS) and double labelling immunofluorescence was performed. Retinas were permeated in PBS containing 2% triton and blocked with 3% normal horse serum in PBS containing 1% triton. Retinas were incubated overnight at 4°C with a combination of primary antibodies diluted in the blocking solution (Table 2.1). Retinas were washed twice in PBS and incubated for 3 hours at room temperature with combination of AlexaFluor-488 and -594 conjugated secondary antibodies diluted in the blocking solution (1:250; Invitrogen, Carlsbad, CA). Retinas were rinsed in PBS and mounted with the photoreceptor side facing up using anti-fade mounting medium (Dako, Carpintería, CA).

2.3.2. Transverse Retinal Sections

2.3.2.1. Paraffin Sections

Eyes were immersion fixed in Davidson's solution for 24 hours, then transferred to 70% ethanol until processing. Davidson's solution, which comprises two-parts formaldehyde

(37%), three-parts 100% ethanol, one-part glacial acetic acid, and three-parts water, is the preferred fixative for whole eyes as it provides optimal tissue morphology while avoiding retinal detachment. Whole eyes were hand-processed according to the following schedule: 70% ethanol for 30 min, 3 × 100% ethanol for 30 min, 2 × xylene for 30 min, 50% xylene/50% wax for 30 min at 62°C, 2 × wax for 30 min at 62°C. Globes were embedded sagittally and 5µm serial sections were cut on a microtome. Tissue sections were deparaffinised, rinsed in 100% ethanol and treated for 30 minutes with hydrogen peroxide in absolute methanol to block endogenous peroxidase activity. Antigen retrieval was achieved by microwaving the sections in 1mM EDTA buffer (pH 8.0) for 10 minutes at 95 – 100°C. The microwave used, NEC N702EP, had been previously calibrated such that a stable temperature range of 95 – 100°C was achieved when two preheated plastic containers, each filled with 250 ml of retrieval solution, were microwaved on power setting 2. Tissue sections were blocked with PBS containing 3% normal horse serum and incubated overnight at room temperature in primary antibody containing 3% normal horse serum (Table 2.1). Subsequent to this, consecutive incubations were carried out with biotinylated secondary antibody (1:250; Vector, Burlingame, CA) and streptavidin–peroxidase conjugate (1:1000; Pierce, Rockford, IL). Color development was achieved using NovaRED substrate kit (Vector, Burlingame, CA). Sections were counterstained with hematoxylin, dehydrated, cleared in histolene, and mounted in DPX. For double labelling immunofluorescence of transverse retinal sections, samples were treated exactly as described above except that a combination of primary antibodies was used (Table 2.1). Detection of signal was achieved by incubation with the appropriate combination of AlexaFluor-488 and -594 conjugated secondary antibodies (1:250; Invitrogen, Carlsbad, CA) instead of streptavidin–peroxidase conjugate. Sections were mounted using anti-fade mounting medium (Dako, Carpintería, CA).

2.3.2.2. Resin Sections

Eyes were fixed by immersion in 2.5% glutaraldehyde with 4% paraformaldehyde in 0.1 M

phosphate buffer (pH 7.4) for 24 hours at 4°C. Eyes were then placed in 2% osmium tetroxide in saline overnight and washed with cacodylate buffer at room temperature. Subsequently, the eyes were dehydrated in graded alcohols and embedded in epoxy resin (TAAB, Aldermaston Berks, UK) for transverse sectioning. Transverse sections (0.5 µm) were cut on an ultramicrotome and mounted on glass slides.

Table 2.1. List of primary antibodies used

Target	Host	Dilution	Clone/Cat. No.	Source*
OPN1SW	Goat	1:1000	sc-14363	Santa Cruz
R/G opsin	Rabbit	1:1000	AB5405	Millipore
Cone arrestin	Rabbit	1:3000	AB15282	Millipore
PNA	Biotinylated	1:3000	B-1075	Vector
RET-P1	Mouse	1:1000 ⁺	ab3267	Abcam
Iba1	Rabbit	1:5000, 1:10 000 ⁺	019-19741	Wako
CD68	Mouse	1:500	MCA1957	Serotec
GFAP	Mouse	1:40 000, 1:10 000 ⁺	6F2	Dako

* Wako Pure Chemicals, Osaka, Japan; AbD Serotec, East Brisbane, QLD, Australia; Santa Cruz Biotechnology, CA, USA; Merck Millipore, Kilsyth, VIC, Australia; Vector Laboratories, Burlingame, CA, USA; Abcam, Cambridge, UK; Dako, Carpintería, CA, USA

⁺ 3-step colourimetric immunohistochemistry

2.4. Autoradiography

Transverse paraffin sections and resin sections from animals injected with carbon-14 (C-14) glucose were used for autoradiographic analysis. Sections were prepared as described above. After the sections were cut, they were dipped in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, NY) diluted 1:1 with distilled water at 40°C. The preparations were exposed in dark for 6 – 24 weeks at 4°C, then developed in Kodak D-19 developer and fixed in Ilford Hypam Rapid Fixer which had been diluted 1:4 with distilled water for 2 min at 20°C. Paraffin

sections were washed in water, counterstained with hematoxylin, dehydrated, cleared in histolene, and mounted in DPX. Epoxy resin sections were washed in water without any counterstain.

2.5. Real-Time PCR

Retinas were dissected, total RNA was isolated, and first strand cDNA was synthesized from DNase-treated RNA samples. Real-time PCR reactions were carried out in 96-well optical reaction plates using the cDNA equivalent of 10 ng total RNA for each sample in a total volume of 20 μ l containing 1 $\times$ SYBR Green PCR master mix (BioRad, Gladesville, NSW) forward and reverse primers. Thermal cycling conditions were 95°C for 3 min and 40 cycles of amplification comprising 95°C for 12 s, 63°C for 30 s and 72°C for 30 s. Primer pairs (Table 2.2) were designed from sequences contained in the GenBank database using the primer design software Primer 3 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>) and were selected wherever possible to amplify sequences that spanned at least one intron. Primer sequences were analyzed for T_m (melting temperature), secondary structure and primer-dimer formation with NetPrimer analysis software (<http://www.premierbiosoft.com/netprimer>). PCR assays were performed using the CFX cycler (Bio-Rad) and all samples were run in duplicate. All mRNAs were amplified with high efficiency and linearity during real-time PCR. Mean amplification efficiencies, as determined by plotting cycle threshold as a function of initial cDNA quantity, were in the range of 1.90 – 2.00. Results obtained from the real-time PCR experiments were, therefore, quantified using the comparative threshold cycle (C_T) method ($\Delta\Delta C_T$) for relative quantitation of gene expression, with a minor correction for amplification efficiency²²⁸. All values were normalised using two different endogenous housekeeping genes, GAPDH and cyclophilin, and expressed relative to the earliest time point analysed (14 days).

Table 2.2. Primer sequences for mRNAs amplified by real-time PCR

mRNA	Primer Sequences	Product Size (bp)	Accession No.
GAPDH	5'-TGCACCACCAACTGCTTAGC-3'	87	NM_001289726
	5'-GGCATGGACTGTGGTCATGAG-3'		
Cyclophilin	5'-GTGTTCTTCGACATCACGGCT-3'	82	NM_008907
	5'-CTGTCTTTGGAACCTTTGTCTGCA-3'		
OPN1SW	5'-CCTCTTTCCCTCATCTGCTTCTC-3'	107	NM_007538
	5'-ACCTCCCGTTCAGCCTTTTG-3'		
OPN1MW	5'-CATCCGAGCAGTGGCAAAG-3'	175	NM_008106
	5'-CACAAGAGGGTGAAGGCATAG-3'		

2.6. Image acquisition

Retinal whole-mounts and immunofluorescence transverse sections were examined under a fluorescence microscope (BX-61; Olympus, Mount Waverly, VIC, Australia). Whole-mount images were taken using a Hamamatsu NanoZoomer 2.0-HT fluorescence module and viewed using the Hamamatsu NanoZoomer Digital Pathology system. Transverse retinal sections were photographed using a light microscope.

2.7. Quantification of Photomicrographs

Photomicrographs measuring 720 μm x 540 μm were taken using the fluorescence microscope on whole mounts retinas. Four photomicrographs of the central retina were taken directly superior, temporal, inferior, and nasal to the centre of the optic nerve. Four photomicrographs of the peripheral retina were taken 1.5 mm superior, temporal, inferior, and nasal to the center of the optic nerve. This process was performed using an excitation wavelength of 488 nm to detect FITC and an excitation wavelength of 594 nm to detect Texas Red. This yielded a total of 16 photomicrographs per retina. The number of cone cell bodies and cone outer segments

in each of the 16 photomicrographs was calculated using an Image-J macro. The same method was used to quantify Iba1 and CD68 labelled microglia.

2.8. Optomotor testing

This test measures the tendency of an animal to follow a moving object using their head and eyes. Optomotor testing is an established method for quantifying visual function in mice ²²⁹. Animals were placed on a platform positioned in the middle of an arena created by a quad square of computer monitors. Vertical sine wave gratings (100% contrast) were projected on the computer monitors. The spatial frequencies tested were 0.05, 0.075, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 cycles per degree, at a constant speed of 12 degrees/s. A camera was positioned directly above the platform in order to observe the animal's head movements. Mice were placed one at a time on the platform and allowed to move freely while the experimenter followed the mouse's head. When a grating perceptible to the mouse was projected on the cylinder wall and the cylinder was rotated (12 degrees /s), the mouse normally stopped moving its body and would begin to track the grating with reflexive head movements in concert with the rotation. An experimenter assessed whether the animals tracked the cylinder by monitoring in the video window the image of the cylinder and the animal simultaneously. The spatial frequency of the grating was systematically increased until the animal no longer responded. Occasionally, during testing, sudden reversals of grating drift direction were interspersed with the grating presentations to induce the animal to stop moving, which facilitated more rapid testing. The process of incrementally changing the spatial frequency of the test grating was repeated until the highest spatial frequency that the mouse could track was identified as the threshold. A threshold for each direction of rotation was assessed this way, and the highest spatial frequency tracked in either direction was recorded as the threshold. Whenever possible, experimenters were blind to the treatment and age of the animals. All animals were habituated before the outset of testing with handling and by placing them on the

platform for a few minutes at a time.

2.9. Sample Size

We chose three mice at each time point in Experiment One to provide comparison between time points, and allow for variability in the quality of the autoradiograms. The number of mice required for Experiment Two was established based on the SD/mean ratio of cone degeneration in other studies^{27,163}. We estimated that a sample size of five animals at each time point would provide quantitative comparison between time points, allowing for inter-animal variation within time points. Then, based on the effect size of neuroprotective agents in other Rd1 studies^{27,163}, we estimated that group sizes of twelve animals in Experiment Three would provide 80% power at an alpha value of 0.05.

2.10. Statistical Analysis

In Experiment Two, statistical analysis on real-time PCR data was performed using one-way ANOVA followed by post-hoc Dunnett's Multiple Comparison Test. The null hypothesis tested was that C_T differences between target and housekeeping genes would be the same at each time point. In Experiment Three, paired t-tests were used to compare vitreous glucose concentrations between glucose injected and saline injected eyes. Paired t-tests were also used to compare cone cell concentrations between saline injected eyes and contralateral un-injected eyes. Unpaired t-tests were used to compare cone cell concentrations and optomotor testing results between glucose treated eyes and saline treated eyes, and also between creatine treated mice and standard diet mice.

3. EXPERIMENT ONE

3.1. Experimental Design

The aim of this experiment was to investigate the role of glucose in the retina and to provide an explanation for the high rate of aerobic glycolysis observed in the retina. Cancer cells are the only other cell types that display high rates of aerobic glycolysis⁷⁸. In cancer cells, the dumping of excess carbon as lactate allows faster incorporation of carbon into biomass, which in turn facilitates rapid cell division²³⁰. We hypothesized that radiolabeled glucose would be incorporated, to a high degree, into the photoreceptor outer segments of mice, which are composed mainly of visual pigment proteins. This finding would suggest that the mouse retina, like cancer cells, uses glucose for biomass synthesis. This would provide an explanation for the high rate of aerobic glycolysis in the retina. Six-week old C57BL/6J mice (n = 24) were injected with intraperitoneal C-14 glucose in aqueous solution at 10 μ Ci/mouse (PerkinElmer, Waltham, MA, USA) under inhaled isoflurane sedation. Mice were kept in a radiation safety room and killed in groups of three at 30 minutes, 1 hour, 3 hours, 8 hours, 24 hours, 48 hours, 1 week and 4 weeks (Fig. 3.1). Eyes were enucleated and processed into transverse retinal sections. Right eyes were embedded in paraffin and cut into 5 μ m sections. Left eyes were embedded in epoxy resin and cut into 0.5 μ m sections. All sections were prepared into autoradiograms and examined under a light microscope.

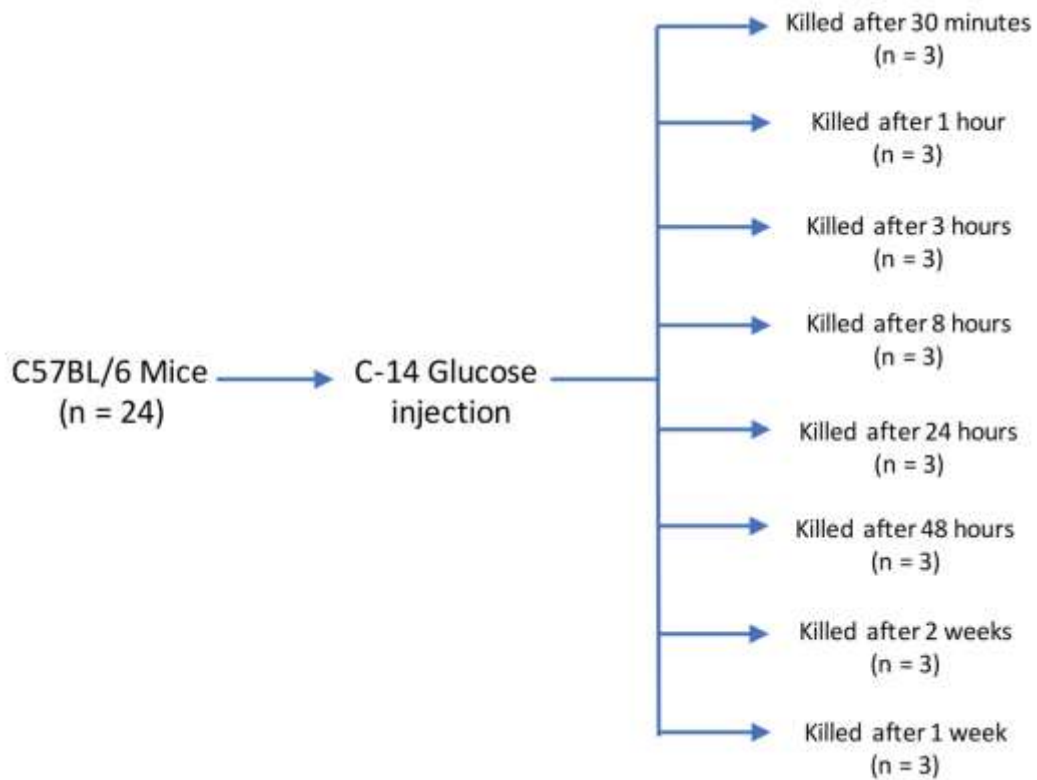


Figure 3.1. Flow chart of the experimental plan in Experiment One.

3.2. Results

3.2.1. Background Staining

The autoradiographic reaction was observed by the visualization of silver granules in transverse retinal sections of mice injected with C-14 glucose. Background staining was observed by deposition of granules into areas that did not contain biological tissue. The optical density of the autoradiographic staining was qualitatively compared between biological tissue samples and background levels. In all sections, paraffin and resin, the density of granules in tissue samples was markedly greater than the density of granules in background images, demonstrating that tissue samples were displaying an autoradiographic reaction to the C-14 glucose.

3.2.2. Autoradiographic Reaction in Paraffin Sections

In paraffin sections there was marked autoradiographic staining throughout the retina 30 minutes after C-14 injection (Fig. 3.2). The density of autoradiographic staining gradually declined thereafter until returning to near background levels at 4 weeks after C-14 injection (Fig. 3.2).

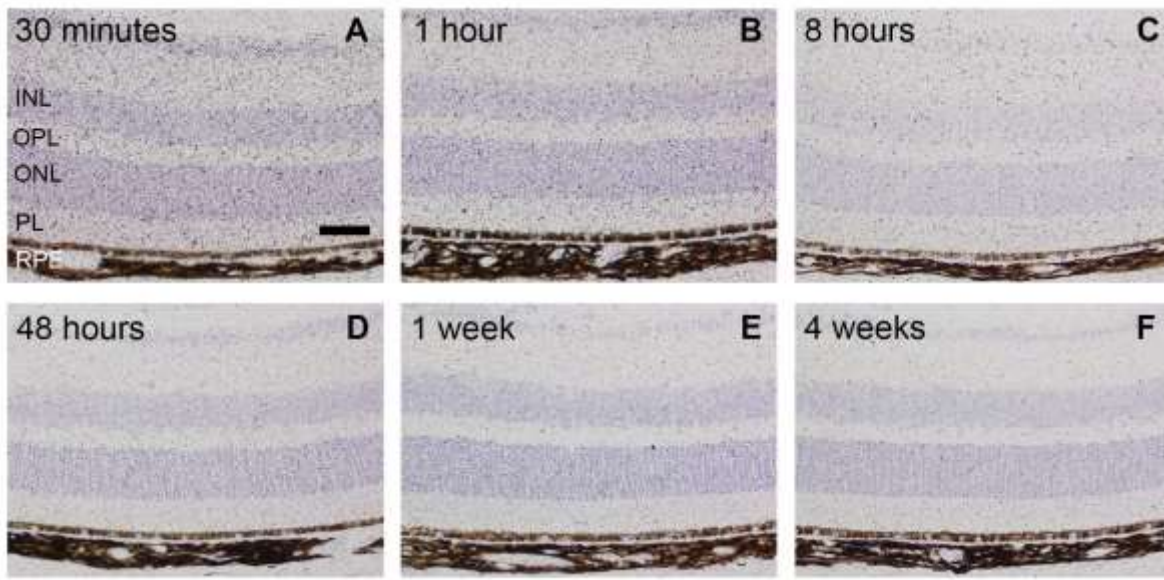


Figure 3.2. Photomicrographs showing the autoradiographic reaction in transverse paraffin sections of the C57BL/6J mouse retina. Representative images are taken from animals killed at 30 minutes to 4 weeks after systemic C-14 glucose injection. The pigment granules are autoradiographic deposits. There is diffuse autoradiographic reaction throughout the retina 30 minutes after C-14 glucose injection (A). The amount of autoradiographic staining decreases over time until reaching background levels at 4 weeks (F). INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer; PL = photoreceptor layer; RPE = retinal pigment epithelium. Scale bar 50 μ m.

3.2.3. Autoradiographic Reaction in Resin Sections

In resin sections, there was marked autoradiographic staining 30 minutes after C-14 injection (Figs. 3.3-3.4). The density of autoradiographic staining followed a distinct gradient from high density staining in the outer retina to lower density staining in the inner retina (Figs. 3.3-3.4). Resin sections were not counterstained with toluidine blue since this masked the

autoradiographic reaction. In order to further localise the distribution of staining, cyto-architectural comparison was made with the paraffin sections. This revealed that the high-density band of autoradiographic staining was localized mainly to the photoreceptors. The overall density of autoradiographic staining decreased over time in resin sections. The staining gradient between inner and outer retina was maintained until 1 week after C-14 glucose injection.

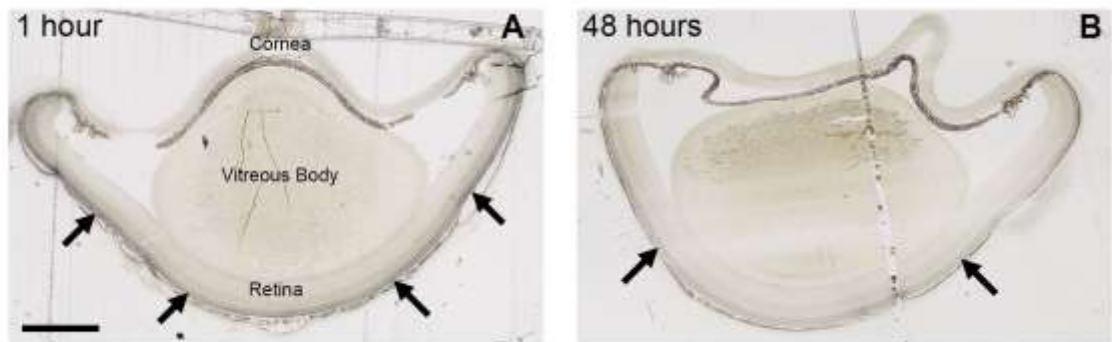


Figure 3.3. Low power photomicrographs showing transverse resin sections of the whole C57BL/6J mouse eye. Representative images are taken from animals killed at 1 hour (A) and 48 hours (B) after C-14 glucose injection. A dark band of autoradiographic activity is observed in the outer retina after 1 hour (arrows) and is concentrated in the apical processes of the photoreceptors. The intensity of this autoradiographic band is decreased by 48 hours. Scale bar 500 μ m.

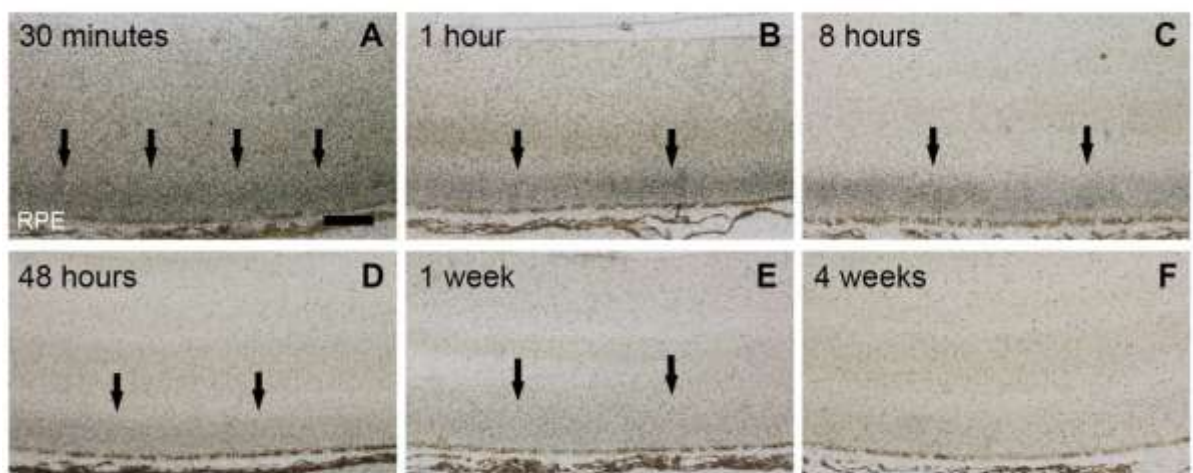


Figure 3.4. High power photomicrographs of transverse resin sections from the C57BL/6J mouse retina. Representative images are taken from animals killed 30 minutes to 4 weeks after C-14 glucose injection. The density of autoradiographic labelling is greatest after 30 minutes (A). The density of autoradiographic labelling

gradually decreases thereafter until reaching background levels at 4 weeks (F). The brown retinal pigment epithelial (RPE) layer is seen at the bottom of each image. The apical portion of photoreceptor outer segments insert into the RPE. The dark autoradiographic bands (arrows), which represent the area of greatest autoradioactivity, are localised to the apical processes of the photoreceptors. The density of autoradiographic labelling in the outer nuclear layer is similar to the rest of the neural retina. The discrete dark bands are observed in the photoreceptor layer until 1 week after C-14 glucose injection (E). Scale bar 50 μm .

3.3. Discussion

3.3.1. C-14 Glucose was Preferentially Distributed in Photoreceptors

Autoradiography is a bio-analytical technique used to visualise the distribution of radioactively labeled substances in a biological sample. Autoradiography can be used for qualitative study, by characterizing the location and distribution of radioactive material, and for quantitative estimation of radioactive material by calculating and comparing optical density. In this experiment, the radioactive reaction observed in the tissue sections was due to radiolabeled C-14. The C-14 was presumably contained within products of glucose metabolism. There was also mild background radiographic staining in all sections, illustrated by radioactive reaction outside of the tissue specimens. The optical density of radioactive staining in tissue sections was greater than the background staining, demonstrating that C-14 glucose was taken up by the retina.

The distribution of radioactive staining was generalized throughout all layers of the retina in the paraffin transverse sections. In the resin sections, the density of radioactive staining was greater in the outer retina compared to the inner retina, and maximal in the photoreceptor layer and outer nuclear layer. Photoreceptor cell bodies are located in the outer nuclear layer and photoreceptor inner and outer segments are located in the photoreceptor layer. This suggests C-14 glucose was preferentially distributed in photoreceptors. This supports the idea that photoreceptors are the most metabolically active cells in retina and

consume the most glucose. The optical density of radioactive staining in paraffin and resin sections was greatest after 30 minutes and gradually decreased thereafter until returning to background levels after 4 weeks as C-14 glucose was cleared by the retina. The disparity in radioactive staining between thin resin sections and semi-thin paraffin sections occurs because the resolution of autoradiographs varies as a function of the thickness of the sections used ²³¹. Grain density decreases as section thickness increases, and becomes minimal at a section thickness of 5 μm ²³¹.

3.3.2. C-14 Glucose was Not Preferentially Incorporated into Outer Segment Discs

We hypothesized that C-14 glucose would be incorporated into the production of outer segment discs which are mainly composed of visual pigment proteins. Outer segment discs migrate in a scleral direction and are renewed at a rate of 80 – 90 discs per day. Therefore, if C-14 glucose was incorporated into outer segment discs, we would observe a distinct radioactive reaction band at the base of the outer segment that would gradually be displaced towards the RPE over time. Previous studies have demonstrated that radiolabeled amino acids are incorporated into photoreceptor outer segments discs in this fashion ^{91,92}.

In this experiment, we did not observe a radioactive reaction band at the base of the outer segments. This suggested C-14 glucose was not preferentially incorporated into the production of outer segment discs. These data do not support the hypothesis that the carbon atoms in glucose are used for biomass synthesis in photoreceptors. Therefore, the question of why the retina displays a high rate of aerobic glycolysis remains unanswered.

3.3.3. Alternative Explanations for Aerobic Glycolysis in the Retina

There are two other possible explanations for the high rate of aerobic glycolysis in the retina. Firstly, the speed of ATP generation in glycolysis is quicker than in oxidative

phosphorylation. The retina may have evolved to utilise the faster glycolytic pathway which is likely better suited for the energy demands of the retina ⁸⁶. Additional sources of energy provided by the fast phosphocreatine shuttle system would help correct the mismatch in energy supply and demand produced by the lack of ATP from glycolysis ^{5,44,86}. This hypothesis has also been proposed as an alternative explanation for aerobic glycolysis in cancer cells ^{232,233}.

A second possible explanation to why the retina exhibits high rates of aerobic glycolysis is that it allows the retina to survive longer when subjected to hypoperfusion ²³⁴. Such conditions occur in cancer, as a result of rapid cell proliferation that outpaces the growth of oxygen-supplying blood vessels. This slows or incapacitates the oxygen-dependent mitochondrial ATP generating system and forces the glycolytic system to compensate for this loss in ATP production ²³⁵. The retina is very susceptible to injury during periods of hypoperfusion. Previous investigations in our laboratory have demonstrated that short-term hyperglycemia is neuroprotective against hypoperfusion induced retinal damage in rats ^{115-117,121}. This may suggest that the mechanism of cell damage during hypoperfusion is dependent on insufficient glucose supply more than hypoxia. In this way, photoreceptors may have developed aerobic glycolysis to allow them to utilise glucose when oxygen is scarce. However, this explanation seems tenuous because photoreceptor energy delivery is critically based on blood supply. Photoreceptors, like all neurons, do not synthesize large amounts of glycogen and consequently cannot utilise intracellular fuels during periods of poor nutrient supply ^{236,237}.

3.3.4. Conclusion

This experiment has not shed light on the reason why the retina displays a high rate of aerobic glycolysis. An improved understanding of retinal energy metabolism will help in understanding retinal diseases, such as RP, that may involve a component energy failure. We

have designed another experiment to examine the biosynthetic fates of radiolabeled glucose and amino acids in retinal cells using mass spectrometry analysis. This study is currently being conducted in collaboration with the Adelaide Proteomics Centre.

4. EXPERIMENT TWO

4.1. Experimental Design

The aim of this experiment was to characterize the spatial and temporal distribution of cone degeneration in the Rd1 mouse model and provide baseline data for the final neuroprotection experiment. Groups of C3H/HeJArc mice were sacrificed at postnatal day (P) 7 (n = 5), P14 (n = 7), P21 (n = 5), P30 (n = 7), P45 (n = 7), P60 (n = 7), P75 (n = 4), P90 (n = 6), P150 (n = 5) and P300 (n = 5) (Fig. 4.1). Six C57BL/6J mice were sacrificed at P60. Right eyes were collected for whole-mount fluorescent immunohistochemistry. Left eyes were collected for transverse retinal sections and real-time PCR analysis. Cone density and microglial density was measured as previously described.

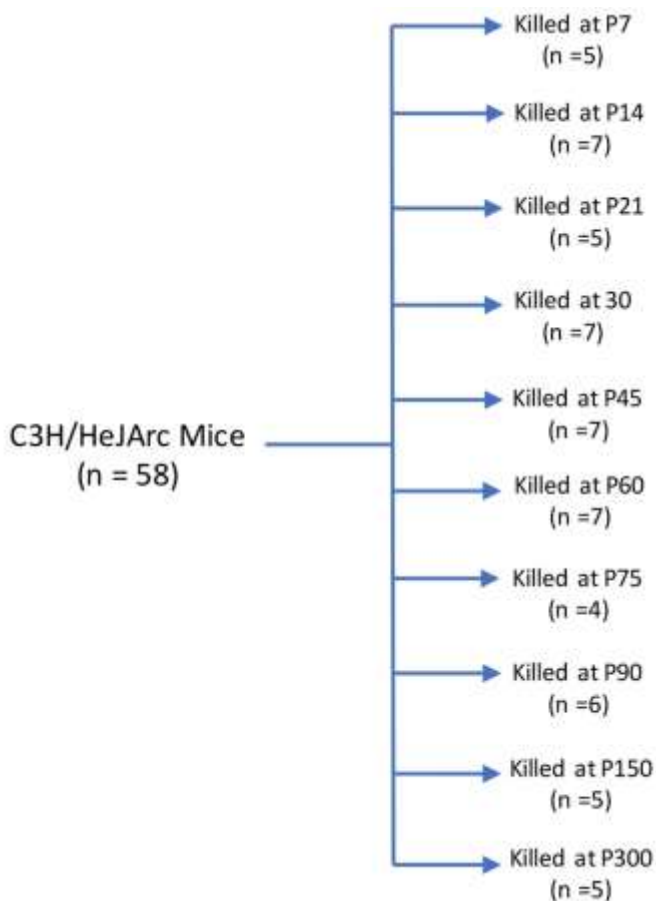


Figure 4.1. Flow chart of the experimental plan in Experiment 2.

4.2. Results

4.2.1. Quantification of S-cones and M/L-cones in the Rd1 Retina

Cones were identified by immunodetection of their cell bodies and outer segments in whole-mount retinas. Mice possess two types of cones that are distinguished by the portion of visible light spectrum to which they are sensitive. Short wavelengths stimulate S-cones and medium to long wavelengths stimulate M/L-cones²³⁸. OPN1SW labels the ultraviolet sensitive opsin in S-cones. Anti-R/G opsin labels the red and green sensitive M/L-cones. Cone cell bodies were identified by their wide ovoid morphology and labelled with moderate fluorescent intensity (Fig. 4.2). Cone outer segments were identified by their narrow bundle-like appearance and labelled with greater fluorescent intensity (Fig. 4.2).

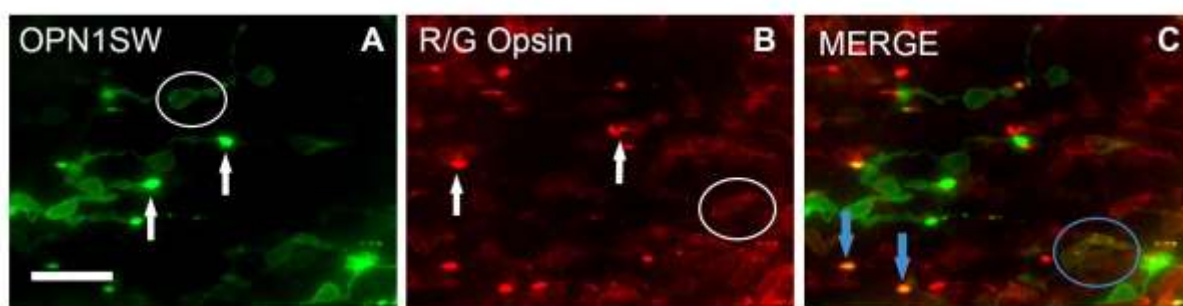


Figure 4.2. Ultra-high power immunofluorescent photomicrographs from Rd1 mice. S-cones have been labelled using OPN1SW (A) and M/L-cones have been labelled using anti-R/G opsin (B). Outer segments are identified by high fluorescent intensity (white arrows). In comparison, cell bodies and their adjacent inner segments label with lower fluorescent intensity (white circles). These different cellular structures can therefore be analysed and quantified separately. There are also “dual” cones that labelled with both OPN1SW and anti-R/G opsin (C; blue arrows). Scale bar 20 μm .

The mean concentration (number of cells per 0.3888 mm^2) of OPN1SW and R/G-labelled cone outer segments decreased from P7 to P300 (Fig. 4.3, Table 4.2, Table 4.4). The mean concentration of OPN1SW-labelled cone outer segments was 2926 ± 376 at P7 and 1.4 ± 1.6 at P300. The mean concentration of R/G-labelled cone outer segments was 1344 ± 516 at P7 and 0.4 ± 0.5 at P300. In comparison, the mean concentration of OPN1SW-labelled cone

outer segments in C57BL/6J control mice was 3108 ± 490 and the mean concentration of R/G-labelled cone outer segments in C57BL/6J mice was 3424 ± 771 . Cone outer segment degeneration in Rd1 mice occurred rapidly between P7 and P45. Greater than 90% of OPN1SW-positive and R/G-positive outer segments had degenerated by P45. The high concentration of intensely labelled outer segments at P7 and P14 made cone cell body identification difficult. Therefore, cone cell body concentration was only accurately measured from P21. The mean concentration of OPN1SW and R/G-labelled cone cell bodies decreased from P21 to P300 (Fig. 4.3, Table 4.1, Table 4.3). The mean concentration of OPN1SW-labelled cone cell bodies was 2545 ± 237 at P21 and 129 ± 78 at P300. The mean concentration of R/G-labelled cone cell bodies was 1260 ± 444 at P21 and 80 ± 70 at P300. Cone degeneration progressed more rapidly in the central retina than in the peripheral retina (Fig. 4.4). Cone cell body degeneration occurred slowly compared to outer segment degeneration (Fig. 4.3).

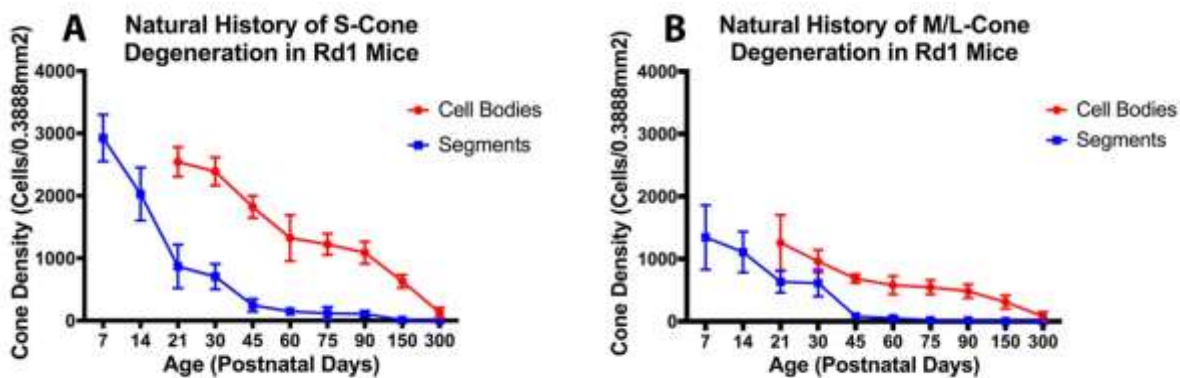


Figure 4.3. Quantification of the natural history of OPN1SW-labelled S-cone (A) and R/G-labelled M/L-cone (B) degeneration over time in Rd1 retinas. X-axes indicate age and Y-axes indicate the mean density of cones per retina. Cell bodies and outer segments were analysed and quantified separately. S-cone and M/L-cone outer segments degenerated earlier than cell bodies. Data represent mean $\pm$ SD.

Table 4.1. Natural history of S-cone cell body degeneration in Rd1 mice

Age	<i>n</i>	Number of S-Cone Cell Bodies Per 0.388mm² (mean ± SD)		
		<i>Central Retina</i>	<i>Peripheral Retina</i>	<i>Whole Retina</i>
21	7	2286 ± 275	2803 ± 220	2545 ± 237
30	8	2160 ± 268	2622 ± 279	2391 ± 227
45	7	1324 ± 146	2323 ± 277	1823 ± 178
60	5	592 ± 283	2056 ± 464	1324 ± 367
75	5	471 ± 91	1978 ± 283	1224 ± 169
90	5	340 ± 135	1839 ± 276	1090 ± 179
150	10	81 ± 38	1128 ± 367	631 ± 103
300	10	17 ± 14	240 ± 146	129 ± 78

Table 4.2. Natural history of S-cone outer segment degeneration in Rd1 mice

Age	<i>n</i>	Number of S-Cone Segments Per 0.388mm² (mean ± SD)		
		<i>Central Retina</i>	<i>Peripheral Retina</i>	<i>Whole Retina</i>
7	7	2944 ± 340	2909 ± 612	2926 ± 376
14	7	1722 ± 537	2339 ± 595	2031 ± 425
21	7	166 ± 102	1572 ± 628	869 ± 349
30	8	123 ± 142	1290 ± 370	707 ± 202
45	7	78 ± 48	417 ± 213	247 ± 103
60	5	49 ± 42	247 ± 200	148 ± 21
75	5	37 ± 3.3	200 ± 40	119 ± 101
90	5	18 ± 13	199 ± 98	109 ± 48
150	10	0.9 ± 2.9	22 ± 18	12 ± 8.4
300	10	0 ± 0	2.8 ± 3.2	1.4 ± 1.6
C57	7	3282 ± 810	2935 ± 345	3108 ± 490

Table 4.3. Natural history of M/L-cone cell body degeneration in Rd1 mice

Age	<i>n</i>	Number of M/L-Cone Cell Bodies Per 0.388mm² (mean ± SD)		
		<i>Central Retina</i>	<i>Peripheral Retina</i>	<i>Whole Retina</i>
21	6	1129 ± 666	1392 ± 309	1260 ± 444
30	6	708 ± 284	1213 ± 211	961 ± 182
45	5	335 ± 141	1035 ± 51	685 ± 71
60	5	162 ± 149	994 ± 175	578 ± 146
75	5	101 ± 14	990 ± 236	545 ± 114
90	5	71 ± 40	895 ± 189	483 ± 110
150	7	17 ± 13	604 ± 210	310 ± 107
300	7	9 ± 5.9	151 ± 136	80 ± 70

Table 4.4. Natural history of M/L-cone outer segment degeneration in Rd1 mice

Age	<i>n</i>	Number of M/L-Cone Segments Per 0.388mm² (mean ± SD)		
		<i>Central Retina</i>	<i>Peripheral Retina</i>	<i>Whole Retina</i>
7	7	1154 ± 647	1535 ± 236	1344 ± 516
14	6	953 ± 479	1263 ± 426	1108 ± 327
21	6	36 ± 21	1232 ± 358	634 ± 174
30	6	9.7 ± 7.6	1214 ± 426	612 ± 211
45	5	7.2 ± 3.2	156 ± 83	81 ± 42
60	5	5.6 ± 2.2	95 ± 54	50 ± 28
75	5	5.2 ± 2.0	34 ± 28	20 ± 14
90	5	4.1 ± 3.6	25 ± 19	14 ± 10
150	7	0 ± 0	6.4 ± 4.4	3.2 ± 2.2
300	7	0 ± 0	0.8 ± 1.0	0.4 ± 0.5
C57	4	2472 ± 1288	2935 ± 345	3424 ± 771

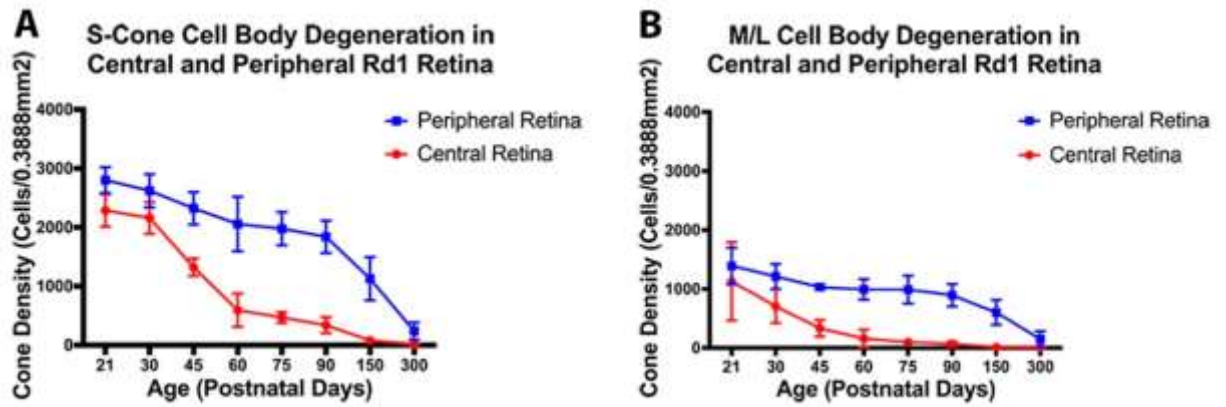


Figure 4.4. Quantification of the natural history of OPN1SW-labelled S-cone (A) and R/G-labelled M/L-cone (B) cell body degeneration in the central Rd1 retina compared to the peripheral Rd1 retina. Cone cell bodies degenerated earlier in the central retina and later in the peripheral retina. Data represent mean $\pm$ SD.

4.2.2. Distribution of S-cones and M/L-cones in the Rd1 Retina

M/L-cones were distributed homogenously throughout the Rd1 retina at P7 (Figs. 4.5, 4.6). However, there was a gradient of S-cone distribution, from sparsely populated cones in the superior retina to a higher density of cones in the inferior retina (Figs. 4.5, 4.6). There was hemispheric asymmetry in the rate of S-cone and M/L-cone degeneration. The concentration of M/L-cones decreased at a faster rate in the inferior hemisphere (Figs. 4.5-4.7). In contrast, the concentration of S-cones decreased at a faster rate in the superior hemisphere (Figs. 4.5-4.7). Therefore, a unique topographic separation of the two spectral classes occurred, which became more pronounced as cone degeneration progressed (Figs. 4.5-4.7). By P300, small quantities of M/L-cones survived in the peripheral part of the superior retina and nasal retina and small quantities of S-cones remained in the peripheral part of the inferior and nasal retina (Figs. 4.5-4.7).

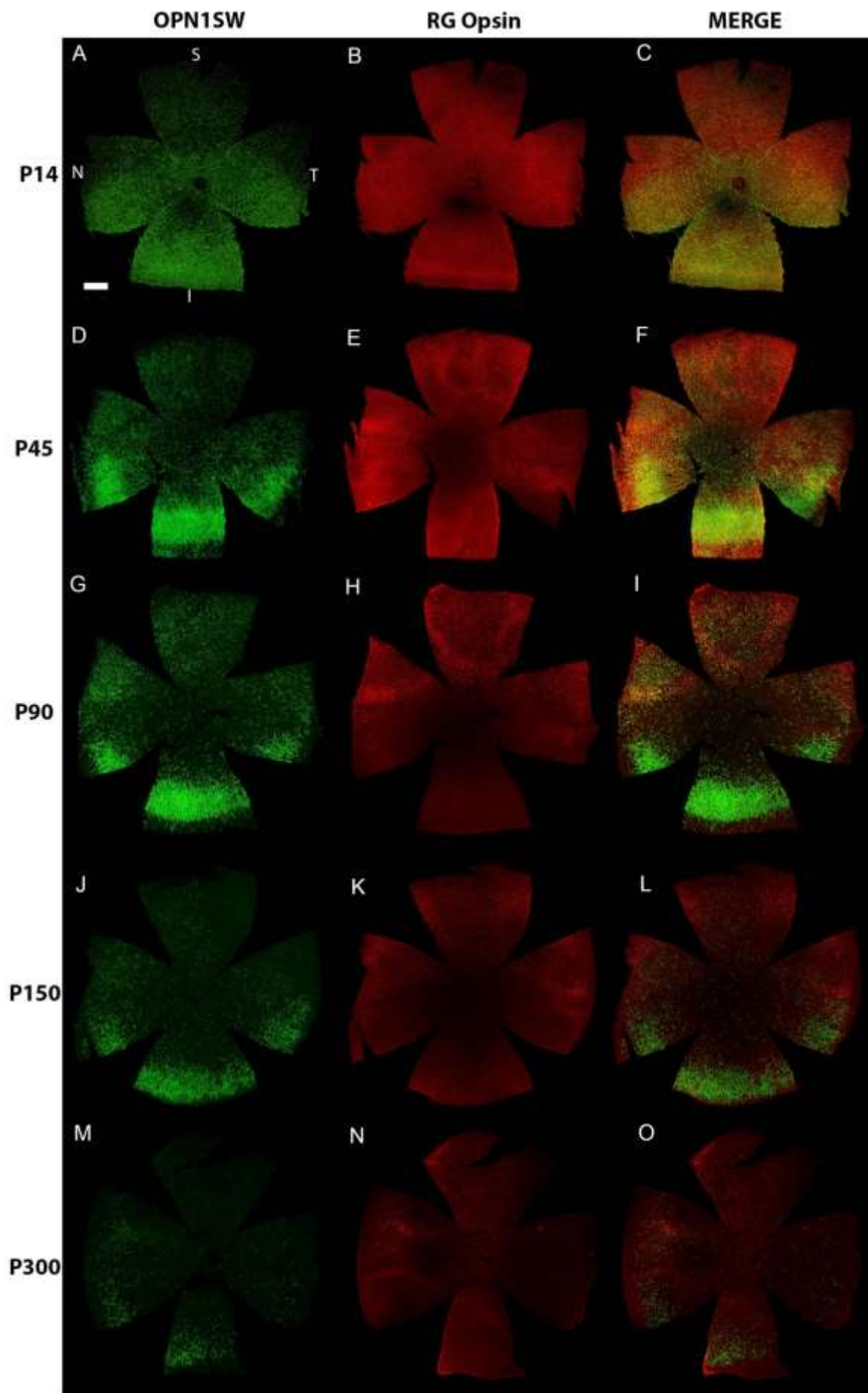
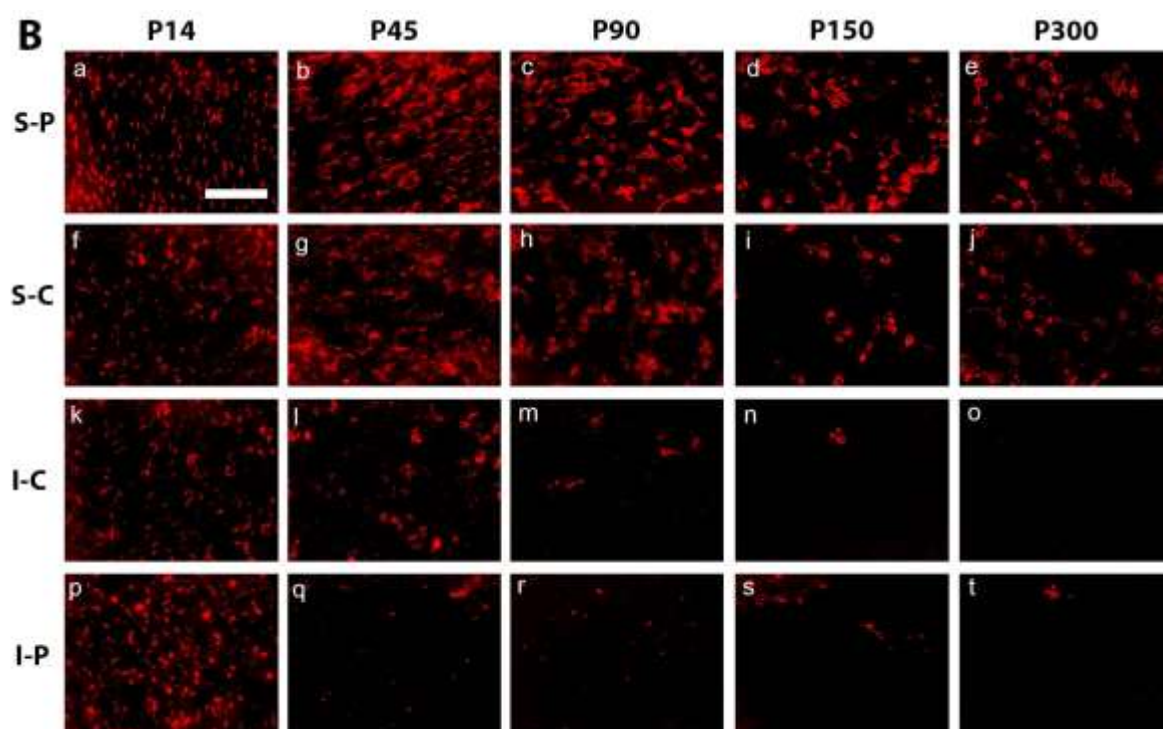
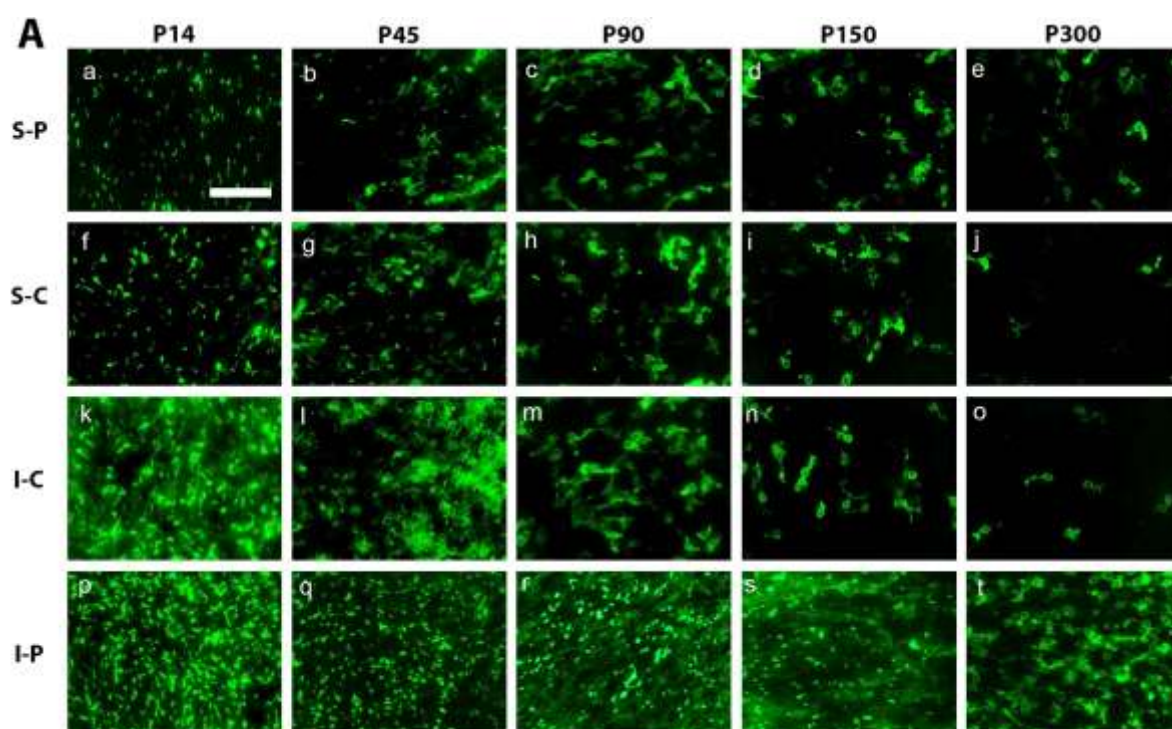


Figure 4.5. Double labelled immunofluorescence of whole-mount Rd1 retinas from postnatal day (P) 14 to P300. Immunodetection has been achieved using OPN1SW for S-cones (A, D, G, J, M) and anti-R/G opsin for M/L-cones (B, E, H, K, N). Cell bodies and outer segments cannot be readily differentiated in these low magnification images. However, the topographical distribution of cones at each time point is readily apparent. S-cones degenerated from superior to inferior, while M/L-cones degenerated from inferior to superior. Few S-cones remain in the inferior and nasal quadrants of the peripheral retina at P300 (M) while few M/L-cones remain in the superior and nasal quadrants of the peripheral retina at P300 (N). Abbreviations in (A): S = superior, I = inferior, N = nasal, T = temporal. Scale bar 500 μm .



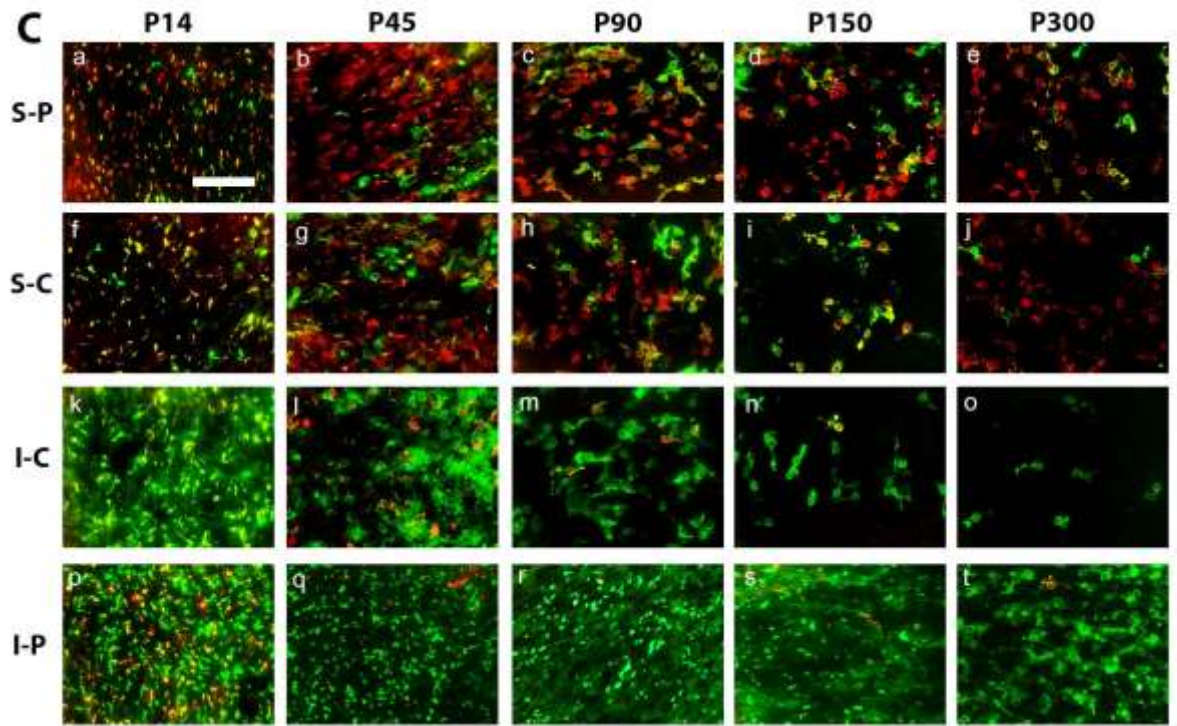


Figure 4.6. High magnification double labelled immunofluorescence photomicrographs of Rd1 retinas from postnatal day (P) 14 to P300. OPN1SW labelled S-cones (A, green) and R/G-labelled M/L-cones (B, red) are shown. The merge image (C) is a composite of A and B. For quantification of cones, four photomicrographs of the central Rd1 retina were taken directly superior, temporal, inferior, and nasal to the centre of the optic nerve. Another four photomicrographs of the peripheral Rd1 retina were taken 1.5 mm superior, temporal, inferior, and nasal to the center of the optic nerve. Representative images of the superior (a-j) and inferior (k-t) Rd1 retina are shown from areas that were photographed for quantification. The numbers of both spectral cone types decreased over time. High fluorescent intensity outer segments were visualised at P14 (a, f, k, p) which obscured the lower fluorescent intensity cell bodies. Large numbers of cone outer segments had degenerated by P45 (b, g, l, q), rendering cone cell bodies more easily visible. The topographical separation of the two spectral cone types is readily appreciated in C. S-P: superior-peripheral retina, S-C: superior-central retina, I-C: inferior-central retina, I-P: inferior-peripheral retina. Scale bar 50 μ m.

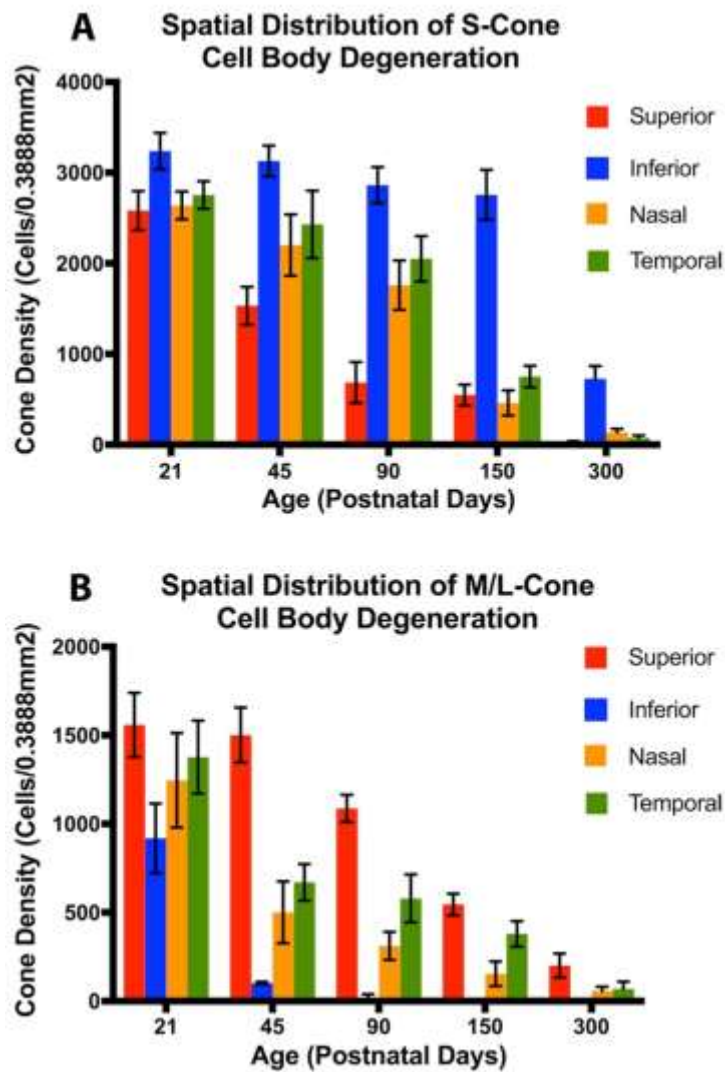


Figure 4.7. Quantification of the spatio-temporal degeneration of S-cones (A) and M/L-cones (B) in Rd1 retinas. Cone density is broken down into quadrants. Data represent mean $\pm$ SD.

4.2.3. Cone Labelling in Rd1 Transverse Retinal Sections

Immunohistochemistry of transverse retinal sections showed progressive thinning of the photoreceptor layer from P7 onwards (Figs. 4.8, 4.12). Similar to the data from whole-mount Rd1 retinas, the number of S-cones decreased over time in the transverse retinal sections (Fig. 4.8). S-cone outer segments degenerated rapidly between P7 and P14 and were barely detectable by P21 (Fig. 4.8). S-cone cell bodies remained after the cone outer segments had degenerated and died at a slower rate (Fig. 4.8). Immunolabelling of cones with peanut agglutinin (PNA) was also performed (Fig. 4.9). PNA is a lectin with high affinity for

galactose-galactosamine disaccharide residues and binds to all cone photoreceptors. PNA labelling was localized to cone outer segments only (Fig. 4.9).

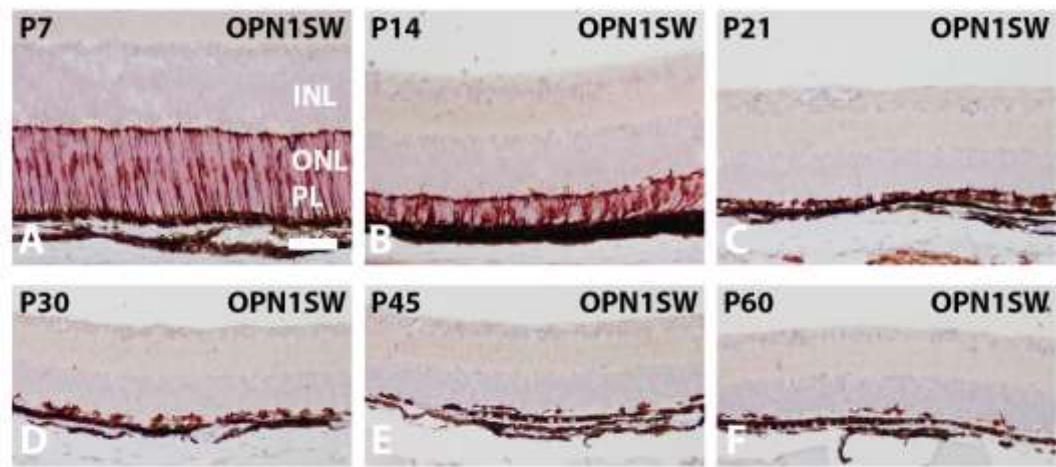


Figure 4.8. Photomicrographs of OPN1SW immunolabelling in transverse sections of the Rd1 mouse retina from postnatal day (P) 7 to P60. OPN1SW labelling of the S-cones is demonstrated in the outer nuclear layer (ONL) and the photoreceptor layer (PL). S-cone outer segment degeneration is complete by P21. S-cone cell body degeneration progresses at a slower rate. The thickness of the ONL and PL also decreases over time. Scale bar 50 μ m. INL: inner nuclear layer.

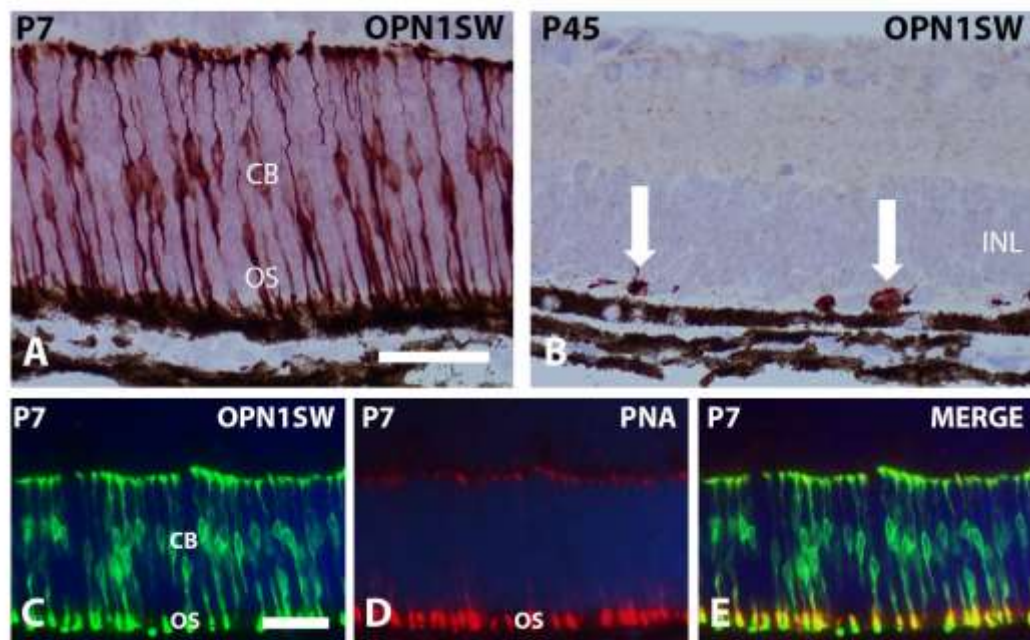


Figure 4.9. High magnification photomicrographs of OPN1SW immunolabelling in transverse sections of the Rd1 mouse retina at postnatal day (P) 7 and P45 (A, B). The white arrows in B point to OPN1SW-labelled S-cones. Immunofluorescent photomicrographs of OPN1SW (C) and PNA immunolabelling (D) in transverse

sections at P7 are also shown. OPN1SW labels both S-cone and dual cone cell bodies (CB) and S-cone and dual cone outer segments (OS). The outer nuclear layer and photoreceptor layer are severely diminished by P45. PNA, which labels both spectral cone classes, is detected in cone OS but not cone CB (D). Scale bar (A – B) 20 μ m. Scale bar (D – F) 20 μ m.

4.2.4. Real-Time PCR using Cone Specific mRNAs in the Rd1 Retina

Real-time PCR was used to examine the levels of cone-specific mRNAs in Rd1 retinas. The levels of OPN1SW and OPN1MW (M/L-cone) mRNAs decreased gradually from P14 until P90 (Table 4.5). The decreases in S-cone mRNA expression were statistically significant at all time points after P14. The decreases in M/L-cones mRNA expression were statistically significant from P45 onwards. There was a five-fold decrease in the levels of OPN1SW mRNA expression and OPN1MW mRNA expression from P14 to P90 (Fig. 4.10). The data obtained from real-time PCR was therefore concordant with that obtained from fluorescent immunohistochemistry and transverse retinal sections.

Table 4.5. OPN1SW and OPN1MW mRNA levels in Rd1 retinas expressed relative to the P14 time point

Age	Transcript	mRNA Level (mean $\pm$ SEM)	
	<i>OPN1SW</i>	Normalised to GAPDH	Normalised to Cyclophilin
14		1.0 $\pm$ 0.07	1.0 $\pm$ 0.09
30		0.56 $\pm$ 0.05	0.74 $\pm$ 0.05
45		0.32 $\pm$ 0.02	0.55 $\pm$ 0.02
60		0.29 $\pm$ 0.03	0.35 $\pm$ 0.03
90		0.18 $\pm$ 0.01	0.19 $\pm$ 0.01
	<i>OPN1MW</i>		
14		1.0 $\pm$ 0.07	1.0 $\pm$ 0.10
30		0.56 $\pm$ 0.05	0.73 $\pm$ 0.06
45		0.35 $\pm$ 0.04	0.60 $\pm$ 0.05
60		0.32 $\pm$ 0.02	0.37 $\pm$ 0.02
90		0.20 $\pm$ 0.03	0.21 $\pm$ 0.03

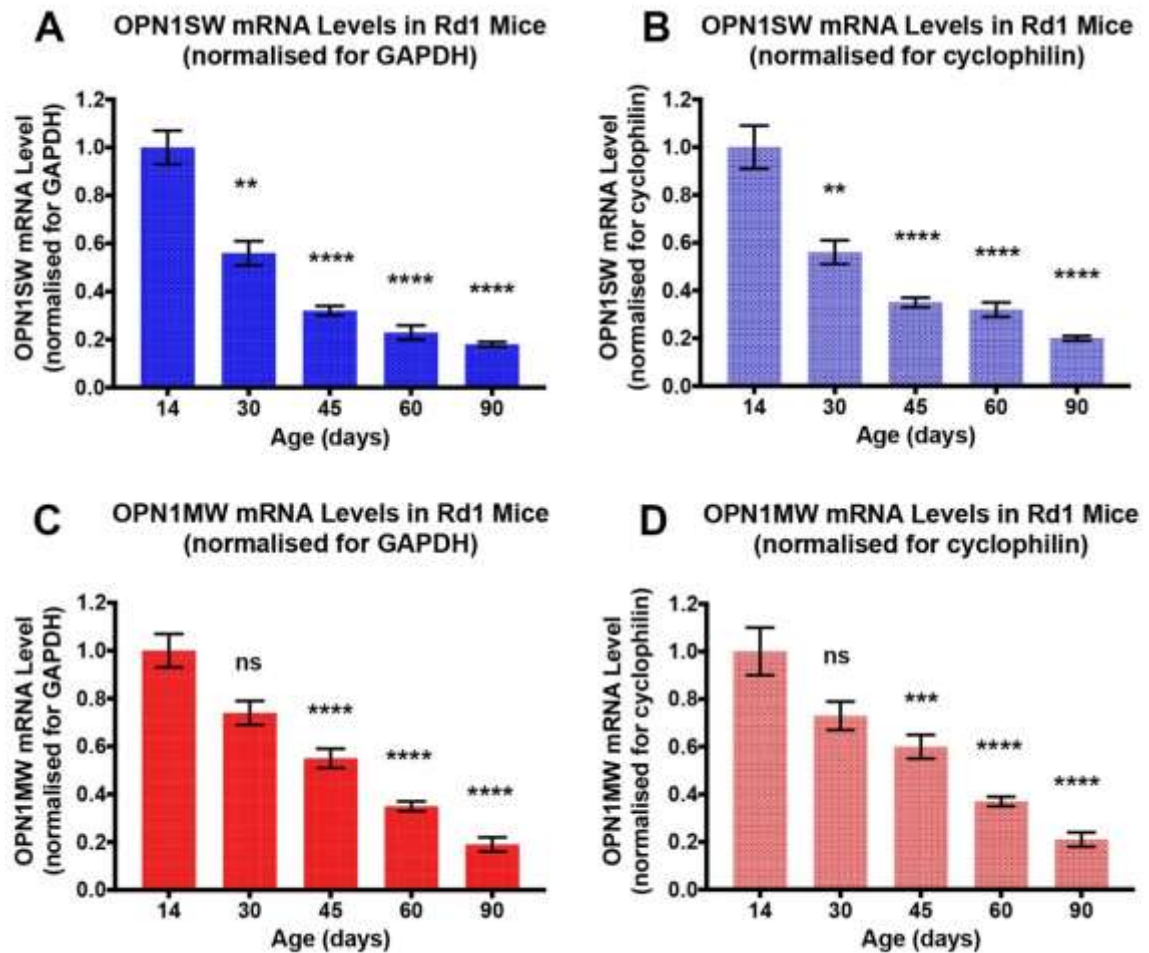


Figure 4.10. Quantification of S-cone (A, B) and M/L-cone (C, D) mRNA levels in Rd1 retinas from P14 to P90. Results were normalised using two different endogenous housekeeping genes, GAPDH (A, C) and cyclophilin (B, D), and expressed relative to P14 time point. Data represent mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.2.5. Cone Arrestin Immunolabelling in Whole-Mount Rd1 Retinas

Whole-mount Rd1 retinas were also labelled with cone arrestin (Fig. 4.11). Cone arrestin is a regulatory protein that is expressed by all mouse cones. Qualitative analysis of the degeneration of cones that expressed cone arrestin occurred at a faster rate in the central retina compared to the peripheral retina. As expected, this was similar to the pattern of degeneration in S-cones and M/L-cones (Fig. 4.5).

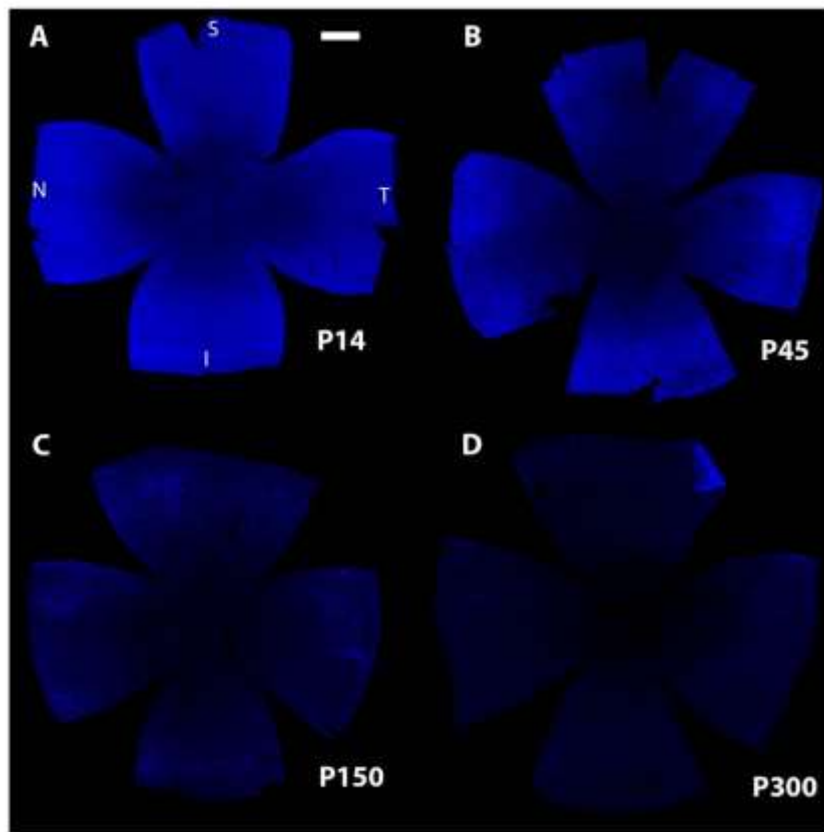


Figure 4.11. Immunofluorescent photomicrographs of whole-mount Rd1 retinas from postnatal day (P) 14 to P300. Immunodetection has been achieved using cone arrestin which labels both S-cones and M/L-cones. The density of cones decreases early in the central retina and later in the peripheral retina. S: superior, I: inferior; N: nasal, T: temporal. Scale bar 500 μm .

4.2.6. Labelling of Rods in Rd1 Transverse Retinal Sections

Immunodetection of rod photoreceptors, which comprise the majority (95%) of photoreceptors in the mouse retina, was performed using an anti-rhodopsin antibody in transverse retinal sections (Fig. 4.12). Rhodopsin immunolabelling was abundant at P7, greatly reduced at P14, and minimal by P21 (Fig. 4.12). The progressive degeneration of rods correlated with the sudden and severe thinning of the outer nuclear layer and photoreceptor layer between P7 and P21 (Fig. 4.12). Thinning of the outer nuclear layer proceeded at a slower rate from P21 to P60 (Fig. 4.8).

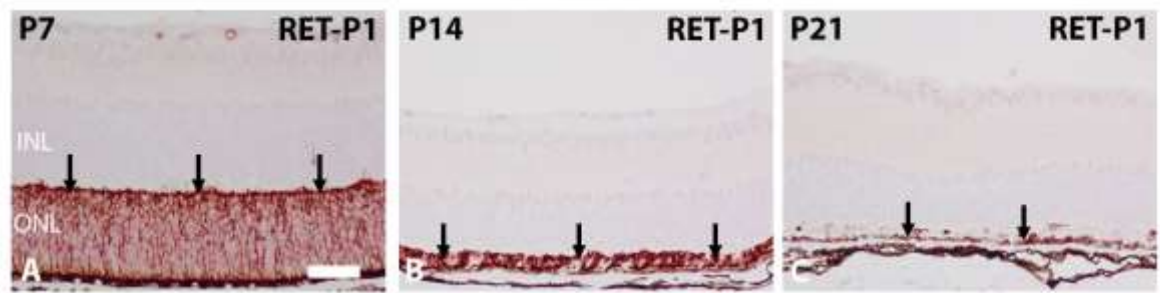


Figure 4.12. Photomicrographs of RET-P1 immunolabelling in transverse sections of the Rd1 mouse retina from postnatal day (P) 7 to P21. RET-P1 is a monoclonal antibody that detects rhodopsin, which is expressed by rods. Rhodopsin immunolabelling (arrows) was abundant at P7 (A), greatly reduced at P14 (B), and minimal by P21 (C). There is corresponding thinning of the outer nuclear and photoreceptor layers between P7 and P21. INL: inner nuclear layer, ONL: outer nuclear layer. Scale bar 50 μ m.

4.2.7. Microglial Activation in the Rd1 Retina

Microglial activation has been previously demonstrated in Rd1 mice²¹⁷⁻²²². Iba1 is a calcium-binding protein specifically expressed by quiescent as well as activated microglia and macrophages. CD68 is a glycoprotein expressed by activated, phagocytic microglia and macrophages. Immunodetection of microglia in whole-mount Rd1 retinas was performed using Iba1 and CD68 immunolabelling and in transverse retinal sections using Iba1 immunolabelling (Figs. 4.13, 4.14). The mean concentration of Iba1-labelled microglia in whole-mount Rd1 retinas decreased between P21 and P300 from 240 ± 39 at P7 to 81 ± 47 at P300 (Figs. 4.13, 4.14). The mean concentration of CD68-labelled microglia decreased between P21 and P300 from 127 ± 31 at P21 to 2.5 ± 0.5 at P300 (Figs. 4.13, 4.14). The distribution of Iba1-labelled microglia and CD68-labelled microglia between the four quadrants of the Rd1 retina was similar at all ages (Fig. 4.13).

In transverse sections, background levels of quiescent Iba1-positive microglia were present at P7 (Fig. 4.15). Iba1-labelled microglia were prominent in the nerve fibre layer and inner plexiform layer, and a smaller population was present in the outer plexiform layer. At

P14, Iba1-labelled microglia had largely migrated to the outer nuclear layer and photoreceptor layer (Fig. 4.15). The density of Iba1 labelling in the outer nuclear layer had decreased by P21 and was minimal at P60 (Fig. 4.15).

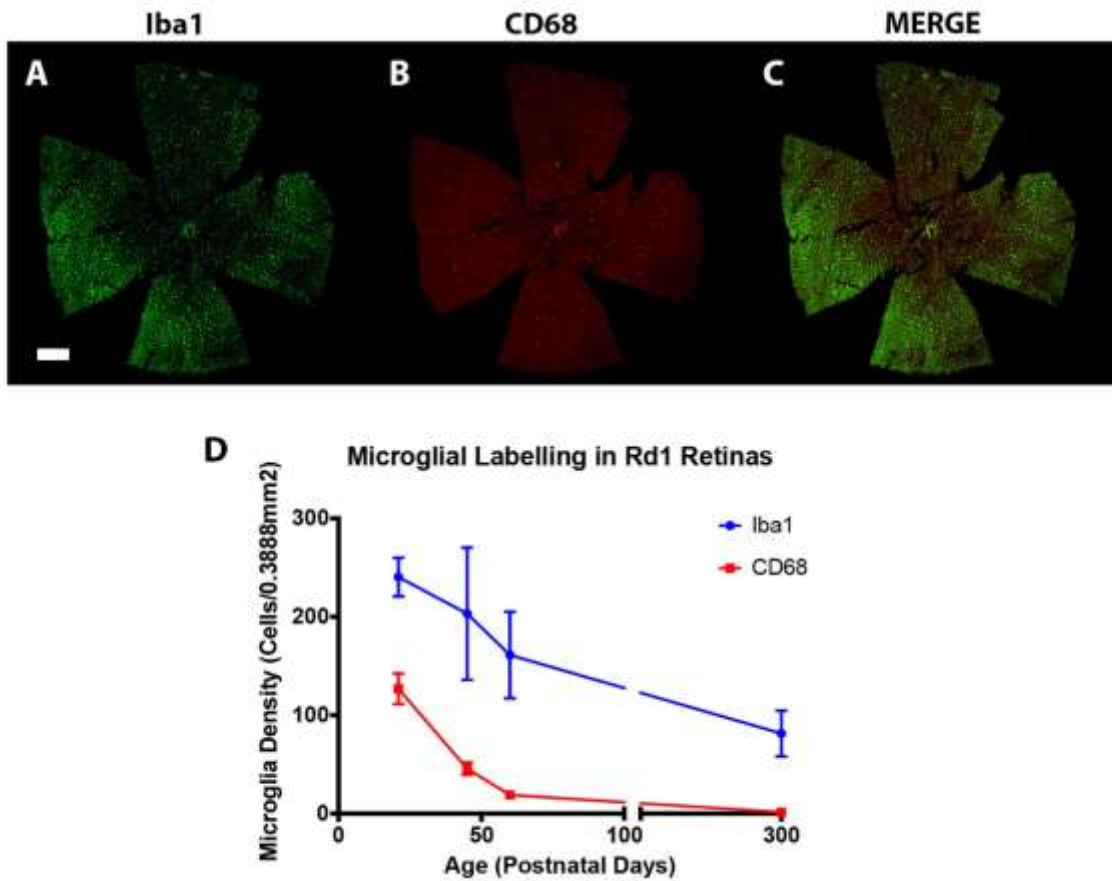


Figure 4.13. (A-C) Double labelled immunofluorescent photomicrographs of a whole-mount retina from a 45-day-old Rd1 mouse. Immunodetection of microglia was achieved using Iba1 and CD68. There is labelling of both Iba1-positive microglia and CD68-positive microglia throughout the retina. The distribution of microglia was similar at all time points, i.e. microglia were not preferentially activated in any particular quadrant of the Rd1 retina. Scale bar 500 μ m. (D) Quantification of microglial labelling in the Rd1 retina over time. The density of microglial labelling decreases over time. Data represent mean $\pm$ SD.

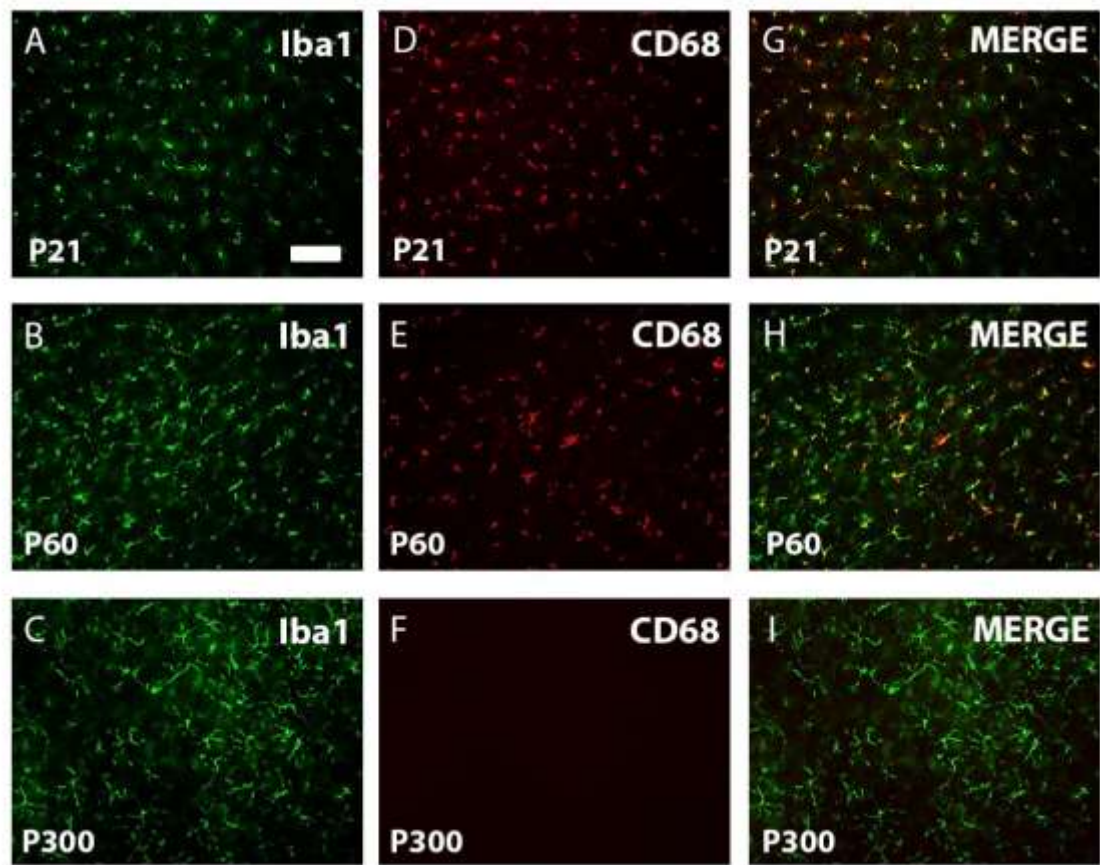


Figure 4.14. Higher magnification double labelled immunofluorescent photomicrographs of Rd1 retinas from postnatal day (P) 21, P60 and P300. Immunodetection of microglia was achieved using Iba1 (A – C) and CD68 (D – F). The number of Iba1-positive microglia decreases over time from P21 (A) to P300 (C). The morphology of Iba1-positive microglia changes from an activated amoeboid shape with retracted processes at P21 (A) to a quiescent appearance with ramified processes by P300 (C). The morphological appearance correlates with the extent of photoreceptor degeneration: there is a high amount of photoreceptor loss early in the disease process. The number of CD68-positive microglia also decreases from P21 (D) to P60 (E), and is minimal by P300 (F). The peak level of CD68 activity likewise correlates with the high amount of photoreceptor degeneration that occurs early in the disease process. Scale bar 100 μ m.

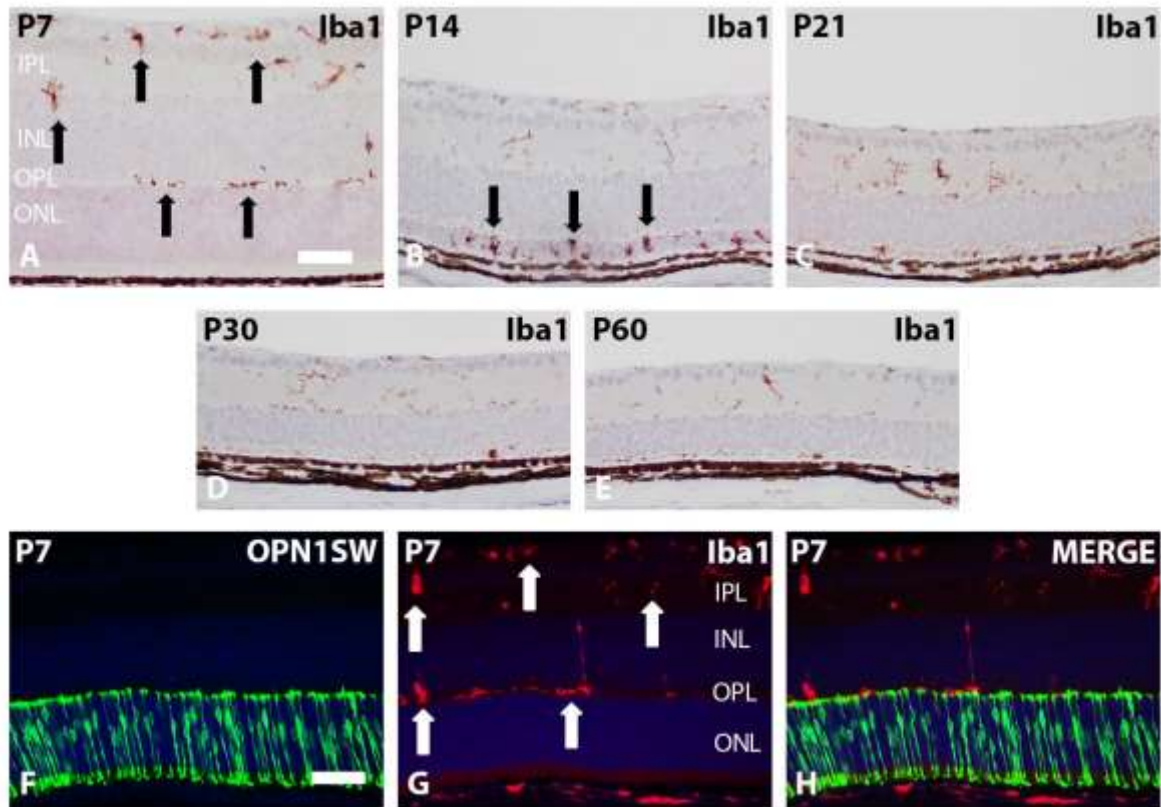


Figure 4.15. (A – E) Photomicrographs of Iba1 immunolabelling in transverse sections of the Rd1 mouse retina from postnatal day (P) 7 to P60. Iba-positive microglia are present in the nerve fibre layer, inner and outer plexiform layers at P7 (A, G, arrows). Iba1-positive microglia migrate to the outer nuclear layer (ONL) at P14 (B, arrows) which coincides with photoreceptor degeneration. The density of Iba1 labelling in the ONL is lower by P21 (C) and minimal at P30 (D) and P60 (E). (F – H) Double labelled immunofluorescent photomicrographs of OPN1SW (F) and Iba1 immunolabelling (G) in transverse sections at P7 are shown. There is no co-localization between OPN1SW immunolabelling and Iba1 immunolabelling (F – H). IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer. Scale bars: A – E = 50 μ m; F – H = 50 μ m.

4.2.8. Muller Cell Activity in the Rd1 Retina

GFAP is an intermediate filament protein expressed by Muller cell endfeet and astrocytes in the retina. Retinal injury leads to GFAP upregulation throughout Muller cells. At P7, GFAP immunolabelling was restricted to the nerve fibre layer (Fig. 4.16A). At P14, GFAP expression was observed in Muller cell processes spanning the retina (Fig. 4.16B). GFAP expression increased between P14 and P21 and remained elevated until P90 (Fig. 4.16C – E).

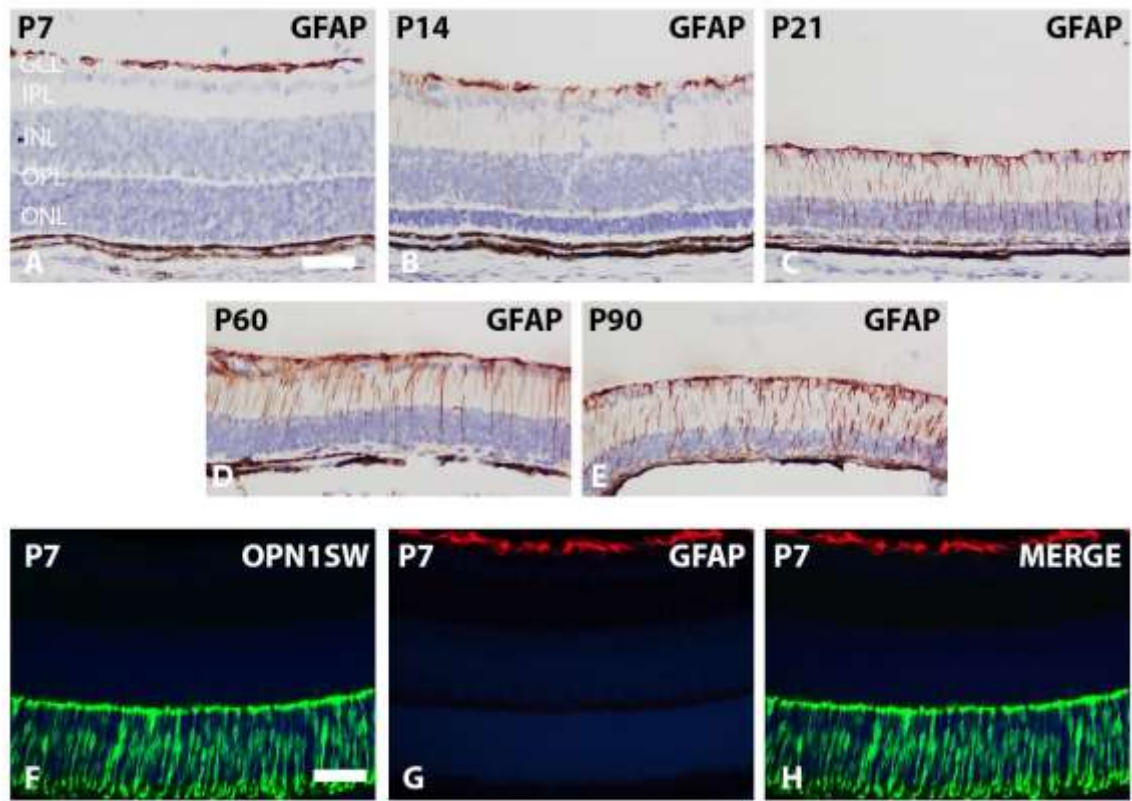


Figure 4.16. (A – E) Photomicrographs of GFAP immunolabelling in transverse sections of the Rd1 mouse retina from postnatal day (P) 7 to P60. GFAP-positive astrocytes are localised to the nerve fibre layer at P7 (A, G). Müller cells, which can be identified by their processes that span the thickness of the retina, upregulate GFAP expression from P14 onwards. GFAP-positive Müller cells can be seen throughout the retina from P14 to P60 (B – E). (F – H) Double labelled immunofluorescence photomicrographs of OPN1SW (F) and GFAP (G) in transverse sections at P7 are shown. There is no co-localization between OPN1SW and GFAP (H). GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer. Scale bars: A – E = 50µm; F – H = 50µm.

4.3. Discussion

4.3.1. Cone Outer Segments Degenerated Rapidly Compared to Cone Cell Bodies

The Rd1 mouse has been widely used as a model for human RP²³⁹. Carter-Dawson *et al*²⁴⁰ first described the time course of photoreceptor degeneration in transverse sections of the Rd1 mouse retina using light microscopy and electron microscopy. A rapid loss of rod cell nuclei was followed by a slower loss of cone cell nuclei over the remaining lifetime of the animal.

Similar results have been reported in studies by Jimenez *et al*²⁴¹ and LaVail *et al*²⁴². In the present study, we have demonstrated that rod degeneration was almost complete by P21 and cone degeneration proceeded gradually thereafter.

Cones have four primary structural regions: outer segments, inner segments, cell bodies, and synaptic terminals. The opsin antibodies used in this study label proteins located in the outer segments of cones. However, these antibodies were also found to label other structural parts of the cone, including cell bodies and inner segments. This phenomenon does not occur in the wild type mouse retina²⁴³. To validate this observation, we co-labelled cones with OPN1SW and PNA (Fig. 4.9). OPN1SW was expressed in all parts of the cone cell while PNA was only expressed in outer segments. Additionally, we double-labelled transverse retinal sections with OPN1SW and Iba1 to determine whether the OPN1SW-positive cell bodies seen in whole-mount retinas belonged to microglia (Fig. 4.15). We found no overlap between Iba1 labelling and OPN1SW labelling which suggested that the OPN1SW-positive cell bodies quantified in whole-mount images belonged to S-cones and not to microglia.

Previous studies of cone degeneration in Rd mice have measured the numbers of surviving cones²⁴⁰⁻²⁴². Recent experiments using cone-specific antibodies on whole-mount Rd retinas have identified immunolabelling of two distinct parts of the cell^{27,163,243}. These studies observed that cone outer segments are compromised early in the degeneration process, while cone inner segments and cell bodies are maintained. The differences in rates of degeneration between cone cell bodies and cone outer segments have not been previously described. We have quantified the rate, and described the spatial distribution, of cone outer segment and cone cell body degeneration in whole-mount Rd1 retinas using fluorescent immunodetection of S-cones and M/L-cones. Our results confirm that cone outer segments degenerate faster than cone cell bodies and that there is hemispheric asymmetry in the rate of S-cone and M/L-cone degeneration.

4.3.2. Implications for Other Studies

The quantitative data presented in this study will prove useful for other researchers that employ the Rd1 model to examine cone loss and preservation strategies. Many studies use cone quantification as a primary outcome to determine the effect of potential therapies^{27,28,163,244}. In many of these studies, it is unclear if cone outer segments or cone cell bodies are quantified^{163,244}. This distinction becomes important when considering the timing of therapy. For example, by P21 more than 50% of cone outer segments have degenerated. Therefore, if a therapy was initiated at P21, the effect of the therapy on cone outer segment quantification will be masked by the low baseline number of cone outer segments. Since cone cell bodies survive longer than outer segments, we believe quantification of cell bodies presents a more practical method of measuring the effect of cone preservation therapies.

4.3.3. Topographical Differences in S-cone and M/L-cone Distribution

Antibodies that label both S-cones and M/L-cones have been used in previous studies that examined the topographical distribution of cone degeneration in Rd mice^{241,245}. These studies also found that cone loss advances from the central to the peripheral retina. Ogilvie *et al*²⁴⁵ used PNA labelling and reported that cone degeneration began nasally, then extended inferiorly and lastly affected the supero-temporal retina. Jimenez *et al*²⁴¹ used a polyclonal cone antibody that labels both cone types and reported that cone degeneration began in the inferior hemisphere then extended into the superior hemisphere along the dorsal-ventral axis. Szél *et al*²⁴⁶ studied cone distribution in different laboratory mouse strains and reported that M/L-cones were present exclusively in the supero-temporal retina while S-cones were primarily in the infero-nasal retina. Given these results, Ogilvie *et al*²⁴⁵ and Jimenez *et al*²⁴¹ suggested that M/L-cones survived longer than S-cones in the Rd1 retina. However, using antibodies specific to the two spectral classes of cones, we observed S-cones primarily in the inferior hemisphere of the Rd1 retina and M/L-cones distributed throughout the entire Rd1

retina. This distribution is similar to the distribution of S-cones and M/L-cones in the wild type C57BL/6J retina^{238,247}. By P300, a few M/L-cones survived in the superior Rd1 retina and a few S-cones survived in the inferior Rd1 retina. These topographical differences are of relevance to studies that only quantify either S-cones or M/L-cones when measuring neuroprotection. Due to the differences in number and distribution of the two cone types, the results of cone preservation therapy on a single spectral class may not necessarily be applicable to the other spectral class.

4.3.4. Reasons for the Differences Between S-cone and M/L-cone Numbers

The number of S-cones was approximately two-fold greater than the number of M/L-cones in the Rd1 retina at all time points when quantified either by outer segment number or cell body number. In comparison, M/L-cones outnumbered S-cones in C57BL/6J control mice when quantified by outer segment number. We suspect the reason for the apparent low density of M/L-cones relative to S-cones on the whole-mount images is methodological and relates to differing signal-to-noise ratios for the M/L- and S-cone antibodies. The S-cone antibody offers superior immunolabelling and we suspect that M/L-cones have been under-counted in the Rd1 retina. The relative difference in the two antibodies is not apparent in C57BL/6J control mice as the segments label so brightly. However, it can also be argued that there are simply fewer M/L-cones when compared to S-cones in the Rd1 retina. Both antibodies label cone segments brightly, and yet substantially lower numbers of M/L-cones compared to S-cones were quantified at each time point. Future work should examine whether different M/L-cone antibodies provide different results.

4.3.5. Justification for the Method of Cone Quantification

Methods of cone quantification vary across different studies. Ortin-Martinez *et al*²³⁸ proposed

an automated method to quantify the total number of cones in the mouse retina by sampling areas of high, medium and low cone density. However, this approach is difficult to replicate in studies of cone degeneration because areas of high, medium and low cone density change with age and, therefore, researcher bias may be introduced when choosing sampling areas. Also, areas of high, medium and low cone density differ for the two spectral cone types. The approach used in this study is similar to most other studies that use the Rd1 model. This current approach is more easily replicated and less susceptible to researcher bias since it uses standard distances from the optic nerve head.

4.3.6. Real-time PCR Provided Complementary Data to Immunohistochemistry

In addition to histologic methods, reliable and sensitive assessment of photoreceptor injury can be achieved by measurement of the total retinal content of mRNAs encoding cone-specific markers ²⁴⁸. In the present study, there was a 95% reduction in S-cone segments from P14 to P90 and 99% reduction in M/L cone segments from P14 to P90. In comparison, there was a five-fold decrease in S-cone and M/L-cone mRNA levels during the same time period. These data suggest cones can still continue to synthesize opsin proteins while their outer segments degenerate ²⁴³. Therefore, cone opsin mRNA expression can serve as another useful quantitative marker of cone degeneration in studies that require a long therapeutic window for intervention.

4.3.7. Role of Microglia in Cone Degeneration

Microglial activation has been reported to occur during the development of photoreceptor degeneration in Rd1 mice ²¹⁷⁻²²². In the present study, microglia started to migrate to the photoreceptor layer at P14, coinciding with the period of rod degeneration. Similar results were obtained by Zeng *et al* ²¹⁹ who demonstrated that the peak in microglial activity and expression of pro-inflammatory chemokines occurs between P12 and P14 in the Rd1 retina.

In order to understand the relationship between microglial activity and cone degeneration, we quantified the number of activated microglia after rod degeneration was complete (i.e. P21). The concentration of CD68-labelled microglia decreased rapidly between P21 and P60, then proceeded gradually until P300. Thus the profile of microglial activity correlated with the temporal pattern of cone death, implying that microglial cells are engaged with the cone degeneration process.

Growing evidence suggests that microglial activation may contribute to photoreceptor degeneration in retinal degeneration models^{218,219,221,222}. However, recent studies have mostly examined microglial activation in relation to rod degeneration, making it difficult to draw conclusions about the effects of microglial activation on cones^{218,219,221}. Roque *et al*²²¹ demonstrated that retina-derived microglial cells accelerate photoreceptor apoptosis *in vitro*. Peng *et al*²²² demonstrated that Cx3cr1 deficiency, which is a microglia-specific chemokine receptor, exacerbated rod death without affecting cone density in Rd10 mice. However, photopic ERG b-wave amplitudes were significantly smaller and cone morphology was severely damaged in Cx3cr1 deficient mice compared to Rd10 controls, indicating functional decline of cones. Peng *et al*²²² also demonstrated that microglial inhibition reduced pro-inflammatory cytokine expression and preserved rods in Rd10 mice. ERG testing revealed increased scotopic b-wave amplitudes, which suggested functional preservation of rod photoreceptors, but photopic b-wave amplitudes were not significantly different to control animals. The effect of microglial inhibition on cone degeneration therefore remained unclear.

4.3.8. Role of Muller Cells in Cone Degeneration

Muller cells provide homeostatic and metabolic support to other retinal cells. Muller cells also produce neurotrophic factors such as BDNF and CNTF which promote the survival of retinal neurons^{249,250}. Early studies showed that intravitreal injection of the neurotrophic factors BDNF, CNTF, and FGF-2 resulted in a rapid increase in phosphorylated extracellular signal-

regulated kinase and c-fos immunoreactivity in Müller cells ^{251,252}. The protective effect of these neurotrophic factors on photoreceptors was therefore suggested to be mediated via the activation of Muller cells. Recent studies have also demonstrated that Muller cells undergo reactive gliosis in photoreceptor degeneration models ²⁵³⁻²⁵⁵. Reactive gliosis is characterized by the hypertrophy of the terminal processes of Muller cells, a dramatic increase in intermediate filament expression and increased deposition of proteoglycans ²⁵⁶.

We found that Muller cells were upregulated during the acute stage of photoreceptor degeneration at P14. This likely helped to protect the inner retina from further degeneration by limiting the site of degeneration ²⁵³. However, prolonged upregulation of Muller cells can interfere with neuronal survival and regeneration ²⁵⁴. Additionally, gliosis may act as a physical barrier to therapies administered to the retina ²⁵⁷⁻²⁵⁹. Barber *et al* ²⁵⁷ found photoreceptor transplant integration was impaired in murine retinal degeneration models with higher levels of gliosis. Kinouchi *et al* ²⁵⁹ demonstrated that mice that were deficient in GFAP and vimentin allowed better cellular integration of transplanted retinal neurons compared with wild-type mice. Gruter *et al* ²⁵⁸ demonstrated that administration of proteoglycan-digesting enzymes to the subretinal space of adult mice in conjunction with lentiviral vector delivery improved viral diffusion and viral vector transduction, suggesting that gliosis-mediated proteoglycan deposition was acting as a physical barrier. An improved understanding of the role of Muller cells in retinal degeneration models could therefore enable the development of strategies that promote photoreceptor survival and regeneration.

4.3.9. Conclusion

In this experiment we have characterized the spatial and temporal distribution of cones in the Rd1 mouse, and quantified the rate of cone outer segment degeneration and cone cell body degeneration. These data will be used to provide baseline data for the following experiment

and will also be useful for future studies that examine cone responses to neuroprotective strategies in this mouse model.

5. EXPERIMENT THREE

5.1. Experimental Design

The aim of this experiment was to determine if bio-energetic therapies can protect cones in the Rd1 mouse model of RP. The experiment was divided into three parts. The first part involved examining the effect of subconjunctival glucose administration on the retina in order to establish that the subconjunctival route would provide adequate drug delivery to photoreceptors. The second part involved examining the effects of subconjunctival glucose administration on cone survival in the Rd1 retina and on functional visual responses in Rd1 mice. For this study, we hypothesized that subconjunctival glucose administration would preserve cones in Rd1 mice. The third part involved examining the effects of systemic oral creatine treatment on cone survival in the Rd1 retina and on functional visual responses in Rd1 mice. For this study, we hypothesized that oral creatine treatment would preserve cones in Rd1 mice.

5.1.1. Preliminary Study

In order to establish that subconjunctival glucose is delivered to the retina, a preliminary investigation was performed to measure the vitreous glucose concentration after subconjunctival injection. Subconjunctival drug administration bypasses the conjunctival epithelial barrier, which is a rate-limiting factor for the permeation of water-soluble drugs²⁶⁰. However, various dynamic, static, and metabolic barriers limit drug access to the posterior segment and retina. Dynamic barriers include conjunctival blood and lymphatic circulation. Drug elimination from the subconjunctival space becomes a major determinant of the vitreous drug levels following subconjunctival administration. The molecules that escape the conjunctival vasculature permeate through the sclera and choroid to reach the vitreous humor, and eventually the retina and photoreceptor cells²⁶¹.

The vitreous humour is a transparent gel located behind the lens and extends to the retinal cup. It consists of 98% water and is acellular. Molecules in the vitreous humour are eliminated either by the anterior route or posterior route. The anterior elimination route involves diffusion across the vitreous into the aqueous humor through zonular spaces followed by elimination through aqueous turnover and uveal blood flow. The posterior elimination pathway involves permeation across the blood–retinal barrier and requires optimum passive permeability or active transport mechanisms ²⁶¹. Glucose in the vitreous humour diffuses across the blood–retinal barrier via glucose transporters and is delivered to photoreceptors. Therefore, the presence of a high level of glucose in the vitreous humour after subconjunctival glucose injection suggests glucose delivery to photoreceptor cells, which are the target cells in this experiment.

5.1.2. Subconjunctival Injections

A single injection of 20 µl of 50% glucose was administered into the subconjunctival space in the right eyes of C57BL/6J mice (n = 9). This procedure involved lifting the inside of the eyelid with fine forceps and delivering a subconjunctival injection using a 33G needle. Use of an operating microscope facilitated the procedure. Injections were performed under isoflurane sedation and topical amethocaine 0.5% anaesthetic. During every injection, visualisation of a subconjunctival bleb provided confirmation that the injection had been successfully administered. Left eyes were treated with an injection of osmotically matched 8.4% saline. Mice were killed after 1 hour, 8 hours and 16 hours (see Fig. 5.1 for experimental plan). Eyes were enucleated and the vitreous was removed and sonicated in a 1 ml Eppendorf tube. 10 µl of sonicated vitreous was removed from each sample and made up to 100 µl with distilled water. The samples were then analysed using a glucose hexokinase assay kit (Sigma-Aldrich Corp., St Louis, MO, USA) and absorbances read with a microplate reader (Fluostar Optima; BMG Labtech, Mornington, VIC, Australia). The final vitreous glucose

concentration (mmol/L) was determined by allowing for the 10-fold dilution factor, and comparing the absorbance with a previously constructed calibration curve.

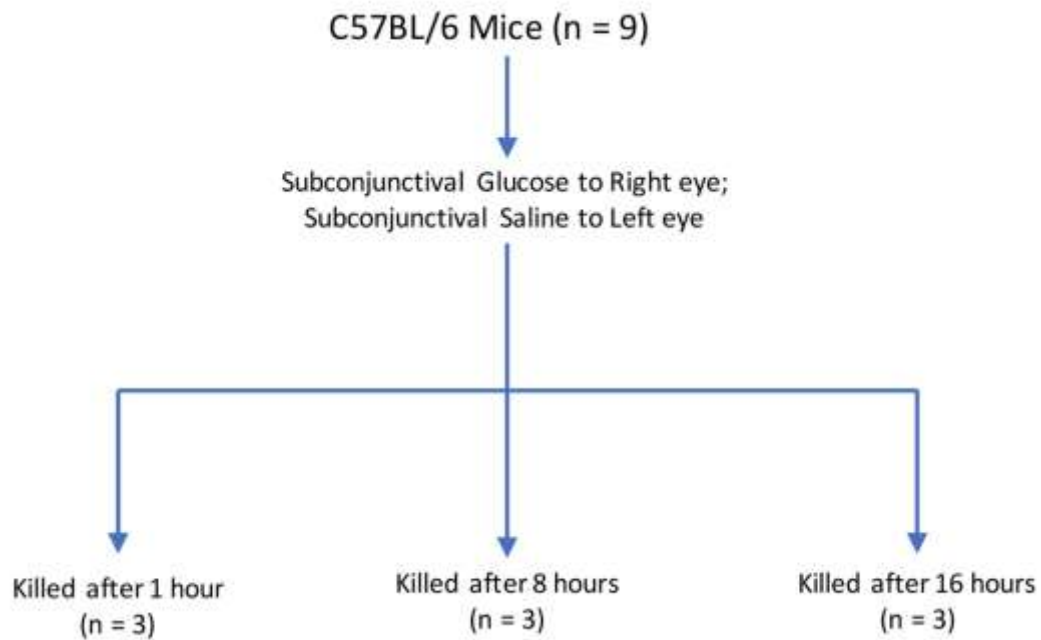


Figure 5.1. Flow chart of the experimental plan in the preliminary glucose vitreous study.

5.1.3. Subconjunctival Glucose Treatment

C3H/HeJArc mice were randomly divided into experimental (n = 19) and control (n = 17) groups. At the starting age of P14, the right eyes were treated with an injection of 20 μ l of 50% glucose into the subconjunctival space as described above. Injections were repeated at 24-hourly intervals. The left eye remained as an untreated control. Mice in the control group receive a subconjunctival injection of osmotically matched 8.4% saline. Again, the fellow eye served as an untreated control. Injections were continued for 30 days in both groups. Optomotor testing was performed 10 days into the treatment regimen at P24. Mice were killed at either P45 or P60 (see Fig. 5.2 for experimental plan).

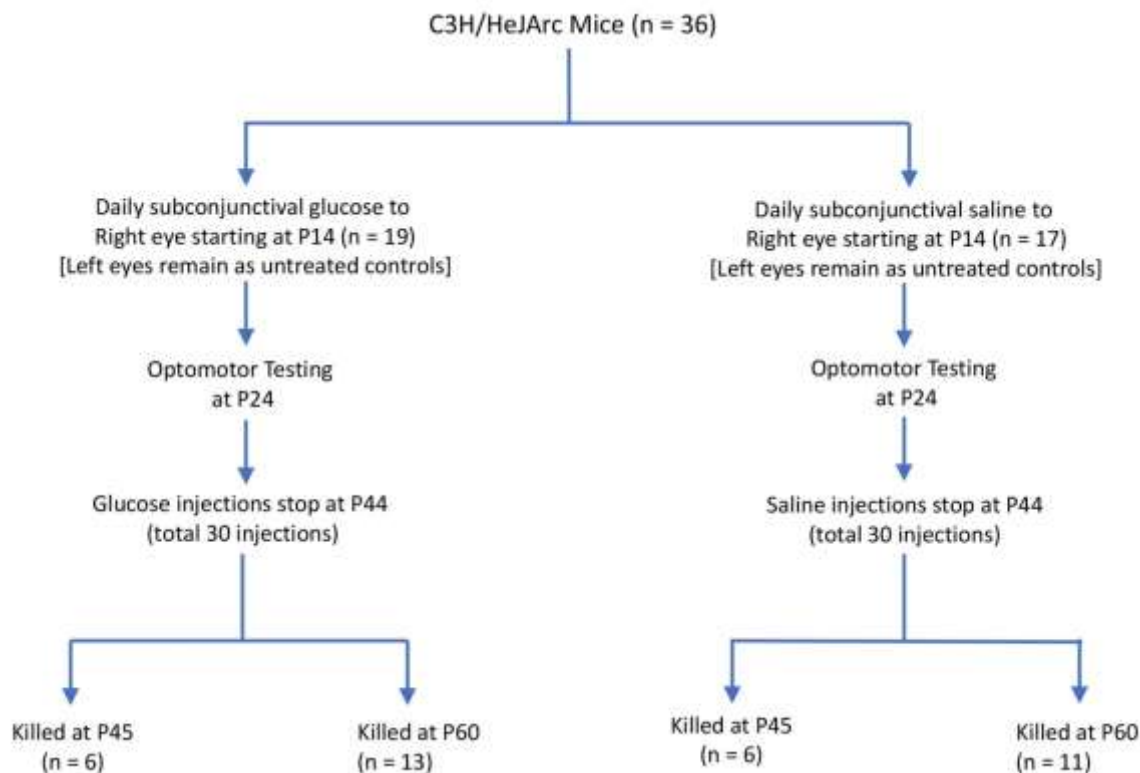


Figure 5.2. Flow chart of the experimental plan in the glucose study in Rd1 mice.

5.1.4. Oral Creatine Treatment

C3H/HeJArc mice were randomly divided into experimental (n = 30) and control (n = 27) groups. The experimental group was placed on a 2% oral creatine diet starting at the age of P21. Mice litters are weaned from their parents at P21 and this was the earliest age to commence oral treatment. Control animals were given an equicaloric standard mouse diet, without creatine. Mice were carefully observed to monitor their eating. Because the creatine was incorporated in the food, and mice were kept at 4 – 5 per cage, it was difficult to accurately assess how much food each mouse consumed. Daily weighing of mice was performed, and satisfactory weight gain was used as an indicator of appropriate food consumption. Optomotor testing was performed 9 days into the treatment regimen at P30. Mice in experimental and control groups continued their diet until they were killed at either P45 or P60 (see Fig. 5.3 for experimental plan).

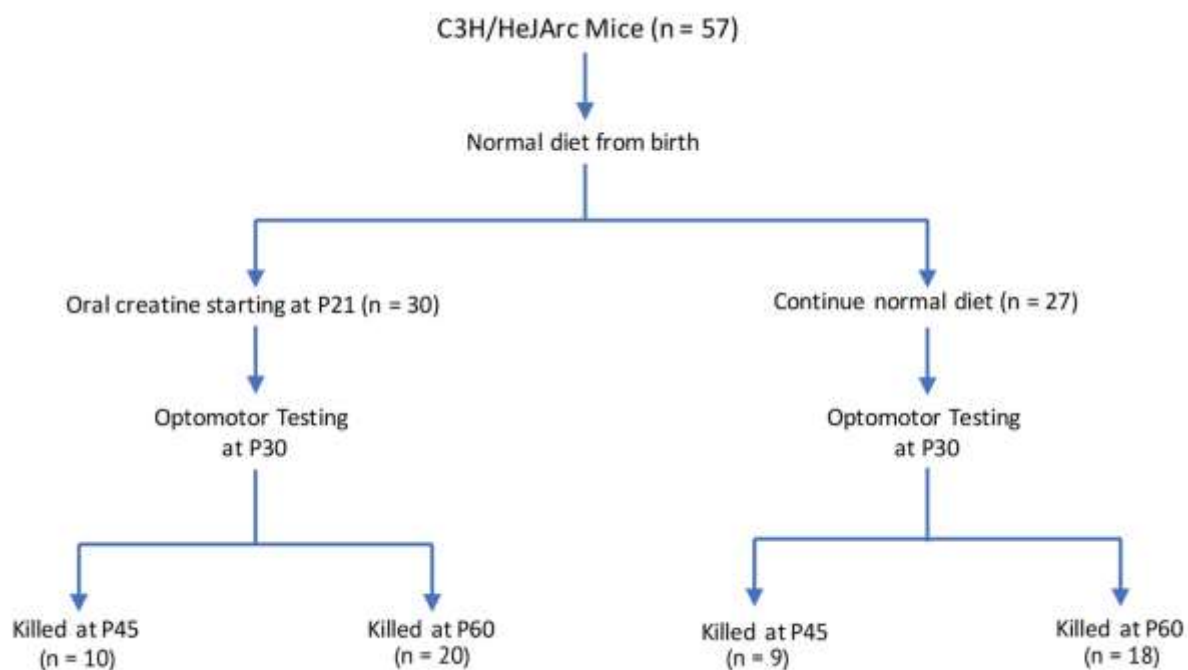


Figure 5.3. Flow chart of the experimental plan in the creatine study in Rd1 mice.

5.2. Results

5.2.1. Vitreous Glucose Study

We hypothesized that subconjunctival glucose injections would elevate vitreous glucose concentrations compared to osmotically matched saline injections. The mean ($\pm$ SEM) vitreous glucose concentration 1 hour after subconjunctival injection was greater for glucose than saline treated eyes (7.96 ± 0.72 vs 2.47 ± 0.74 mmol/L [$n = 3$, $p = 0.006$; paired t-test]). The mean ($\pm$ SEM) vitreous glucose concentration 8 hours after subconjunctival injection was also greater for glucose than saline treated eyes (4.44 ± 0.35 vs 2.2 ± 0.51 mmol/L [$n = 3$, $p = 0.02$; paired t-test]). The mean ($\pm$ SEM) vitreous glucose concentration was not significantly different in glucose-injected eyes compared to saline-injected eyes after 16 hours (3.07 ± 0.30 vs 2.03 ± 0.51 mmol/L [$n = 3$, $p = 0.15$; paired t-test]). These data showed that subconjunctival glucose injection resulted in an increased vitreous glucose concentration for at least 8 hours after injection (Fig. 5.4).



Figure 5.4. Quantification of the vitreous glucose concentration in the C57BL/6J mouse eye at 1 – 16 hours after subconjunctival injection of either glucose or saline. Subconjunctival glucose injection resulted in a significant elevation of the vitreous glucose concentration after 1 hour. The effect was maintained after 8 hours and had returned to baseline after 16 hours. Error bars indicate SEM; ** $p = 0.006$; * $p = 0.02$.

5.2.2. *Effect of Subconjunctival Injections on Cone Degeneration*

First, we hypothesized that daily subconjunctival injections would protect cones in Rd1 mice. The number of cone cell bodies in saline-injected eyes was compared to contralateral eyes. Next, we hypothesized that daily subconjunctival glucose injections would protect cones in Rd1 mice compared to osmotically-matched saline injections. The number of cone cell bodies in glucose-injected eyes was compared to saline-injected eyes.

The mean ($\pm$ SEM) density (number of cells per 0.3888 mm²) of OPN1SW-labelled cone cell bodies at P45 was 1880 ± 178 in glucose-injected eyes ($n = 6$) and 1828 ± 85 in saline-injected eyes ($n = 6$). The mean ($\pm$ SEM) concentration of OPN1SW-labelled cone cell bodies at P60 was 1455 ± 120 in glucose-injected eyes ($n = 13$) and 1442 ± 71 ($n = 11$) in saline-injected eyes. The mean ($\pm$ SEM) concentration of OPN1SW-labelled cone cell bodies in contralateral eyes from saline-injected mice was 1824 ± 118 at P45 ($n = 6$) and 1319 ± 45 at P60 ($n = 11$) (Fig. 5.5-5.7; Table 5.1). There were no significant differences between mean densities of OPN1SW-labelled cone cell bodies in saline-injected eyes compared to contralateral eyes at P45 ($p = 0.96$; paired t-test) or P60 ($p = 0.059$; paired t-test). There were also no significant differences in mean density of OPN1SW-labelled cone cell bodies between glucose-injected eyes and saline-injected eyes at P45 ($p = 0.80$; unpaired t-test) or P60 ($p = 0.94$; unpaired t-test).

The mean ($\pm$ SEM) concentration of R/G-labelled cone cell bodies at P45 was 822 ± 35 ($n = 6$) in glucose-injected eyes and 791 ± 67 ($n = 6$) in saline-injected eyes. The mean ($\pm$ SEM) concentration of R/G-labelled cone cell bodies at P60 was 603 ± 67 ($n = 13$) in glucose-injected eyes and 619 ± 36 ($n = 11$) in saline-injected eyes. The mean ($\pm$ SEM) concentration of R/G-labelled cone cell bodies in contralateral eyes from saline-treated mice was 677 ± 52 at P45 ($n = 6$) and 525 ± 19 at P60 ($n = 11$) (Fig. 5.5-5.7; Table 5.2). The mean concentration of R/G-labelled cone cell bodies was significantly greater in saline-injected eyes compared to

contralateral eyes at P45 ($p = 0.023$; paired t-test) and P60 ($p = 0.045$; paired t-test). There was no significant difference in the mean concentration of R/G-labelled cone cell bodies between glucose-injected eyes and saline-injected eyes at P45 ($p = 0.69$; unpaired t-test) or P60 ($p = 0.83$; unpaired t-test).

Table 5.1. Quantification of OPN1SW labelled S-cones in glucose-injected and saline-injected Rd1 mice

Group	<i>n</i>	S-Cone Cell Bodies Per 0.388mm² (mean ± SEM)		
<i>P45</i>		<i>Central</i>	<i>Peripheral</i>	<i>Whole</i>
Glucose injected	6	1410 ± 185	2350 ± 172	1880 ± 178
Saline injected	6	1361 ± 156	2297 ± 121	1829 ± 85
Un-injected (glucose)	6	1368 ± 176	2341 ± 156	1854 ± 148
Un-injected (saline)	6	1305 ± 189	2342 ± 74	1824 ± 118
<i>P60</i>				
Glucose injected	13	825 ± 114	2080 ± 145	1455 ± 120
Saline injected	11	713 ± 50	2172 ± 113	1443 ± 71
Un-injected (glucose)	13	587 ± 65	2032 ± 92	1310 ± 72
Un-injected (saline)	11	598 ± 55	2040 ± 58	1319 ± 45

Table 5.2. Quantification of R/G-labelled M/L-cones in glucose-injected and saline-injected Rd1 mice

Group	<i>n</i>	M/L-Cone Cell Bodies Per 0.388mm² (mean ± SEM)		
<i>P45</i>		<i>Central</i>	<i>Peripheral</i>	<i>Whole</i>
Glucose injected	6	539 ± 66	1105 ± 59	822 ± 35
Saline injected	6	495 ± 108	1086 ± 50	791 ± 67
Un-injected (glucose)	6	349 ± 77	1065 ± 108	707 ± 70
Un-injected (saline)	6	310 ± 55	1045 ± 100	677 ± 52
<i>P60</i>				
Glucose injected	13	215 ± 44	999 ± 118	602 ± 67
Saline injected	11	221 ± 42	1018 ± 49	619 ± 36
Un-injected (glucose)	13	123 ± 20	913 ± 35	518 ± 18
Un-injected (saline)	11	130 ± 22	920 ± 29	525 ± 19

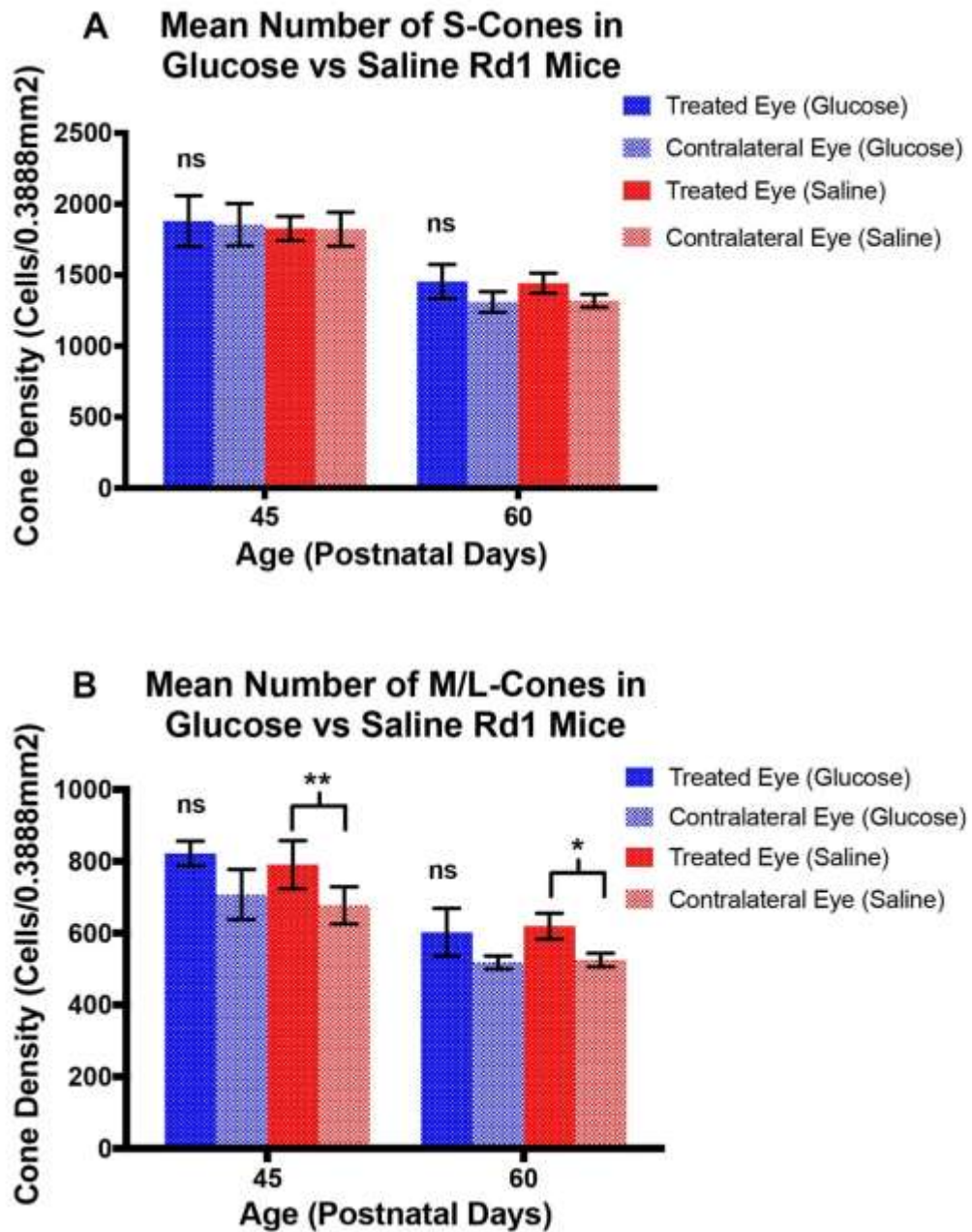


Figure 5.5. Quantification of the mean density of OPN1SW-labelled S-cones (A) and R/G-labelled M/L-cones (B) in the Rd1 retina in glucose-injected eyes and saline-injected eyes, as well their respective contralateral un-injected eyes. Data are shown from animals analysed at P45 and P60. There were no significant differences in the mean densities of S-cones or M/L-cones in glucose-injected eyes versus saline-injected eyes at P45 or P60. However, M/L-cone density was significantly greater in saline-injected eyes compared to contralateral un-injected eyes at P45 and P60. Error bars indicate SEM. ** $p = 0.023$, * $p = 0.045$.

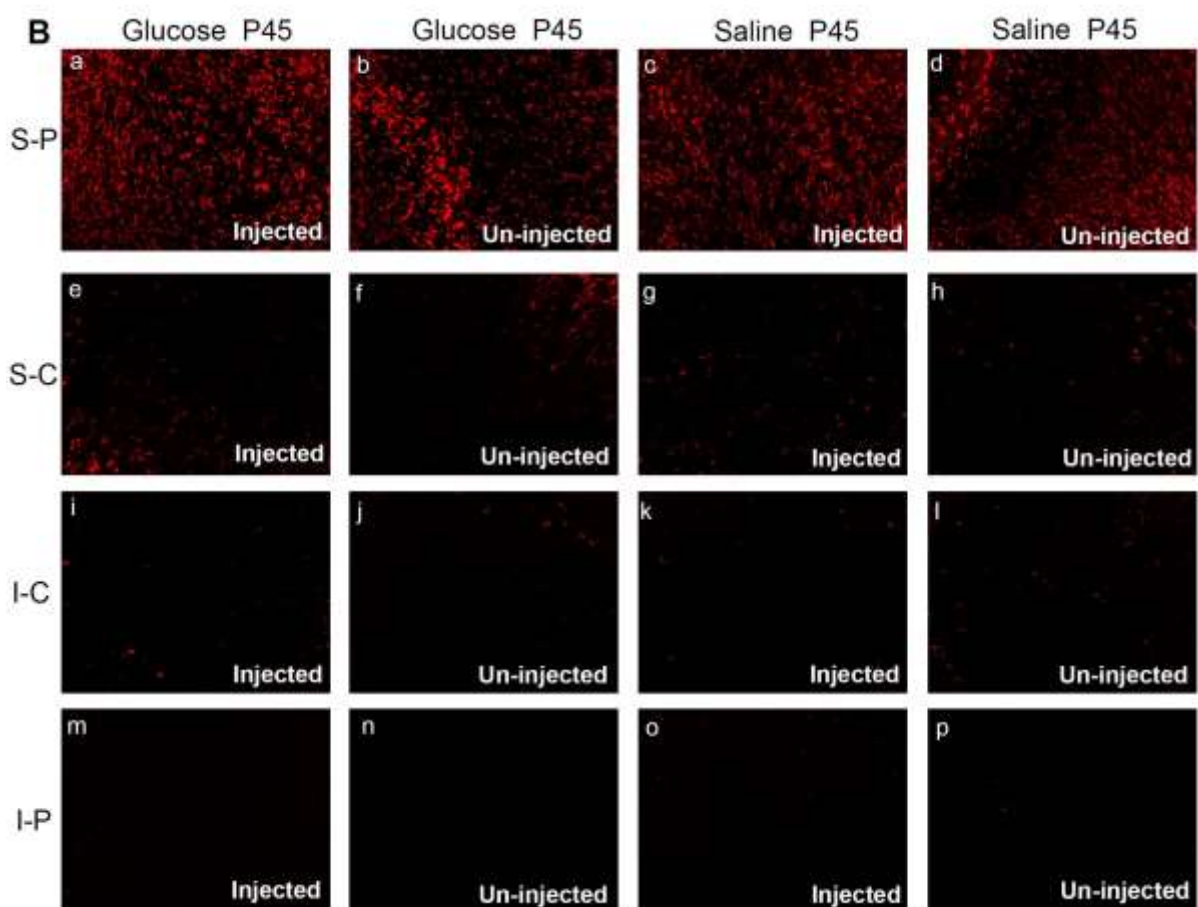
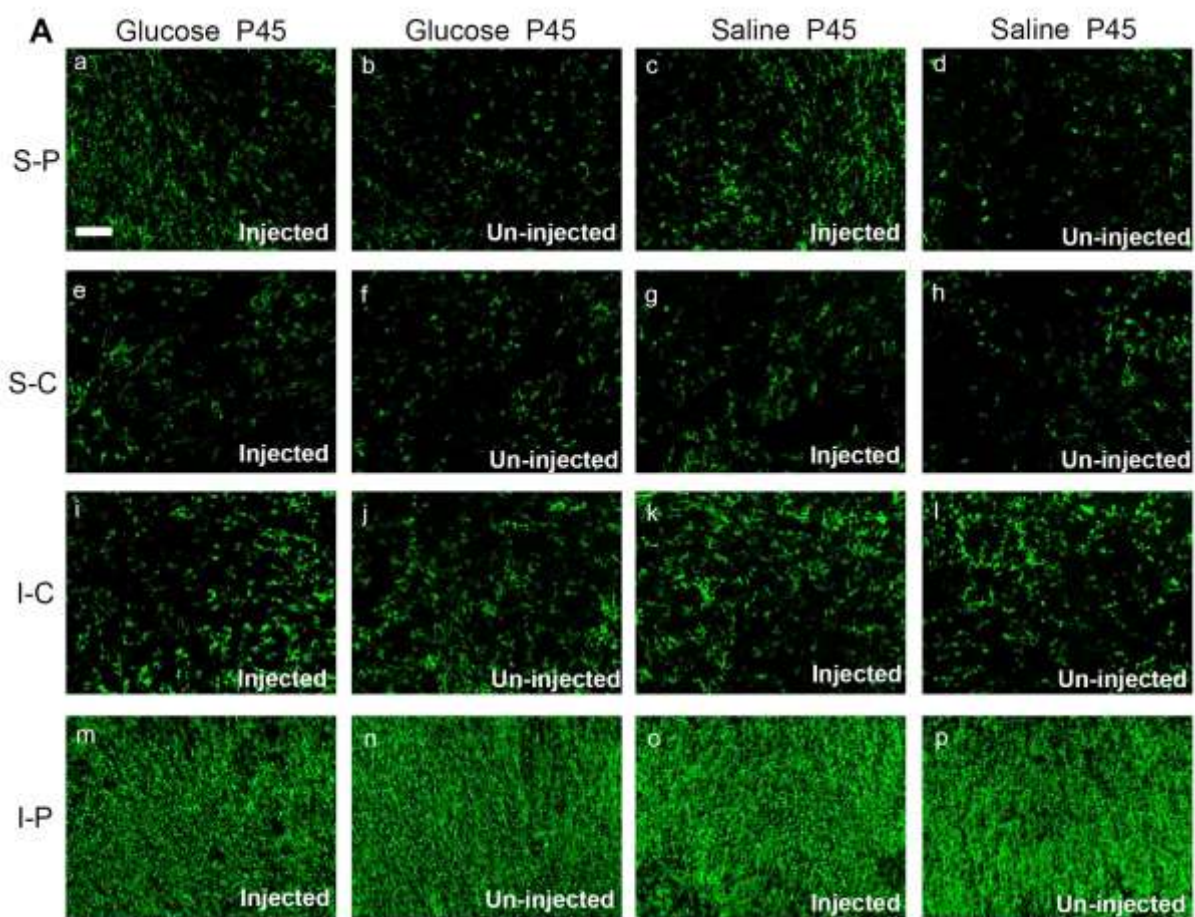


Figure 5.6. Medium power immunofluorescent photomicrographs of whole-mount Rd1 retinas analysed at postnatal day (P) 45. OPN1SW-labelled S-cones (A) and R/G-labelled M/L-cones (B) are shown. Representative images from glucose-injected eyes (a, e, i, m), saline-injected eyes (c, g, k, o) and contralateral un-injected eyes are shown. Images in the top-most (a-d) and bottom-most (m-p) rows correspond respectively to the peripheral portion of the superior and inferior quadrants of the retina. Images in the second (e-h) and third (i-l) rows correspond respectively to the central portion of the superior and inferior quadrants of the retina. The overall distribution of S-cones and M/L-cones is consistent with the data reported in Experiment Two. S-P: superior-peripheral retina, S-C: superior-central retina, I-C: inferior-central retina, I-P: inferior-peripheral retina. Scale bar 100 μ m.

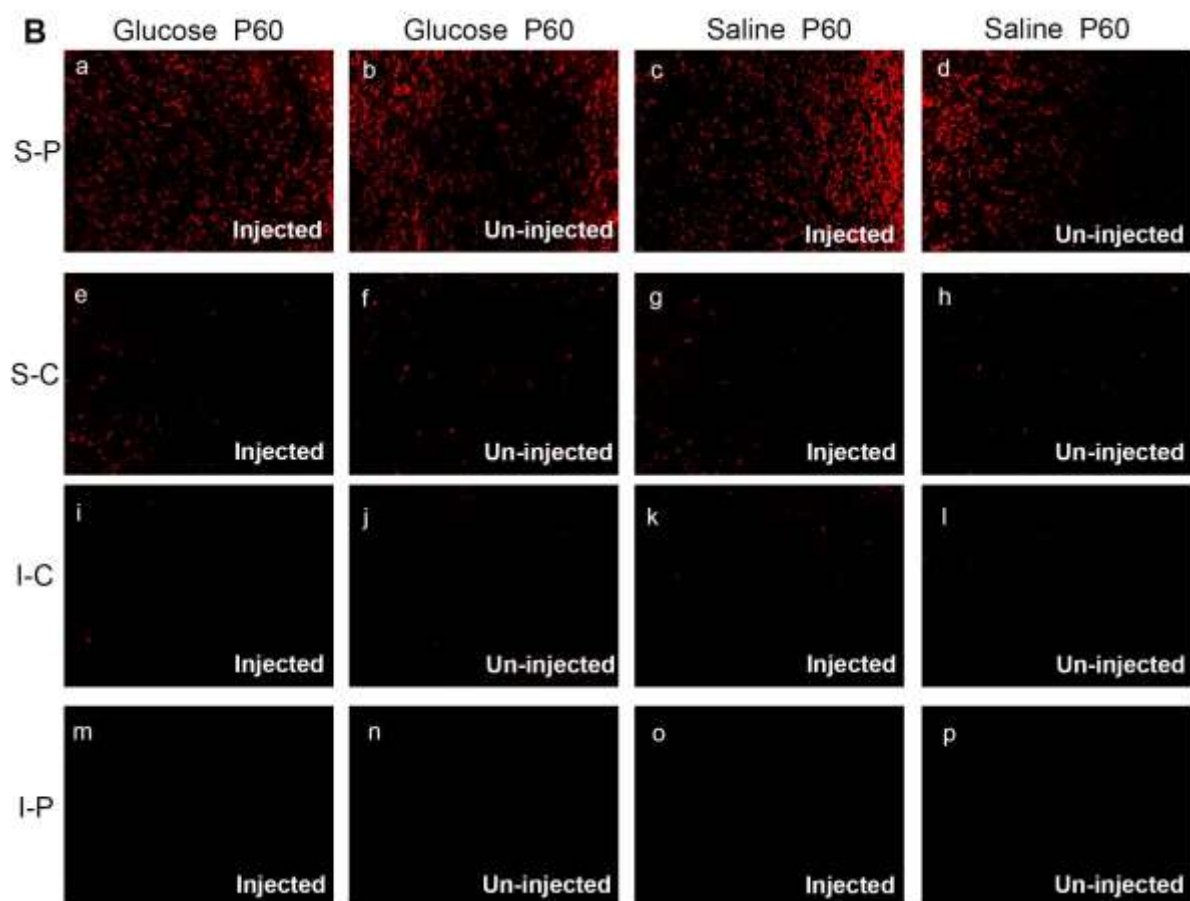
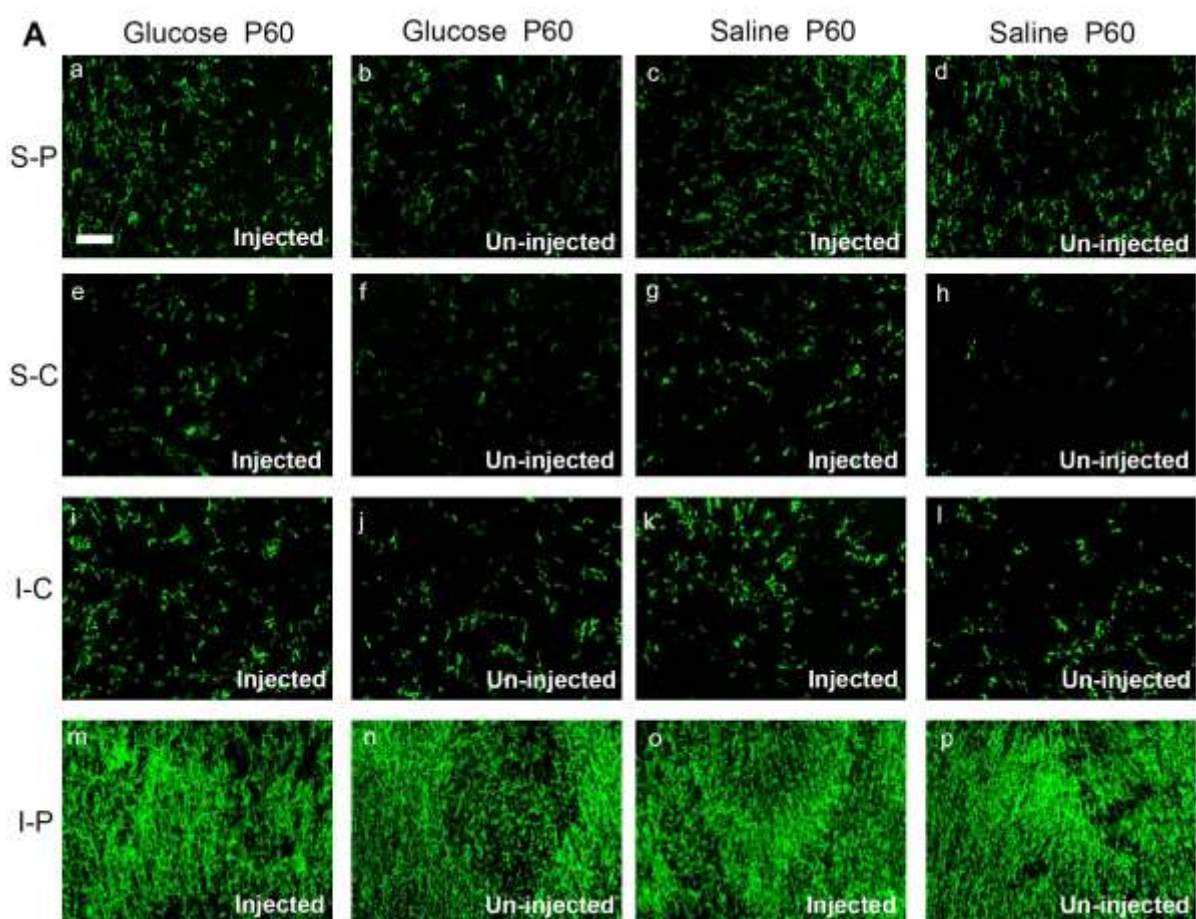


Figure 5.7. Medium power immunofluorescent photomicrographs of whole-mount Rd1 retinas analysed at postnatal day (P) 60. OPN1SW-labelled S-cones (A) and R/G-labelled M/L-cones (B) are shown. Representative images from glucose-injected eyes (a, e, i, m), saline-injected eyes (c, g, k, o) and contralateral un-injected eyes are shown. The layout of images is the same as described for Figure 5.6. S-P: superior-peripheral retina, S-C: superior-central retina, I-C: inferior-central retina, I-P: inferior-peripheral retina. Scale bar 100 μ m. Scale bar 100 μ m.

5.2.3. Effect of Oral Creatine on Cone Degeneration

We hypothesized that oral creatine supplementation would protect cones in Rd1 mice. The mean concentration of cone cell bodies in creatine-fed mice was compared to standard diet mice. The mean ($\pm$ SEM) concentration of OPN1SW-labelled cone cell bodies in creatine-fed mice was 1903 ± 76 ($n = 10$) at P45 and 1517 ± 53 ($n = 21$) at P60. The mean ($\pm$ SEM) concentration of OPN1SW-labelled cone cell bodies in standard diet mice was 1849 ± 91 ($n = 9$) at P45 and 1332 ± 46 ($n = 18$) at P60. The mean concentration of OPN1SW-labelled cone cell bodies was significantly greater in creatine-fed mice compared to standard diet mice at P60 ($p = 0.013$; unpaired t-test) but not at P45 ($p = 0.65$; unpaired t-test) (Figs. 5.8-5.10; Table 5.3).

The mean ($\pm$ SEM) concentration of R/G-labelled cone cell bodies at P45 was 823 ± 32 ($n = 10$) in creatine-fed mice and 694 ± 45 ($n = 9$) in standard diet mice. The mean ($\pm$ SEM) concentration of R/G-labelled cone cell bodies at P60 was 696 ± 33 ($n = 10$) in creatine-fed mice and 571 ± 24 ($n = 9$) in standard diet mice. The mean concentration of R/G-labelled cone cell bodies was significantly greater in creatine-fed mice compared to standard diet mice at P45 ($p = 0.032$; unpaired t-test) and at P60 ($p = 0.007$; unpaired t-test) (Figs. 5.8-5.10; Table 5.4).

Table 5.3. Quantification of OPN1SW-labelled S-cones in creatine treated and control Rd1 mice

Group	<i>n</i>	S-Cone Cell Bodies Per 0.388mm^2 (mean $\pm$ SEM)		
<i>P45</i>		<i>Central</i>	<i>Peripheral</i>	<i>Whole</i>
Creatine	10	1358 ± 87	2448 ± 106	1903 ± 76
Control	9	1317 ± 109	2380 ± 91	1849 ± 91
<i>P60</i>				
Creatine	20	661 ± 51	2374 ± 65	1517 ± 53
Control	18	581 ± 28	2082 ± 72	1332 ± 46

Table 5.4. Quantification of R/G-labelled M/L-cones in creatine treated and control Rd1 mice

Group	n	M/L-Cone Cell Bodies Per 0.388mm² (mean ± SEM)		
<i>P45</i>		<i>Central</i>	<i>Peripheral</i>	<i>Whole</i>
Creatine	10	548 ± 90	1097 ± 43	823 ± 32
Control	9	334 ± 73	1055 ± 53	694 ± 46
<i>P60</i>				
Creatine	20	279 ± 57	1113 ± 32	696 ± 33
Control	18	158 ± 26	983 ± 32	571 ± 24

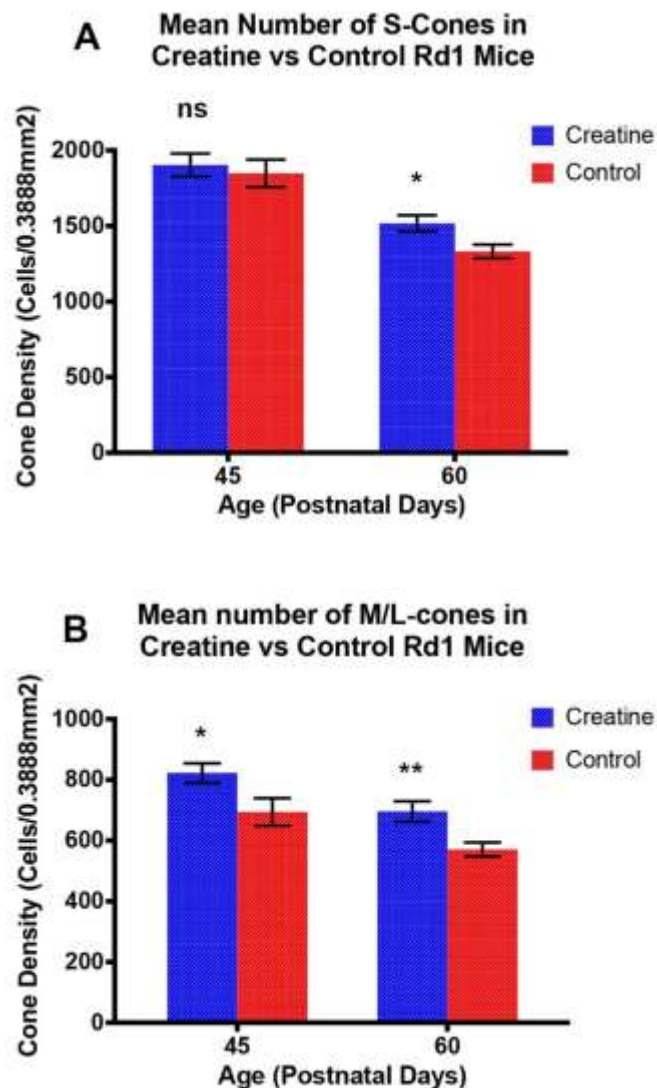


Figure 5.8. Quantification of the mean density of OPN1SW-labelled S-cones (A) and R/G-labelled M/L-cones (B) in creatine diet and standard diet Rd1 mice. Data are shown from animals analysed at P45 and P60. The mean density of S-cones was greater in creatine-treated mice compared to control mice at P60. The mean density of M/L-cones was greater in creatine-treated mice compared to control mice at P45 and P60. Error bars indicate SEM. * $p < 0.05$, ** $p = 0.007$.

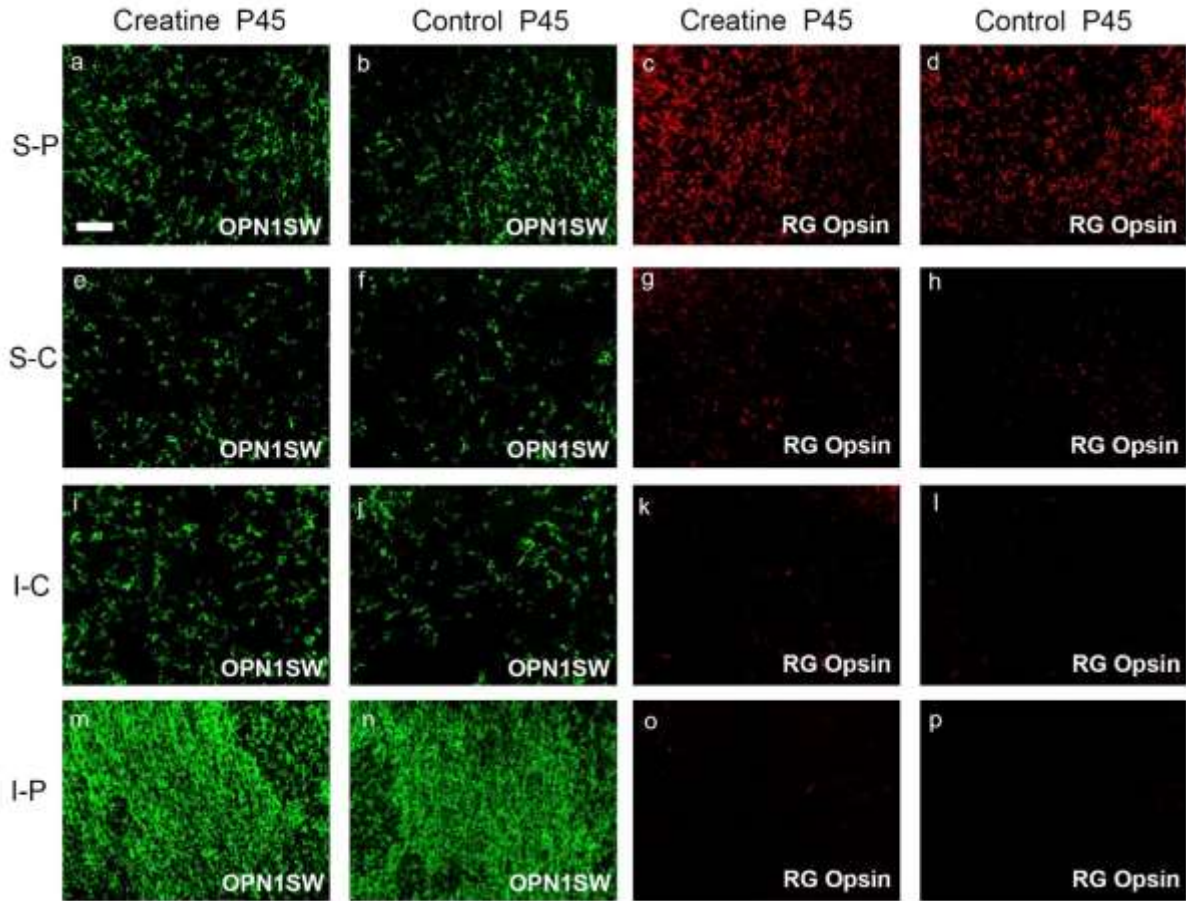


Figure 5.9. Medium power immunofluorescent photomicrographs of whole-mount Rd1 retinas analysed at postnatal day (P) 45. OPN1SW-labelled S-cones (a-b, e-f, i-j, m-n) and R/G-labelled M/L-cones (c-d, g-h, k-l, o-p) are shown. Representative images from creatine-treated animals (a, e, i, m, c, g, k, o) and control animals (b, f, j, n, d, h, l, p) are shown. The layout of images is the same as described for Figure 5.6. S-P: superior-peripheral retina, S-C: superior-central retina, I-C: inferior-central retina, I-P: inferior-peripheral retina. Scale bar 100 μ m.

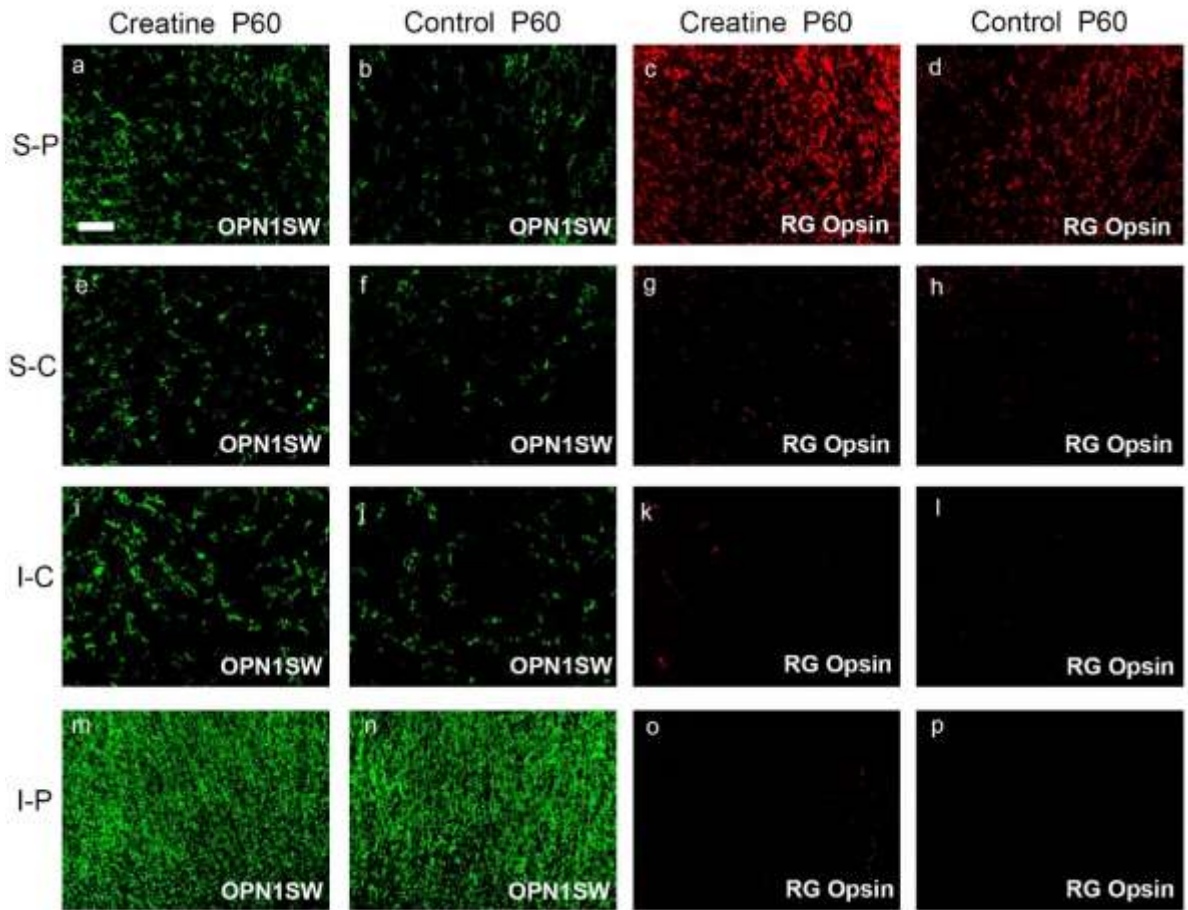


Figure 5.10. Medium power immunofluorescent photomicrographs of whole-mount Rd1 retinas analysed at postnatal day (P) 60. OPN1SW-labelled S-cones (a-b, e-f, i-j, m-n) and R/G-labelled M/L-cones (c-d, g-h, k-l, o-p) are shown. Representative images from creatine-treated animals (a, e, i, m, c, g, k, o) and control animals (b, f, j, n, d, h, l, p) are shown. The layout of images is the same as described for Figure 5.6. S-P: superior-peripheral retina, S-C: superior-central retina, I-C: inferior-central retina, I-P: inferior-peripheral retina. Scale bar 100 μ m.

5.2.4. Optomotor Testing

The mean ($\pm$ SEM) threshold spatial frequency (cycles per degree) was 0.068 ± 0.004 ($n = 8$) in saline-injected eyes and 0.056 ± 0.004 ($n = 8$) in contralateral eyes of saline-injected animals. The mean ($\pm$ SEM) threshold spatial frequency in glucose-injected eyes was 0.066 ± 0.007 ($n = 8$). The mean threshold spatial frequency was 0.016 ± 0.011 ($n = 8$) in standard diet mice and 0.075 ± 0.007 ($n = 8$) in creatine-fed mice. Mean threshold spatial frequency was significantly greater in saline-injected eyes compared to contralateral eyes ($p = 0.049$; paired

t-test) and in creatine-fed mice compared to standard diet mice ($p = 0.0003$; unpaired t-test). There was no significant difference in mean threshold spatial frequency between saline-injected eyes and glucose-injected eyes ($p = 0.25$; unpaired t-test) (Table 5.5, Fig. 5.11).

Table 5.5. Performance of Rd1 mice in the optomotor tracking test

Group	<i>n</i>	Threshold Spatial Frequency (mean cycles/degree $\pm$ SEM)
<i>P24</i>		
Glucose injected	8	0.066 ± 0.007
Glucose un-injected	8	0.059 ± 0.011
Saline injected	8	0.069 ± 0.004
Saline un-injected	8	0.056 ± 0.004
<i>P30</i>		
Creatine	8	0.075 ± 0.007
Control	8	0.016 ± 0.011

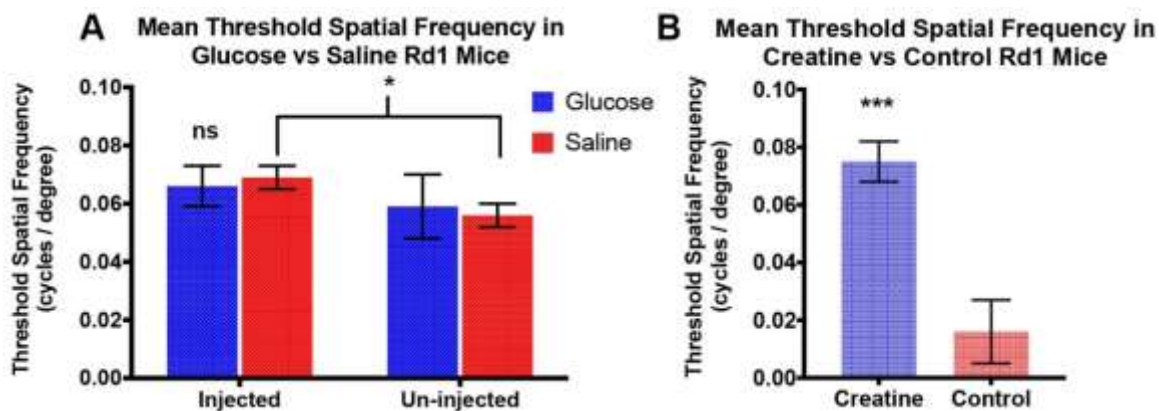


Figure 5.11. Performance of Rd1 mice in the optomotor tracking test. Mean threshold spatial frequency can be used as a marker of visual function. Glucose-injected eyes were compared with saline-injected eyes at P24 (A) and creatine-treated mice were compared with standard diet mice at P30 (B). Saline injections had a minor protective effect on visual function compared to un-injected eyes (A). Creatine treatment had a marked positive effect on visual function compared to controls (B). Error bars indicated SEM. * $p = 0.049$, *** $p = 0.0003$.

5.3. Discussion

5.3.1. Major Findings

There is growing evidence to support the theory that cones in RP ultimately die from a mechanism that involves energy failure. Recent studies by Ait-Ali *et al*²⁸ and Punzo *et al*²⁷ suggest that the mechanism of cone degeneration is related to compromised glucose uptake. We therefore hypothesized that increasing glucose supply to the retina would protect cones in Rd1 mice. In addition, there is increasing evidence that demonstrates the reliance of photoreceptor outer segment and synaptic energy supply on the phosphocreatine shuttle pathway^{5,44}. We therefore also hypothesized that increasing creatine supply to the retina would protect cones in Rd1 mice.

The primary outcomes in the present study were differences in OPN1SW-labelled S-cone densities and R/G-labelled M/L-cone densities between groups, which were analysed using fluorescent immunohistochemistry on whole-mount retinas. The secondary outcome was difference in visual function between groups, assessed using the optomotor tracking test. The major findings in this experiment were as follows: (1) once daily local glucose delivery did not improve cone survival compared to saline treatment at any time point; (2) local saline injections preserved M/L-cones, but not S-cones, compared to un-injected eyes; (3) oral creatine treatment improved survival of both S- and M/L-cones compared to standard diet treatment at P60 and improved survival of M/L-cones only at P45. Creatine treatment also improved visual function in Rd1 mice.

5.3.2. Glucose Injections Did Not Protect Cones in Rd1 Mice

The concept of glucose as a neuroprotective agent in RP may seem counter-intuitive. Long-term hyperglycemia has deleterious effects on the retina, resulting in damage to the microvasculature, which can lead to vision-threatening complications²⁶². This process is

clinically exemplified in patients with diabetic retinopathy. We believe the ineffectiveness of local glucose treatment in this study may be due to a number of factors. A single subconjunctival injection of glucose resulted in significant elevation of the vitreous glucose concentration, which was maximal after 1 hour, and decreased gradually thereafter. This suggested that subconjunctival administration of glucose likely reaches the photoreceptor layer, since molecules in the vitreous are transported to the retina. However, other retinal neurons and glial cells would also be exposed to the glucose before it reaches the photoreceptors, reducing the total amount of glucose delivered to photoreceptors. Also, glucose would likely only be significantly higher in the retina for limited number of hours following the daily injection. This might be insufficient supply if there is a bio-energetic deficit in cones. Prolonged, slow release, glucose delivery could prove a better option.

An additional factor that may account for the inability of glucose to protect against cone loss relates to glucose uptake. In Rd1 mice, the decreased production of RdCVF from rod degeneration leads to decreased activity of GLUT-1²⁸. Normally, GLUT-1 exists in an equilibrium between its homodimeric and homotetrameric quaternary structures²⁶³. RdCVF binding to BSG-1 displaces the equilibrium toward the tetramer, accelerating GLUT-1 transport function and stimulating glucose uptake by cones²⁸. Extracellular glucose enters cones through glucose transport carriers, in particular GLUT-1 but also GLUT-3 and GLUT-4. In the Rd1 model, where GLUT-1 becomes defective, increasing extracellular glucose alone may not satisfactorily elevate the intracellular glucose concentration. Therefore, local administration of glucose may not reach the intended intracellular cone metabolic structures. Overall, this finding does not necessarily detract from the hypothesis that cones die from glucose deficiency. However, these data suggest that subconjunctival glucose administration is unlikely to provide benefit if there is a perpetual energy deficit in cones or if the GLUT-1 transporter remains defective. Improved glucose delivery methods or therapies that target GLUT-1, and its binding partner BSG-1, might prove more beneficial for cone survival in RP.

5.3.3. Saline Injections had a Protective Effect on Cones in Rd1 Mice

The finding that saline injections had a protective effect on M/L-cones and on functional visual outcomes was not entirely unexpected. Silverman and Hughes ²⁶⁴ first demonstrated that subretinal saline injections preserved photoreceptors in RCS rats. Further studies by Faktorovich *et al* ^{265,266} confirmed this finding. These studies demonstrated that the act of inserting a needle into the subretinal space, with or without saline, had a protective effect on photoreceptors. Faktorovich *et al* ²⁶⁶ hypothesized that the injurious effect of the needle in the eye could trigger the release of survival factors, such as FGF-2, from intraocular sources. Subsequent studies demonstrated the release of FGF-2 following injury in CNS tissue ²⁶⁷, thereby lending weight to this hypothesis. However, in the studies by Faktorovich *et al* ^{265,266}, the rescue effect of saline injections was limited compared to the rescue produced by injection of neurotrophic factors.

5.3.4. Creatine Protected Cones in Rd1 Mice

Creatine supplementation has been shown to be efficacious in a variety of animal models of neurodegenerative diseases including Alzheimer disease ¹²³, Parkinson's disease ¹²⁴, amyotrophic lateral sclerosis ¹²⁵, and Huntington's disease ¹²⁶⁻¹²⁸. In the present study, creatine supplementation preserved S-cones and M/L-cones in Rd1 mice at P60, and preserved M/L-cones at P45. Creatine treatment also improved functional visual outcomes in Rd1 mice at P30. At the start of creatine treatment, approximately 70% of S-cone outer segments and 50% of M/L-cone outer segments had completely degenerated (data from Experiment Two). This suggests that clinically useful cone function might be preserved even when cone degeneration has progressed to a late stage.

The mechanism of creatine-induced cone preservation has not been elucidated in this study. In other animal studies, exogenous creatine supplementation has been shown to increase the total intracellular phosphocreatine pool ¹²⁹. In photoreceptors, it has been

suggested that the phosphocreatine system provides energy to outer segments for photo-transduction and synaptic terminals for neurotransmission ^{5,44}. We believe the primary mechanism of creatine-induced cone preservation involved increasing the available phosphocreatine pool, which would have provided more energetic fuel for the cell. This would support the idea that the phosphocreatine shuttle system plays an important role in energy supply in cones and that cone degeneration in RP occurs due to a mechanism that involves energy failure.

In other animal studies, creatine has also been shown to exert its effects via a number of other pathways ^{123,129,268}. Creatine supplementation has been shown in skeletal muscle cells to activate 5' adenosine monophosphate-activated protein kinase (AMPK) signaling ²⁶⁸. AMPK is a regulator of metabolism that is activated during conditions of low ATP production. Activation of AMPK regulates a large number of downstream pathways that increase energy production (increased oxidation of fatty acids and glucose), and decrease energy utilization (decreased glycogenesis, decreased fatty acid synthesis, and decreased protein synthesis) ²⁶⁹. Activated AMPK also inhibits the mTOR pathway, which is downregulated in mouse models of RP ²⁷. Recent studies have showed a neuroprotective effect of AMPK activation in animal models of retinal inflammation ^{270,271}. Creatine supplementation might also activate AMPK signaling in Rd1 mice, increasing cone survival by promoting downstream energy production.

In addition to the bio-energetic effects, creatine administration in animal and cellular models of neurodegenerative diseases has also been shown to protect against oxidative stress ^{123,272}. Growing evidence suggests that oxidative stress may play a role in cone degeneration in RP ^{163,180,198-201}. The CK enzyme, which catalyses the reversible phosphorylation of creatine to phosphocreatine, is highly sensitive to oxidative stress due to the presence of cysteine residues that are readily modified by an oxidative insult ²⁷³. Creatine supplementation has been shown to protect against oxidative mediated conversion of octomeric mitochondrial CK to the dysfunctional dimeric mitochondrial CK, which ameliorates the ROS-induced

inactivation of CK^{123,129}. In this way, creatine treatment in Rd1 mice might also protect cones from oxidative damage. Further investigation will help to determine the precise mechanisms by which creatine protects cones in the Rd1 mouse model.

6. GENERAL DISCUSSION AND CONCLUSIONS

Preservation of cones would represent a major medical breakthrough that would greatly increase the quality of life in patients suffering from photoreceptor degenerating conditions such as RP. A limited understanding of the mechanisms that lead to cone degeneration has previously plagued efforts directed towards developing cone preservation therapies. The overriding premise of this thesis was to further our understanding of photoreceptor energy metabolism and manipulate photoreceptor energy metabolism for clinical advantage using the Rd1 mouse model of retinitis pigmentosa. The work presented here demonstrates that cone degeneration in RP likely occurs due to a mechanism that involves energy failure and that bio-energetic treatments, such as creatine, may play a role as future therapies for RP.

6.1. Experiment One

We hypothesized that C-14 glucose would be preferentially incorporated into the photoreceptor outer segments of mice and move in a scleral direction as outer segment discs are turned over and phagocytosed by the RPE. This finding has been demonstrated in previous studies where radiolabeled amino acids were injected into mice ^{91,92}. Photoreceptor outer segment discs are composed mainly of visual pigment proteins. If C-14 glucose was incorporated into visual pigment proteins, it would demonstrate that glucose was used for carbon biomass synthesis in photoreceptors. Our results showed widespread, diffuse C-14 glucose uptake in photoreceptors. This supports the idea of photoreceptors as metabolically active cells, but does not advance the idea that glucose is used for carbon biomass synthesis.

Therefore, the findings have not provided an explanation for the high rate of aerobic glycolysis in the retina. Recently, Hoisos *et al* performed a study to compare the relative contribution of radiolabeled glucose, glutamine and amino acids to carbon biomass synthesis in cultured cancer cells ²⁷⁴. They found most of the carbon mass in proteins came from amino

acids, not from glucose. This finding contradicts the notion that glucose contributes to carbon biomass synthesis in cancer cells and is consistent with data from our first experiment. However, the authors suggested rapid consumption of glucose need not correlate with cell mass incorporation and the large fluxes through glycolysis may support proliferation in ways other than supplying carbon. For example, duplication of nucleic acids is needed for cell proliferation and this requires ribose from glucose or another bioavailable carbohydrate ²⁷⁴.

We have designed a similar experiment to the one performed by Hoisos *et al.* We propose to examine the biosynthetic fates of radiolabeled glucose and amino acids in retinal cells using mass spectrometry analysis at the Adelaide Proteomics Centre. We hypothesize that amino acids will account for the majority of carbon in retinal cells, and the rates of biosynthesis will be similar to those reported in cultured cancer cells by Hoisos *et al.* This result would support the notion that glucose metabolism in the retina is similar to cancer cells and would suggest that the high rate of aerobic glycolysis in the retina facilitates the generation of metabolic products beyond carbon biomass synthesis.

6.2. Experiment Two

Many studies use cone quantification as a primary outcome to determine the effect of potential therapies ^{27,28,163,244}. In many of these studies, it is not clear if cone outer segments or cone cell bodies are quantified ^{163,244}. The differences in rates of degeneration between cone cell bodies and cone outer segments has not been previously described. We described the temporal and spatial distribution of S-cones and M/L-cones in Rd1 mice over time. Our results demonstrated that cone outer segments degenerate faster than cone cell bodies and that there is hemispheric asymmetry in the rate of S-cone and M/L-cone degeneration. This provided the baseline data for the third experiment and will be useful for other studies that use the Rd1 model to examine cone preservation strategies.

6.3. Experiment Three

We hypothesized that bio-energetic treatment would preserve cones in the Rd1 model of RP. Local glucose injections had no protective effect on cones. However, local saline injections had a mild protective effect on M/L-cones. Also, oral creatine improved cone survival and optomotor tracking performances in Rd1 mice. The mechanism by which creatine preserved cones was not elucidated. Given the bio-energetic effects of creatine in other models of disease, we believe creatine treatment likely exerted its neuroprotective effect via a mechanism that improved the overall bio-energetic function of cones.

The promising results obtained from creatine supplementation in mouse models of other neurodegenerative diseases has prompted its use in human clinical trials ²⁷⁵⁻²⁷⁹. Creatine supplementation has been tested in a randomized double-blind futility phase II clinical trial in early Parkinson's disease patients ²⁷⁶. Creatine treatment improved mood, slowed disease rate progression by almost 50% at 1 year, and led to a smaller dose increase of dopaminergic therapy ^{275,276}. Additionally, creatine supplementation has been tested in a randomized double-blind placebo control phase II clinical trial in Huntington's disease patients ²⁷⁷. Creatine treatment reduced serum levels of 8-hydroxy-2-deoxyguanosine, which is a marker of oxidative damage, and slowed the progression of cortical atrophy ²⁷⁷.

The safety and tolerability of low doses (8 g/day) of oral creatine supplementation in humans has been established by these clinical trials ²⁷⁷. Higher doses (30 g/day) have demonstrated greater neuroprotective effects ²⁷⁷. However, high doses of creatine may have potential adverse effects on the kidney and liver ²⁸⁰. The development of a targeted ocular delivery system, which bypasses the systemic circulation, would present a safe long-term route of administration for RP.

We plan to develop a pathway from creatine treatment in mice to clinical translation in human patients. We will begin by examining the effect of dietary creatine supplementation

on different animal models of RP, such as the Rd10 mouse and the RCS rat. These models exhibit slower rates of photoreceptor degeneration and can allow for longer therapeutic treatment windows. We can then examine the effect of dietary creatine supplementation on visual function in patients in RP. If this is successful, we can then proceed to development of a targeted ocular delivery system that would facilitate higher doses of creatine to the retina with fewer systemic complications. In addition, we plan to investigate the effect of creatine treatment in other retinal diseases where energy failure plays a role, such as glaucoma, diabetic retinopathy and age-related macular degeneration.

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